Processing nanopore reads

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Preprocess reads

Dorado doesn't support demultiplexing of dual indexes at 5' and 3' ends. Additionally, library reads are able to ligate in either orientation. Most straightforward approach to demultiplex reads, is to utilise cutadapt. You can demultiplex index pairs using linked adapters approach in forward and reverse orientation, then process with scripts to reverse complement reverse read files and merge each of them with forward reads.

Extracting forward reads to fastq file can be performed with following command

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

Command will extract barcodes defined in barcodes.fasta file and output matches into individual files in fdemuxed subdirectory. Minimum length is set in example to 1200 bp.

Extracting reverse reads using reverse complemented barcodes.fasta file

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

Reads are demultiplexed into separate directory

Tip! O, e, m and M parameters can be used to reduce chances of misaligned matches

Next we use bash script that will process each reverse read file and reverse complement them using basic command

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

Final step is merging. You can use also for this simple bash script that merge files with same base name from two separate directories using basic command

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

Trimming PCR amplification primers

Finally, you can use cutadapt and bash scripts to trim forward and reverse PCR primers from sequence files.

Import set1 to R

Load libraries

```
library(dada2);packageVersion("dada2")
```

```
[1] '1.32.0'
```

```
library(knitr);packageVersion("knitr")
```

```
[1] '1.48'
```

```
library(Biostrings);packageVersion("Biostrings")
```

```
[1] '2.72.1'
```

```
library(DECIPHER);packageVersion("DECIPHER")
```

```
[1] '3.0.0'
```

```
library(phyloseq);packageVersion("phyloseq")
```

```
[1] '1.48.0'
```

```
library(tidyverse);packageVersion("tidyverse")
```

```
[1] '2.0.0'
```

```
library(kableExtra);packageVersion("kableExtra")
```

```
[1] '1.4.0'
```

```
library(mia);packageVersion("mia")
```

```
[1] '1.12.0'
```

```
library(qiime2R);packageVersion("qiime2R")
```

```
[1] '0.99.6'
```

Set variables

```
# Path variables
path <- "data/processed/set1"
training <- "~/feature_classifiers/SILVA_SSU_r138_2019.RData"
meta_file <- "data/set1_meta.tsv"
exportloc <- "results_set1/"
# Variables: truncation length, phix (Illumina)
truncation <- 1400
#Creates results directory
dir.create(exportloc)
#metadata
metadata <- data.frame(read_tsv(meta_file))
```

For the project, we take advantage of computing power of CSC puhti server and import already executed data objects from there. R code is unaltered and can can be executed by turning eval to TRUE.

```
#List files inside directory
list.files(path)
```

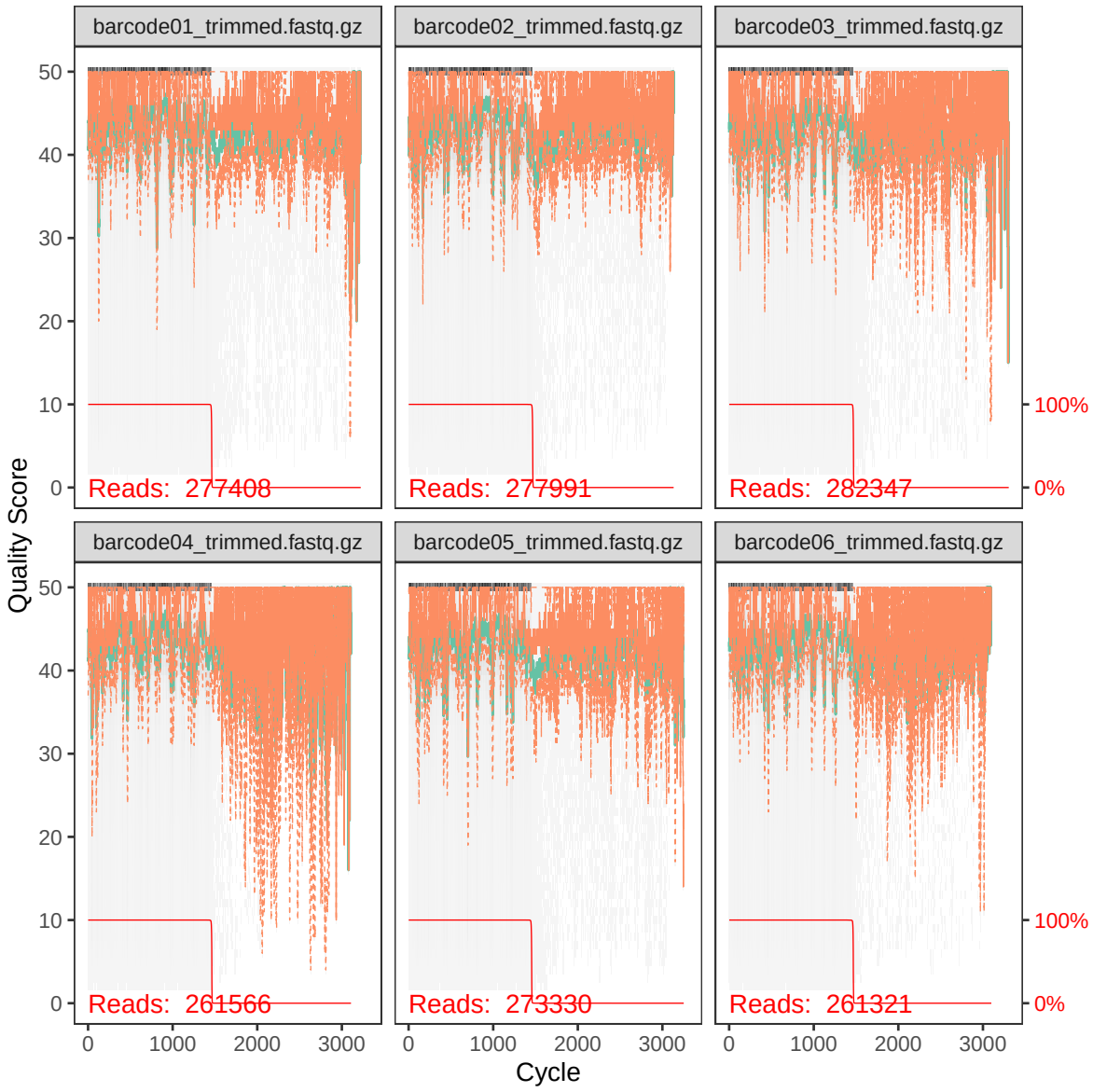
```
[1] "barcode01_trimmed.fastq.gz" "barcode02_trimmed.fastq.gz"
[3] "barcode03_trimmed.fastq.gz" "barcode04_trimmed.fastq.gz"
[5] "barcode05_trimmed.fastq.gz" "barcode06_trimmed.fastq.gz"
```

```
# Forward fastq filenames have format: SAMPLENAME_R1_001.fastq
fnFs <- sort(list.files(path, pattern="_trimmed_all.fastq.gz", full.names = TRUE))
# Extract sample names, assuming filenames have format: SAMPLENAME_XXX.fastq
sample.names <- sapply(strsplit(basename(fnFs), "_"), `[`, 1)
```

Plot sequence quality profile for samples

```
# Base quality plot
prsetI <- plotQualityProfile(fnFs[1:6])
prsetI
```

```
prsetI <- readRDS("rds/set1_rds/prsetI.rds")
prsetI
```



Filter sequence data

Filtering reads (maxEE ≈ 1 error/200 bp sequence is good starting point for this amplicons)

```
# Filtered files are placed in filtered subdirectory
filtFs <- file.path(path, "filtered", paste0(sample.names,
                                             "_F_filt.fastq.gz"))
# For single end data sets without phix control
names(filtFs) <- sample.names
out <- filterAndTrim(fnFs, filtFs, truncLen=truncation,
                     maxN = 0, maxEE = 7 , truncQ = 2,
                     compress = TRUE, multithread = TRUE,
                     rm.phix = FALSE)
```

```
out <- readRDS("rds/set1_rds/out.rds")
```

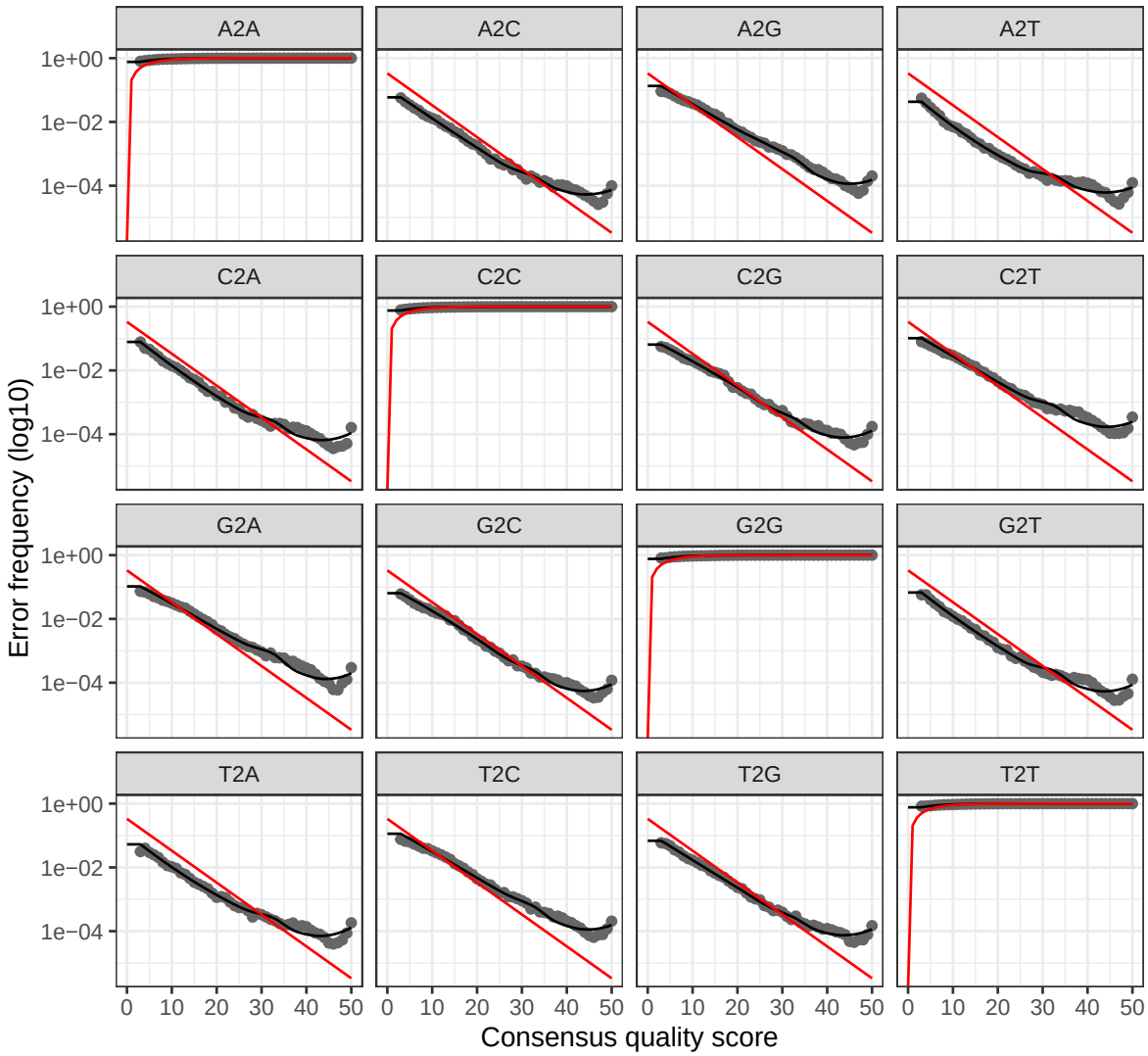
Learn error rates

```
# Forward read error rate
errF <- learnErrors(filtFs, multithread = TRUE)
```

```
errF <- readRDS("rds/set1_rds/errF.rds")
```

Plot error rates

```
# Plotting error rate profile for forward reads
plotErrors(errF, nominalQ = TRUE)
```



Denoise

```
dadaFs <- dada(derepFs, err = errF, multithread = TRUE)
```

```
dadaFs <- readRDS("rds/set1_rds/dadaFs.rds")
```

Build asv table

```
seqtab <- makeSequenceTable(dadaFs)
# Dimensions of ASV table
dim(seqtab)
```

```
[1]  6 72
```

Chimera removal

```
seqtab.nochim <- removeBimeraDenovo(seqtab, method = "consensus",
                                    multithread = TRUE, verbose = TRUE)
dim(seqtab.nochim)
```

```
[1]  6 63
```

Summary

```
getN <- function(x) sum(getUniques(x))
track <- cbind(out, sapply(dadaFs, getN), rowSums(seqtab.nochim),
               rowSums(seqtab.nochim != 0))
#If processing a single sample, remove the sapply calls
colnames(track) <- c("Input", "Filtered", "DenoisedF", "Nonchimeric",
                     "N:o of variants")
rownames(track) <- metadata$Name
kable(track, caption="Summary table")  %>%
  kable_styling(latex_options=c("striped", "HOLD_position")) %>%
                row_spec(0,background = "teal", color = "ivory")
```

Table 1: Summary table

	Input	Filtered	DenoisedF	Nonchimeric	N:o of variants
RMM1	277408	225976	225599	223766	19
RMM2	277991	223708	223395	223395	12
MMY1	282347	221198	220613	220613	11
MMY2	261566	215868	215550	210277	8
MMY3	273330	203550	203212	203212	16
MMY4	261321	204075	203814	202829	8

Taxonomy assignment

IdTaxa from DECIPHER package

```
#Create a DNAStringSet from the ASV sequences
repseq <- DNAStringSet(getSequences(seqtab.nochim))
# CHANGE TO THE PATH OF YOUR TRAINING SET
load(training)
ids <- IdTaxa(repseq, trainingSet, strand = "top",
              processors = 3, verbose = FALSE,
              threshold = 50)
ranks <- c("domain", "phylum", "class", "order", "family",
           "genus", "species")
# Convert the output to a matrix analogous to the output from assignTaxonomy
taxid <-t(sapply(ids, function(x) {
        m <- match(ranks, x$rank)
        taxa <- x$taxon[m]
        taxa[startsWith(taxa, "unclassified_")] <- NA
        taxa
}))
colnames(taxid) <- ranks; rownames(taxid) <- getSequences(seqtab.nochim)
```

```
taxid <- readRDS("rds/set1_rds/taxid.rds")
```

Create phyloseq object

```
pseq <- phyloseq(otu_table(seqtab.nochim, taxa_are_rows = FALSE),
                 tax_table(taxid))
row.names(metadata) <- sample_names(pseq)
sample_data(pseq) <- metadata
pseq
```

```
phyloseq-class experiment-level object
otu_table()   OTU Table:         [ 63 taxa and 6 samples ]
sample_data() Sample Data:       [ 6 samples by 2 sample variables ]
tax_table()   Taxonomy Table:    [ 63 taxa by 7 taxonomic ranks ]
```

Sequence data is stored as taxa_names. We will store sequences as refseq and create numbered variant names

```
#create DNA object and store sequences
seqs <- DNAStringSet(taxa_names(pseq))
names(seqs) <- taxa_names(pseq)
pseq <- merge_phyloseq(pseq, seqs)
#new variant names
taxa_names(pseq) <- paste0("ASV", seq(ntaxa(pseq)))
#capitalise taxonomic ranks
colnames(tax_table(pseq)) <- c("Kingdom", "Phylum", "Class",
  "Order", "Family", "Genus", "Species")
```

Remove non-bacterial taxa

```
#pseq <- subset_taxa(pseq, Kingdom == c("Bacteria", "Archaea"), na.rm = TRUE)
```

Write results to files

Abundance table is transponed and written as tsv file

```
#variant names in rows
ASV_names <- taxa_names(pseq)
#sample names will be columns
ASV_counts <- t(otu_table(pseq))
ASVdf <- (data.frame(ASV_names,ASV_counts))
#write
write_tsv(ASVdf, paste0(exportloc,"asvs.tsv"))
```

Likewise taxonomy table is saved as tsv

```
#variant names in rows
ASV_names <- taxa_names(pseq)
#taxonomy ranks in columns
taxonomy <- (data.frame(ASV_names, tax_table(pseq)))
#write
write_tsv(taxonomy,paste0(exportloc,"taxonomy.tsv"))
```

Variant sequences are saved into fasta file

```
pseq %>% refseq() %>% writeXStringSet(paste0(exportloc,"repseq.fasta"),
                                      append = FALSE, compress = FALSE,
                                      format = "fasta")
```

Vsearch@97%

Data has been processed in qiime, except feature classification

```
#read qiime2 table
vs97 <- readQZA("data/vsearch/97/filtered-table.qza")
#reorder samples
vs97 <- vs97[, order(colnames(vs97))]
#read taxonomy
otu_taxonomy <- readRDS("data/vsearch/97/taxid.rds")
pseq_vsearch97 <- phyloseq(otu_table(vs97, taxa_are_rows = TRUE),
                  tax_table(otu_taxonomy))
#change names
taxa_names(pseq_vsearch97) <- paste0("ASV", seq(ntaxa(pseq_vsearch97)))
#capitalise taxonomic ranks
colnames(tax_table(pseq_vsearch97)) <- c("Kingdom", "Phylum", "Class",
  "Order", "Family", "Genus", "Species")
#create DNA object and store sequences
seqs <- readDNAStringSet("data/vsearch/97/dna-sequences.fasta")
names(seqs) <- taxa_names(pseq_vsearch97)
pseq_vsearch97 <- merge_phyloseq(pseq_vsearch97, seqs)
#prune non-bacterial taxa
#pseq_vsearch97 <- subset_taxa(pseq_vsearch97, Kingdom == c("Bacteria", "Archaea"))
```

Write vsearch97 after data wrangling

```
#create_directory
results <- "results_vsearch97/"
dir.create(results)
#variant_table
ASV_names <- taxa_names(pseq_vsearch97)
ASV_counts <- otu_table(pseq_vsearch97)
ASVdf <- (data.frame(ASV_names,ASV_counts))
write_tsv(ASVdf, paste0(results,"asvs_set1.tsv"))
#taxonomy
ASV_names <- taxa_names(pseq_vsearch97)
taxonomy <- (data.frame(ASV_names, tax_table(pseq_vsearch97)))
write_tsv(taxonomy,paste0(results,"taxonomy_set1.tsv"))
#sequences
refseq(pseq_vsearch97) %>% writeXStringSet(paste0(results,"repseq_set1.fasta"),
                                    append = FALSE, compress = FALSE,
                                    format = "fasta")
```

Vsearch@99%

```
#read qiime2 table
vs99 <- readQZA("data/vsearch/99/filtered-table.qza")
#reorder samples
vs99 <- vs99[, order(colnames(vs99))]
#read taxonomy
otu_taxonomy <- readRDS("data/vsearch/99/taxid.rds")
pseq_vsearch99 <- phyloseq(otu_table(vs99, taxa_are_rows = TRUE),
                  tax_table(otu_taxonomy))
#change names
taxa_names(pseq_vsearch99) <- paste0("ASV", seq(ntaxa(pseq_vsearch99)))
#capitalise taxonomic ranks
colnames(tax_table(pseq_vsearch99)) <- c("Kingdom", "Phylum", "Class",
  "Order", "Family", "Genus", "Species")
#create DNA object and store sequences
seqs <- readDNAStringSet("data/vsearch/99/dna-sequences.fasta")
names(seqs) <- taxa_names(pseq_vsearch99)
pseq_vsearch99 <- merge_phyloseq(pseq_vsearch99, seqs)
#prune non-bacterial taxa
#pseq_vsearch99 <- subset_taxa(pseq_vsearch99, Kingdom == c("Bacteria", "Archaea"))
```

Write vsearch99 files after data wrangling

```
#create_directory
results <- "results_vsearch99/"
dir.create(results)
#variant_table
ASV_names <- taxa_names(pseq_vsearch99)
ASV_counts <- otu_table(pseq_vsearch99)
ASVdf <- (data.frame(ASV_names,ASV_counts))
write_tsv(ASVdf, paste0(results,"asvs_set1.tsv"))
#taxonomy
ASV_names <- taxa_names(pseq_vsearch99)
taxonomy <- (data.frame(ASV_names, tax_table(pseq_vsearch99)))
write_tsv(taxonomy,paste0(results,"taxonomy_set1.tsv"))
#sequences
refseq(pseq_vsearch99) %>% writeXStringSet(paste0(results,"repseq_set1.fasta"),
                                    append = FALSE, compress = FALSE,
                                    format = "fasta")
```

Observations

Low bacterial diversity in the samples is most likely explanation why denoising seemingly produces good results for long 16S rRNA. Error rate plot looks in this case flawless. However, it is notable that all samples contain over 150 k unique reads.

On the other hand, vsearch produced very high number of variants, over 900 and 9000, respectively.