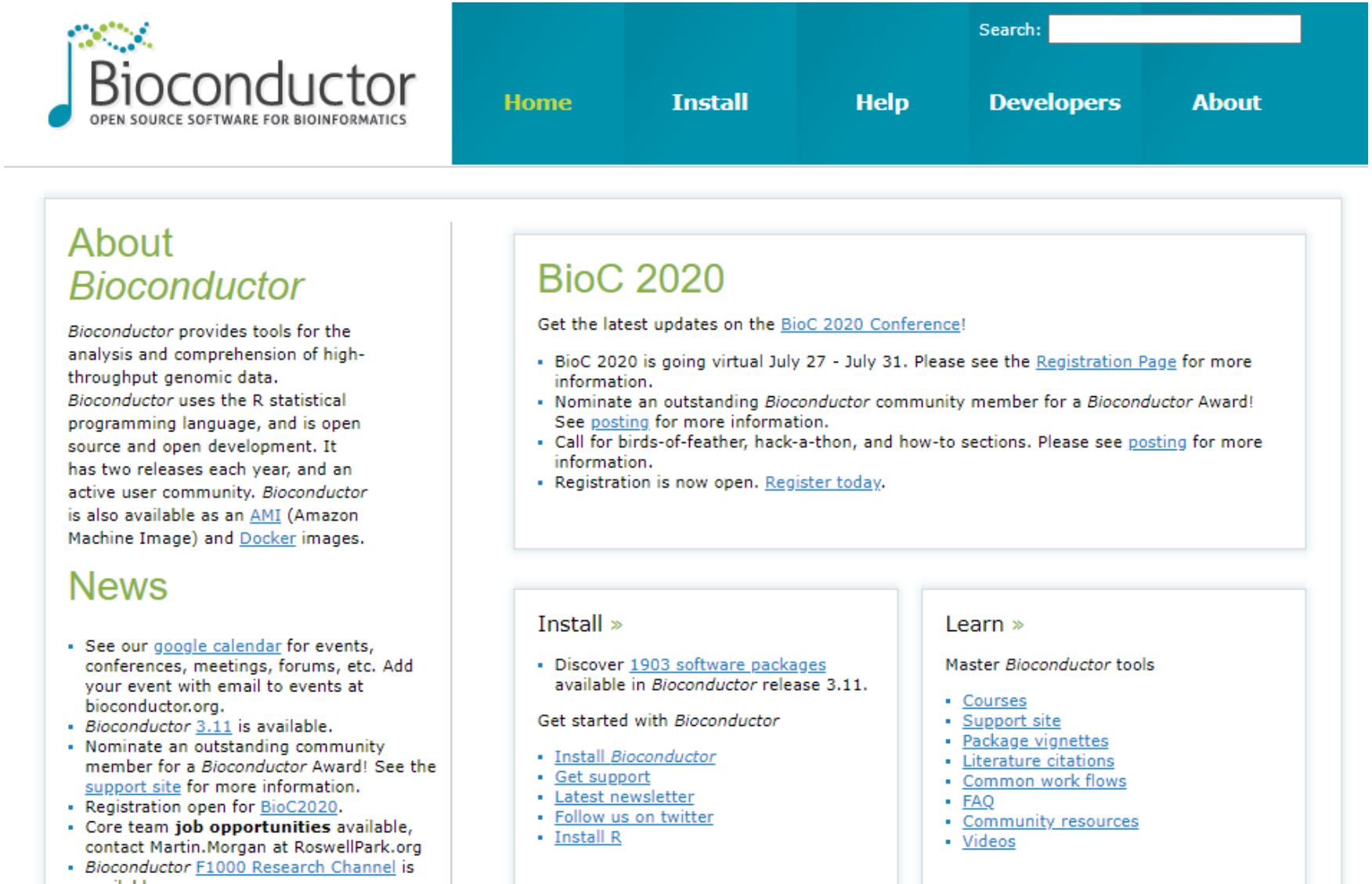
Practical Approaches to Bioanalysis in R

Day 4 – Bioconductor packages

Extending R through packages: There's a package for everything

Bio-specific R packages are available on Bioconductor



Common Bioconductor Methods/Classes

Class – Blueprint for an object. If I say “data.frame”, what comes to mind?
Method – Function that ”reacts” to class of input. as.data.frame( ) is a simple example.

Importing

- GTF, GFF, BED, BigWig, etc., – rtracklayer::import()
- VCF – VariantAnnotation::readVcf()
- SAM / BAM – Rsamtools::scanBam(), GenomicAlignments::readGAlignment*()
- FASTA – Biostrings::readDNAStringSet()
- FASTQ – ShortRead::readFastq()
- MS data (XML-based and mgf formats) – MSnbase::readMSData(), MSnbase::readMgfData()

Common Classes

- Rectangular feature x sample data – SummarizedExperiment::SummarizedExperiment() (RNAseq count matrix, microarray, …)
- Genomic coordinates – GenomicRanges::GRanges() (1-based, closed interval)
- DNA / RNA / AA sequences – Biostrings::*StringSet()
- Gene sets – GSEABase::GeneSet() GSEABase::GeneSetCollection()
- Multi-omics data – MultiAssayExperiment::MultiAssayExperiment()
- Single cell data – SingleCellExperiment::SingleCellExperiment()
- Mass spec data – MSnbase::MSnExp()

https://bioconductor.org/developers/how-to/commonMethodsAndClasses/

You can install Bioconductor packages using `BiocManager::install()` in RStudio

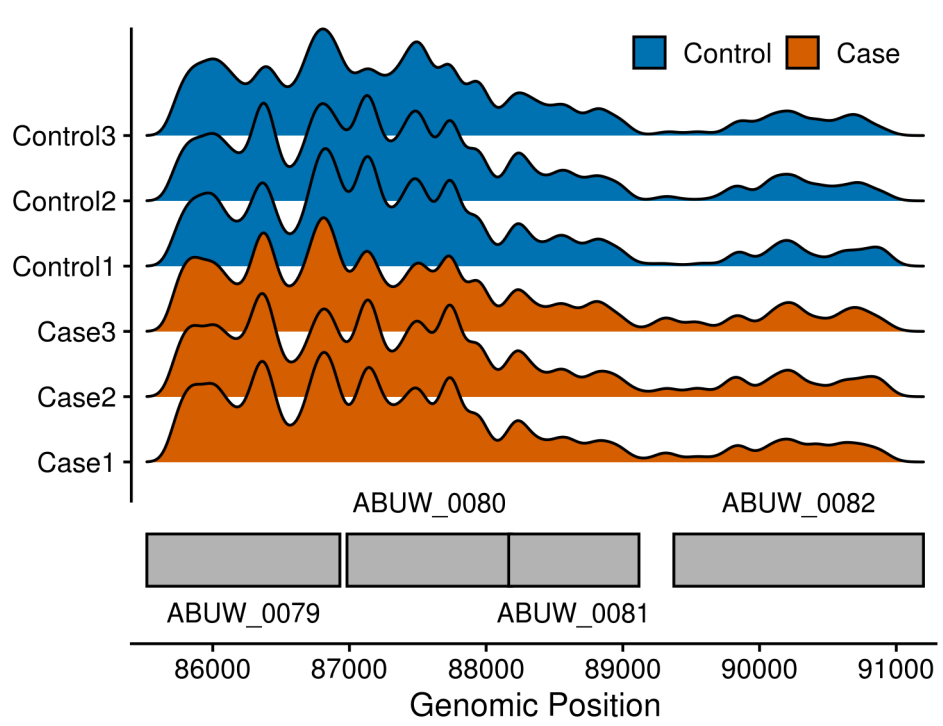
Run this once

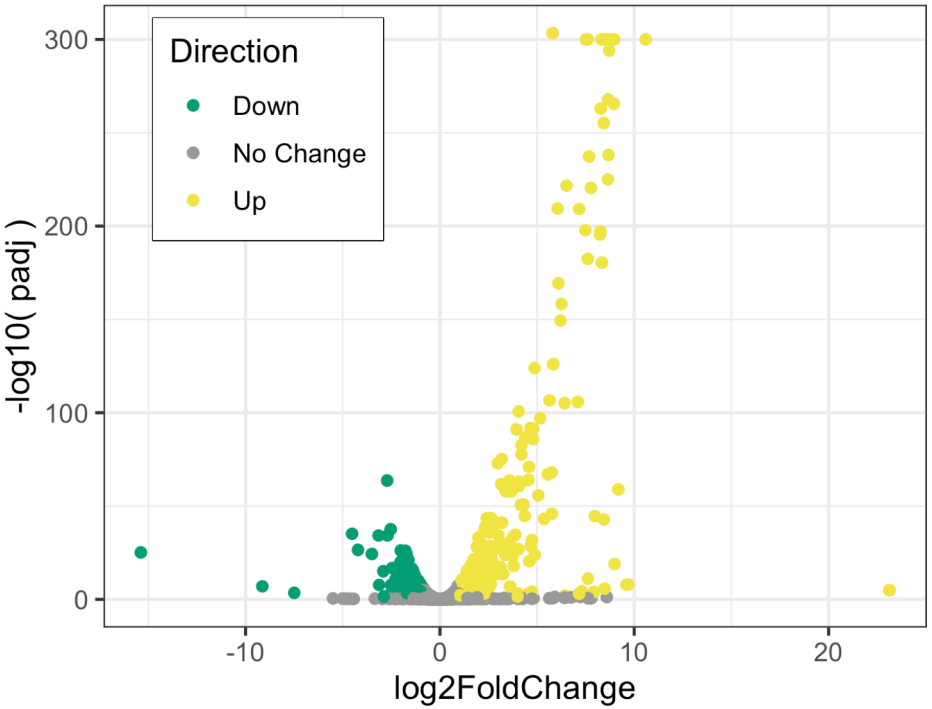
```
if (!requireNamespace("BiocManager", quietly = TRUE))
    install.packages("BiocManager")

BiocManager::install("DESeq2")
```

DESeq2: Differential Expression of Sequencing data

DESeq2: Differential Expression of Sequencing data

Go from transcript abundances to normalized log2 fold changes (with p-values).





Following slides adapted from DESeq2 vignette:
http://bioconductor.org/packages/devel/bioc/vignettes/DESeq2/inst/doc/DESeq2.html

DESeq2: Minimum example

```
dds <- DESeqDataSetFromMatrix(countData = cts,
                              colData = coldata,
                              design= ~ batch + condition)
dds <- DESeq(dds)
resultsNames(dds) # lists the coefficients
res <- results(dds, name="condition_trt_vs_untrt")
```

DESeq2: Minimum example

Matrix with tx count data

GLM defining variables of interest.
NOTE: For more experiments,
ask a statistician for help here

data.frame with sample info

```
dds <- DESeqDataSetFromMatrix(countData = cts,
                              colData = coldata,
                              design= ~ batch + condition)
dds <- DESeq(dds)
resultsNames(dds) # lists the coefficients
res <- results(dds, name="condition_trt_vs_untrt")
```

data.frame with log2FC and stats

colData	4 obs. of 3 variables
cts	num [1:4427, 1:4] 1441 3004 9734 380 3492 ...
dds	Large DESeqDataSet (4427 elements, 3.6 Mb)
res	Large DESeqResults (6 elements, 587.8 Kb)

An aside on r data types

dds	Large DESeqDataSet (4427 elements, 3.6 Mb)

```
dds                                  Large DESeqDataSet (4427 elements, 3.6 Mb)
  ..@ design :Class 'formula' language ~phenotype
  .. .. ..- attr(*, ".Environment")=<environment: R_GlobalEnv>
  ..@ dispersionFunction:function (q)
  .. ..- attr(*, "coefficients")= Named num [1:2] 0.0245 10.8751
  .. .. ..- attr(*, "names")= chr [1:2] "asymptDisp" "extraPois"
  .. ..- attr(*, "fitType")= chr "parametric"
  .. ..- attr(*, "varLogDispEsts")= num 1.83
  .. ..- attr(*, "dispPriorVar")= num 0.777
  ..@ rowRanges :Formal class 'CompressedGRangesList' [package "GenomicRanges"] with 5 slots
  .. .. ..@ unlistData :Formal class 'GRanges' [package "GenomicRanges"] with 7 slots
  .. .. .. .. ..@ seqnames :Formal class 'Rle' [package "S4Vectors"] with 4 slots
  .. .. .. .. .. .. ..@ values : Factor w/ 0 levels:
  .. .. .. .. .. .. ..@ lengths : int(0)
  .. .. .. .. .. .. ..@ elementMetadata: NULL
  .. .. .. .. .. .. ..@ metadata : list()
  .. .. .. .. ..@ ranges :Formal class 'IRanges' [package "IRanges"] with 6 slots
```

“It’s in the data object!”

dds	S4 [4427 x 4] (DESeq2::DESec	S4 object of class DESeqDataSet
design	formula	~phenotype
dispersionFunction	function	function(q) { ... }
rowRanges	S4 (GenomicRanges::Compres	S4 object of class CompressedGRangesList
unlistData	S4 (GenomicRanges::GRanges	S4 object of class GRanges
elementMetadata	S4 [4427 x 22] (S4Vectors::DF	S4 object of class DFrame
rownames	NULL	Pairlist of length 0
nrows	integer [1]	4427
listData	list [22]	List of length 22
baseMean	double [4427]	1665 3673 11815 410 3211 11135 ...
baseVar	double [4427]	13138 439407 9864711 13807 543332 5011277 ...
allZero	logical [4427]	FALSE FALSE FALSE FALSE FALSE FALSE ...
dispGeneEst	double [4427]	1.84e-03 1.00e-08 1.42e-02 6.48e-02 7.65e-02 6.41e-03 ...
dispGeneIter	double [4427]	9 2 2 2 3 2 ...

```
dds@rowRanges@elementMetadata$baseMean
```

DESeq2: Loading in real data

```
library(tidyverse)
library(tximport) # Bioconductor
library(rhdf5) # Bioconductor
library(DESeq2) # Bioconductor
```

```
kallisto_dir <- "kallisto_results"
kallisto_df <- read_csv("kallisto_results/SraRunTable.csv")
row.names(kallisto_df) <- kallisto_df$Run

# Filter to include only one condition
coldata <- kallisto_df %>%
  filter(od600_nm == 2) %>%
  select(Run,
         od600_nm,
         phenotype)
# Put abundance file locations into a list
kallisto_files <- file.path(kallisto_dir,
                            coldata$Run,
                            "abundance.tsv")
txi <- tximport(kallisto_files, type="kallisto", txOut = TRUE)
cts <- txi$counts # This is a count matrix like the one in the example
```

DESeq2: Read normalization & contrasts

```
# Import tx object to DESeq format
dds <- DESeqDataSetFromTximport(txi,
                                colData = coldata,
                                design = ~ phenotype)

dds <- DESeq(dds) # This runs the standard DESeq normalization pipeline
resultsNames(dds) # Show contrast levels
res <- results(dds, contrast = c("phenotype", "HNS.KO", "WT")) # Get log2FC
results_df <- as.data.frame(res)
```

DESeq2: Differential expression

	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
VC1130|ID:1735915|hns|	1.024319e+04	-15.390456	1.4180686	-10.8531110	1.927509e-27	7.838538e-26
VCSEN_bncRNA561	4.964988e+01	-9.139446	1.5808870	-5.7812140	7.416347e-09	1.164257e-07
VIBCH10482|ID:1733969|	1.570148e+01	-7.494045	1.8264345	-4.1031008	4.076496e-05	3.635394e-04
VC1854|ID:1736307|ompT|	1.335629e+05	-5.533453	0.1802948	-30.6911446	7.471610e-207	1.329324e-204
VCSEN_ancRNA266	4.001725e+00	-5.502976	3.6938425	-1.4897700	1.362847e-01	3.452647e-01
VCSEN_ancRNA274	2.807449e+00	-5.010165	4.0839363	-1.2267980	2.198985e-01	4.623174e-01
VCSEN_ancRNA71	2.547773e+00	-4.854970	4.2120729	-1.1526319	2.490615e-01	4.992923e-01
VCSEN_ancRNA324	2.296628e+00	-4.714862	4.3528026	-1.0831785	2.787292e-01	5.358729e-01
VCSEN_ancRNA301	2.039622e+00	-4.539884	4.5269179	-1.0028642	3.159264e-01	5.774853e-01
VC1333|ID:1736020|	1.241901e+03	-4.519809	0.3528569	-12.8091853	1.456557e-37	7.584755e-36
VCSEN_bncRNA580	1.891270e+00	-4.431406	4.6511926	-0.9527463	3.407186e-01	6.041813e-01
VC1334|ID:1736021|	3.406184e+03	-4.216178	0.3784948	-11.1393297	8.072463e-29	3.481759e-27

DESeq2: Checking low count data

```
counts_df <- counts(dds) # Make a df with counts for each read
colnames(counts_df) <- coldata$Run # Fix column names
results_df <- cbind(results_df, counts_df) # Bind counts and results dfs
```

DESeq2: Checking low count data

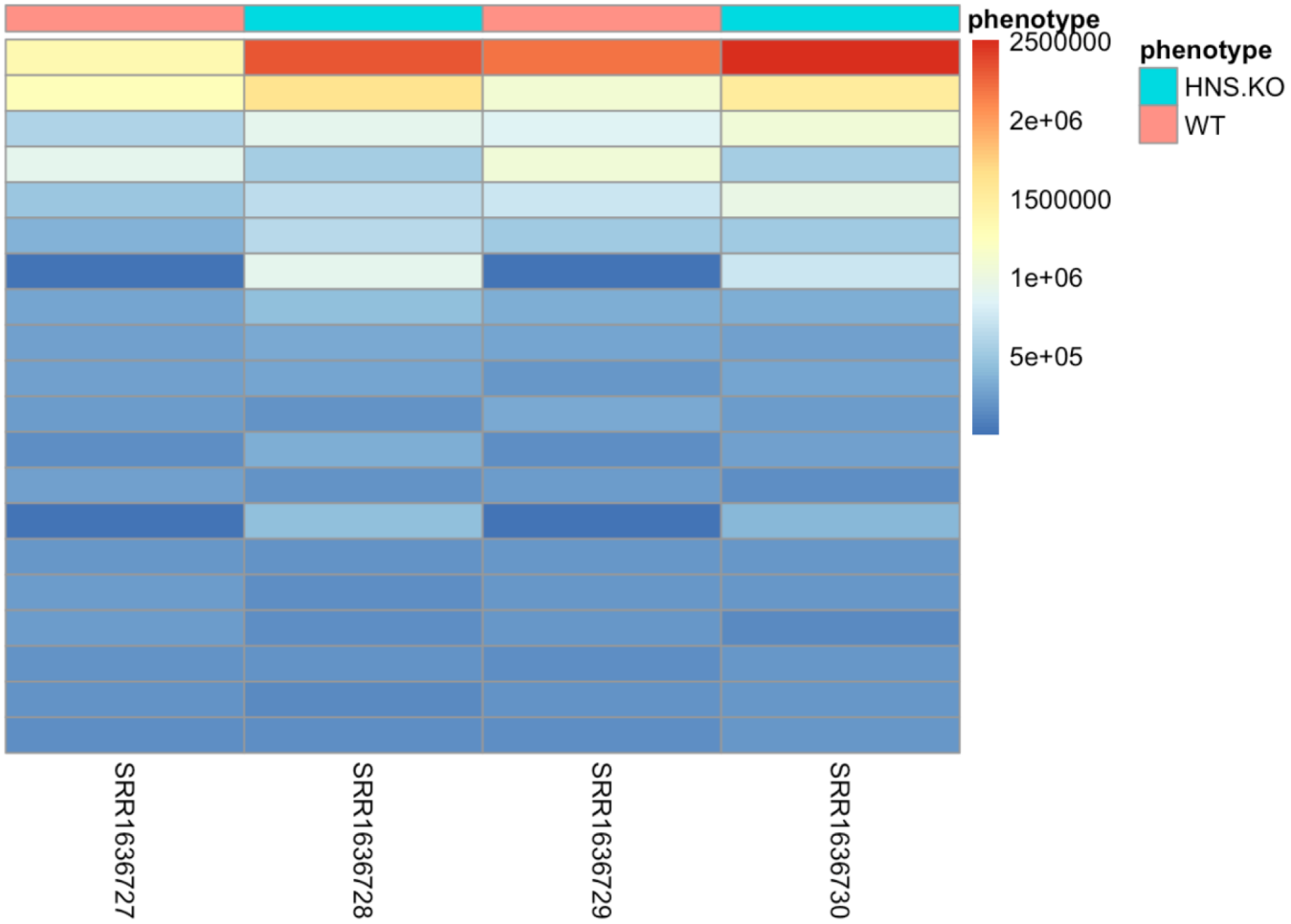
```
counts_df <- counts(dds) # Make a df with counts for each read
colnames(counts_df) <- coldata$Run # Fix column names
results_df <- cbind(results_df, counts_df) # Bind counts and results dfs
```

	SRR1636727	SRR1636728	SRR1636729	SRR1636730	baseMean	log2FoldChange
VC1130|ID:1735915|hns|	20960	0	18141	1	1.024319e+04	-15.390456
VCSEN_bncRNA561	77	0	114	0	4.964988e+01	-9.139446
VIBCH10482|ID:1733969|	22	0	38	0	1.570148e+01	-7.494045
VC1854|ID:1736307|ompT|	265941	6399	237758	5352	1.335629e+05	-5.533453
VCSEN_ancRNA266	15	0	0	0	4.001725e+00	-5.502976
VCSEN_ancRNA274	0	0	11	0	2.807449e+00	-5.010165
VCSEN_ancRNA71	0	0	10	0	2.547773e+00	-4.854970
VCSEN_ancRNA324	0	0	9	0	2.296628e+00	-4.714862
VCSEN_ancRNA301	0	0	8	0	2.039622e+00	-4.539884
VC1333|ID:1736020|	2550	86	2007	125	1.241901e+03	-4.519809
VCSEN_bncRNA580	7	0	0	0	1.891270e+00	-4.431406

DESeq2: Making a heatmap

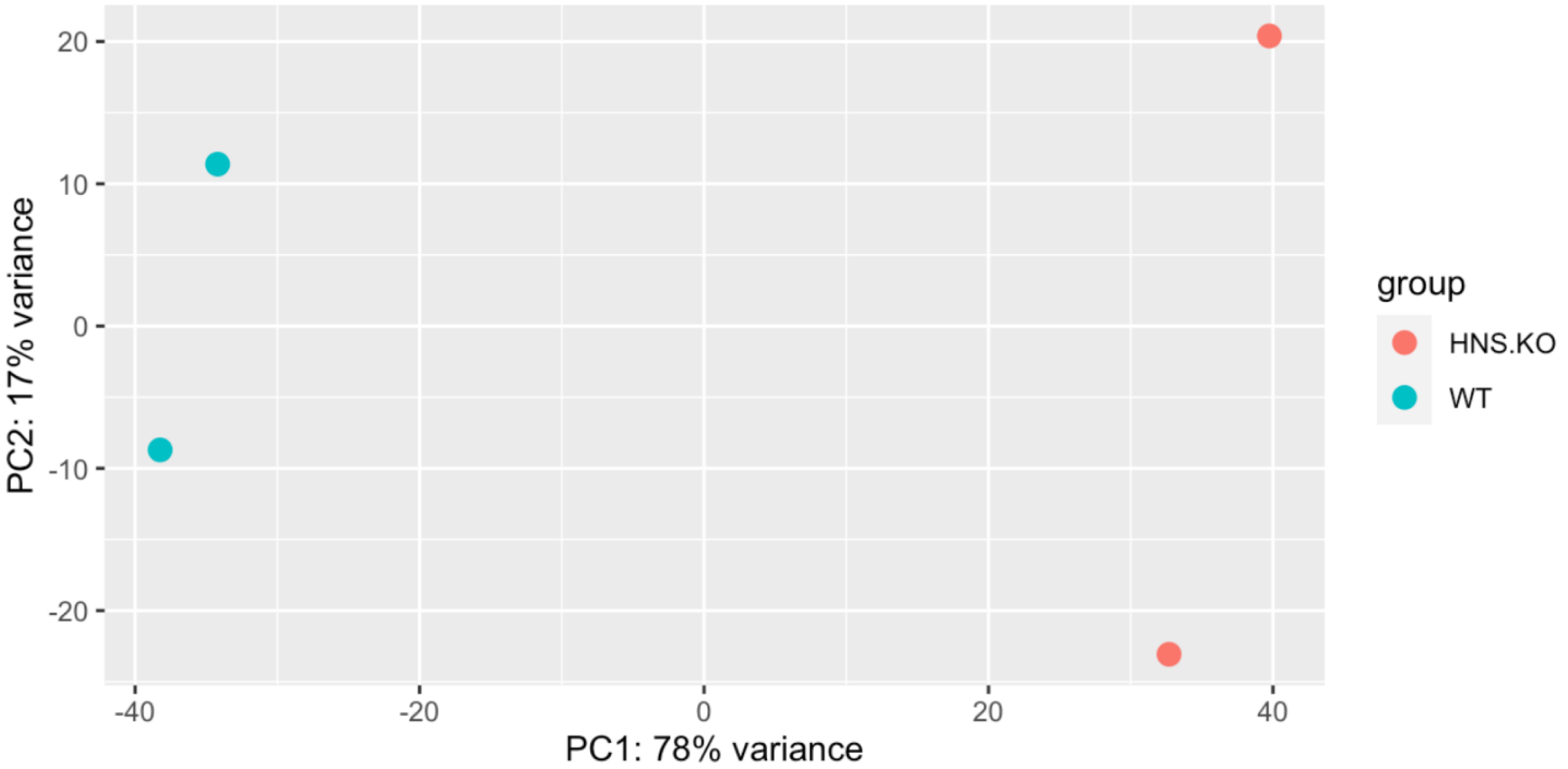
```
select <- order(rowMeans(counts_df), decreasing=TRUE)[1:20] # Select top 20 genes
df <- as.data.frame(colData(dds)[,c("phenotype")]) #
rownames(df) <- coldata$Run # Fix rownames (must matach counts_df columns)
colnames(df) <- "phenotype" # Fix column name
# Make a heatmap
pheatmap(counts_df[select,],
         cluster_rows=FALSE,
         show_rownames=FALSE,
         cluster_cols=FALSE, annotation_col=df)
```

DESeq2: Making a heatmap



DESeq2: Making a PCA plot

```
ntd <- normTransform(dds) # Normalizes to gives log2(n + 1)
plotPCA(ntd, intgroup=c("phenotype")) # Plot PCA
```



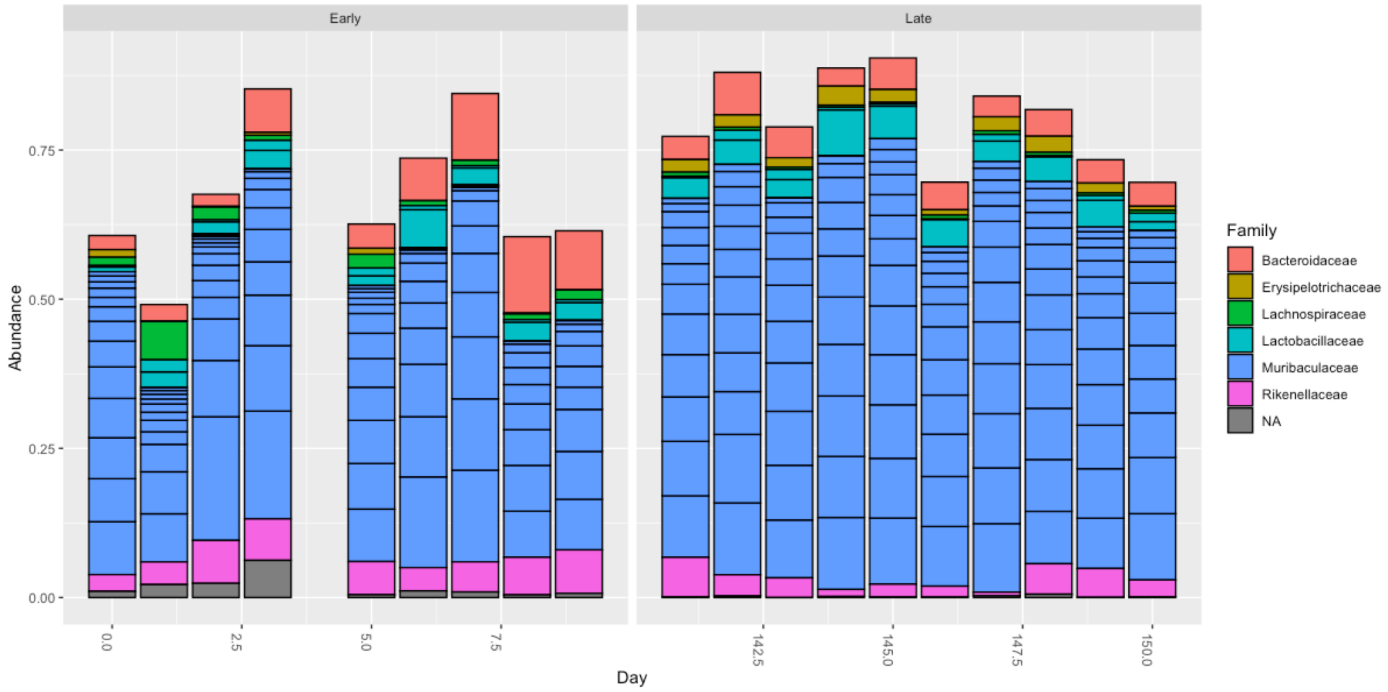
DADA2: Analyzing 16S data

DADA2: Analyzing 16S data

From raw fastqs…

@READ_1
GATTTGGGGTTCAAAGCAGTATCGATCAAATAGTAAATCCATTTGTTCAACTCACAGTTT
+
!''*((((***+))%%%++)(%%%%).1***-+*''))**55CCF>>>>>>CCCCCCC65

to OTUs (Operational Taxonomic Units)!



Following slides adapted from DADA2 vignette: https://benjjneb.github.io/dada2/tutorial.html

DADA2: Import data from fastqs

```
library(dada2) # Bioconductor
library(phyloseq) # Bioconductor
library(Biostrings) # Bioconductor
library(ggplot2)
```

```
path2dada2 <- "MiSeq_SOP" # Name directory with .fastq files
# Forward and reverse fastq filenames have format: SAMPLENAME_R1_001.fastq and SAMPLENAME_R2_001.fastq
fnFs <- sort(list.files(path2dada2, pattern="_R1_001.fastq", full.names = TRUE))
fnRs <- sort(list.files(path2dada2, pattern="_R2_001.fastq", full.names = TRUE))
# Extract sample names, assuming filenames have format: SAMPLENAME_XXX.fastq
sample.names <- sapply(strsplit(basename(fnFs), "_"), `[`, 1)

filtFs <- file.path(path2dada2, "filtered", paste0(sample.names, "_F_filt.fastq.gz"))
filtRs <- file.path(path2dada2, "filtered", paste0(sample.names, "_R_filt.fastq.gz"))
names(filtFs) <- sample.names
names(filtRs) <- sample.names

# Filter and trim input .fastq files
out <- filterAndTrim(fnFs, filtFs, fnRs, filtRs, truncLen=c(240,160),
              maxN=0, maxEE=c(2,2), truncQ=2, rm.phix=TRUE,
              compress=TRUE, multithread=TRUE)
```

DADA2: Learn error rates

```
# These function "learn" errror rates. By using the distribution of nucleotides
# paired with underlying quality scores, dada2 can make a best guess at PCR or sequencing errors
# using machine learning.
errF <- learnErrors(filtFs, multithread=TRUE)
```

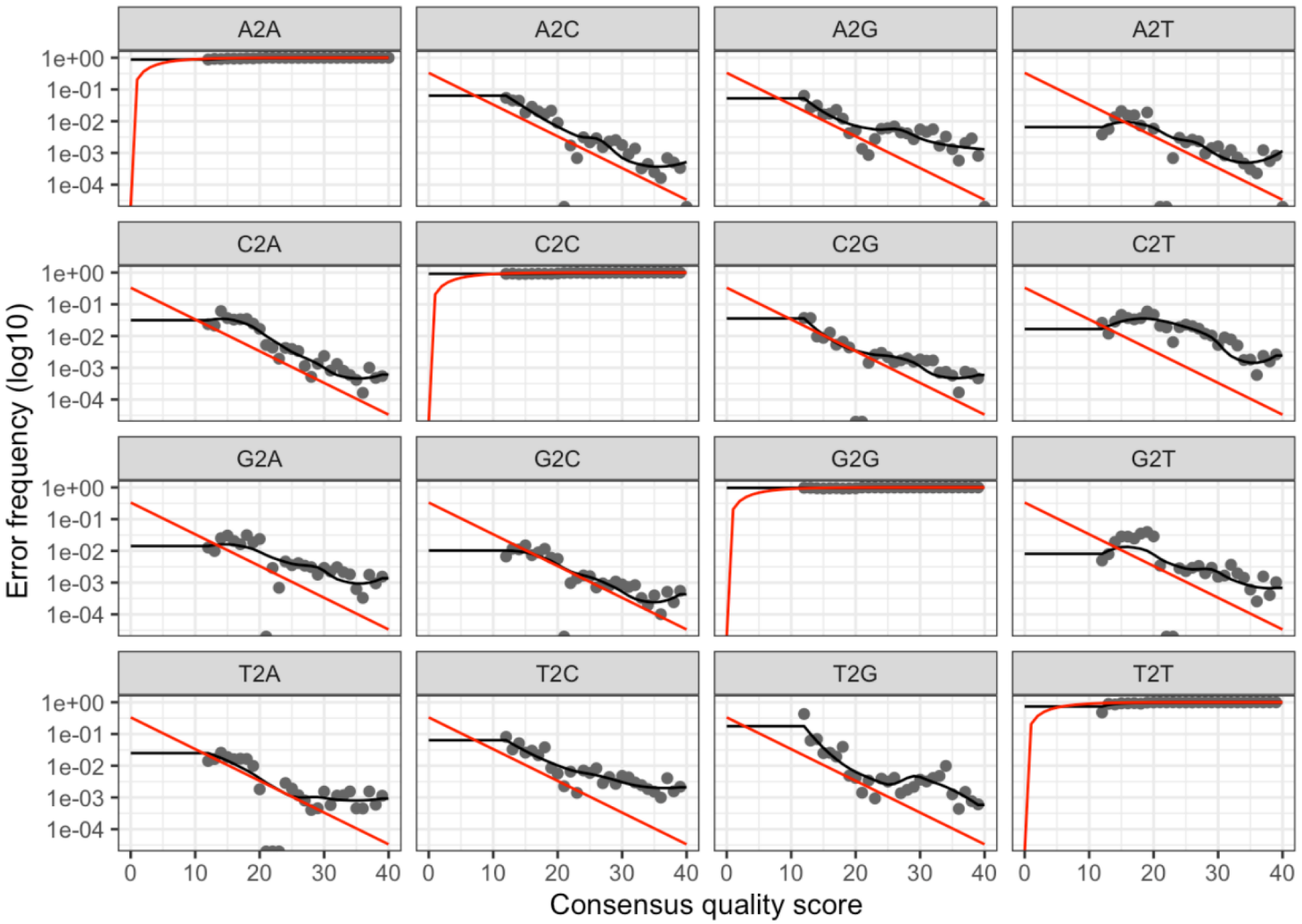
```
## 33514080 total bases in 139642 reads from 20 samples will be used for learning the error rates.
```

```
errR <- learnErrors(filtRs, multithread=TRUE)
```

```
## 22342720 total bases in 139642 reads from 20 samples will be used for learning the error rates.
```

DADA2: Learn error rates

```
plotErrors(errF, nominalQ=TRUE)
```



DADA2: Group reads into 16s amplicons

```
# Sample inference (group reads into 16s amplicons)
dadaFs <- dada(filtFs, err=errF, multithread=TRUE)
```

```
dadaRs <- dada(filtRs, err=errR, multithread=TRUE)
```

```
dadaFs[["F3D0"]] # Summary for first read pair of first read (F3D0)
```

```
## dada-class: object describing DADA2 denoising results
## 128 sequence variants were inferred from 1979 input unique sequences.
## Key parameters: OMEGA_A = 1e-40, OMEGA_C = 1e-40, BAND_SIZE = 16
```

```
dadaFs[[1]] # Same as previous line
```

```
## dada-class: object describing DADA2 denoising results
## 128 sequence variants were inferred from 1979 input unique sequences.
## Key parameters: OMEGA_A = 1e-40, OMEGA_C = 1e-40, BAND_SIZE = 16
```

DADA2: Merge reads

```
# Merge paired end sequences
mergers <-
  mergePairs(dadaFs,
             filtFs,
             dadaRs,
             filtRs,
             verbose=TRUE)

head(mergers[[1]]) # Inspect the merger date from first sample
```

DADA2: Merge reads

```
                     sequence
## 1 TACGGAGGATGCGAGCGTTATCCGGATTTATTGGGTTTAAAGGGTGCGCAGGCGGAAGATCAAGTCAGCGGTAAAATTGAGAGGCTCAACCTCTTCGAGCCGTTGAAA
CTGGTTTTCTTGAGTGAGCGAGAAGTATGCGGAATGCGTGGTGTAGCGGTGAAATGCATAGATATCACGCAGAACTCCGATTGCGAAGGCAGCATACCGGCGCTCAACTGACG
CTCATGCACGAAAGTGTGGGTATCGAACAGG
## 2 TACGGAGGATGCGAGCGTTATCCGGATTTATTGGGTTTAAAGGGTGCGTAGGCGGCCTGCCAAGTCAGCGGTAAAATTGCGGGGCTCAACCCCGTACAGCCGTTGAAA
CTGCCGGGCTCGAGTGGGCGAGAAGTATGCGGAATGCGTGGTGTAGCGGTGAAATGCATAGATATCACGCAGAACCCCGATTGCGAAGGCAGCATACCGGCGCCCTACTGACG
CTGAGGCACGAAAGTGCGGGGATCAAACAGG
## 3 TACGGAGGATGCGAGCGTTATCCGGATTTATTGGGTTTAAAGGGTGCGTAGGCGGGCTGTTAAGTCAGCGGTCAAATGTCGGGGCTCAACCCCGGCCTGCCGTTGAAA
CTGGCGGCCTCGAGTGGGCGAGAAGTATGCGGAATGCGTGGTGTAGCGGTGAAATGCATAGATATCACGCAGAACTCCGATTGCGAAGGCAGCATACCGGCGCCCGACTGACG
CTGAGGCACGAAAGCGTGGGTATCGAACAGG
## 4 TACGGAGGATGCGAGCGTTATCCGGATTTATTGGGTTTAAAGGGTGCGTAGGCGGGCTTTTAAGTCAGCGGTAAAAATTCGGGGCTCAACCCCGTCCGGCCGTTGAAA
CTGGGGGCCTTGAGTGGGCGAGAAGAAGGCGGAATGCGTGGTGTAGCGGTGAAATGCATAGATATCACGCAGAACCCCGATTGCGAAGGCAGCCTTCCGGCGCCCTACTGACG
CTGAGGCACGAAAGTGCGGGGATCGAACAGG
## 5 TACGGAGGATGCGAGCGTTATCCGGATTTATTGGGTTTAAAGGGTGCGCAGGCGGACTCTCAAGTCAGCGGTCAAATCGCGGGGCTCAACCCCGTTCCGCCGTTGAAA
CTGGGAGCCTTGAGTGCGCGAGAAGTAGGCGGAATGCGTGGTGTAGCGGTGAAATGCATAGATATCACGCAGAACTCCGATTGCGAAGGCAGCCTACCGGCGCGCAACTGACG
CTCATGCACGAAAGCGTGGGTATCGAACAGG
## 6 TACGGAGGATGCGAGCGTTATCCGGATTTATTGGGTTTAAAGGGTGCGTAGGCGGGATGCCAAGTCAGCGGTAAAAAAGCGGTGCTCAACGCCGTCGAGCCGTTGAAA
CTGGCGTTCTTGAGTGGGCGAGAAGTATGCGGAATGCGTGGTGTAGCGGTGAAATGCATAGATATCACGCAGAACTCCGATTGCGAAGGCAGCATACCGGCGCCCTACTGACG
CTGAGGCACGAAAGCGTGGGTATCGAACAGG
##   abundance forward reverse nmatch nmismatch nindel prefer accept
## 1       579       1       1    148          0      0      1   TRUE
## 2       470       2       2    148          0      0      2   TRUE
## 3       449       3       4    148          0      0      1   TRUE
## 4       430       4       3    148          0      0      2   TRUE
## 5       345       5       6    148          0      0      1   TRUE
## 6       282       6       5    148          0      0      2   TRUE
```

DADA2: Look into amplicon distributions

```
seqtab <- makeSequenceTable(mergers) # Makes table with counts for amplicon sequence variants
dim(seqtab) # How many species (amplicon sequence variants) do we see?
```

```
## [1]  20 293
```

```
table(nchar(getSequences(seqtab))) # Inspect distribution of sequence lengths
```

```
##
## 251 252 253 254 255
##   1  88 196   6   2
```

DADA2: Remove chimeras

```
# Remove chimeras (i.e. r1 from one sequence, r2 from another)
seqtab.nochim <-
  removeBimeraDenovo(seqtab,
                     method="consensus",
                     multithread=TRUE,
                     verbose=TRUE)
sum(seqtab.nochim)/sum(seqtab)
```

```
## [1] 0.964064
```

```
dim(seqtab.nochim)
```

```
## [1]  20 232
```

```
seqtab <- makeSequenceTable(mergers) # Makes table with counts for amplicon sequence variants
dim(seqtab) # How many species (amplicon sequence variants) do we see?
```

```
## [1]  20 293
```

DADA2: Pipeline summary

```
# Track read counts through pipeline
getN <- function(x) sum(getUniques(x)) # Function to get unique reads
track <-
  cbind(out,
        sapply(dadaFs, getN),
        sapply(dadaRs, getN),
        sapply(mergers, getN),
        rowSums(seqtab.nochim)) # Get unique reads for each setp

colnames(track) <- c("input", "filtered", "denoisedF", "denoisedR", "merged", "nonchim")
rownames(track) <- sample.names
head(track)
```

```
##         input filtered denoisedF denoisedR merged nonchim
## F3D0     7793     7113      6976      6979   6540    6528
## F3D1     5869     5299      5227      5239   5028    5017
## F3D141   5958     5463      5331      5357   4986    4863
## F3D142   3183     2914      2799      2830   2595    2521
## F3D143   3178     2941      2822      2867   2552    2518
## F3D144   4827     4312      4151      4228   3627    3488
```

DADA2: Assign taxonomies

```
# Assign taxonomy
taxa <- assignTaxonomy(seqtab.nochim,
                       refFasta = "tax/silva_nr_v138_train_set.fa.gz",
                       multithread=TRUE)
taxa.print <- taxa # Removing sequence rownames for display only
rownames(taxa.print) <- NULL
head(taxa.print)
```

```
##      Kingdom    Phylum          Class          Order           Family
## [1,] "Bacteria" "Bacteroidota" "Bacteroidia" "Bacteroidales" "Muribaculaceae"
## [2,] "Bacteria" "Bacteroidota" "Bacteroidia" "Bacteroidales" "Muribaculaceae"
## [3,] "Bacteria" "Bacteroidota" "Bacteroidia" "Bacteroidales" "Muribaculaceae"
## [4,] "Bacteria" "Bacteroidota" "Bacteroidia" "Bacteroidales" "Muribaculaceae"
## [5,] "Bacteria" "Bacteroidota" "Bacteroidia" "Bacteroidales" "Bacteroidaceae"
## [6,] "Bacteria" "Bacteroidota" "Bacteroidia" "Bacteroidales" "Muribaculaceae"
##      Genus
## [1,] NA
## [2,] NA
## [3,] NA
## [4,] NA
## [5,] "Bacteroides"
## [6,] NA
```

DADA2: Format for phyloseq

```
# Format data for phyloseq
samples.out <- rownames(seqtab.nochim)
subject <- sapply(strsplit(samples.out, "D"), `[`, 1)
gender <- substr(subject,1,1)
subject <- substr(subject,2,999)
day <- as.integer(sapply(strsplit(samples.out, "D"), `[`, 2))
samdf <- data.frame(Subject=subject, Gender=gender, Day=day)
samdf$When <- "Early"
samdf$When[samdf$Day>100] <- "Late"
rownames(samdf) <- samples.out
```

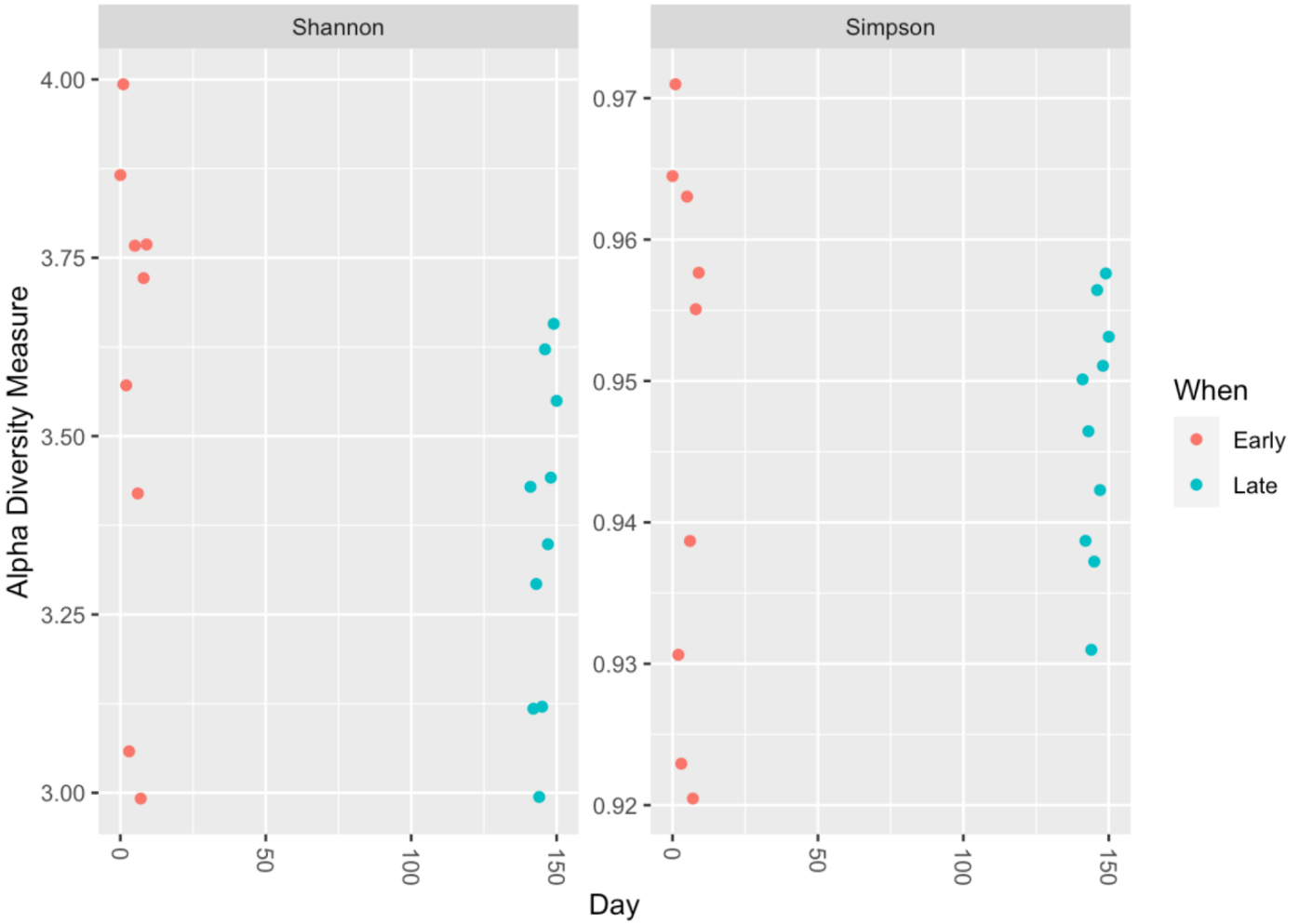
DADA2: Format for phyloseq

```
# Construct phyloseq object
ps <- phyloseq(otu_table(seqtab.nochim, taxa_are_rows=FALSE),
               sample_data(samdf),
               tax_table(taxa))
ps <- prune_samples(sample_names(ps) != "Mock", ps) # Remove mock sample


# Store full DNA sequences and give ASVs short names to more easily visualize
dna <- Biostrings::DNAStringSet(taxa_names(ps))
names(dna) <- taxa_names(ps)
ps <- merge_phyloseq(ps, dna)
taxa_names(ps) <- paste0("ASV", seq(ntaxa(ps)))
ps
```

```
## phyloseq-class experiment-level object
## otu_table()   OTU Table:         [ 232 taxa and 19 samples ]
## sample_data() Sample Data:       [ 19 samples by 4 sample variables ]
## tax_table()   Taxonomy Table:    [ 232 taxa by 6 taxonomic ranks ]
## refseq()      DNAStringSet:      [ 232 reference sequences ]
```

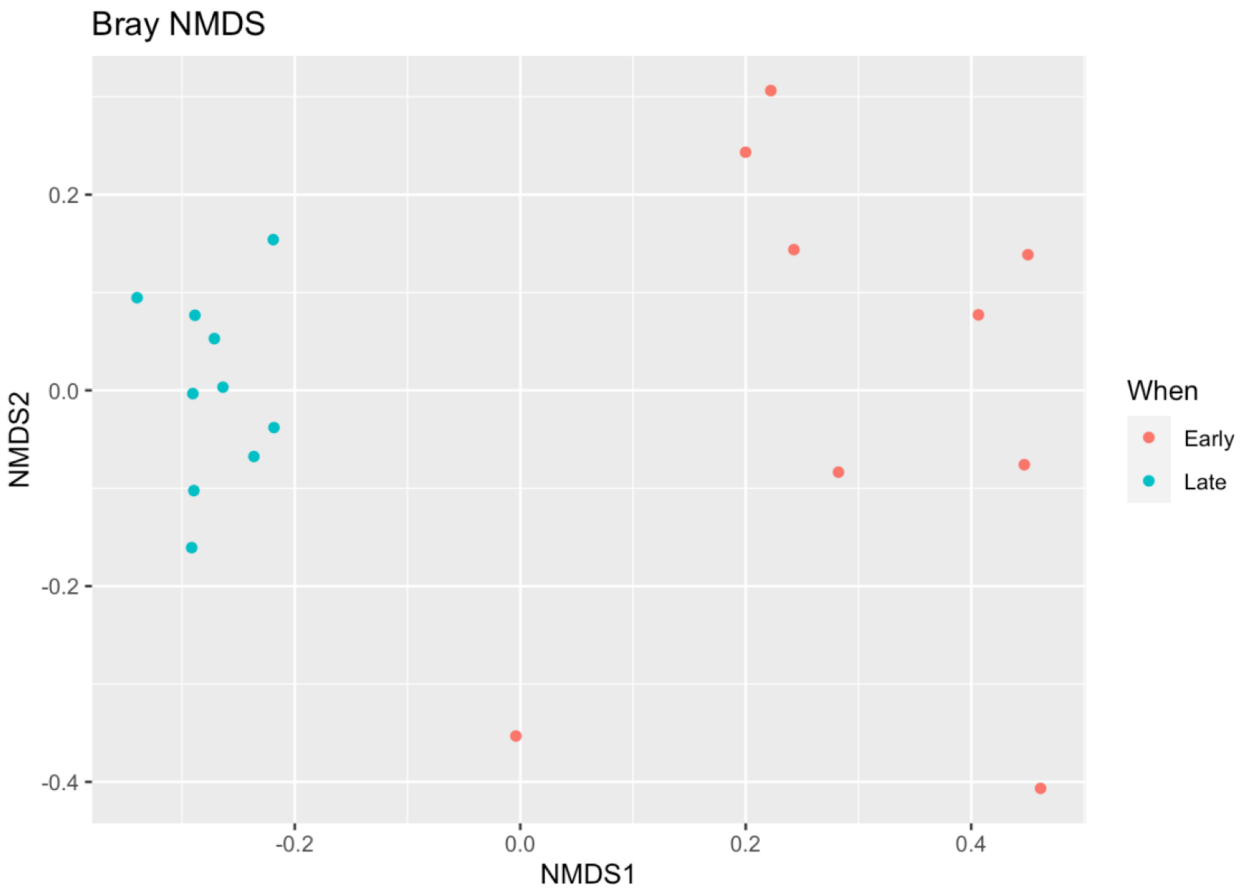
DADA2: Plot alpha diversity

```
# Alpha diversity
plot_richness(ps, x="Day", measures=c("Shannon", "Simpson"), color="When")
```



DADA2: Plot ordination (PCA)

```
# Transform data to proportions as appropriate for Bray-Curtis distances
# (conceptually similar to PCA)
ps.prop <- transform_sample_counts(ps, function(otu) otu/sum(otu))
ord.nmds.bray <- ordinate(ps.prop, method="NMDS", distance="bray")
plot_ordination(ps.prop, ord.nmds.bray, color="When", title="Bray NMDS")
```



DADA2: Plot species abundances

```
top20 <- names(sort(taxa_sums(ps), decreasing=TRUE))[1:20]
ps.top20 <- transform_sample_counts(ps, function(OTU) OTU/sum(OTU))
ps.top20 <- prune_taxa(top20, ps.top20)
plot_bar(ps.top20, x="Day", fill="Family") + facet_wrap(~When, scales="free_x")
```