Microbiome analysis using nanopore reads

Marko Suokas

Preprocessing reads

Dorado does not support demultiplexing custom dual indexes located on both the 5' and 3' ends. In ligated libraries, these sequences can appear in either orientation. To address this, we use cutadapt for demultiplexing. Index pairs are identified using the linked adapters approach in both forward and reverse orientations. Afterward, a script is applied to reverse complement sequences in the reverse orientation. Finally, the forward and reverse reads are merged for each sample.

Extract Forward Reads

Forward reads can be demultiplexed using following command:

```
cutadapt -e 0 -O 12 -g file:~/scripts/barcodes.fasta --trimmed-only \
-m 1200 -o "fdemuxed/{name}.fastq.gz" reads.fastq.gz
```

This command extracts barcodes defined in the `barcodes.fasta` file and outputs matching reads into individual files within the `fdemuxed` subdirectory. The minimum read length is set to 1200 bp for 16S amplicons.

Extract Reverse Reads

In order to extract reverse reads, the reverse-complemented barcode file is used:

```
cutadapt -e 0 -O 12 -g file:~/scripts/rev_barcodes.fasta --trimmed-only \
-m 1200 -o "rdemuxed/{name}.fastq.gz" reads.fastq.gz
```

The reads are demultiplexed into a rdemuxed directory.

Tip: Parameters `-O`, `-e`, `-m`, and `-M` can help reduce the chances of mismatched alignments.

Reverse Complement Reverse Reads

Next, we use a bash script to process each reverse file and reverse complement them using the following command:

```
seqkit seq -rp --seq-type DNA -o reverse_comp.fastq.gz reverse_out.fastq.gz
```

Merging Forward and Reverse Reads

Subsequent step is to merge forward and reverse reads with the same base name from two directories. Here's a simple bash command for that:

```
zcat forward_out.fastq.gz reverse_comp.fastq.gz > merged_reads.fastq.gz
```

Trimming Primers

Finally, `cutadapt` and bash scripts can be employed to trim forward and reverse PCR primers from the sequence reads.

Read quality

Nanopore sequencing Phred scores should be interpreted with caution, as per-base accuracy cannot be directly determined from the electrical signal. Instead, scores are estimates based on the confidence of the basecalling model rather than a direct probability of sequencing error. As a result, traditional quality filtering approaches may not always be appropriate.

ONT instead calculates an estimated cumulative error rate and converts it into an average quality score per read. The following code computes this metric and generates a violin plot showing the distribution of average sequence quality per read for each sample.

```
# Define path
source_dir <- "data/reads/set1"
# A function to calculate so called AvgQual
extract_nano_qscores <- function(file) {
    # Read fastq
    fq <- readFastq(file)
    # Extract q-values to a matrix
    qmat <- as(quality(fq), "matrix")
    # Convert values to error probabilities
    error_probs <- 10^(-qmat/10)
    # Compute number of expected errors per read
    total_errors <- rowSums(error_probs, na.rm = T)
    # Length per read
    read_lenghts <- rowSums(!is.na(qmat))
```

```
    # Compute "ONT-style" Q-score
    ont_qscores <- -10*log10(total_errors/read_lenghts)
    # Trim file names
    sampleid <- sub("\\.fastq\\.gz$", "", basename(file))

    # Create a dataframe
    qdata <- tibble(
        nanopore_qscore = ont_qscores,
        sampleid = sampleid)
    return(qdata)
}

# Create list of files
files <- list.files(source_dir, pattern = "\\.fastq\\.gz$",
                    full.names = T)
# Apply function and use map_dfr to combine dataframes together
qscore_data <- map_dfr(files, extract_nano_qscores)
# Save result to a rds file
saveRDS(qscore_data, "set1/nanopore_quality.rds")
```

Violin plot

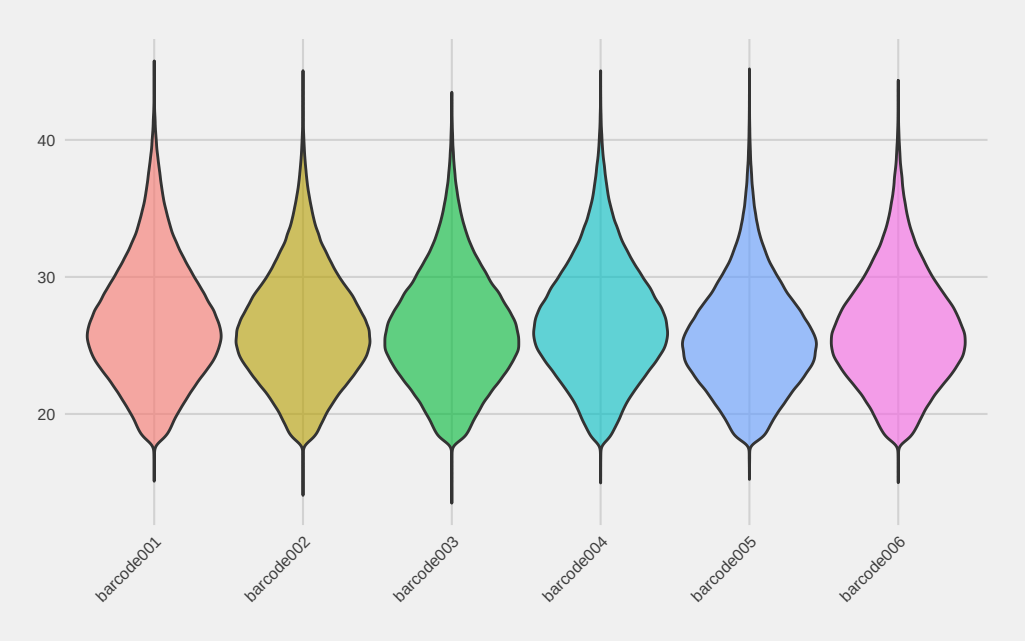
```
# Read data
qdata <- readRDS("set1/nanopore_quality.rds")

# Divide samples to groups of 12
sample_list <- unique(qdata$sampleid)
sample_groups <- split(sample_list, ceiling(seq_along(sample_list) / 12))

# Generate a separate plot for each group
plots <- map(sample_groups, function(group_samples) {
  ggplot(filter(qdata, sampleid %in% group_samples),
         aes(x = sampleid, y = nanopore_qscore, fill = sampleid)) +
    geom_violin(scale = "width", alpha = 0.6) +
    theme_fivethirtyeight(base_size=8) +
    labs(x = "Sample", y = "Nanopore Q-score") +
    theme(legend.position = "none", axis.text.x = element_text(angle = 45, hjust = 1))
})

# Combine all plots into a single vertical layout
final_plot <- wrap_plots(plots, ncol = 1)

# Show the plot
print(final_plot)
```



Import sequence data to Qiime 2

QIIME2 import requires manifest.csv that defines names and paths of sequence files.

```
# Activate qiime environment
export PATH="/homedir04/msuokas/miniconda3/bin:$PATH"
source activate qiime-2024.10

# Import sequence data
qiime tools import \
  --type 'SampleData[SequencesWithQuality]' \
  --input-path data/reads/set1/manifest.csv \
  --output-path data/set1/demux.qza \
  --input-format SingleEndFastqManifestPhred33
```

Dereplicate sequences

Dereplication will remove sequence redundancy

```
# Activate qiime environment
export PATH="/homedir04/msuokas/miniconda3/bin:$PATH"
source activate qiime-2024.10
```

```
# Dereplicate sequences with vsearch plugin
qiime vsearch dereplicate-sequences \
    --p-min-seq-length 1200 \
    --o-dereplicated-table data/set1/derep_table.qza \
    --o-dereplicated-sequences data/set1/derep_sequences.qza \
    --i-sequences data/set1/demux.qza
```

Pick otus using closed-otu-picking from Silva reference

For low diversity samples closed-otu-picking seems good approach.

```
# Activate qiime environment
export PATH="/homedir04/msuokas/miniconda3/bin:$PATH"
source activate qiime-2024.10

# Closed reference otu clustering  command
qiime vsearch cluster-features-closed-reference \
   --i-sequences data/set1/derep_sequences.qza \
   --i-table data/set1/derep_table.qza \
   --i-reference-sequences ~/reference/silva-138-99-seqs.qza \
   --p-strand plus --p-threads 10 --p-perc-identity 0.97 \
   --o-clustered-table data/set1/clustered_table \
   --o-clustered-sequences data/set1/clustered_seq.qza \
   --o-unmatched-sequences data/set1/unmatched.qza
```

Remove rare features

Our sampling is higher than typically and chosen minimum frequency was selected accordingly.

```
# Activate qiime environment
export PATH="/homedir04/msuokas/miniconda3/bin:$PATH"
source activate qiime-2024.10

# QIIME 2 command to filter rare features
qiime feature-table filter-features \
--i-table data/set1/clustered_table.qza \
--p-min-frequency 10 \
--o-filtered-table data/set1/n10_table.qza

# QIIME 2 command to filter sequence file
qiime feature-table filter-seqs \
   --i-data data/set1/clustered_seq.qza \
   --i-table data/set1/n10_table.qza \
   --o-filtered-data data/set1/n10_seq.qza
```

Detect and filter chimeric features

Detecting chimeric features after removal of rare features will save computing resources.

```
# Activate qiime environment
export PATH="/homedir04/msuokas/miniconda3/bin:$PATH"
source activate qiime-2024.10

# Detect chimeras
qiime vsearch uchime-denovo \
--i-sequences data/set1/n10_seq.qza \
--i-table data/set1/n10_table.qza \
--o-chimeras data/set1/chimeras.qza \
--o-stats data/set1/uchime-stats.qza \
--o-nonchimeras data/set1/nonchimeras.qza

# Keep nonchimeric features in the table
qiime feature-table filter-features \
--i-table data/set1/n10_table.qza \
--m-metadata-file data/set1/nonchimeras.qza \
--o-filtered-table data/set1/otu_table.qza

# Keep nonchimeric sequences
qiime feature-table filter-seqs \
   --i-data data/set1/n10_seq.qza \
   --i-table data/set1/otu_table.qza \
   --o-filtered-data data/set1/otu_seq.qza
```

Import qiime files and metadata

We will import feature table along with representative sequences and metadata to a TSE object.

```
#sequence file
tse <- importQIIME2(featureTableFile = "data/set1/otu_table.qza",
                    refSeqFile = "data/set1/otu_seq.qza")
tse <- tse[, sort(colnames(tse))]
#add metadata
metadata <- data.frame(read_tsv("data/set1_meta.tsv",
                                show_col_types = F))
metadata <- column_to_rownames(metadata, "Sampleid")
colData(tse) <- DataFrame(metadata)
tse
```

```
class: TreeSummarizedExperiment
dim: 95 6
metadata(0):
assays(1): counts
```

```
rownames(95): CP016294.613081.614615 JF312983.1.1520 ...
  FR682931.1.1497 AB294555.1.1541
rowData names(0):
colnames(6): barcode001 barcode002 ... barcode005 barcode006
colData names(1): Name
reducedDimNames(0):
mainExpName: NULL
altExpNames(0):
rowLinks: NULL
rowTree: NULL
colLinks: NULL
colTree: NULL
referenceSeq: a DNAStringSet (95 sequences)
```

Create taxonomy table from Silva

As we have used Silva to our otu picking, we can fetch taxonomic information using Silva sequnence identifiers.

```
# Routine to separate taxonomic ranks
silva_tax <- read_tsv("~/reference/silva_taxonomy.tsv",
                      show_col_types = F)
rows <- data.frame(FeatureID = rownames(tse))
taxonomy <- dplyr::left_join(rows, silva_tax, by = "FeatureID")
taxonomy <- taxonomy %>%
  separate(Taxon,
           into = c("Kingdom", "Phylum", "Class", "Order", "Family", "Genus", "Species"),
           sep = "; ",
           fill = "right")
taxonomy <- taxonomy %>%
  mutate(across(everything(), ~ sub("^[a-z]__", "", .)))
taxonomy <- column_to_rownames(taxonomy, "FeatureID")
```

Species-level information is filtered to remove ambiguous matches.

```
taxonomy <- taxonomy %>%
  mutate(Species = if_else(str_detect(Species, "unknown|uncultured|metagenome"),
                           NA_character_, Species))
```

Add taxonomy to rowData

```
# Add taxonomy
rownames(taxonomy) <- NULL
rowData(tse) <- DataFrame(taxonomy)
# Check object
tse
```

```
class: TreeSummarizedExperiment
dim: 95 6
metadata(0):
assays(1): counts
rownames(95): CP016294.613081.614615 JF312983.1.1520 ...
  FR682931.1.1497 AB294555.1.1541
rowData names(7): Kingdom Phylum ... Genus Species
colnames(6): barcode001 barcode002 ... barcode005 barcode006
colData names(1): Name
reducedDimNames(0):
mainExpName: NULL
altExpNames(0):
rowLinks: NULL
rowTree: NULL
colLinks: NULL
colTree: NULL
referenceSeq: a DNAStringSet (95 sequences)
```

Write data to files

Write RDS. It can be reloaded to R

```
saveRDS(tse, "set1/tse.rds")
```

Write abundance table.

```
#FeatureID will be rowname
abd <- data.frame(FeatureID = rownames(tse),assays(tse)$counts)
#Write
write_tsv(abd, "set1/otu_table.tsv")
```

Write taxonomy table.

```
#FeatureID will be rowname
taxt <- data.frame(FeatureID = rownames(tse), rowData(tse))
#Write
write_tsv(taxt, "set1/taxonomy.tsv")
```

Write sequences to fasta file.

```
writeXStringSet(referenceSeq(tse), "set1/repseq.fasta",
                                        append = F, compress = F,
                                        format = "fasta")
```

Write metadata file.

```
metadf <- data.frame(colData(tse)) %>% rownames_to_column(var="Sampleid")
#write
write_tsv(metadf, "set1/metadata.tsv")
```

Microbe data analysis

Agglomerate taxonomy to genus-level and count relative abundance

```
# Agglomerate
tse <- agglomerateByRank(tse, rank = "Genus",
                              onRankOnly = T, na.rm = F)
# Relabundance
tse <- transformAssay(tse, assay.type = "counts", method = "relabundance")
```

Pick most abundant features

```
#top features
top10 <- getTop(tse, top = 7, method = "mean",
                        assay.type = "relabundance")
#create and filter table
table <- data.frame(assays(tse)$relabundance)
table <- table %>% rownames_to_column(var = "Genus") %>%
  filter(Genus %in% top10)
```

Composition table

```
kable(table, digits=5) %>%
  kable_styling(latex_options = c("HOLD_position", "striped"),
                font_size = 8) %>%
  row_spec(0, background = "teal", color = "white")
```

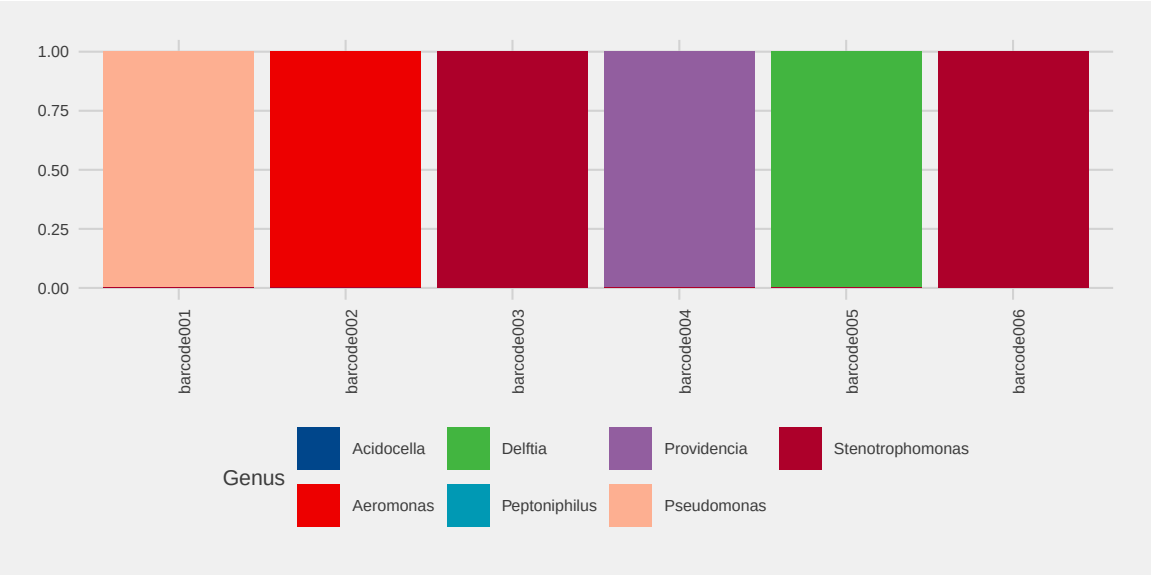
Genus	barcode001	barcode002	barcode003	barcode004	barcode005	barcode006
Acidocella	0.00002	0.00000	0.00000	0.00000	0.00001	0.00000
Aeromonas	0.00001	0.99997	0.00001	0.00000	0.00000	0.00002
Delftia	0.00000	0.00000	0.00000	0.00000	0.99999	0.00001
Peptoniphilus	0.00000	0.00000	0.00000	0.00003	0.00000	0.00000
Providencia	0.00001	0.00000	0.00000	0.99996	0.00001	0.00000
Pseudomonas	0.99995	0.00000	0.00000	0.00000	0.00000	0.00000
Stenotrophomonas	0.00000	0.00003	0.99999	0.00000	0.00000	0.99997

Composition plot

Convert data first to long table format and then plot.

```
# Long dataframe
df_long <- table %>% pivot_longer(cols = starts_with("barcode"),
names_to = "Sample", values_to = "Abundance")

# Plot stacked barplot
ggplot(df_long, aes(x = Sample, y = Abundance, fill = Genus)) +
geom_bar(stat = "identity") + theme_fivethirtyeight(base_size = 8) + scale_fill_lancet() + theme(axis
element_text(angle = 90))
```



Summary of closed reference otu picking strategy

Count first number of sequences in raw files.

```
# List all compressed fastq files in the folder
fastq_files <- list.files("data/reads/set1", pattern = "\\.fastq\\.gz$", full.names = T)

# Function to count sequences in a compressed FASTQ file
count_sequences_in_fastq <- function(file) {
  fq <- readFastq(file)
  return(length(fq))
}
# Apply the function to each file and store the counts
sequence_counts <- sapply(fastq_files, count_sequences_in_fastq)
```

```
# Print the result
raw_data <- data.frame(File = fastq_files, Sequences = sequence_counts)
```

Create summary table

```
summary <- data.frame(Sample = colnames(tse), Raw_Counts = raw_data$Sequences, Counts = colSums(assay
summary <- summary %>% mutate(Percentage = Counts/Raw_Counts)
rownames(summary) <- NULL
kable(summary, digits=2) %>%
  kable_styling(latex_options = c("HOLD_position", "striped"),
                font_size = 12) %>%
  row_spec(0, background = "teal", color = "white")
```

Sample	Raw_Counts	Counts	Percentage
barcode001	391434	308345	0.79
barcode002	394282	362506	0.92
barcode003	401407	400226	1.00
barcode004	370675	358410	0.97
barcode005	388126	382881	0.99
barcode006	371125	359805	0.97

Results

Results show that using SUP basecalling with additional quality filtering produce sequence accuracy that is enough for OTU picking stategies via sequence clustering. Most of nanopore studies use mapping strategies that might not always recognize all biological features.

The samples were purified cultures, and a closed-reference OTU-picking strategy was chosen accordingly. Our results confirm that all samples were effectively pure cultures. In fact, exceptionally low noise level at genus level results suggest that predicted 0.5-1 bp errors / 100 bp sequence is realistic.

Additionally, proportion of matched sequences against database was high across all samples suggesting that strategy is a viable option when analysing microbial communities with nanopore sequencer.