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] '1.30.0'

library(knitr);packageVersion("knitr")

[1] '1.48'

library(Biostrings);packageVersion("Biostrings")

[1] '2.70.2'

library(DECIPHER);packageVersion("DECIPHER")

[1] '2.30.0'

library(phyloseq);packageVersion("phyloseq")

[1] '1.46.0'

library(tidyverse);packageVersion("tidyverse")

[1] '2.0.0'

library(kableExtra);packageVersion("kableExtra")

[1] '1.4.0'

library(mia);packageVersion("mia")

[1] '1.10.0'

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)

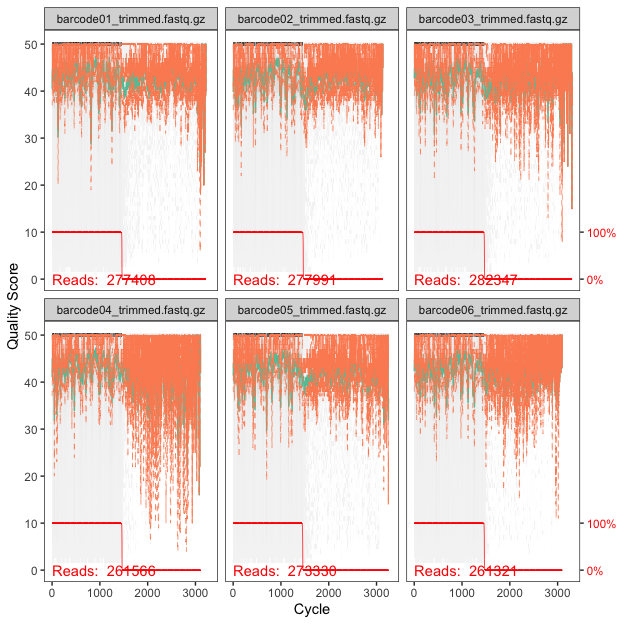
[1] "barcode01\_trimmed.fastq.gz" "barcode02\_trimmed.fastq.gz"  
[3] "barcode03\_trimmed.fastq.gz" "barcode04\_trimmed.fastq.gz"  
[5] "barcode05\_trimmed.fastq.gz" "barcode06\_trimmed.fastq.gz"  
[7] "filtered"

# 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")

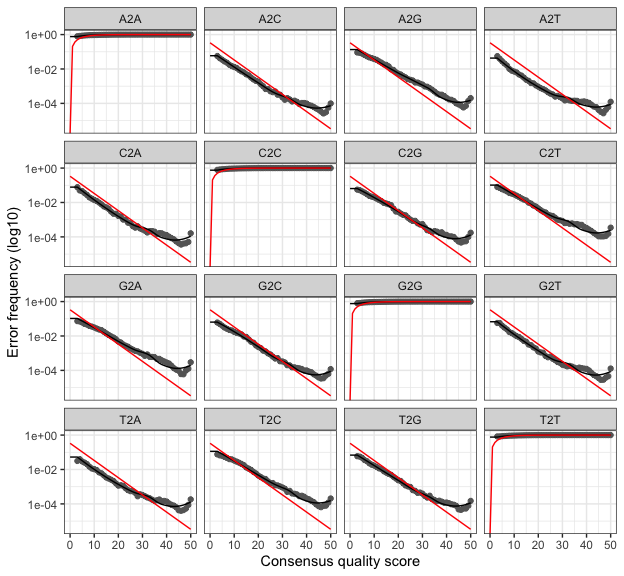
#### Learn error rates

# 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")

Summary table

|  | Input | Filtered | DenoisedF | Nonchimeric | N:o of variants |
| --- | --- | --- | --- | --- | --- |
| PR01 | 277408 | 225976 | 225599 | 223766 | 19 |
| PR02 | 277991 | 223708 | 223395 | 223395 | 12 |
| PR03 | 282347 | 221198 | 220613 | 220613 | 11 |
| PR04 | 261566 | 215868 | 215550 | 210277 | 8 |
| PR05 | 273330 | 203550 | 203212 | 203212 | 16 |
| PR06 | 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.