Oct 16, 2019

Creating Differential Transcript Expression Results with DESeq2

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In Development dx.doi.org/10.17504/protocols.io.799hr96

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ABSTRACT

Differential expression analysis of transcript count tables using DESeq2

GUIDELINES

Note: this is a demonstrative experimental protocol for a specific differential expression analysis; variables, file names and some other code will need to be changed for your own circumstances

BEFORE STARTING

You should have gene-annotated transcript count tables for multiple sequencing libraries, renamed to match the form of "<library_identifier>_wide_transcript_counts_LAST.csv". See here for how to create these tables from nanopore cDNA transcripts.

You should also have a sample metadata file that matches library/barcode pairs to experimental conditions, with at least "SampleID" and "Label" fields. The SampleID field should be of the form "<library_identifier>.BCXX"; this will be replaced by the "Label" field when results are output to a file.

Collating count data

1 Combine the transcript count data into a single data structure. To reduce confusion when this protocol is run multiple times, we declare an *analysisDate* variable to be used for output file names:

```
countDate <- format(Sys.Date(), "%Y-%b-%d");
```

We also load libraries that will be used in the protocol:

```
## steps 1-2
library(dplyr);    # data table manipulation
library(tidyr);    # data table cleaning
library(readr);    # reading csv files into tbl
library(magrittr); # additional pipe operators
## steps 3-8
library(DESeq2);   # differential expression analysis
library(ashr);     # for log fold change shrinking
## step 9
options(java.parameters = "-Xmx10G"); # may be needed to avoid Excel errors
library(xlsx);                        # writing to an Excel file
```

1.1 Collect a list of the count data files:

```
data.files <- list.files(pattern = "_wide_transcript_counts_LAST.csv$");
names(data.files) <- sub("_wide_transcript_counts_LAST.csv$","",data.files);
```

CHECK - print out the file names to make sure they're correct:

```
data.files

## example output
#                                  CGAug18_004
#"CGAug18_004_wide_transcript_counts_LAST.csv"
#                                      CGDec17
#    "CGDec17_wide_transcript_counts_LAST.csv"
#                                  CGJan19_006
#"CGJan19_006_wide_transcript_counts_LAST.csv"
#                                  CGMar19_009
#"CGMar19_009_wide_transcript_counts_LAST.csv"
#                                  CGNov18_005
#"CGNov18_005_wide_transcript_counts_LAST.csv"
```

1.2 Set up the skeleton structures for creating the combined table. This is created in two parts:

1. A gene lookup table, containing gene metadata
2. A count table, containing transcript counts

```
geneLookup.tbl <- NULL;
counts.raw.tbl <- tibble(tdir=character());
```

1.3 The skeleton structures are then populated with the data from individual library files:

```
for(dfi in seq_along(data.files)){
  data.files[dfi] %>%
    read_csv %>%
    mutate(tdir = paste0(transcript, "_", dir)) ->
      counts.sub.tbl;
  ## append columns without barcode names to gene table
  geneLookup.tbl %<>%
      rbind(select(counts.sub.tbl,
                   -starts_with("BC"),
                   -starts_with("RB")));
  ## append columns *with* barcode names to count table
  counts.sub.tbl %<>%
      select("tdir", starts_with("BC"), starts_with("RB"));
  ## add file label to barcode name column
  bcCols <- grep("^(BC|RB)", colnames(counts.sub.tbl));
  colnames(counts.sub.tbl)[bcCols] %<>%
    paste0(names(data.files)[dfi], ".", .);
  counts.raw.tbl %<>%
      full_join(counts.sub.tbl, by="tdir");
}
## Remove duplicates from the gene table
geneLookup.tbl %<>% unique;
```

CHECK - make sure that the column headings of the aggregated count table match the expected names:

```
colnames(counts.raw.tbl)

## Example output
# [1] "tdir"             "CGAug18_004.BC04" "CGAug18_004.BC05" "CGAug18_004.BC06"
# [5] "CGDec17.BC06"     "CGDec17.BC07"     "CGJan19_006.BC03" "CGJan19_006.BC04"
# [9] "CGJan19_006.BC05" "CGJan19_006.BC06" "CGJan19_006.BC07" "CGJan19_006.BC08"
#[13] "CGMar19_009.BC07" "CGMar19_009.BC08" "CGMar19_009.BC09" "CGMar19_009.BC10"
#[17] "CGNov18_005.BC01" "CGNov18_005.BC02" "CGNov18_005.BC03" "CGNov18_005.BC04"
#[21] "CGNov18_005.BC05" "CGNov18_005.BC06"
```

2 Do some cleaning / reordering of the data, then create an intermediate aggregate count table

2.1 The sample metadata file is read in, mostly as factors; the sample ID is converted to a character vector:

```
read.csv("metadata.csv") %>%
    mutate(SampleID = as.character(SampleID)) ->
    meta.df;
```

Metadata rows are subset and re-ordered to match the order of the count table:

```
meta.df <- meta.df[match(colnames(counts.raw.tbl)[-1], meta.df$SampleID),];
```

2.2 Missing values for genes are set to counts of zero, and the count table is appended to the genes table:

```
counts.raw.tbl %>%
    replace(is.na(.), 0) %>%
    left_join(geneLookup.tbl, ., by="tdir") ->
    counts.withGenes.tbl;
```

2.3 The combined table is output to an intermediate file, using the analysis date as a file name:

```
counts.withGenes.tbl %>%
    write_csv(sprintf("raw_counts_%s.csv", countDate));
```

Differential Expression

3 Set up variables to change output file names and behaviour:

Note: the countDate *is not "today" because different explorations of differential expression could be done on the same count data.*

```
countDate <- "2019-Oct-16"; # date of count aggregation
l2FCShrink <- TRUE; # whether the Log2FC values should be shrunk
analysisDate <- format(Sys.Date(), "%Y-%b-%d"); # date of DESeq analysis
resultSource <- "GRCm38_CG_4T1"; # descriptive label for results
excluded.factors <- "Treatment"; # factors to exclude from statistical model
```

Read in the intermediate aggregated count file and the metadata file:

```
sprintf("raw_counts_%s.csv", countDate) %>%
    read_csv ->
    count.tbl;

read.csv("metadata.csv") %>%
    mutate(SampleID = as.character(SampleID)) %>%
    ## Make sure metadata information only includes samples in the count table
    filter(SampleID %in% colnames(count.tbl)) ->
    meta.df;
```

4 Carry out metadata filtering (i.e. sample exclusion) and count filtering (e.g. gene / sample QC)

4.1 Filter the metadata table to only include the desired samples:

Note: this step will be situation specific

```
meta.df %<>%
    ## Only keep 4T1 strain data
    filter(Strain == "4T1") %>%
    ## Sort by cell line, then replicate
    arrange(Line, Replicate);
```

4.2 Make sure the count table containing only genes with a total count across all samples of at least *minCount*, have at least *nonZeroThreshold* genes with nonzero counts, and filter to choose only Sample IDs that are in the metadata table:

```
count.tbl %<>%
    pivot_longer(cols=c(-transcript, -Chr, -Strand, -Start, -End,
                 -Description, -Gene, -dir, -tdir),
                 names_to = "SampleID", values_to = "count") %>%
    ## only keep transcripts with a total count of 2 or more
    group_by(SampleID) %>%
    filter(sum(count) >= 2) %>%
    filter(sum(count > 0) > (length(count) * 0.25)) %>%
    ## convert to integer
    mutate(count = as.integer(count)) %>%
    ## restore wide format
    ungroup %>%
    pivot_wider(names_from = SampleID, values_from = count) %>%
    ## reorder to match metadata table
    select(transcript, Chr, Strand, Start, End, Description, Gene, dir, tdir,
           match(meta.df$SampleID, colnames(.)))
```

4.3 Filter the metadata file to match the count table (i.e. removing any samples filtered out in the previous step), and exclude any columns that have single values:

Refactor the metadata structure to remove missing values:

```
for(x in colnames(meta.df)){
    if(is.factor(meta.df[[x]])){
        meta.df[[x]] %<>% factor;
    }
}
```

CHECK - make sure the SampleIDs in the column names match the exact order of the metadata table:

```
colnames(count.tbl)[-(1:9)] == meta.df$SampleID

## Example output
# [1] TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE
#[16] TRUE TRUE
```

5 Create DESeq2 data structure, and run a differential expression analysis

5.1 Identify factors for the statistical model from the metadata file:

```
setdiff(colnames(metasub.df),
        c(c("SampleID","Label","Replicate","Notes"), excluded.factors)) ->
    factorNames;
```

5.2 Create the transcript count matrix:

```
count.mat <- as.matrix(count.tbl[-(1:9)]);
rownames(count.mat) <- count.tbl$tdir;
```

5.3 Convert to DESeq2 structure and run DESeq2:

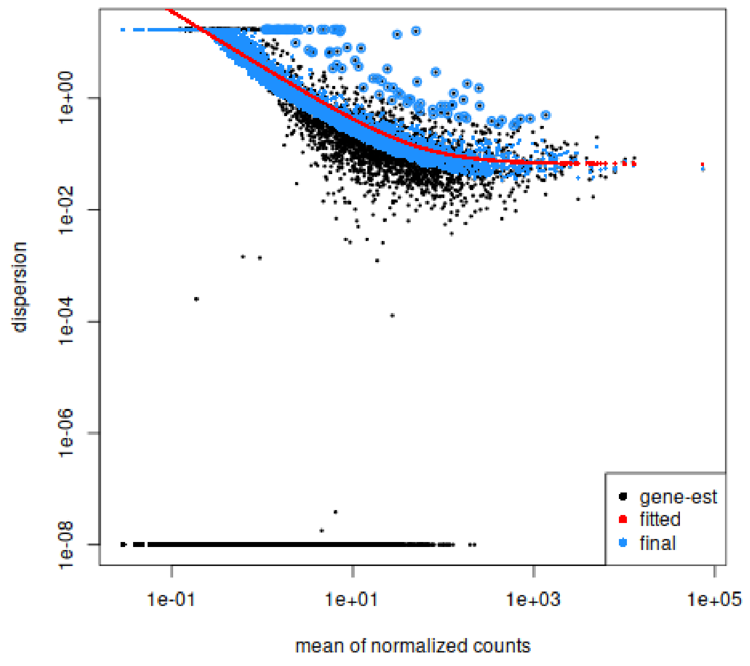
```
DESeqDataSetFromMatrix(count.mat, meta.df,
        as.formula(paste0("~ ", paste(factorNames, collapse=" + ")))) %>%
    DESeq ->
    dds;
```

CHECK - make sure dispersion and result names look reasonable:

Note: The result names will not indicate every possible comparison; just a subset from which all comparisons can be derived from.

```
plotDispEsts(dds);
resultsNames(dds);

## Example output
# [1] "Intercept"                    "Experiment_CG005_vs_CG004"
# [3] "Experiment_CG006_vs_CG004"    "Experiment_CG009_vs_CG004"
# [5] "Experiment_CGDec17_vs_CG004" "Line_p0D25_vs_p0"
# [7] "Line_p0SC_vs_p0"              "Line_p0SCL_vs_p0"
# [9] "Line_WT_vs_p0"
```



Dispersion plot for nanopore transcript data. Ideally there should be a smooth curve, with higher dispersion for low-count genes, and lower dispersion for high-count genes.

Result Collation

6 Prepare results table skeletons for DESeq2 results

6.1 Collect up comparisons to make:

```
resultList <- NULL;
for(fi in factorNames){
    vn <- unique(meta.df[[fi]]);
    vn <- vn[order(-xtfrm(vn))];
    if(length(vn) == 1){
        next;
    }
    for(fai in seq(1, length(vn)-1)){
        for(fbi in seq(fai+1, length(vn))){
            cat(sprintf("%s: %s vs %s\n", fi, vn[fai], vn[fbi]));
            resultList <- c(resultList,
                            list(c(fi, as.character(vn[fai]), as.character(vn[fbi]))));
        }
    }
}
```

6.2 Create variance-stabilised [log2] count matrix from DESeq2 structure:

```
dds.counts <- assay(vst(dds, blind=FALSE));
```

Rescale VST values to have the same 99th percentile, but a minimum value of zero. This makes the scaled counts resemble more closely the actual read counts:

```
dds.quantile99 <- quantile(dds.counts[dds.counts > min(dds.counts)], 0.99);
((dds.counts - min(dds.counts)) /
 (dds.quantile99 - min(dds.counts))) * (dds.quantile99) ->
    dds.counts;
```

Replace column names in the VST matrix with labels from the metadata:

Note: the substution removes any initial whitespace from the label

```
colnames(dds.counts)[match(meta.df$SampleID,
                           colnames(dds.counts))] <-
    paste0("adj.",sub("^ +", "", as.character(meta.df$Label)));
```

6.3 Generate base count table:

```
dds.withCounts.tbl <- count.tbl
## replace column names with metadata labels
colnames(dds.withCounts.tbl)[match(meta.df$SampleID,
                                   colnames(dds.withCounts.tbl))] <-
    paste0("raw.",sub("^ +", "", as.character(meta.df$Label)));
```

Tack on VST matrix:

```
dds.withCounts.tbl[,colnames(dds.counts)] <- round(dds.counts,2);
```

Pre-populate wth minimum p-value column:

```
dds.withCounts.tbl$min.p.val <- 0;
```

7 Fetch DESeq2 results for each comparison from the DESeq2 data structure and add to the base table:

```
for(rn in resultList){
    print(rn);
    results.df <-
        if(l2FCShrink){
            as.data.frame(lfcShrink(dds, contrast=rn, type = "ashr"));
        } else {
           as.data.frame(results(dds, contrast=rn));
        }
    results.df$log2FoldChange <- round(results.df$log2FoldChange, 2);
    results.df$pvalue <- signif(results.df$pvalue, 3);
    results.df$padj <- signif(results.df$padj, 3);
    rn.label <- paste(rn, collapse="-");
    results.tbl <- as.tbl(results.df[, c("log2FoldChange", "lfcSE", "pvalue", "padj")]);
    colnames(results.tbl) <- paste0(c("L2FC.", "lfcSE.", "pval.", "padj."), rn.label);
    results.tbl$tdir <- rownames(results.df);
    dds.withCounts.tbl <-
        left_join(dds.withCounts.tbl, results.tbl, by="tdir");
}
```

8 Write results out to a CSV file:

```
dds.withCounts.tbl$min.p.val <-
    apply(dds.withCounts.tbl[,grep("^padj\\.",colnames(dds.withCounts.tbl))],
          1, min, na.rm=TRUE);
write.csv(dds.withCounts.tbl, row.names=FALSE,
          gzfile(sprintf("DE_%s_VST_%s_%s.csv.gz",
                         if(l2FCShrink){"shrunk"} else {"orig"},
                         resultSource, analysisDate)));
```

Excel Worksheet Output

9 Separate results and put into an Excel file

Note: this step will be situation specific

9.1 Split out mitochondrial genes:

```
filtered.dds.tbl <- filter(dds.withCounts.tbl, Chr != "MT");
MT.dds.tbl <- filter(dds.withCounts.tbl, Chr == "MT");
```

9.2 Write split datasets out to the Excel file:

```
write.xlsx2(as.data.frame(filtered.dds.tbl),
            sprintf("DE_%s_VST_%s_%s.xlsx",
                    if(l2FCShrink){"shrunk"} else {"orig"},
                    resultSource, analysisDate),
            sheetName="Genome Data", row.names=FALSE);
write.xlsx2(as.data.frame(MT.dds.tbl),
            sprintf("DE_%s_VST_%s_%s.xlsx",
                    if(l2FCShrink){"shrunk"} else {"orig"},
                    resultSource, analysisDate),
            sheetName="MT Data", append=TRUE, row.names=FALSE);
```

9.3 Add worksheets for differentially-expressed pairs:

```
for(rn in resultList){
    print(rn);
    if(rn[1] == "Experiment"){
        next;
    }
    sheetName <- sprintf("%s; %s vs %s", rn[1], rn[2], rn[3]);
    meta.df <- meta.df[order(meta.df$Line, meta.df$Replicate),];
    cnames <- sub("^ +","",as.character(meta.df$Label[meta.df[[rn[1]]] %in% rn[2:3]]));
    cnames <- c(paste0("raw.",cnames), paste0("adj.",cnames));
    rn.label <- paste(rn, collapse="-");
    res.tbl <- filtered.dds.tbl[,c(colnames(filtered.dds.tbl)[c(6,7,8)],
                               cnames,paste0(c("L2FC.","pval.", "padj."), rn.label))];
    res.tbl <- res.tbl[res.tbl[[paste0("padj.", rn.label)]] <= 0.1,];
    res.tbl <- res.tbl[order(-res.tbl[[paste0("L2FC.", rn.label)]]),];
    write.xlsx2(as.data.frame(res.tbl),
                sprintf("DE_%s_VST_%s_%s.xlsx",
                        if(l2FCShrink){"shrunk"} else {"orig"},
                        resultSource, analysisDate),
               sheetName=sheetName, append=TRUE, row.names=FALSE);
}
```