Processing short nanopore reads with dada2

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This document utilises previously computed objects that are saved as rds files. Result objects are loaded from files. Original code can be executed by changing eval to TRUE.

Preprocess Ion Torrent adapter reads

Trim forward reads with Adapter A and trP1(rc) sequences

cutadapt -g "CCATCTCATCCCTGCGTGTCTCCGACTCAG;o=30...ATCACCGACTGCCCATAGAGAGG;o=23" –trimmed-only -e 0.05 -o ev_forward.fastq.gz ev_reads_hq.fastq.gz

Trim reverse reads with trP1 and Adapter A(rc) sequences

cutadapt -g "CCTCTCTATGGGCAGTCGGTGAT;o=23...CTGAGTCGGAGACACGCAGGGATGAGATGG;o=30" –trimmed-only -e 0.05 -o ev_reverse.fastq.gz ev_reads_hq.fastq.gz

Reverse-complement reverse reads

seqkit seq -rp -t DNA -o ev_rcomp.fasta.gz ev_reverce.fasta.gz

Merge with forward reads

cat ev_forward.fasta.gz ev_rcomp.fasta.gz > raw_005.fasta.gz

Import data to qiime

qiime tools import –type MultiplexedSingleEndBarcodeInSequence –input-path raw_005.fasta.gz –output-path raw_005.qza

Demultiplex

qiime cutadapt demux-single –i-seqs raw_005.qza –m-barcodes-file jt_meta.tsv –m-barcodes-column Barcode_seq –output-dir demuxed –p-error-rate 0 –p-anchor-barcode

Trim pcr primers (519F and 926R)

qiime cutadapt trim-single –i-demultiplexed-sequences per_sample_sequences.qza –p-overlap 15 –p-discard-untrimmed –p-front ACAGCMGCCGCGGTAATWC –o-trimmed-sequences trim1.qza

qiime cutadapt trim-single –i-demultiplexed-sequences trim1.qza –p-adapter AAACTCAAAK-GAATTGACGG –o-trimmed-sequences trimmed-sequences.qza

Decompress read files

unzip trimmed-sequences.qza

Note. Parameters allow one error in sequencing adapters, no errors in barcode sequence and 1 and 2 errors in pcr primers, respectively.

Note. Some options in commands require double dash and are not displayed correctly in rendered documents.

Load libraries

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

```
[1] '1.30.0'
```

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

```
[1] '1.45'
```

```
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'
```

Set parameters

```
# Path variables
path <- "data/reads/"
training <- "~/feature_classifiers/SILVA_SSU_r138_2019.RData"
meta_file <- "data/jt_meta.tsv"
exportloc <- "results/"
# Variables: truncation length, phix (Illumina)
truncation <- 350
#Creates results directory
dir.create(exportloc)
#metadata
metadata <- data.frame(read_tsv(meta_file))
#set knitr cache path
knitr::opts_chunk$set(cache.path = "cache/")
```

Import reads

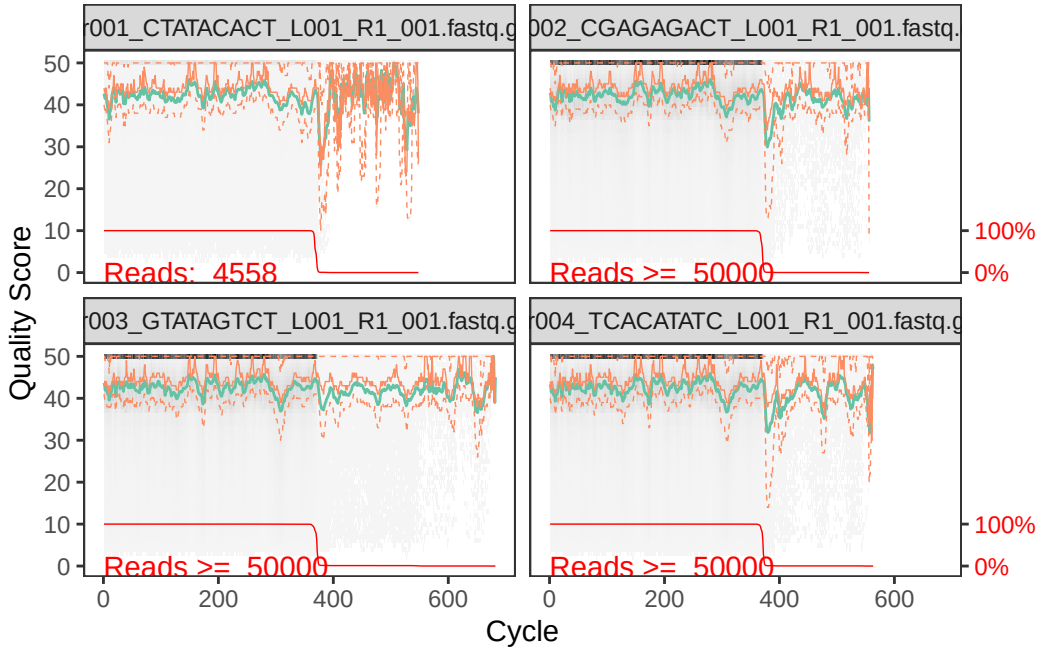
nr072 was removed from dataset (0 reads causing error in denoising step).

```
# Forward fastq filenames have format: SAMPLENAME_R1_001.fastq
fnFs <- sort(list.files(path, pattern="L001_R1_001.fastq.gz", full.names = TRUE))
# Extract sample names, assuming filenames have format: SAMPLENAME_XXX.fastq.gz
sample.names <- sapply(strsplit(basename(fnFs), "_"), `[`, 1)
```

Checking read quality of first samples

```
# Base quality plot
p <- plotQualityProfile(fnFs[1:4], n = 50000)
p
```

```
# Load base quality plot from saved object
p <- readRDS("rds/qplot.rds")
p
```



Filter and trim reads

```
# 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 = 2 , truncQ = 2,
                     compress = TRUE, multithread = FALSE,
                     rm.phix = FALSE)
saveRDS(out, file = "rds/out.rds")
```

```
# 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
# Load previously saved object
out <-readRDS("rds/out.rds")
```

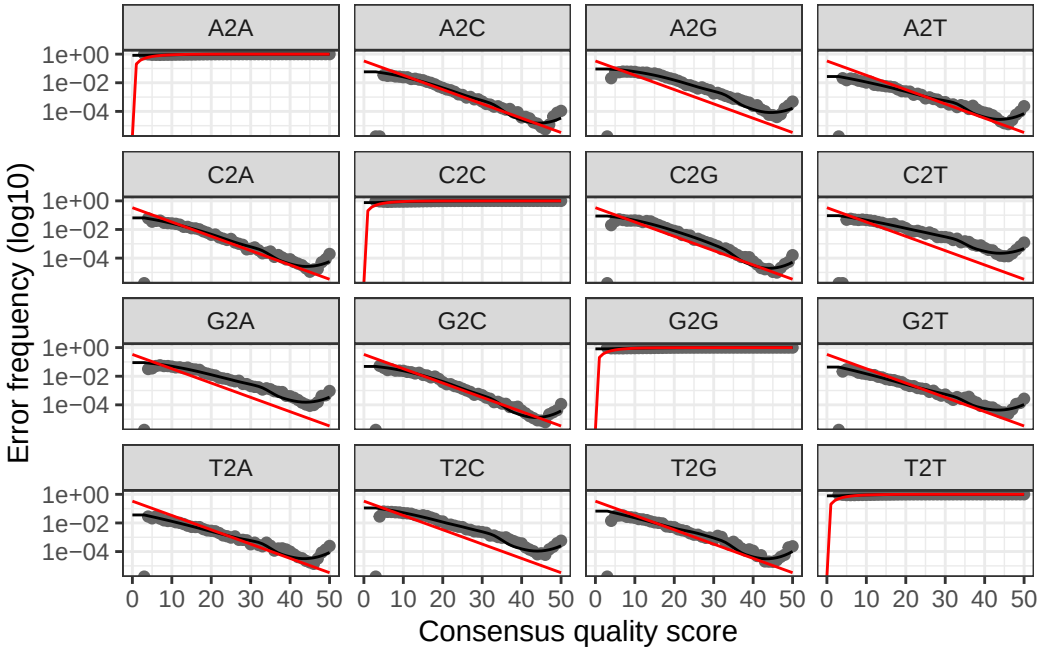
Learn and plot error profile

```
# Forward read error rate
errF <- learnErrors(filtFs, multithread = TRUE)
saveRDS(errF, file = "rds/errF.rds")
```

```
# Load previously saved object
errF <- readRDS("rds/errF.rds")
```

Plot error profile

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



Denoise sequences

```
dadaFs <- dada(filtFs, err = errF, multithread = TRUE, verbose = FALSE)
saveRDS(dadaFs, file = "rds/dadaFs.rds")
```

```
# Load previously saved object
dadaFs <- readRDS("rds/dadaFs.rds")
```

Build ASV table

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

```
[1]  131 8404
```

Remove chimeric variants

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

```
[1]  131 7634
```

Amount of data remaining after chimera removal

```
sum(seqtab.nochim)/sum(seqtab)
```

```
[1] 0.9959133
```

Summary table

```
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) <- sample.names
kable(track, caption="Summary table", booktabs = TRUE, longtable = TRUE)  %>%
  kable_styling(latex_options=c("striped", "HOLD_position", "repeat_header")) %>%
                row_spec(0,background = "teal", color = "ivory")
```

Table 1: Summary table

	Input	Filtered	DenoisedF	Nonchimeric	N:o of variants
nr001	4558	3737	3589	3589	96
nr002	101469	82272	82017	82017	449
nr003	139054	113872	113654	113612	359
nr004	256204	207763	207374	207160	361
nr005	104757	85575	85340	85153	413
nr006	125276	101867	101438	101135	407
nr007	16776	13466	13352	13352	179
nr008	60779	50372	50261	50261	259
nr009	57496	46090	45844	45777	250
nr010	58884	48417	48302	48287	239
nr011	57804	46828	46648	46648	315
nr012	63184	51624	51355	51279	291
nr013	184184	151003	150506	150337	305
nr014	37034	30255	30059	30059	167
nr015	52921	42498	42295	42214	256
nr016	57780	47377	47221	47198	184
nr017	32249	27015	26761	26761	223
nr018	32343	26457	26235	26204	238
nr019	211	182	152	152	8
nr020	17466	14548	14312	14309	271
nr021	13454	10843	10407	10368	298
nr022	36958	30203	29950	29916	330
nr023	5863	4939	4760	4760	169
nr024	39062	32944	32682	32675	287
nr025	18358	15039	14839	14839	234
nr026	54181	45020	44705	44689	461
nr027	57462	46962	46734	46734	281
nr028	6385	5190	4976	4976	135

Table 1: Summary table *(continued)*

	Input	Filtered	DenoisedF	Nonchimeric	N:o of variants
nr029	1149	915	809	809	57
nr030	2978	2463	2280	2280	72
nr031	55110	44610	44399	44191	303
nr032	53814	42979	42625	42284	219
nr033	41001	32923	32685	32664	287
nr034	25124	20580	20298	20296	288
nr035	1028	835	761	761	34
nr036	55738	45478	45334	45302	332
nr037	52126	42022	41630	41550	325
nr038	63365	52054	51762	51391	404
nr039	62495	50482	50215	50090	428
nr040	66486	54623	54394	54325	364
nr041	50049	41199	41048	41028	288
nr042	56406	47601	47412	47302	201
nr043	62973	52026	51849	51794	248
nr044	51860	42460	42297	42212	259
nr045	62931	51359	50996	50953	410
nr046	57953	47598	47331	46941	259
nr047	59138	49654	49488	49442	260
nr048	55998	46407	46245	46231	284
nr049	59015	49029	48942	48940	252
nr050	58770	48128	47929	47881	367
nr051	58199	48129	48011	47938	241
nr052	59722	48648	48421	48387	456
nr053	52295	42909	42812	42810	206
nr054	54222	44000	43814	43761	255
nr055	44896	36482	36250	36211	327
nr056	56564	46213	46048	46037	340
nr057	57220	44611	44384	44384	340
nr058	29071	21680	21628	21628	101
nr059	49673	37678	37585	37585	184
nr060	56441	44838	44765	44720	204
nr061	39464	31746	31592	31558	203
nr062	57638	47417	47311	47311	121
nr063	41622	34042	33991	33847	116
nr064	57665	45751	45672	45672	151
nr065	31057	24009	23923	23923	115
nr066	46974	36323	36192	36192	160
nr067	28068	23098	22969	22967	116
nr068	29823	24238	24203	24203	86
nr069	30168	24183	24129	24127	128

Table 1: Summary table *(continued)*

	Input	Filtered	DenoisedF	Nonchimeric	N:o of variants
nr070	60493	50010	49839	49598	200
nr071	53242	41175	41058	40969	179
nr073	49981	40478	40360	40357	179
nr074	57395	46109	46003	46003	153
nr075	56378	46525	46362	46362	181
nr076	50105	39042	38965	38965	198
nr077	53839	45055	44930	44903	272
nr078	49264	40522	40432	40428	324
nr079	1023	865	775	775	25
nr080	76644	64132	63928	63928	348
nr081	77967	62167	61521	61008	1391
nr082	39055	32857	32731	32731	161
nr083	33607	27830	27742	27719	227
nr084	6891	5655	5515	5515	88
nr085	63220	52028	51855	51853	218
nr086	28118	21641	21539	21515	165
nr087	1547	1240	1214	1214	44
nr088	355	291	248	248	19
nr089	94933	75244	74715	73698	62
nr090	49193	39043	38697	38660	56
nr091	43492	34421	34160	34137	35
nr092	87487	69403	69153	69153	64
nr093	170090	138581	137950	137467	90
nr094	183908	136075	135427	135250	92
nr095	187778	149971	149441	149364	86
nr096	152388	120640	120247	120041	86
nr097	151106	122605	122173	122018	69
nr098	61905	49312	49062	49001	50
nr099	6228	5126	5003	5003	32
nr100	181	137	91	91	11
nr101	118	86	47	47	7
nr102	65	48	23	23	4
nr103	196	145	78	78	9
nr104	198	153	100	100	13
nr105	179	145	82	82	11
nr106	107	83	42	42	5
nr107	176	144	68	68	7
nr108	92	71	29	29	5
nr109	139	113	67	67	9
nr110	447	360	269	269	32
nr111	122	96	52	52	4

Table 1: Summary table *(continued)*

	Input	Filtered	DenoisedF	Nonchimeric	N:o of variants
nr112	212	174	114	114	11
nr113	144	116	74	74	5
nr114	127	103	57	57	7
nr115	502	393	378	378	11
nr116	73	52	16	16	2
nr117	127	103	46	46	6
nr118	120	94	47	47	5
nr119	170311	133480	132659	129636	87
nr120	157	126	64	64	8
nr121	161	131	72	72	11
nr122	199	161	117	117	14
nr123	60	48	14	14	2
nr124	100	83	47	47	10
nr125	84	66	35	35	6
nr126	81	63	25	25	2
nr127	208327	163285	162177	159439	112
nr128	72407	59091	58754	58652	56
nr129	45752	37244	37008	36922	44
nr130	192968	154678	153444	145777	122
nr131	19	17	13	13	2
nr132	14	11	1	1	1

Assign taxonomy

```
#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)
saveRDS(taxid, file = "rds/taxid.rds")
```

```
# Load previously saved object
taxid <- readRDS("rds/taxid.rds")
```

Build 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:         [ 7634 taxa and 131 samples ]
sample_data() Sample Data:       [ 131 samples by 5 sample variables ]
tax_table()   Taxonomy Table:    [ 7634 taxa by 7 taxonomic ranks ]
```

```
#create sequences from rownames to refseq
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 variants

```
#remove non-bacterial taxa
pseq <- subset_taxa(pseq, Kingdom != is.na(Kingdom))
pseq <- subset_taxa(pseq, Kingdom != "Eukaryota")
pseq
```

```
phyloseq-class experiment-level object
otu_table()   OTU Table:         [ 7280 taxa and 131 samples ]
sample_data() Sample Data:       [ 131 samples by 5 sample variables ]
tax_table()   Taxonomy Table:    [ 7280 taxa by 7 taxonomic ranks ]
refseq()      DNAStringSet:      [ 7280 reference sequences ]
```

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")
```

Compatible metadata file as tsv

```
sampleid <- sample_names(pseq)
metafile <- sample_data(pseq)
metadf <- data.frame(sampleid,metafile)
write_tsv(metadf, paste0(exportloc,"metadata.tsv"))
```

Observations

Customised sup basecalling of nanopore sequences produce quality matching Illumina ja Ion Torrent

Error profiles of short amplicon (truncated to 350 bp) follow expected frequency

Proportion of unique reads is smaller when compared to long amplicons

Thus, denoising seem to work normally on shorter read lengths in contrast to 1,5 kbp full-length 16S rRNA gene. This is expected as algorithm relies on error-free reads that are used to build variant clusters. In long reads, even 99,5 % accuracy is simply not enough. At 1400 bp 0,5 % mean error rate means 7 sequencing errors per read

Advantages of nanopore

Read length is not limiting factor while designing amplicon targets

Base quality doesn't decrease as a function of read length

Low diversity libraries are not problem in sequencing

Libraries prepared for other platforms can be conveniently converted to nanopore

Live basecalling allows controlling sequencing throughput and in some cases flow-cell can be reused

Cost per bp in amplicon sequencing is great compared to MiSeq

Disadvantages of nanopore

Homopolymer region accuracy is not quite as good as in Illumina

High accuracy basecalling is computationally intensive

Software tools are not quite at the same level as in other platforms and require more knowledge

Consistency of flow-cells (number of functional pores) and repeatibility of sequencing is so far unclear

Pores might die if library preparation contains contaminants originating from sample (concerns mainly genomic or transcriptomic sequencing)