

Analysis of the CPTAC Spike-in Study

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Contents

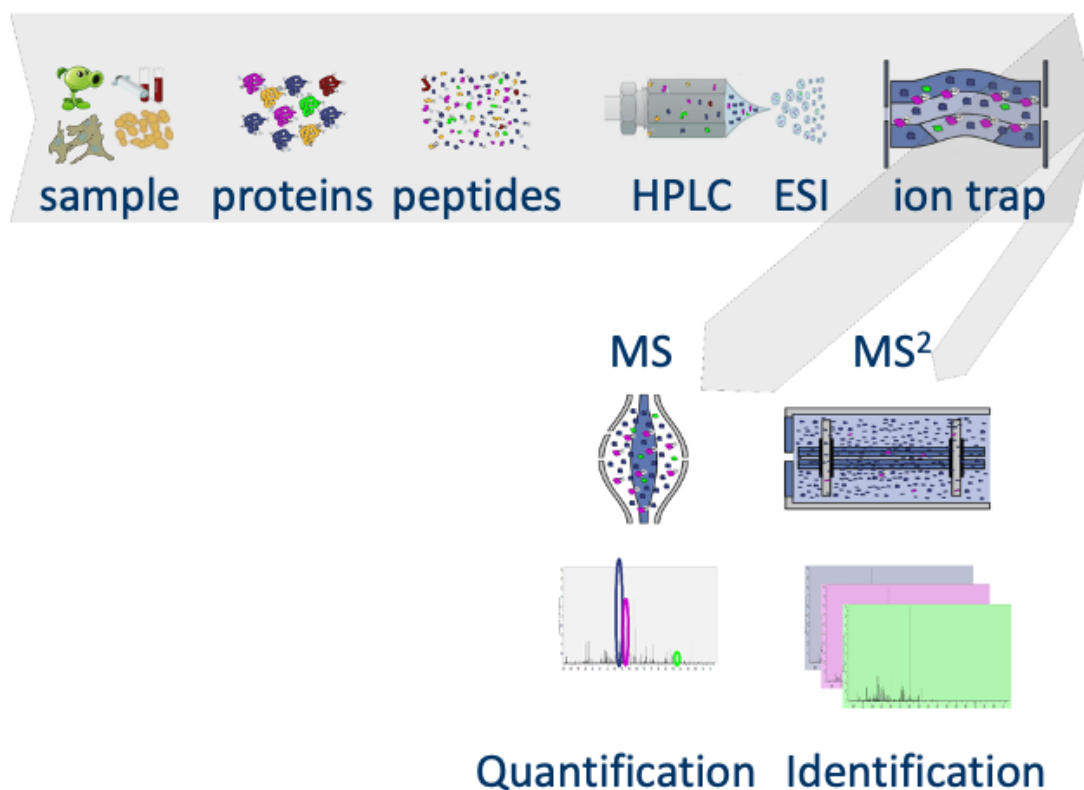
1	Intro: Challenges in Label-Free Quantitative Proteomics	2
1.1	MS-based workflow	2
1.2	Level of quantification	3
1.3	Label-free Quantitative Proteomics Data Analysis Workflows	4
2	QFeatures	4
2.1	Data infrastructure	4
3	Background of the CPTAC Spike-In Study	7
3.1	Import data in R	7
4	Import subset of the CPTAC study	10
4.1	Missingness	10
5	Preprocessing	11
5.1	Log transform the data	11
5.2	Filtering	11
5.3	Normalize the data using median centering	11
5.4	Explore normalized data	12
6	Median summarization	14
6.1	Preprocessing	14
6.2	Data Analysis	15
7	Robust summarization	21
7.1	Preprocessing	21
7.2	Data Analysis	22

8	Where the difference comes from	28
8.1	Import data from the full CPTAC study	28
8.2	Peptide-Level view	30
9	Estimation of differential abundance using peptide level model	38
10	Session Info	39

This is part of the online course [Proteomics Data Analysis \(PDA\)](#)

1 Intro: Challenges in Label-Free Quantitative Proteomics

1.1 MS-based workflow



- Peptide Characteristics
 - Modifications
 - Ionisation Efficiency: huge variability
 - Identification
 - * Misidentification → outliers

- * MS² selection on peptide abundance
- * Context depending missingness
- * Non-random missingness

→ Unbalanced peptide identifications across samples and messy data

1.2 Level of quantification

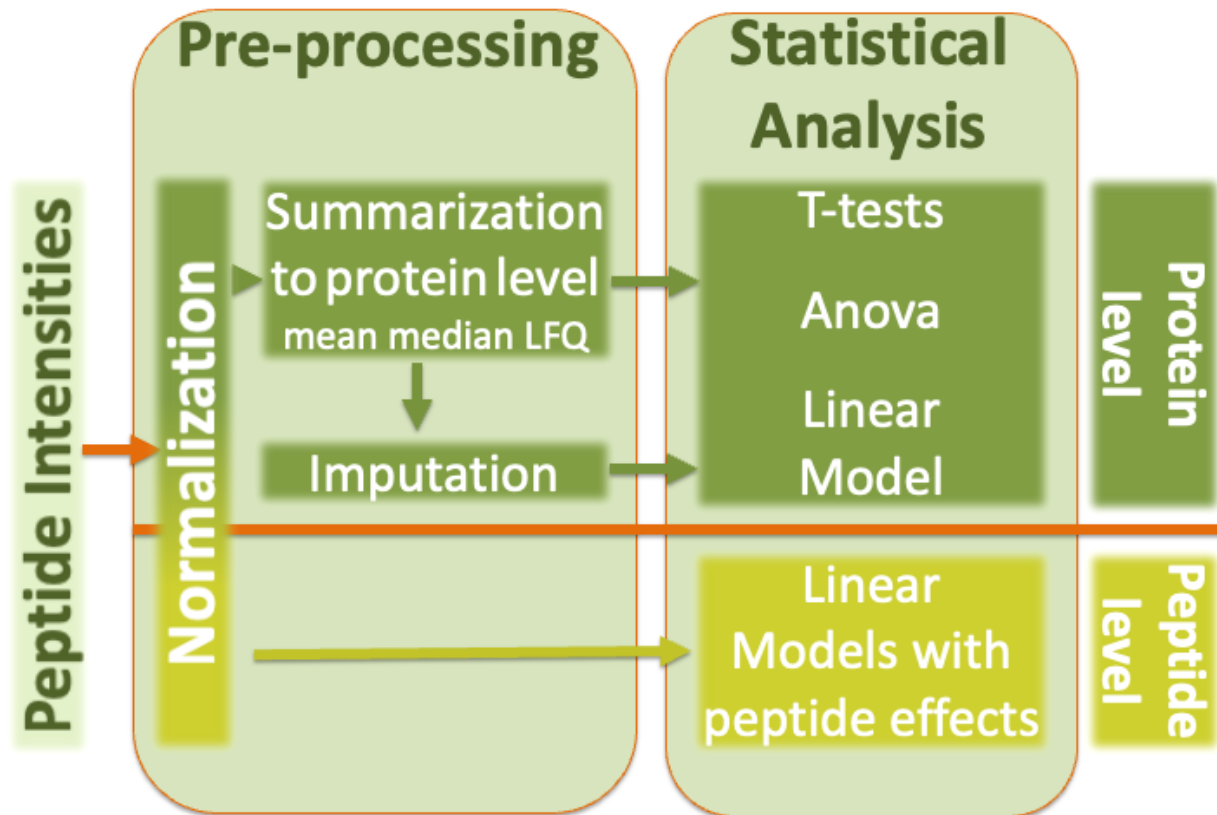
- MS-based proteomics returns peptides: pieces of proteins



- Quantification commonly required on the protein level



1.3 Label-free Quantitative Proteomics Data Analysis Workflows



2 QFeatures

2.1 Data infrastructure

- We use the **QFeatures** package that provides the infrastructure to
 - store,
 - process,
 - manipulate and
 - analyse quantitative data/features from mass spectrometry experiments.
- It is based on the **SummarizedExperiment** and **MultiAssayExperiment** classes.
- Assays in a **QFeatures** object have a hierarchical relation:
 - proteins are composed of peptides,
 - themselves produced by spectra
 - relations between assays are tracked and recorded throughout data processing

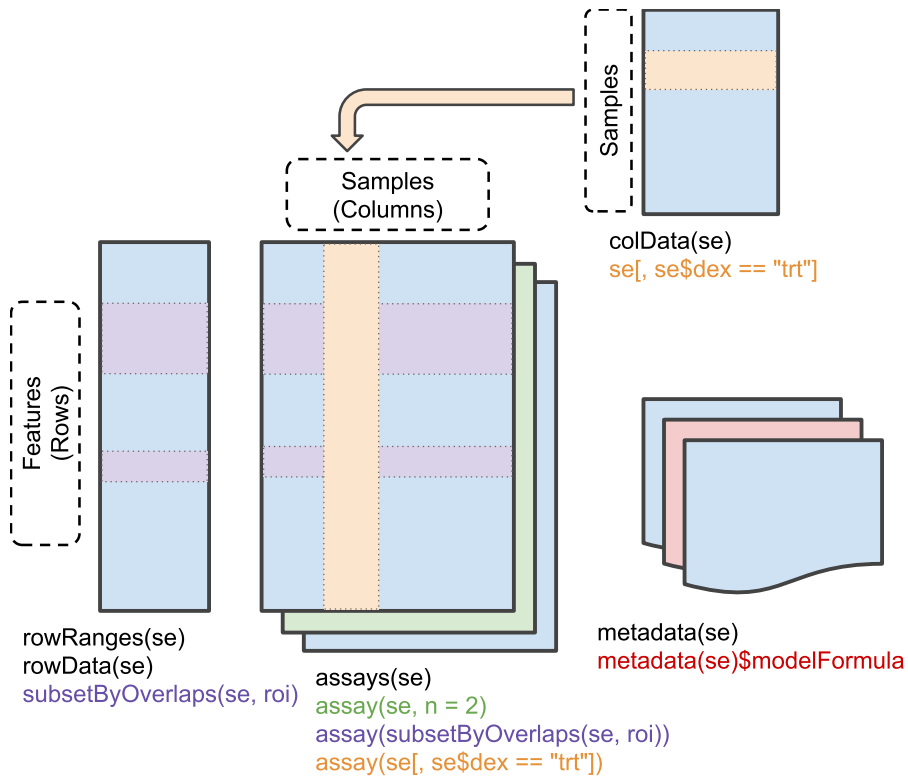


Figure 1: Conceptual representation of a ‘SummarizedExperiment’ object. Assays contain information on the measured omics features (rows) for different samples (columns). The ‘rowData’ contains information on the omics features, the ‘colData’ contains information on the samples, i.e. experimental design etc.

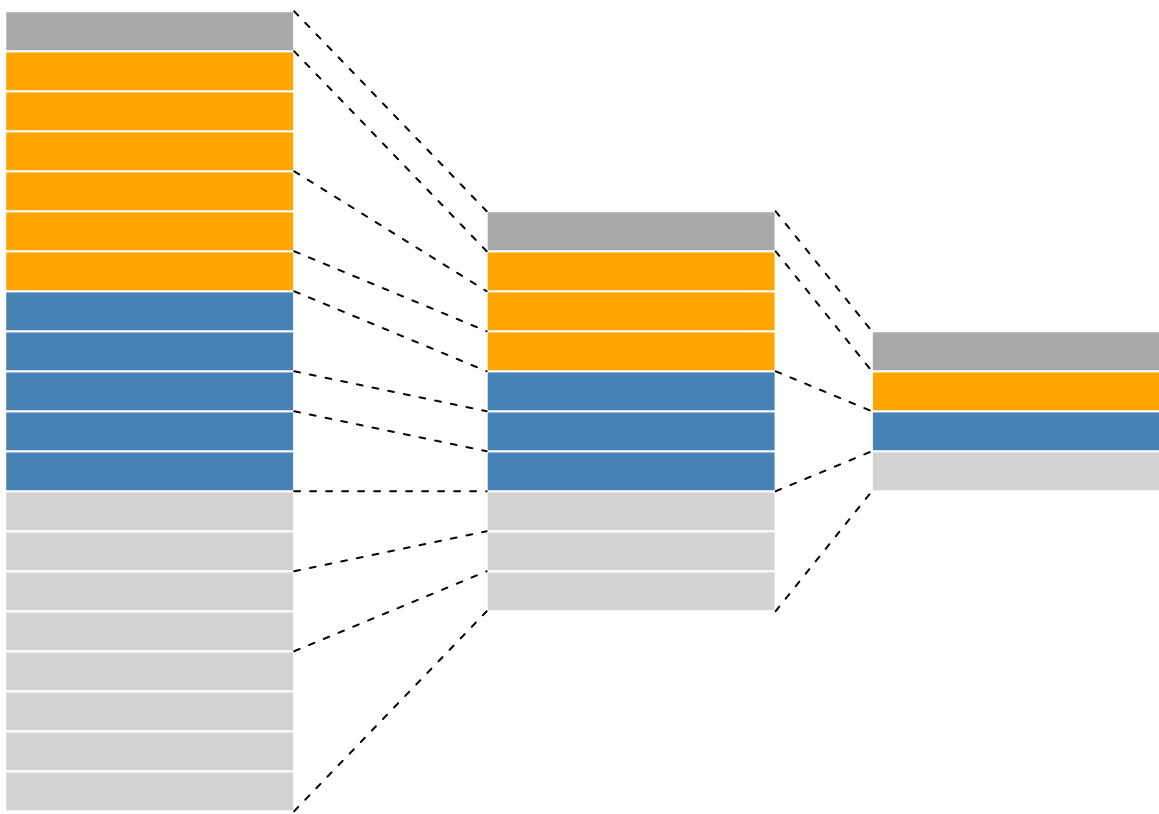


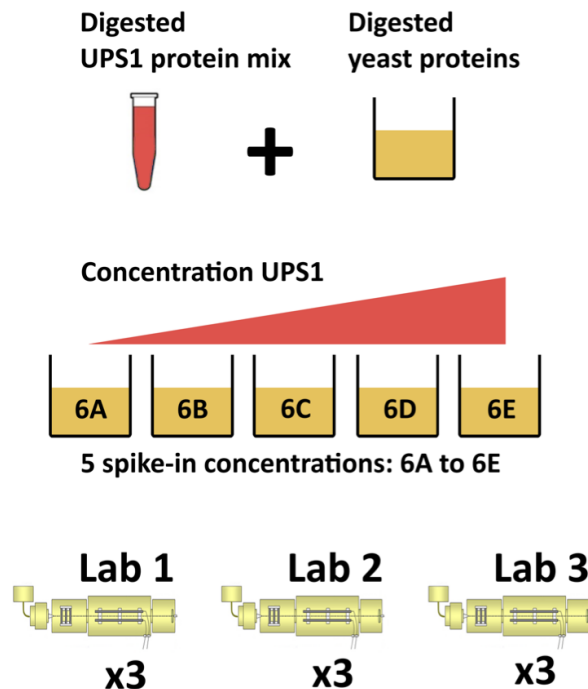
Figure 2: Conceptual representation of a `QFeatures` object and the aggregative relation between different assays.

3 Background of the CPTAC Spike-In Study

This case-study is a subset of the data of the 6th study of the Clinical Proteomic Technology Assessment for Cancer (CPTAC). In this experiment, the authors spiked the Sigma Universal Protein Standard mixture 1 (UPS1) containing 48 different human proteins in a protein background of 60 ng/ μ L *Saccharomyces cerevisiae* strain BY4741.

Five different spike-in concentrations were used: (6A: 0.25 fmol UPS1 proteins/ μ L; 6B: 0.74 fmol UPS1 proteins/ μ L; 6C: 2.22 fmol UPS1 proteins/ μ L; 6D: 6.67 fmol UPS1 proteins/ μ L; and 6E: 20 fmol UPS1 proteins/ μ L) [5].

We limited ourselves to the data of LTQ-Orbitrap W at site 56. The data were searched with MaxQuant version 1.5.2.8, and detailed search settings were described in Goeminne et al. (2016) [1]. Three replicates are available for each concentration.



- After MaxQuant search with match between runs option
 - 41% of all proteins are quantified in all samples
 - 6.6% of all peptides are quantified in all samples

→ vast amount of missingness

3.1 Import data in R

3.1.1 Load libraries

```
library(tidyverse)
library(limma)
library(QFeatures)
library(msqrob2)
```

```
library(plotly)
library(ggplot2)
library(cowplot)
library(gridExtra)
```

3.1.2 Import data from the CPTAC study

1. We use a peptides.txt file from MS-data quantified with maxquant that contains MS1 intensities summarized at the peptide level.

```
peptidesFile <- "https://raw.githubusercontent.com/statOmics/PDA/data/quantification/fullCptacDatasets/MS1/peptides.txt"
```

2. Maxquant stores the intensity data for the different samples in columns that start with Intensity. We can retrieve the column names with the intensity data with the code below:

```
ecols <- grep("Intensity\\.", names(read.delim(peptidesFile)))
```

3. Read the data and store it in QFeatures object

```
pe <- readQFeatures(
  table = peptidesFile,
  fnames = 1,
  ecol = ecols,
  name = "peptideRaw", sep="\t")
```

3.1.3 Explore object

- The rowData contains information on the features (peptides) in the assay. E.g. Sequence, protein, ...

```
head(rowData(pe[["peptideRaw"]]), c("Proteins", "Sequence", "Charges", "Intensity",
                                     "Experiment.6A_7", "Experiment.6A_8", "Experiment.6A_9"))
```

```
## DataFrame with 6 rows and 7 columns
##               Proteins      Sequence      Charges Intensity
##               <character> <character> <character> <numeric>
## AAAAGAGGAGDSGDAVTK sp|P38915|... AAAAGAGGAG...      2  1190800
## AAAALAGGK          sp|Q3E792|...   AAAALAGGK      2 280990000
## AAAALAGGKK         sp|Q3E792|...   AAAALAGGKK      2  33360000
## AAADALSDLEIK       sp|P09938|...   AAADALSDLE...      2  54622000
## AAADALSDLEIKDSK    sp|P09938|...   AAADALSDLE...      3 18910000
## AAAEEFQR           sp|P53075|...   AAAEEFQR       2  1158600
##               Experiment.6A_7 Experiment.6A_8 Experiment.6A_9
##               <integer>      <integer>      <integer>
## AAAAGAGGAGDSGDAVTK           1             1             1
## AAAALAGGK                    2             1             1
## AAAALAGGKK                    1             1             1
## AAADALSDLEIK                  1             1             1
## AAADALSDLEIKDSK               1             1             1
## AAAEEFQR                      NA            NA             1
```


- The colData contains information on the samples

```
colData(pe)
```

```
## DataFrame with 45 rows and 0 columns
```

- No information is stored yet on the design.

```
pe %>% colnames
```

```
## CharacterList of length 1
```

```
## [["peptideRaw"]] Intensity.6A_1 Intensity.6A_2 ... Intensity.6E_9
```

- Note, that the sample names include the spike-in condition.
- They also end on a number.
 - 1-3 is from lab 1,
 - 4-6 from lab 2 and
 - 7-9 from lab 3.
- We update the colData with information on the design

```
colData(pe)$lab <- rep(rep(paste0("lab",1:3),each=3),5) %>% as.factor
colData(pe)$condition <- pe[["peptideRaw"]] %>% colnames %>% substr(12,12) %>% as.factor
colData(pe)$spikeConcentration <- rep(c(A = 0.25, B = 0.74, C = 2.22, D = 6.67, E = 20),each = 9)
```

- We explore the colData again

```
colData(pe)
```

```
## DataFrame with 45 rows and 3 columns
##               lab condition spikeConcentration
##               <factor>  <factor>             <numeric>
## Intensity.6A_1   lab1      A                 0.25
## Intensity.6A_2   lab1      A                 0.25
## Intensity.6A_3   lab1      A                 0.25
## Intensity.6A_4   lab2      A                 0.25
## Intensity.6A_5   lab2      A                 0.25
## ...             ...      ...                 ...
## Intensity.6E_5   lab2      E                 20
## Intensity.6E_6   lab2      E                 20
## Intensity.6E_7   lab3      E                 20
## Intensity.6E_8   lab3      E                 20
## Intensity.6E_9   lab3      E                 20
```

4 Import subset of the CPTAC study

We first import the data from peptideRaws.txt file. This is the file containing your peptideRaw-level intensities. For a MaxQuant search [6], this peptideRaws.txt file can be found by default in the “path_to_raw_files/combined/txt/” folder from the MaxQuant output, with “path_to_raw_files” the folder where the raw files were saved. In this vignette, we use a MaxQuant peptideRaws file which is a subset of the cptac study. This data is available in the `msdata` package. To import the data we use the `QFeatures` package.

We generate the object `peptideRawFile` with the path to the peptideRaws.txt file. Using the `grepEcols` function, we find the columns that contain the expression data of the peptideRaws in the peptideRaws.txt file.

```
peptidesFile <- "https://raw.githubusercontent.com/statOmics/SGA2020/data/quantification/cptacAvsB_lab3/peptideRaws.txt"

ecols <- grep(
  "Intensity\\.\\.",
  names(read.delim(peptidesFile))
)

pe <- readQFeatures(
  table = peptidesFile,
  fnames = 1,
  ecol = ecols,
  name = "peptideRaw", sep = "\t")

colnames(pe)
```

```
## CharacterList of length 1
## [["peptideRaw"]] Intensity.6A_7 Intensity.6A_8 ... Intensity.6B_9
```

In the following code chunk, we can extract the spikein condition from the raw file name.

```
cond <- which(
  strsplit(colnames(pe)[[1]][1], split = "")[[1]] == "A") # find where condition is stored

colData(pe)$condition <- substr(colnames(pe), cond, cond) %>%
  unlist %>%
  as.factor
```

We calculate how many non zero intensities we have per peptide and this will be useful for filtering.

```
rowData(pe[["peptideRaw"]])$nNonZero <- rowSums(assay(pe[["peptideRaw"]]) > 0)
```

Peptides with zero intensities are missing peptides and should be represent with a NA value rather than 0.

```
pe <- zeroIsNA(pe, "peptideRaw") # convert 0 to NA
```

4.1 Missingness

45% of all peptide intensities are missing and for some peptides we do not even measure a signal in any sample.

5 Preprocessing

This section performs preprocessing for the peptide data. This includes

- log transformation,
- filtering and
- summarisation of the data.

5.1 Log transform the data

```
pe <- logTransform(pe, base = 2, i = "peptideRaw", name = "peptideLog")
```

5.2 Filtering

1. Handling overlapping protein groups

In our approach a peptide can map to multiple proteins, as long as there is none of these proteins present in a smaller subgroup.

```
pe <- filterFeatures(pe, ~ Proteins %in% smallestUniqueGroups(rowData(pe[["peptideLog"]])$Proteins))
```

2. Remove reverse sequences (decoys) and contaminants

We now remove the contaminants and peptides that map to decoy sequences.

```
pe <- filterFeatures(pe, ~ Reverse != "+")
pe <- filterFeatures(pe, ~ Potential.contaminant != "+")
```

3. Drop peptides that were only identified in one sample

We keep peptides that were observed at least twice.

```
pe <- filterFeatures(pe, ~ nNonZero >=2)
nrow(pe[["peptideLog"]])
```

```
## [1] 7011
```

We keep 7011 peptides upon filtering.

5.3 Normalize the data using median centering

We normalize the data by subtracting the sample median from every intensity for peptide p in a sample i :

$$y_{ip}^{\text{norm}} = y_{ip} - \hat{\mu}_i$$

with $\hat{\mu}_i$ the median intensity over all observed peptides in sample i .

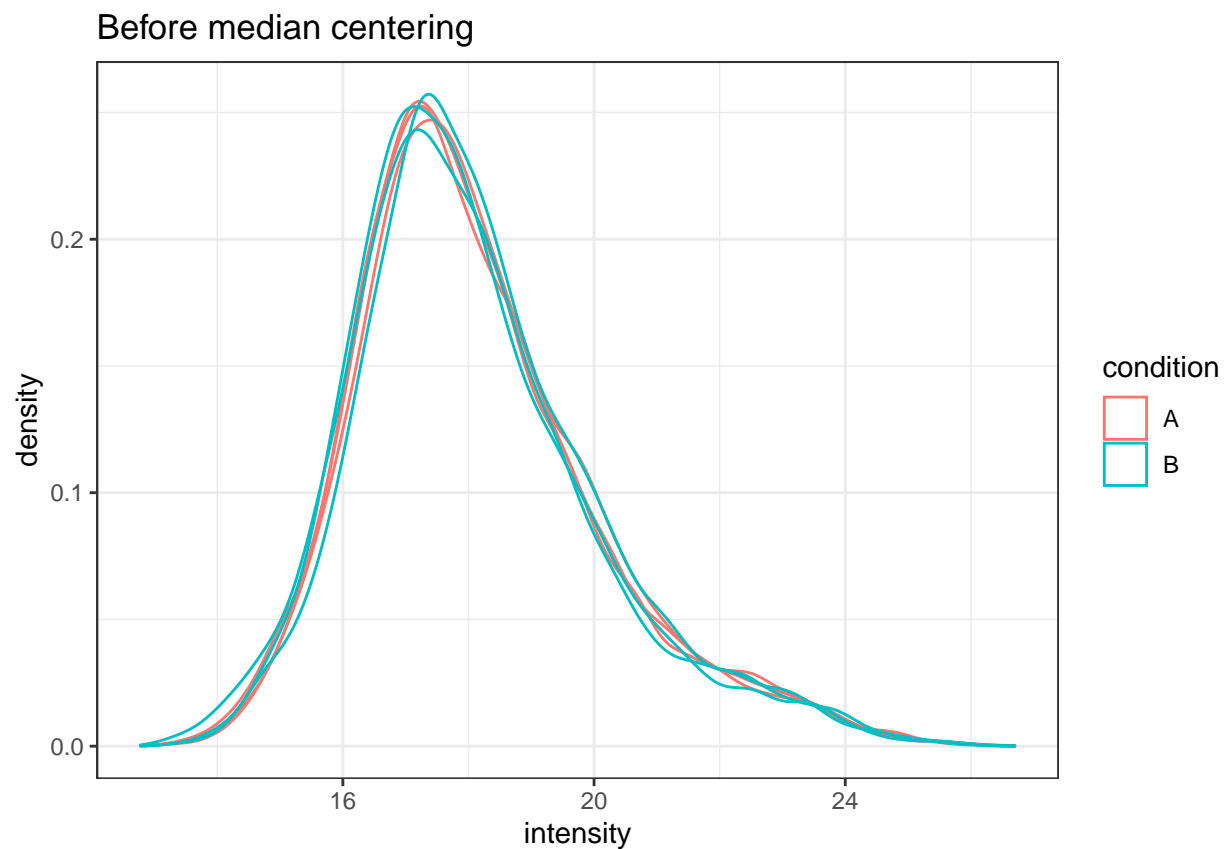
```
pe <- normalize(pe,
  i = "peptideLog",
  name = "peptideNorm",
  method = "center.median")
```

5.4 Explore normalized data

Upon the normalisation the density curves follow a similar distribution.

```
pe[["peptideLog"]] %>%
  assay %>%
  as.data.frame() %>%
  gather(sample, intensity) %>%
  mutate(condition = colData(pe)[sample, "condition"]) %>%
  ggplot(aes(x = intensity, group = sample, color = condition)) +
    geom_density() +
    theme_bw() +
    ggtitle("Before median centering")
```

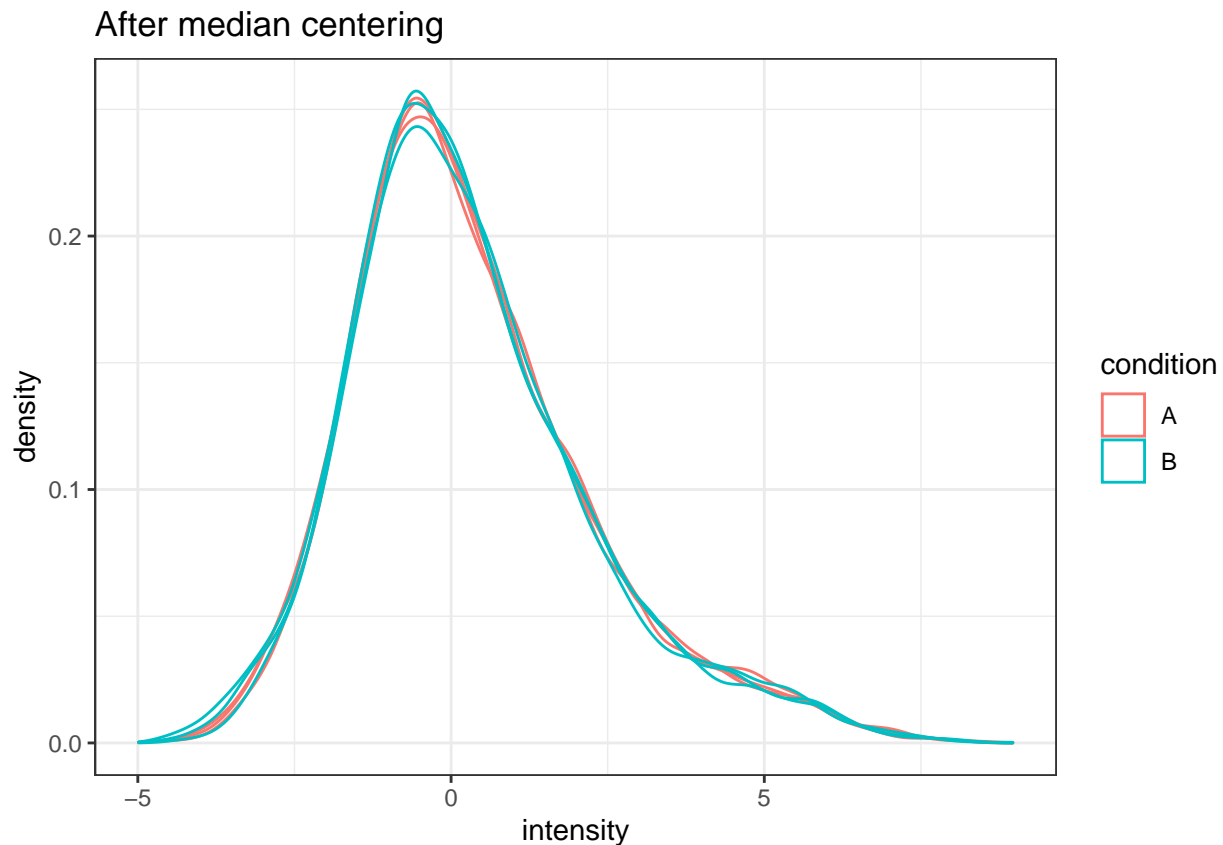
```
## Warning: Removed 8167 rows containing non-finite values (stat_density).
```



```
pe[["peptideNorm"]] %>%
  assay %>%
```

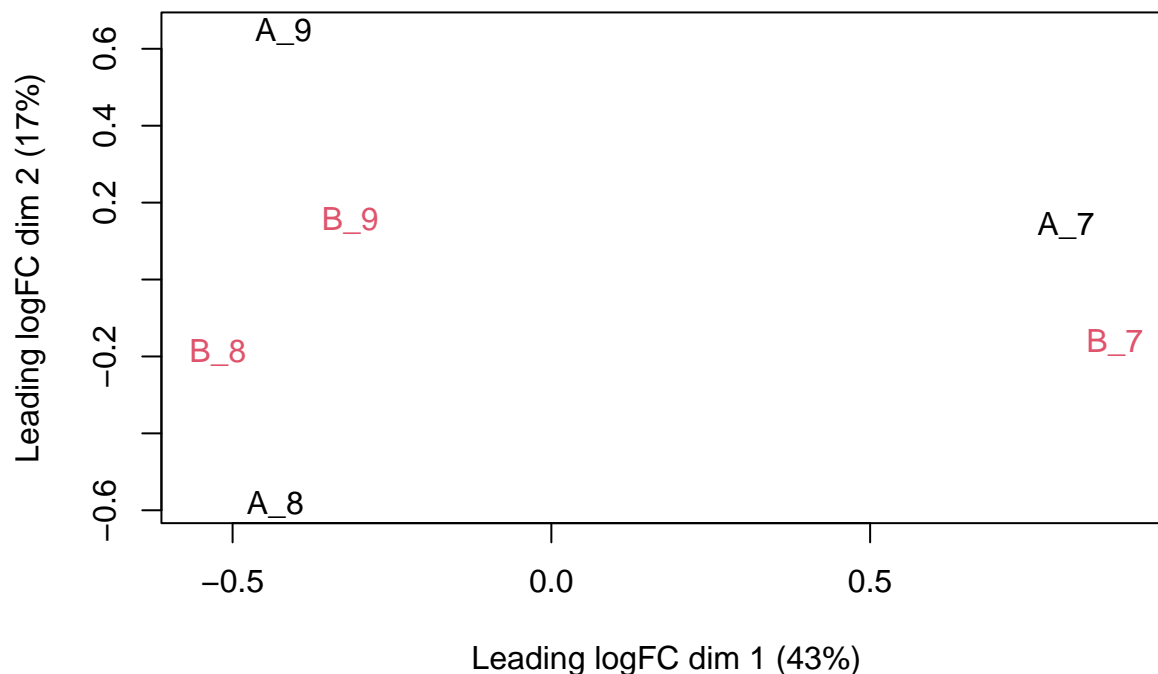
```
as.data.frame() %>%
gather(sample, intensity) %>%
mutate(condition = colData(pe)[sample,"condition"]) %>%
ggplot(aes(x = intensity,group = sample,color = condition)) +
  geom_density() +
  theme_bw()+
  ggtitle("After median centering")
```

Warning: Removed 8167 rows containing non-finite values (stat_density).



We can visualize our data using a Multi Dimensional Scaling plot, eg. as provided by the `limma` package.

```
tmp <- assay(pe[["peptideNorm"]])
colnames(tmp) <- str_replace_all(colnames(tmp), "Intensity.6","")
tmp %>%
  limma::plotMDS(col = as.numeric(colData(pe)$condition))
```



The first axis in the plot is showing the leading log fold changes (differences on the log scale) between the samples.

We notice that the leading differences (log FC) in the peptide data seems to be driven by technical variability. Indeed, the samples do not seem to be clearly separated according to the spike-in condition.

6 Median summarization

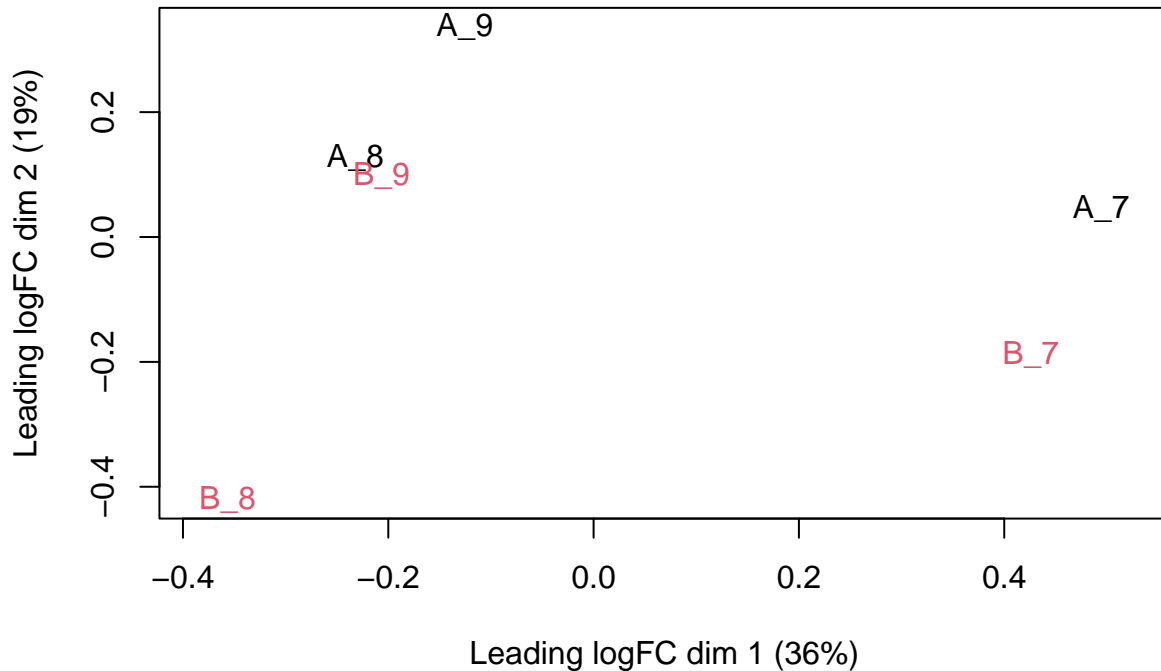
6.1 Preprocessing

- We use median summarization in `aggregateFeatures`.
- Note, that this is a suboptimal normalisation procedure!
- By default robust summarization is used: `fun = MsCoreUtils::robustSummary()`

```
pe <- aggregateFeatures(pe,
  i = "peptideNorm",
  fcol = "Proteins",
  na.rm = TRUE,
  name = "protein_median",
  fun = matrixStats::colMedians)
```

```
## Your quantitative and row data contain missing values. Please read the
## relevant section(s) in the aggregateFeatures manual page regarding the
## effects of missing values on data aggregation.
```

```
tmp <- assay(pe[["protein_median"]])
colnames(tmp) <- str_replace_all(colnames(tmp), "Intensity.6", "")
tmp %>%
  limma::plotMDS(col = as.numeric(colData(pe)$condition))
```



6.2 Data Analysis

6.2.1 Estimation

We model the protein level expression values using `msqrob`. By default `msqrob2` estimates the model parameters using robust regression.

We will model the data with a different group mean. The group is incoded in the variable `condition` of the `colData`. We can specify this model by using a formula with the factor `condition` as its predictor: `formula = ~condition`.

Note, that a formula always starts with a symbol `~`.

```
pe <- msqrob(object = pe, i = "protein_median", formula = ~condition, overwrite=TRUE)
```

```
rowData(pe[["protein_median"]])[,c("Proteins", ".n", "msqrobModels")]
```

```
## DataFrame with 1389 rows and 3 columns
##           Proteins      .n      msqrobModels
```

```
##               <character> <integer>           <list>
## 000762ups|UBE2C_HUMAN_UPS 000762ups|...      2      StatModel:rlm
## P00167ups|CYB5_HUMAN_UPS  P00167ups|...      1 StatModel:fitError
## P00441ups|SODC_HUMAN_UPS  P00441ups|...      3      StatModel:rlm
## P00709ups|LALBA_HUMAN_UPS P00709ups|...      3      StatModel:rlm
## P00915ups|CAH1_HUMAN_UPS  P00915ups|...      1 StatModel:fitError
## ...                      ...              ...
## sp|Q99258|RIB3_YEAST      sp|Q99258|...      4      StatModel:rlm
## sp|Q99260|YPT6_YEAST      sp|Q99260|...      1 StatModel:fitError
## sp|Q99287|SEY1_YEAST      sp|Q99287|...      1      StatModel:rlm
## sp|Q99383|HRP1_YEAST      sp|Q99383|...      3      StatModel:rlm
## sp|Q99385|VCX1_YEAST      sp|Q99385|...      1 StatModel:fitError
```

6.2.2 Inference

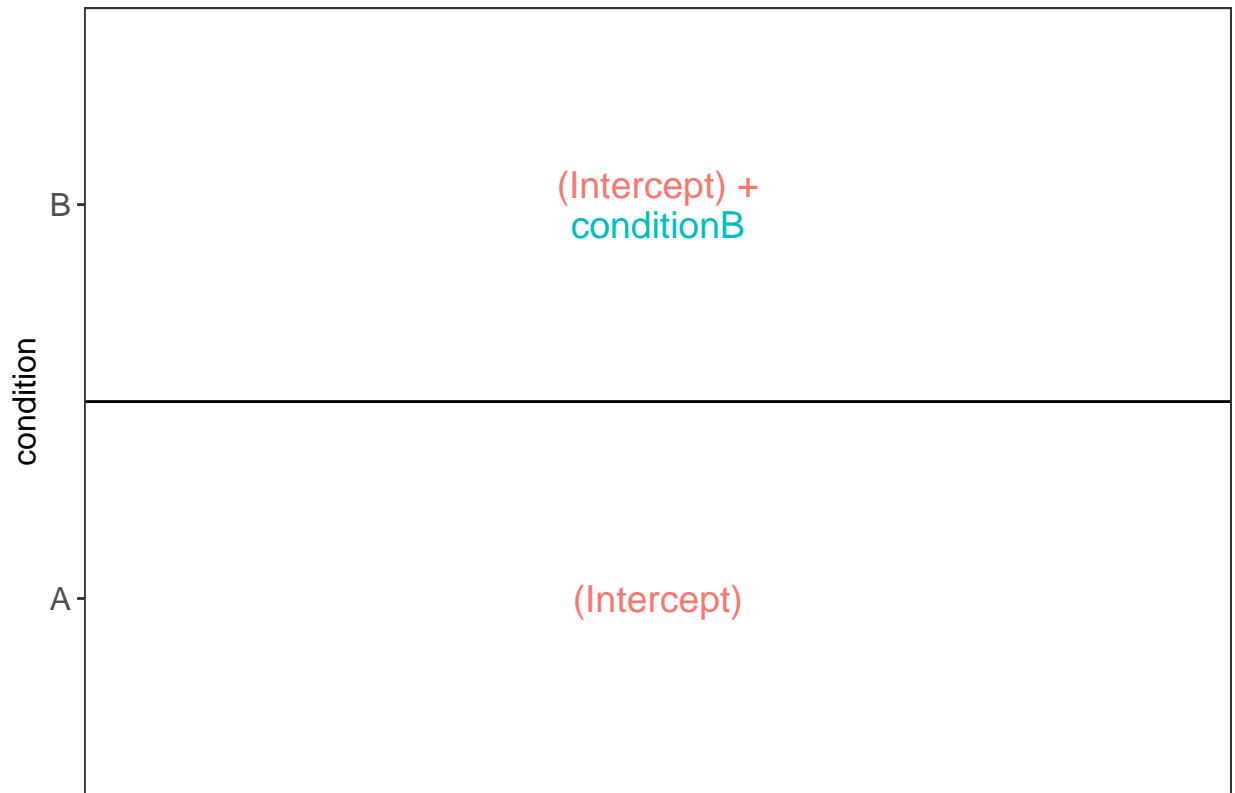
First, we extract the parameter names of the model by looking at the first model. The models are stored in the row data of the assay under the default name `msqrobModels`.

```
getCoef(rowData(pe[["protein_median"]])$msqrobModels[[1]])
```

```
## (Intercept)  conditionB
##   -2.793005    1.541958
```

We can also explore the design of the model that we specified using the the package `ExploreModelMatrix`

```
library(ExploreModelMatrix)
VisualizeDesign(colData(pe),~condition)$plotlist[[1]]
```

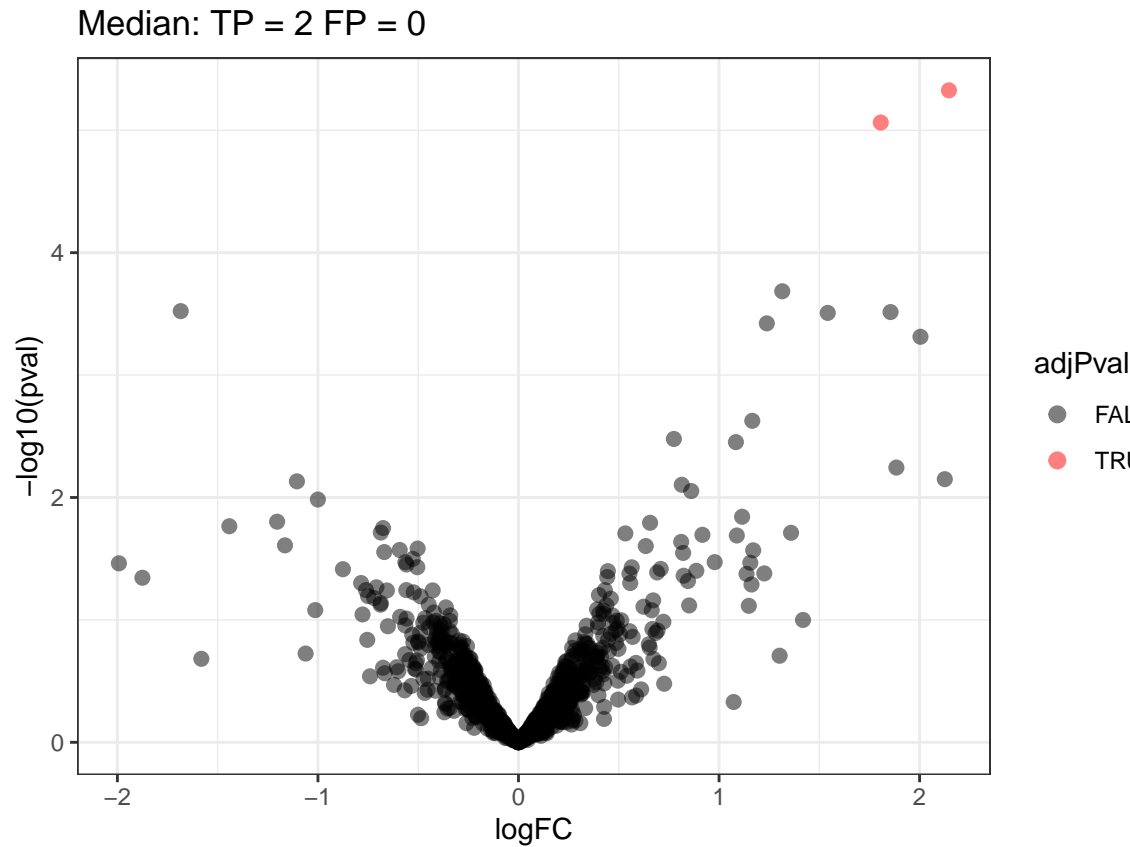
Spike-in condition A is the reference class. So the mean log2 expression for samples from condition A is ‘(Intercept)’. The mean log2 expression for samples from condition B is ‘(Intercept)+conditionB’. Hence, the average log2 fold change between condition b and condition a is modelled using the parameter ‘conditionB’. Thus, we assess the contrast ‘conditionB = 0’ with our statistical test.

```
L <- makeContrast("conditionB=0", parameterNames = c("conditionB"))
pe <- hypothesisTest(object = pe, i = "protein_median", contrast = L)
```

6.2.3 Plots

```
tmp <- rowData(pe[["protein_median"]])$conditionB[complete.cases(rowData(pe[["protein_median"]])$conditionB)]
tmp$shapes <- 16

volcanoMedian<- ggplot(tmp,
  aes(x = logFC, y = -log10(pval), color = adjPval < 0.05)) +
  geom_point(cex = 2.5, shape = tmp$shapes) +
  #geom_point(x =FP$logFC, y = -log10(FP$pval), shape = 8, size = 4 )+
  scale_color_manual(values = alpha(c("black", "red"), 0.5)) +
  theme_bw() +
  ggtitle(paste0("Median: TP = ",sum(tmp$adjPval<0.05&grepl(rownames(tmp),pattern ="UPS"),na.rm=TRUE), "%"))
volcanoMedian
```

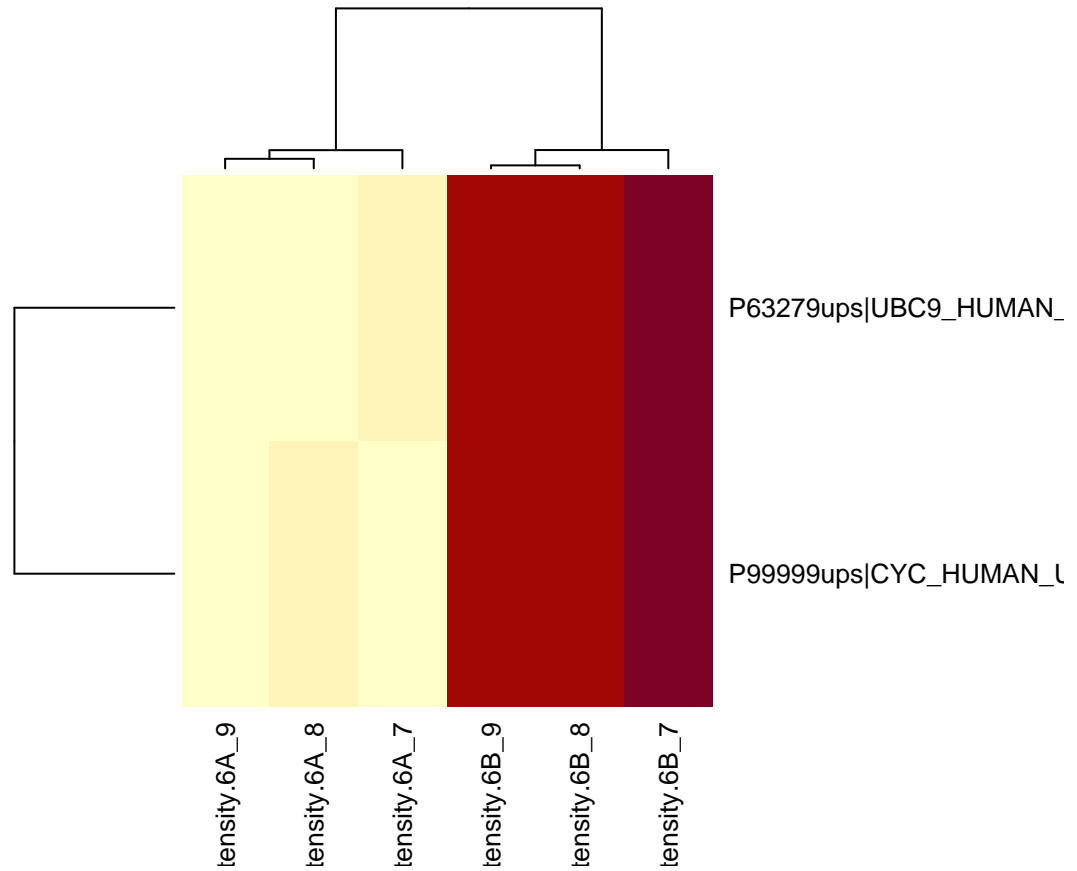


6.2.3.1 Volcano-plot

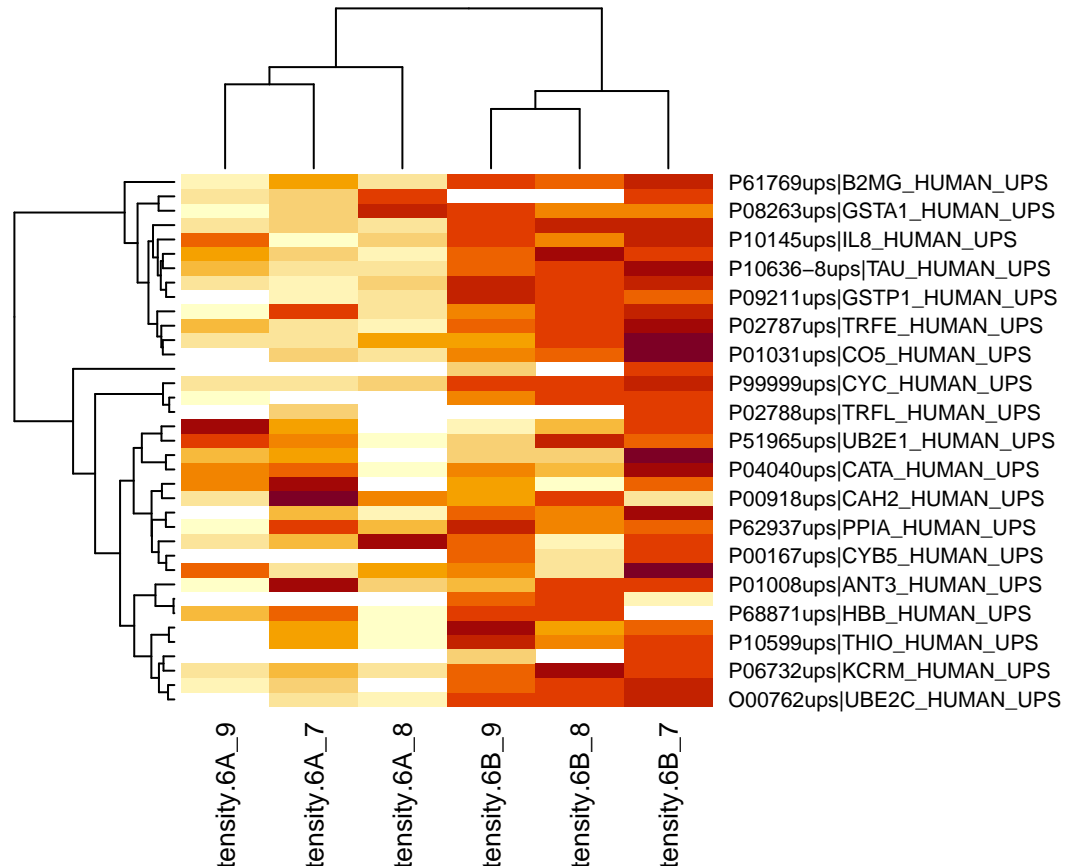
Note, that only 2 proteins are found to be differentially abundant.

6.2.3.2 Heatmap We first select the names of the proteins that were declared significant

```
sigNames <- rowData(pe[["protein_median"]])$conditionB %>%
  rownames_to_column("protein_median") %>%
  filter(adjPval<0.05) %>%
  pull(protein_median)
heatmap(assay(pe[["protein_median"]])[sigNames, ], cexRow = 1, cexCol = 1)
```



```
sigProteins <- rowData(pe[["protein_median"]])$conditionB %>%
  rownames_to_column("protein_median") %>%
  filter(grepl("UPS",protein_median)) %>%
  pull(protein_median)
heatmap(assay(pe[["protein_median"]])[sigProteins, ], cexCol = 1)
```

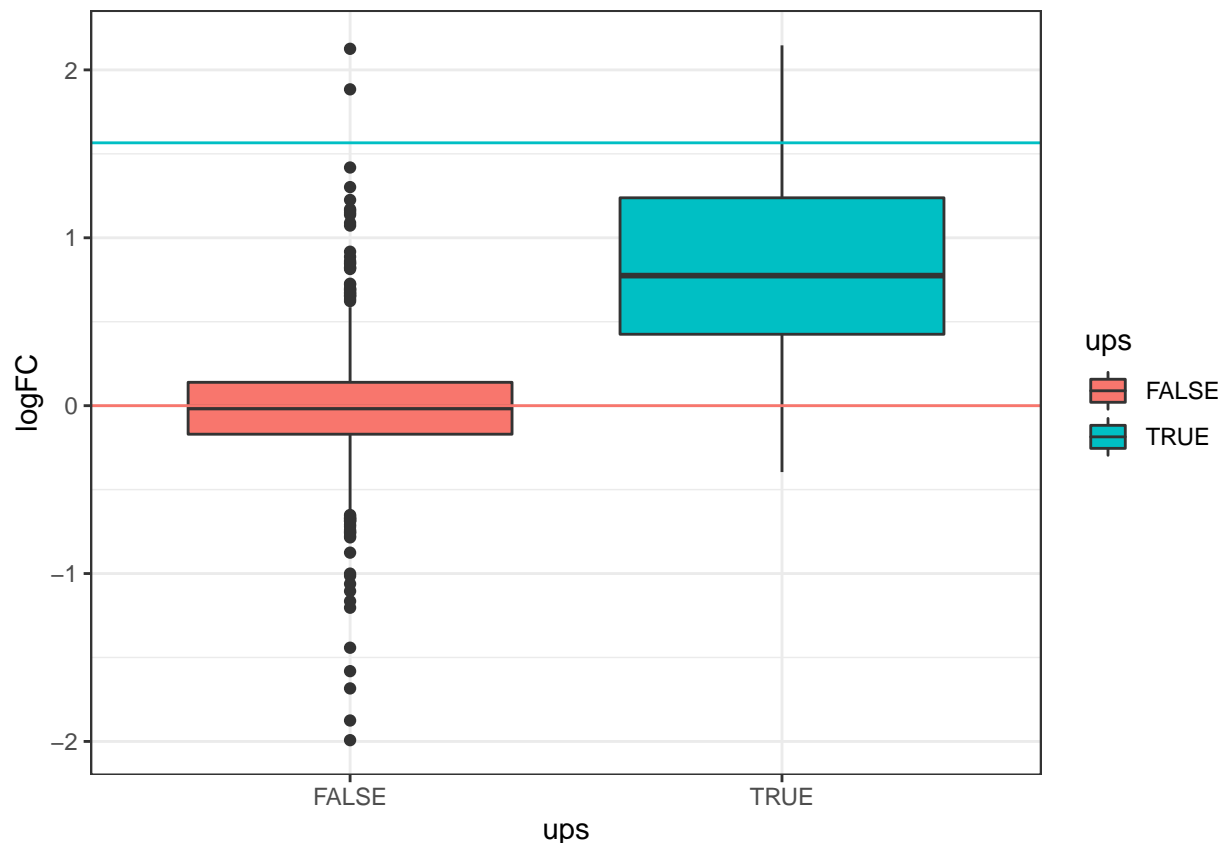


The majority of the proteins are indeed UPS proteins. 1 yeast protein is returned. Note, that the yeast protein indeed shows evidence for differential abundance.

6.2.3.3 Boxplots We create a boxplot of the log2 FC and group according to the whether a protein is spiked or not.

```
rowData(pe[["protein_median"]])$conditionB %>%
  rownames_to_column(var = "protein") %>%
  mutate(ups=grepl("UPS",protein)) %>%
  ggplot(aes(x=ups, y =logFC, fill = ups)) +
  geom_boxplot() +
  theme_bw() +
  geom_hline(yintercept = log2(0.74 / .25), color = "#00BFC4") +
  geom_hline(yintercept = 0, color = "#F8766D")
```

Warning: Removed 166 rows containing non-finite values (stat_boxplot).



7 Robust summarization

7.1 Preprocessing

- By default robust summarization is used: `fun = MsCoreUtils::robustSummary()`
- Structure from QFeatures is usefull here. No need to rerun any of the previous log transformation or normalization.

```
pe <- aggregateFeatures(pe,
  i = "peptideNorm",
  fcol = "Proteins",
  na.rm = TRUE,
  name = "protein_robust",
  fun = MsCoreUtils::robustSummary)
```

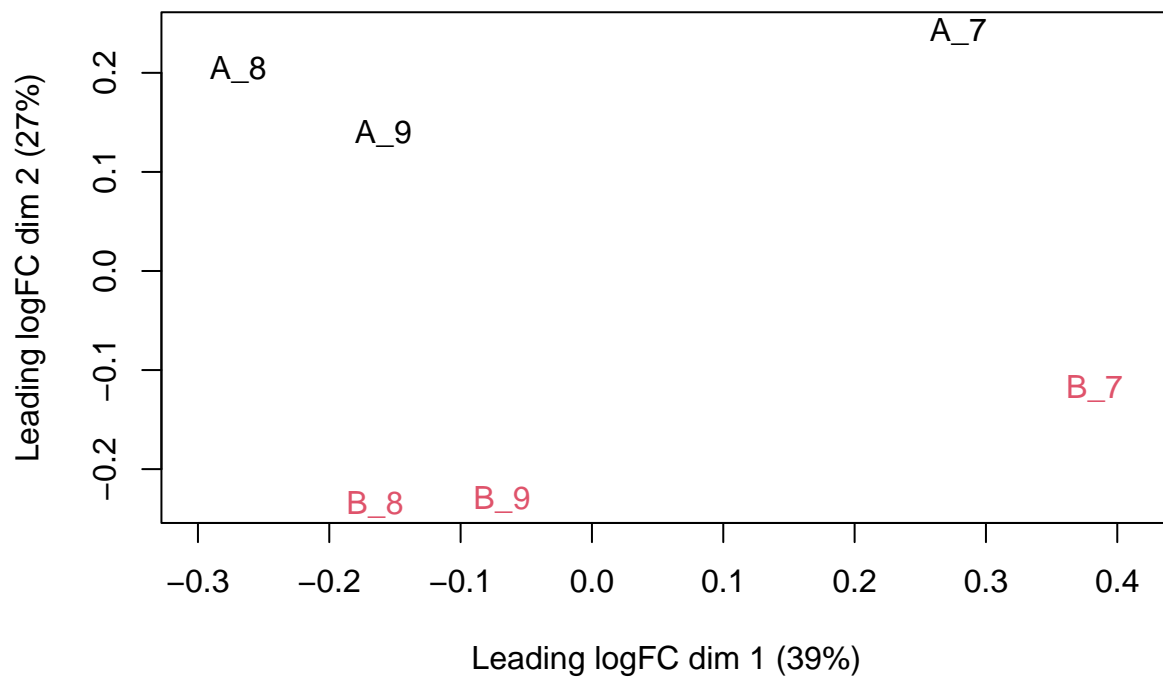
```
## Your quantitative and row data contain missing values. Please read the
## relevant section(s) in the aggregateFeatures manual page regarding the
## effects of missing values on data aggregation.
```

Now we have both the `protein_median` and `protein_robust` in one QFeatures object.

```
pe
```

```
## An instance of class QFeatures containing 5 assays:  
## [1] peptideRaw: SummarizedExperiment with 7011 rows and 6 columns  
## [2] peptideLog: SummarizedExperiment with 7011 rows and 6 columns  
## [3] peptideNorm: SummarizedExperiment with 7011 rows and 6 columns  
## [4] protein_median: SummarizedExperiment with 1389 rows and 6 columns  
## [5] protein_robust: SummarizedExperiment with 1389 rows and 6 columns
```

```
tmp <- assay(pe[["protein_robust"]])  
colnames(tmp) <- str_replace_all(colnames(tmp), "Intensity.6", "")  
tmp %>%  
  limma::plotMDS(col = as.numeric(colData(pe)$condition))
```



Note that the samples upon robust summarisation show a clear separation according to the spike-in condition in the second dimension of the MDS plot.

7.2 Data Analysis

7.2.1 Estimation

We model the protein level expression values using `msqrob`. By default `msqrob2` estimates the model parameters using robust regression.

We will model the data with a different group mean. The group is incoded in the variable `condition` of the `colData`. We can specify this model by using a formula with the factor `condition` as its predictor: `formula = ~condition`.

Note, that a formula always starts with a symbol `'~'`.

```
pe <- msqrob(object = pe, i = "protein_robust", formula = ~condition)
```

7.2.2 Inference

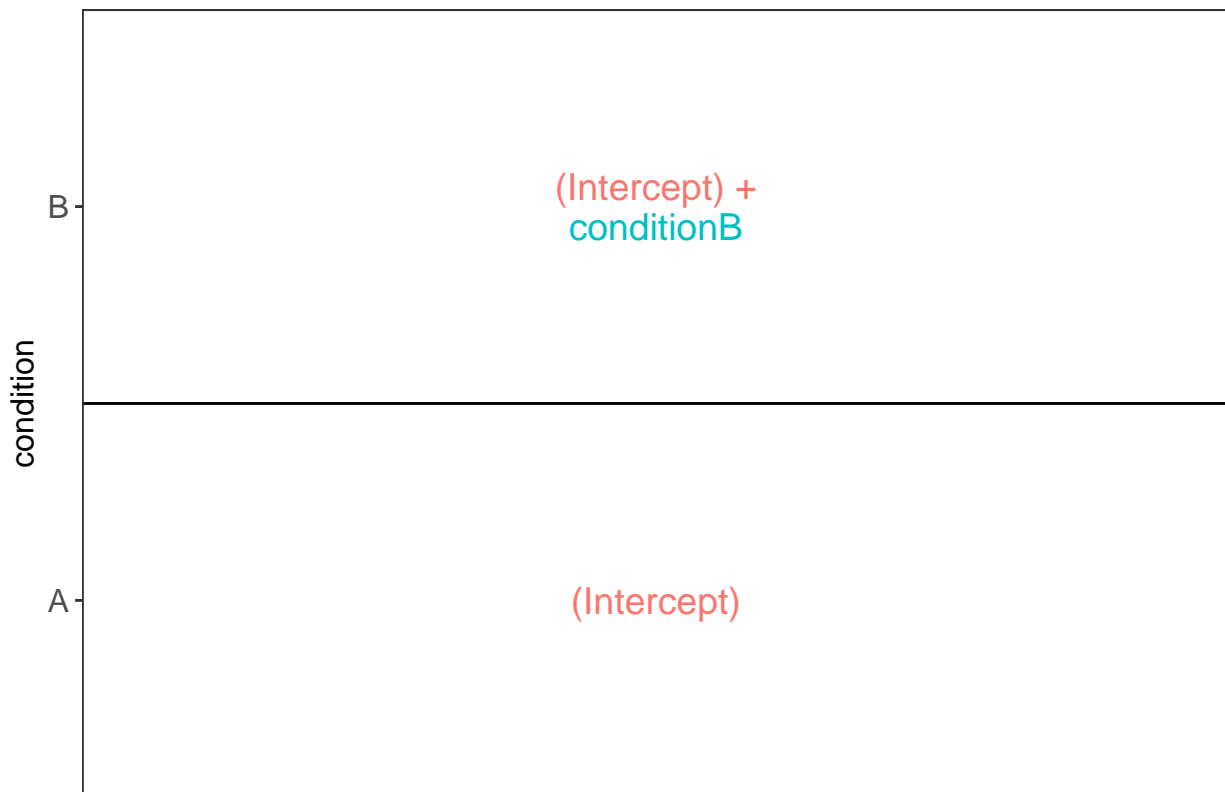
First, we extract the parameter names of the model by looking at the first model. The models are stored in the row data of the assay under the default name `msqrobModels`.

```
getCoef(rowData(pe[["protein_robust"]])$msqrobModels[[1]])
```

```
## (Intercept) conditionB  
## -2.672396 1.513682
```

We can also explore the design of the model that we specified using the the package `ExploreModelMatrix`

```
library(ExploreModelMatrix)  
VisualizeDesign(colData(pe), ~condition)$plotlist[[1]]
```



Spike-in condition A is the reference class. So the mean log2 expression for samples from condition A is `'(Intercept)'`. The mean log2 expression for samples from condition B is `'(Intercept)+conditionB'`. Hence, the

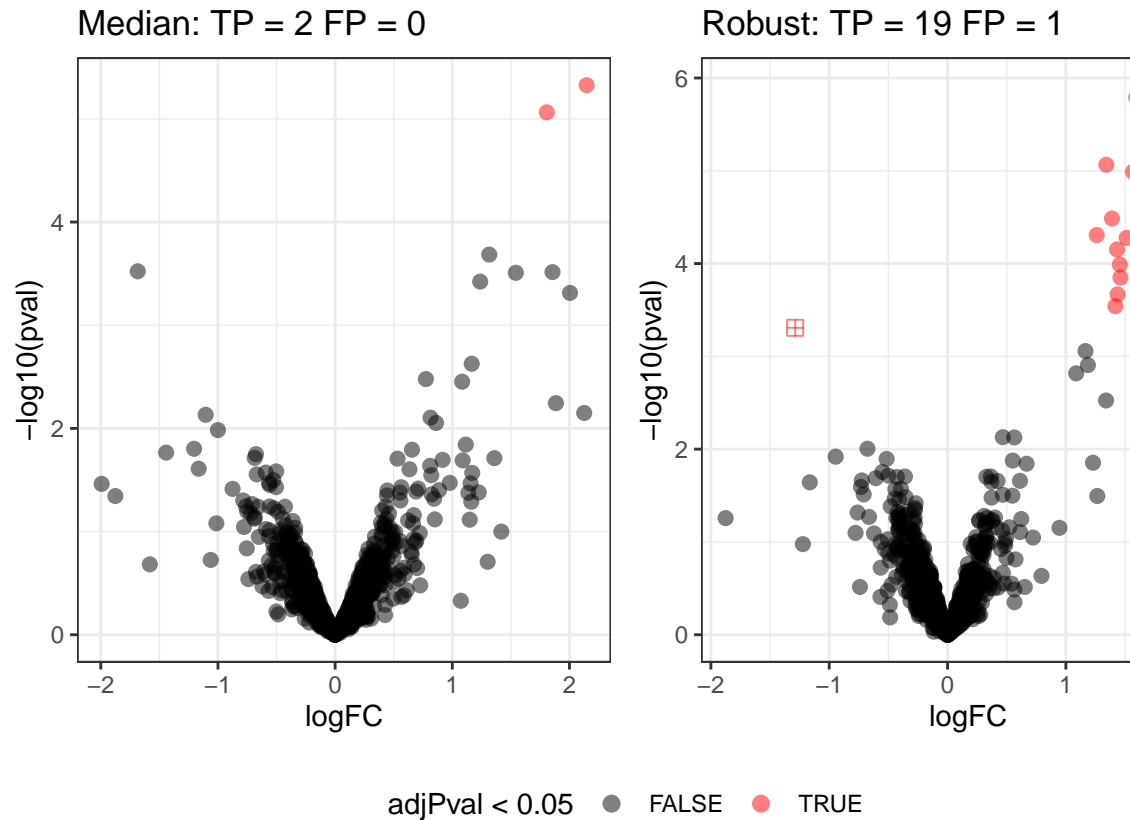
average log2 fold change between condition b and condition a is modelled using the parameter 'conditionB'. Thus, we assess the contrast 'conditionB = 0' with our statistical test.

```
L <- makeContrast("conditionB=0", parameterNames = c("conditionB"))
pe <- hypothesisTest(object = pe, i = "protein_robust", contrast = L)
```

7.2.3 Plots

```
tmp <- rowData(pe[["protein_robust"]])$conditionB[complete.cases(rowData(pe[["protein_robust"]])$conditionB)]
tmp$shapes <- 16
tmp[!grepl("UPS",rownames(tmp)) & tmp$adjPval < 0.05,]$shapes <- 12
#FP <-tmp[!grepl("UPS",rownames(tmp)) & tmp$adjPval < 0.05,]
volcanoRobust<- ggplot(tmp,
  aes(x = logFC, y = -log10(pval), color = adjPval < 0.05)) +
  geom_point(cex = 2.5, shape = tmp$shapes) +
  #geom_point(x =FP$logFC, y = -log10(FP$pval), shape = 8, size = 4 )+
  scale_color_manual(values = alpha(c("black", "red"), 0.5)) +
  theme_bw() +
  ggtitle(paste0("Robust: TP = ",sum(tmp$adjPval<0.05&grepl(rownames(tmp),pattern ="UPS"),na.rm=TRUE), " "))

plot_grid(plot_grid(volcanoMedian+theme(legend.position = "none"),
  volcanoRobust+theme(legend.position = "none")), get_legend(volcanoRobust + theme(legend.position = "none")))
```

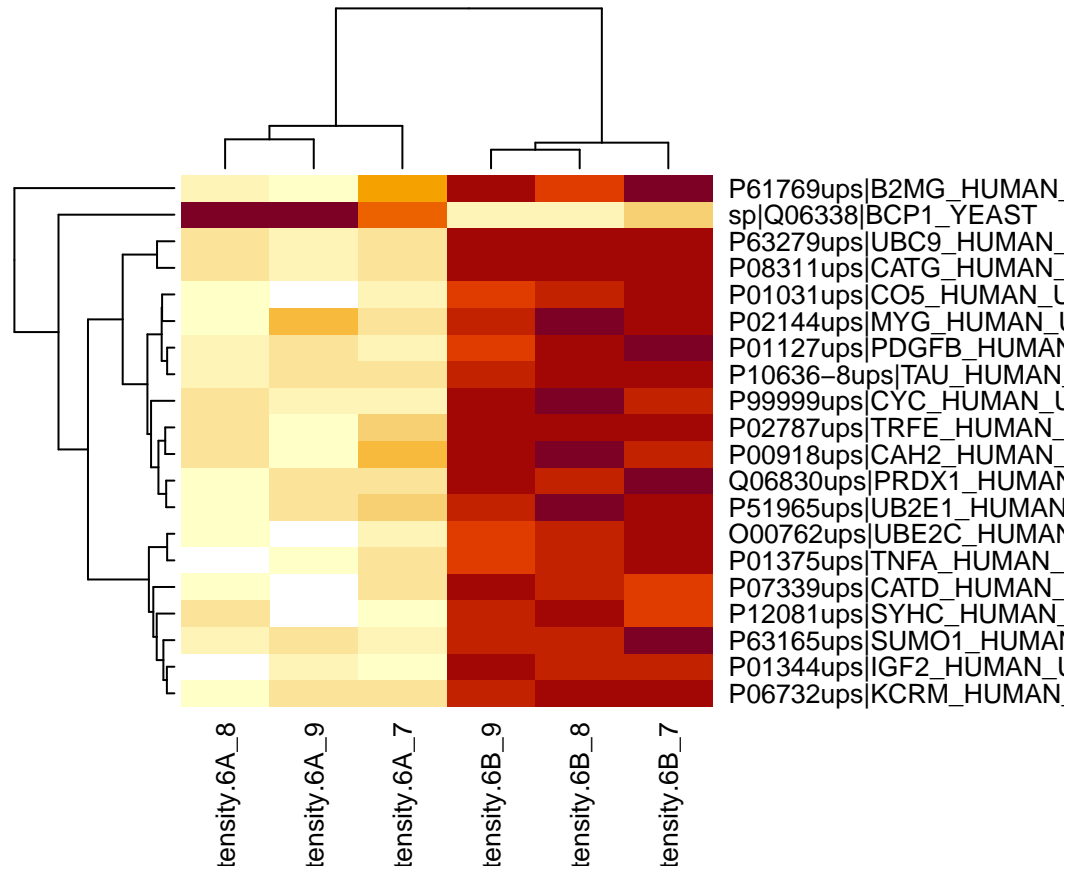



7.2.3.1 Volcano-plot

Note, that 20 proteins are found to be differentially abundant.

7.2.3.2 Heatmap We first select the names of the proteins that were declared significant.

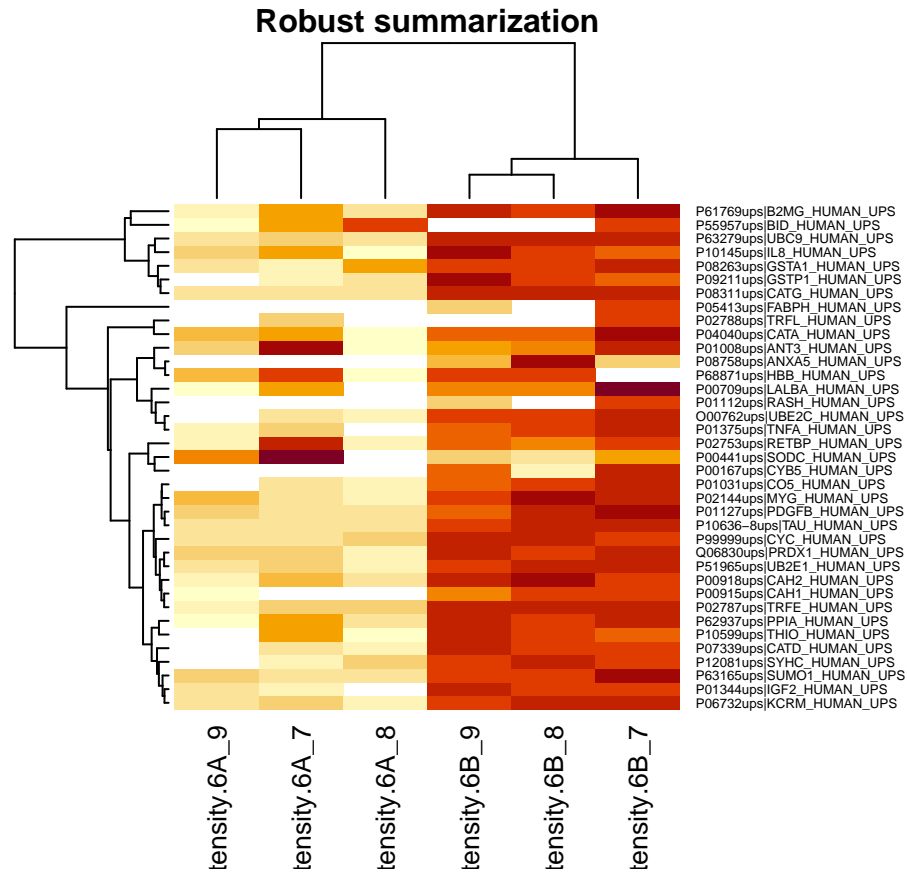
```
sigNames <- rowData(pe[["protein_robust"]])$conditionB %>%
  rownames_to_column("protein_robust") %>%
  filter(adjPval<0.05) %>%
  pull(protein_robust)
heatmap(assay(pe[["protein_robust"]])[sigNames, ],cexCol = 1)
```



The majority of the proteins are indeed UPS proteins. 1 yeast protein is returned. Note, that the yeast protein indeed shows evidence for differential abundance.

heatmaps also show difference between median and robust summarization

```
par(cex.main=.8)
sigProteins <- rowData(pe[["protein_robust"]])$conditionB %>%
  rownames_to_column("protein_robust") %>%
  filter(grepl("UPS",protein_robust)) %>%
  pull(protein_robust)
heatmap(assay(pe[["protein_robust"]])[sigProteins, ], cexCol = 1,cexRow = 0.5, main = "Robust summariza
```



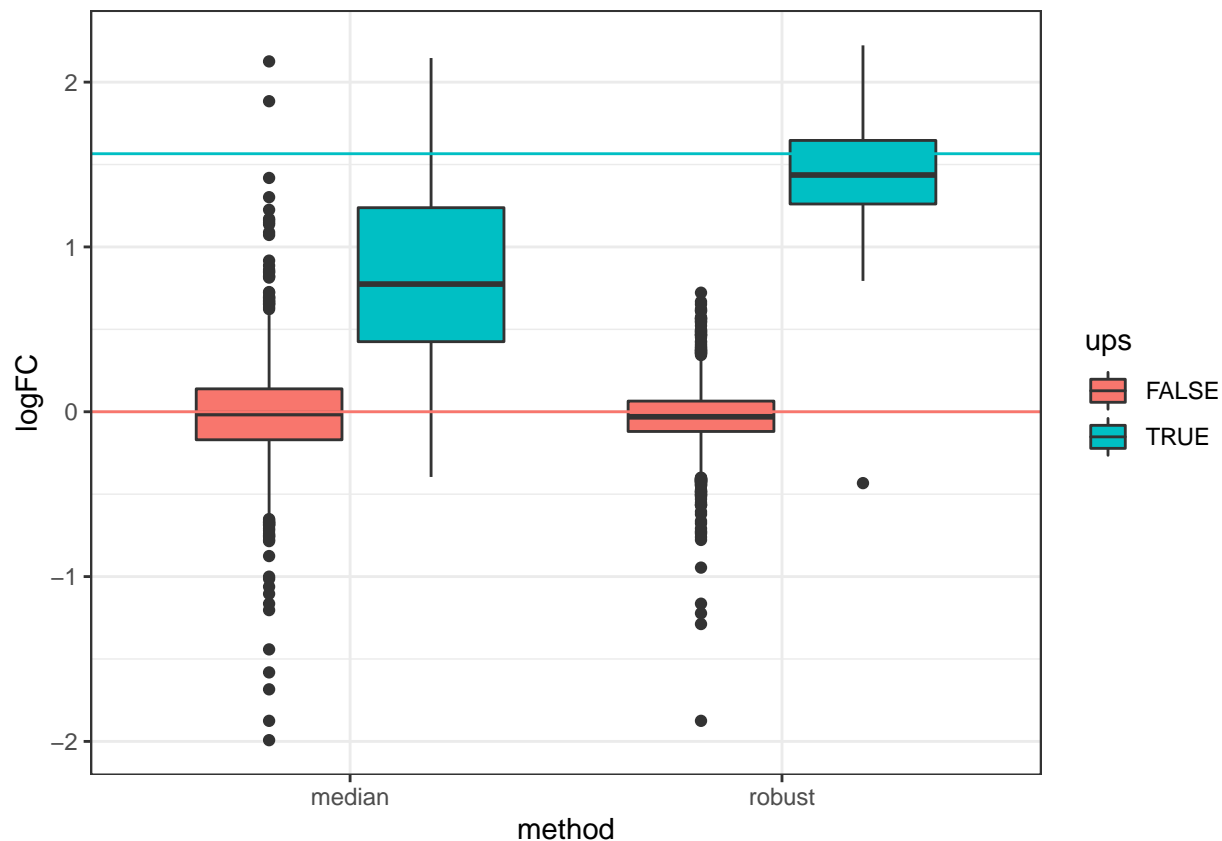
7.2.3.3 Boxplots We make boxplot of the log2 FC and stratify according to the whether a protein is spiked or not.

```

rbind(rowData(pe[["protein_robust"]])$conditionB %>%
  rownames_to_column(var = "protein") %>% mutate(method = "robust"),
  rowData(pe[["protein_median"]])$conditionB %>%
  rownames_to_column(var = "protein") %>% mutate(method = "median")) %>%
  mutate(ups=grepl("UPS",protein)) %>%
  ggplot(aes(x=method, y=logFC, fill = ups)) +
  geom_boxplot() +
  theme_bw() +
  geom_hline(yintercept = log2(0.74 / .25), color = "#00BFC4") +
  geom_hline(yintercept = 0, color = "#F8766D")

```

```
## Warning: Removed 333 rows containing non-finite values (stat_boxplot).
```



8 Where the difference comes from

8.1 Import data from the full CPTAC study

Click to see background and code

1. We use a peptides.txt file from MS-data quantified with maxquant that contains MS1 intensities summarized at the peptide level.

```
peptidesFile <- "https://raw.githubusercontent.com/statOmics/PDA/data/quantification/fullCptacDatasets/MS1/peptides.txt"
```

2. Maxquant stores the intensity data for the different samples in columns that start with Intensity. We can retrieve the column names with the intensity data with the code below:

```
ecols <- grep("Intensity\\.", names(read.delim(peptidesFile)))
```

3. Read the data and store it in QFeatures object

```
pe <- readQFeatures(
  table = peptidesFile,
  fnames = 1,
  ecol = ecols,
  name = "peptideRaw", sep="\t")
```

8.1.1 Design

[Click to see background and code](#)

```
pe %>% colnames
```

```
## CharacterList of length 1  
## [["peptideRaw"]] Intensity.6A_1 Intensity.6A_2 ... Intensity.6E_9
```

- Note, that the sample names include the spike-in condition.
- They also end on a number.
 - 1-3 is from lab 1,
 - 4-6 from lab 2 and
 - 7-9 from lab 3.
- We update the colData with information on the design

```
colData(pe)$lab <- rep(rep(paste0("lab",1:3),each=3),5) %>% as.factor  
colData(pe)$condition <- pe[["peptideRaw"]] %>% colnames %>% substr(12,12) %>% as.factor  
colData(pe)$spikeConcentration <- rep(c(A = 0.25, B = 0.74, C = 2.22, D = 6.67, E = 20),each = 9)
```

- We explore the colData

```
colData(pe)
```

```
## Dataframe with 45 rows and 3 columns  
##           lab condition spikeConcentration  
##           <factor>  <factor>           <numeric>  
## Intensity.6A_1    lab1      A             0.25  
## Intensity.6A_2    lab1      A             0.25  
## Intensity.6A_3    lab1      A             0.25  
## Intensity.6A_4    lab2      A             0.25  
## Intensity.6A_5    lab2      A             0.25  
## ...              ...      ...             ...  
## Intensity.6E_5    lab2      E             20  
## Intensity.6E_6    lab2      E             20  
## Intensity.6E_7    lab3      E             20  
## Intensity.6E_8    lab3      E             20  
## Intensity.6E_9    lab3      E             20
```

8.1.2 Preprocessing

[Click to see R-code to preprocess the data](#)

- We calculate how many non zero intensities we have for each peptide and this can be useful for filtering.

```
rowData(pe[["peptideRaw"]])$nNonZero <- rowSums(assay(pe[["peptideRaw"]]) > 0)
```

- Peptides with zero intensities are missing peptides and should be represent with a NA value rather than 0.

```
pe <- zeroIsNA(pe, "peptideRaw") # convert 0 to NA
```

- Logtransform data with base 2

```
pe <- logTransform(pe, base = 2, i = "peptideRaw", name = "peptideLog")
```

1. Handling overlapping protein groups

In our approach a peptide can map to multiple proteins, as long as there is none of these proteins present in a smaller subgroup.

```
pe <- filterFeatures(pe, ~ Proteins %in% smallestUniqueGroups(rowData(pe[["peptideLog"]])$Proteins))
```

2. Remove reverse sequences (decoys) and contaminants

We now remove the contaminants, peptides that map to decoy sequences, and proteins which were only identified by peptides with modifications.

```
pe <- filterFeatures(pe, ~Reverse != "+")
pe <- filterFeatures(pe, ~ Potential.contaminant != "+")
```

3. Drop peptides that were only identified in one sample

We keep peptides that were observed at least twice.

```
pe <- filterFeatures(pe, ~ nNonZero >=2)
nrow(pe[["peptideLog"]])
```

```
## [1] 10478
```

We keep 10478 peptides upon filtering.

8.1.3 Normalization

Click to see R-code to normalize the data

```
pe <- normalize(pe,
               i = "peptideLog",
               name = "peptideNorm",
               method = "center.median")
```

8.2 Peptide-Level view

8.2.1 Summarization

Click to see code to make plot

```

prot <- "P01031ups|CO5_HUMAN_UPS"
data <- pe[["peptideNorm"]][
  rowData(pe[["peptideNorm"]])$Proteins == prot,
  colData(pe)$lab=="lab3"] %>%
  assay %>%
  as.data.frame %>%
  rownames_to_column(var = "peptide") %>%
  gather(sample, intensity, -peptide) %>%
  mutate(condition = colData(pe)[sample,"condition"]) %>%
  na.exclude
sumPlot <- data %>%
  ggplot(aes(x = peptide, y = intensity, color = condition, group = sample, label = condition), show.legend = FALSE) +
  geom_text(show.legend = FALSE) +
  theme_minimal() +
  theme(axis.text.x = element_text(angle = 90, vjust = 0.5, hjust = 1)) +
  xlab("Peptide") +
  ylab("Intensity (log2)") +
  ggtitle(paste0("protein: ", prot))

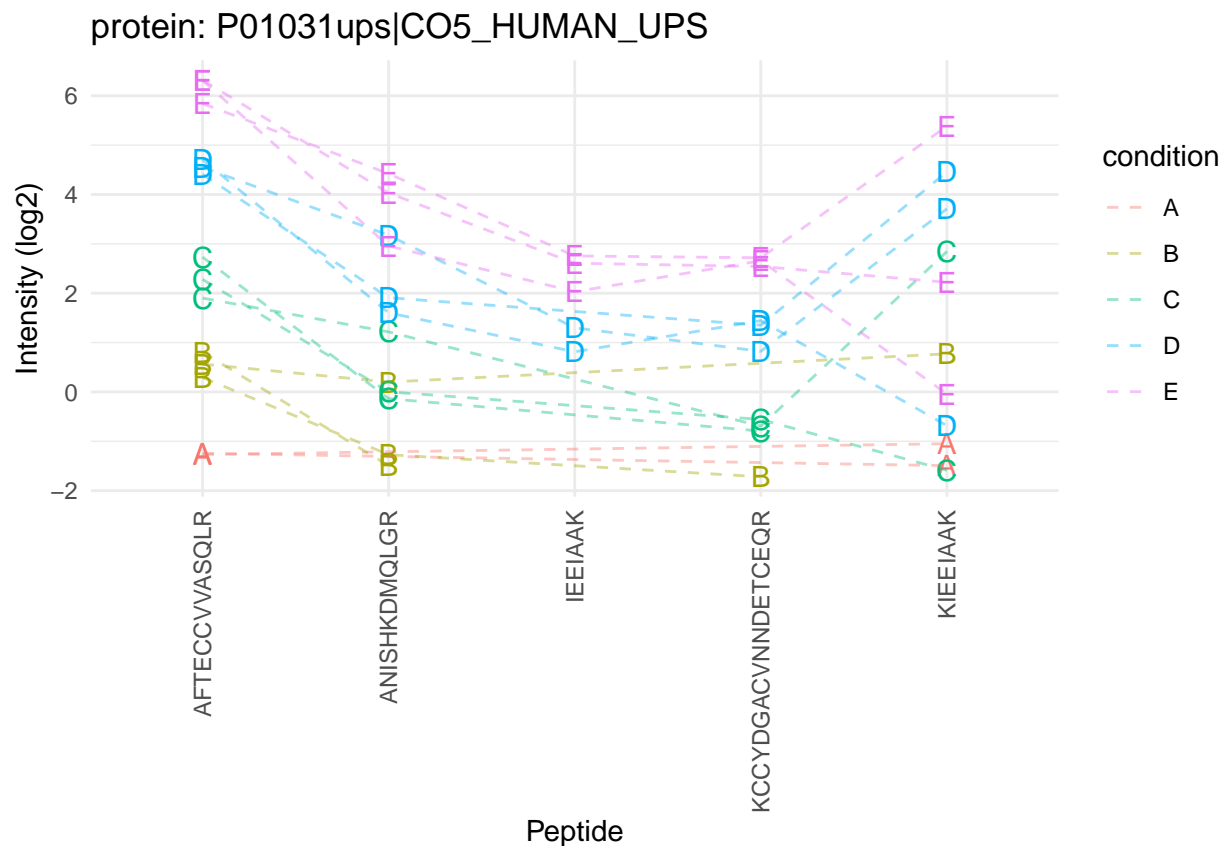
```

Here, we will focus on the summarization of the intensities for protein P01031ups|CO5_HUMAN_UPS from Lab3 for all conditions.

```

sumPlot +
  geom_line(linetype="dashed", alpha=.4)

```



8.2.1.1 Median summarization

We first evaluate median summarization for protein P01031ups|CO5_HUMAN_UPS.

Click to see code to make plot

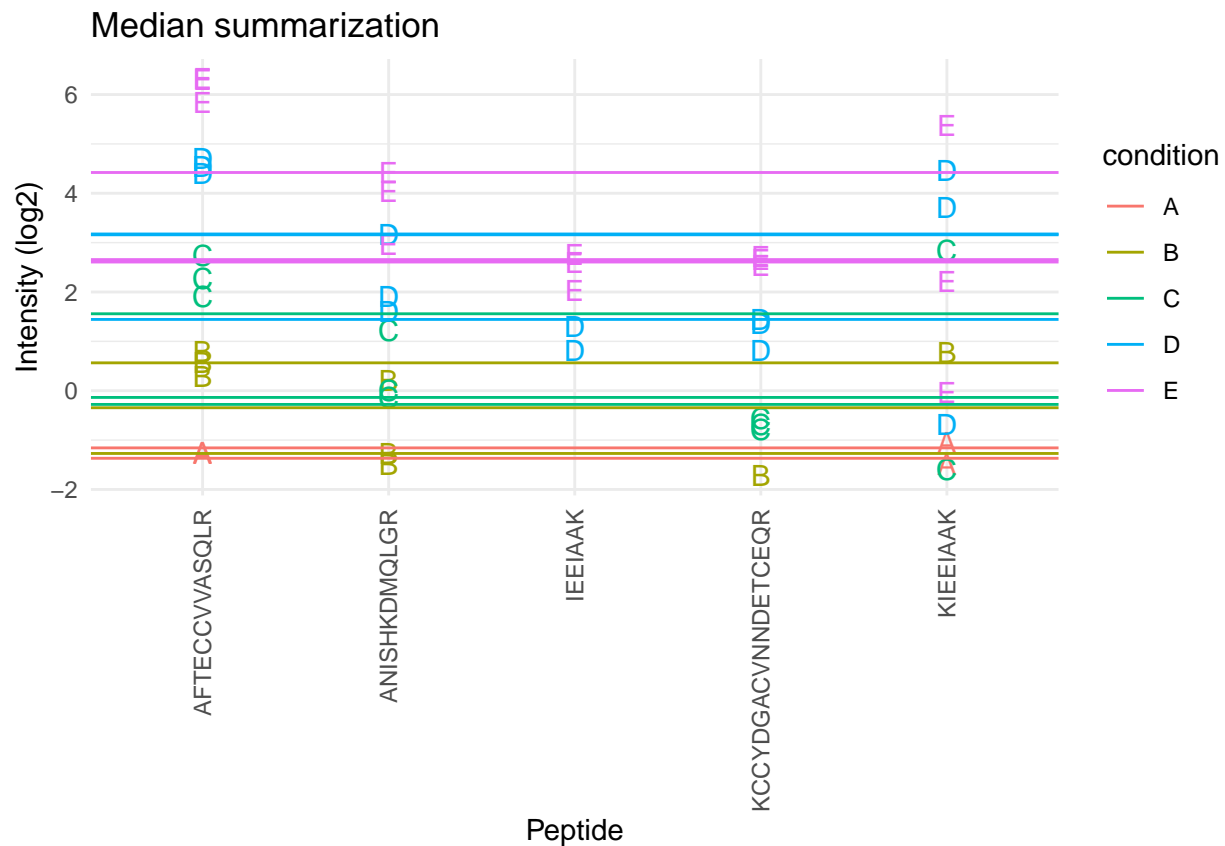
```
dataHlp <- pe[["peptideNorm"]][
  rowData(pe[["peptideNorm"]])$Proteins == prot,
  colData(pe)$lab=="lab3"] %>% assay

sumMedian <- data.frame(
  intensity= dataHlp
  %>% colMedians(na.rm=TRUE)
  ,
  condition= colnames(dataHlp) %>% substr(12,12) %>% as.factor )

sumMedianPlot <- sumPlot +
  geom_hline(
    data = sumMedian,
    mapping = aes(yintercept=intensity,color=condition)) +
  ggtitle("Median summarization")
```

sumMedianPlot

Warning: Removed 1 rows containing missing values (geom_hline).



- The sample medians are not a good estimate for the protein expression value.

- Indeed, they do not account for differences in peptide effects
- Peptides that ionize poorly are also picked up in samples with high spike-in concentration and not in samples with low spike-in concentration
- This introduces a bias.

8.2.1.2 Linear Model based summarization We can use a linear peptide-level model to estimate the protein expression value while correcting for the peptide effect, i.e.

$$y_{ip} = \beta_i^{\text{sample}} + \beta_p^{\text{peptide}} + \epsilon_{ip}$$

Click to see code to make plot

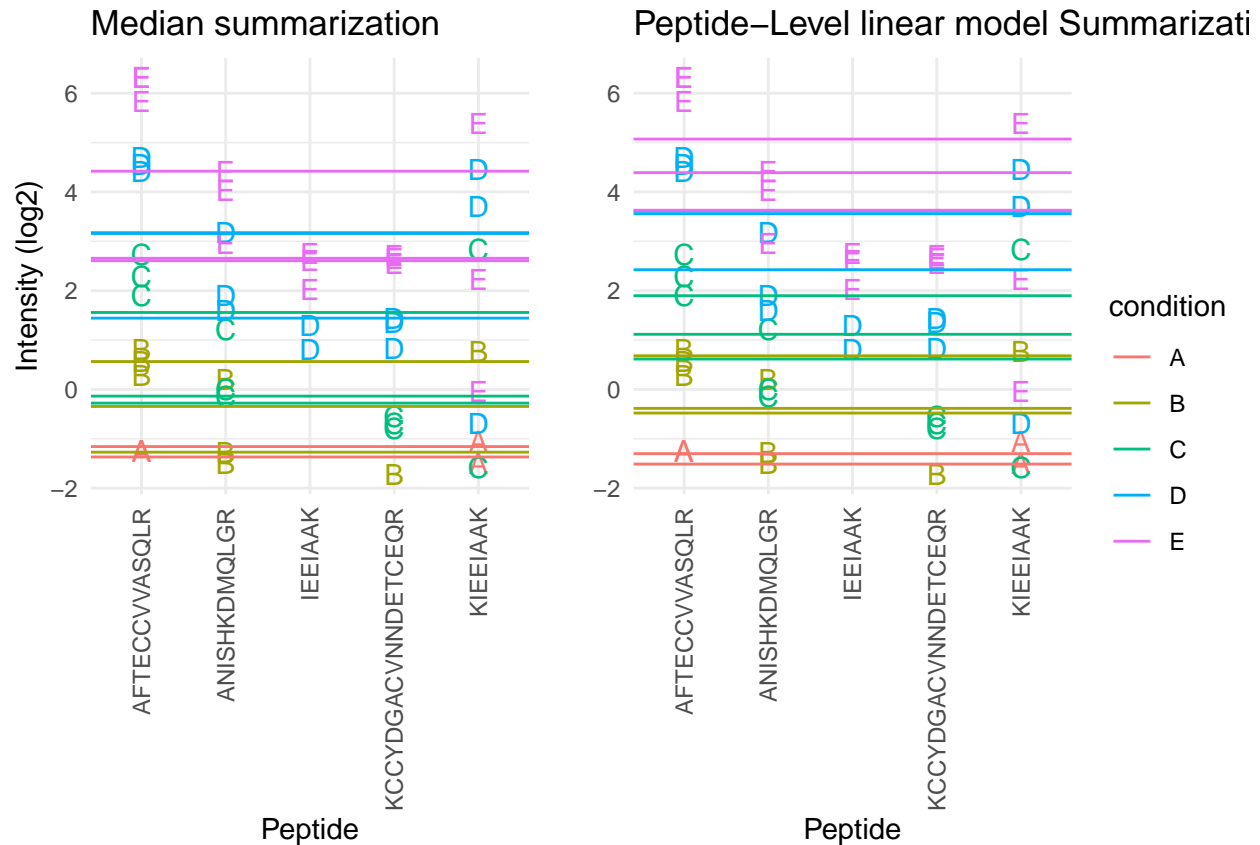
```
sumMeanPepMod <- lm(intensity ~ -1 + sample + peptide, data)

sumMeanPep <- data.frame(
  intensity=sumMeanPepMod$coef[grepl("sample", names(sumMeanPepMod$coef))] + mean(data$intensity) - mean(
  condition= names(sumMeanPepMod$coef)[grepl("sample", names(sumMeanPepMod$coef))] %>% substr(18, 18) %>%

fitLmPlot <- sumPlot + geom_line(
  data = data %>% mutate(fit=sumMeanPepMod$fitted.values),
  mapping = aes(x=peptide, y=fit, color=condition, group=sample)) +
  ggtitle("fit: ~ sample + peptide")
sumLmPlot <- sumPlot + geom_hline(
  data = sumMeanPep,
  mapping = aes(yintercept=intensity, color=condition)) +
  ggtitle("Peptide-Level linear model Summarization")

plot_grid(plot_grid(sumMedianPlot+theme(legend.position = "none"),
  sumLmPlot+theme(legend.position = "none") + ylab("")),
  get_legend(sumLmPlot), ncol = 2, rel_widths = c(1, 0.15))

## Warning: Removed 1 rows containing missing values (geom_hline).
```



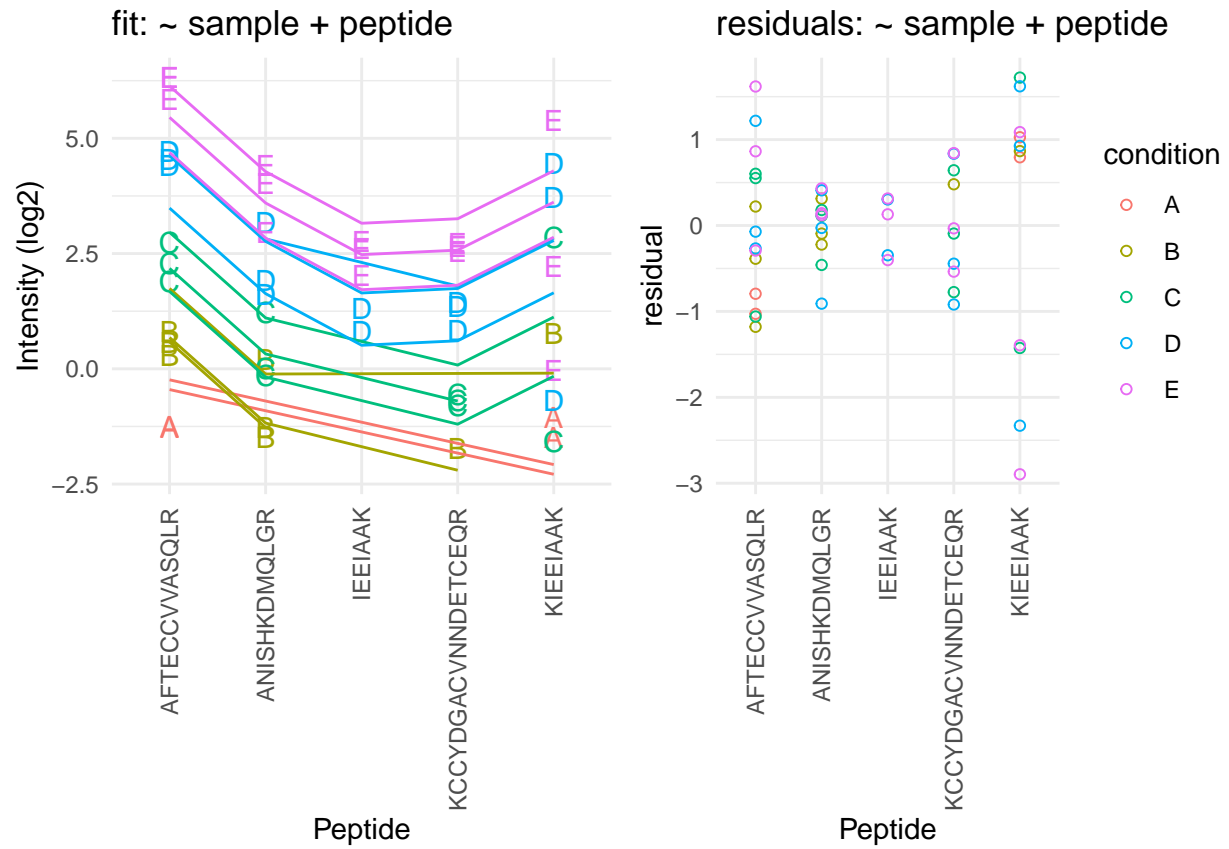
- By correcting for the peptide species the protein expression values are much better separated and better reflect differences in abundance induced by the spike-in condition.
- Indeed, it shows that median and mean summarization that do not account for the peptide effect indeed overestimate the protein expression value in the small spike-in conditions and underestimate that in the large spike-in conditions.
- Still there seem to be some issues with samples for which the expression values are not well separated according to the spike-in condition.

A residual analysis clearly indicates potential issues:

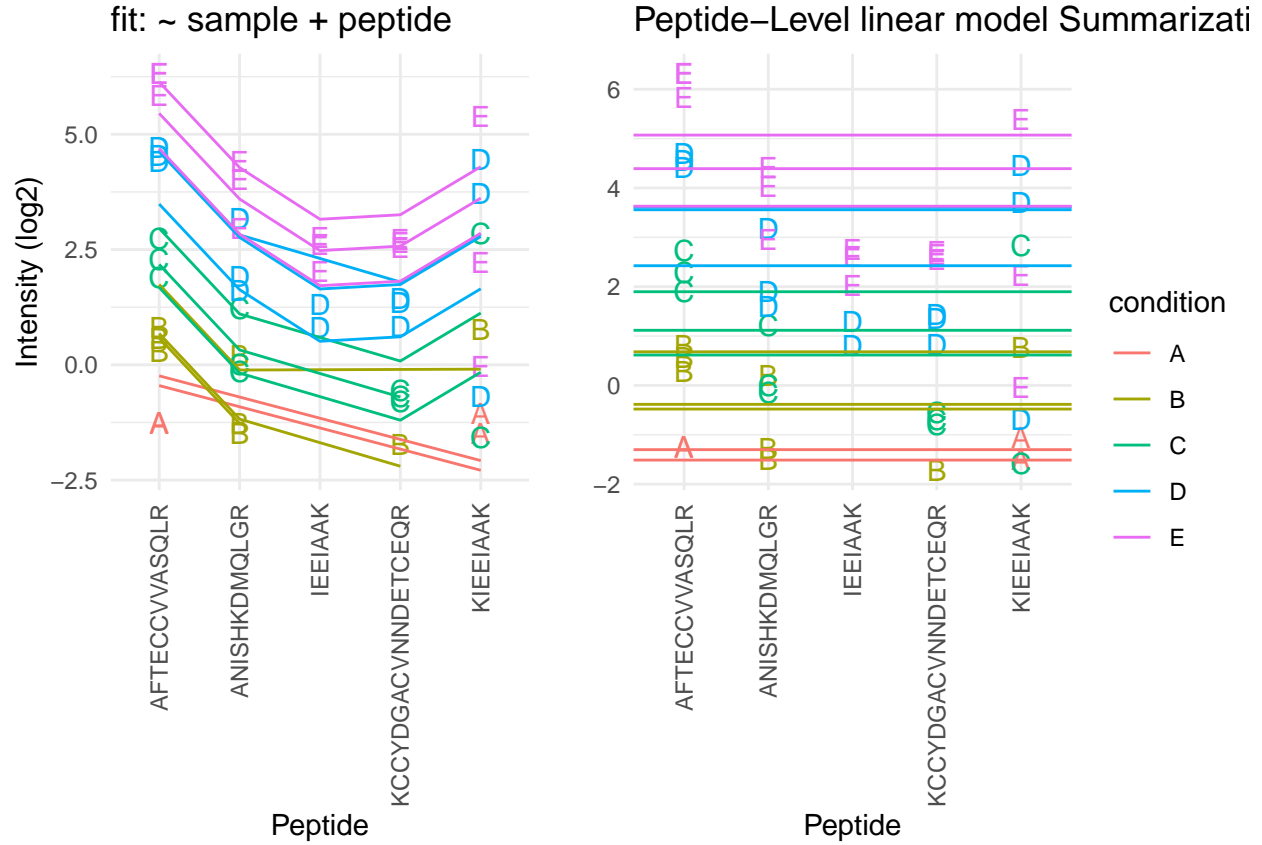
[Click to see code to make plot](#)

```
resPlot <- data %>%
  mutate(res=sumMeanPepMod$residuals) %>%
  ggplot(aes(x = peptide, y = res, color = condition, label = condition), show.legend = FALSE) +
  geom_point(shape=21) +
  theme_minimal() +
  theme(axis.text.x = element_text(angle = 90, vjust = 0.5, hjust = 1)) +
  xlab("Peptide") +
  ylab("residual") +
  ggtitle("residuals: ~ sample + peptide")

grid.arrange(fitLmPlot+theme(legend.position = "none"), resPlot, nrow = 1)
```



```
plot_grid(plot_grid(fitLmPlot+theme(legend.position = "none"),
  sumLmPlot+theme(legend.position = "none") + ylab("")),
  get_legend(sumLmPlot), ncol = 2, rel_widths = c(1,0.15))
```



- The residual plot shows some large outliers for peptide KIEEIAAK.
- Indeed, in the original plot the intensities for this peptide do not seem to line up very well with the concentration.
- This induces a bias in the summarization for some of the samples (e.g. for D and E)

8.2.1.3 Robust summarization using a peptide-level linear model

$$y_{ip} = \beta_i^{\text{sample}} + \beta_p^{\text{peptide}} + \epsilon_{ip}$$

- Ordinary least squares: estimate β that minimizes

$$\text{OLS} : \sum_{i,p} \epsilon_{ip}^2 = \sum_{i,p} (y_{ip} - \beta_i^{\text{sample}} - \beta_p^{\text{peptide}})^2$$

We replace OLS by M-estimation with loss function

$$\sum_{i,p} w_{ip} \epsilon_{ip}^2 = \sum_{i,p} w_{ip} (y_{ip} - \beta_i^{\text{sample}} - \beta_p^{\text{peptide}})^2$$

- Iteratively fit model with observation weights w_{ip} until convergence
- The weights are calculated based on standardized residuals

[Click to see code to make plot](#)

```

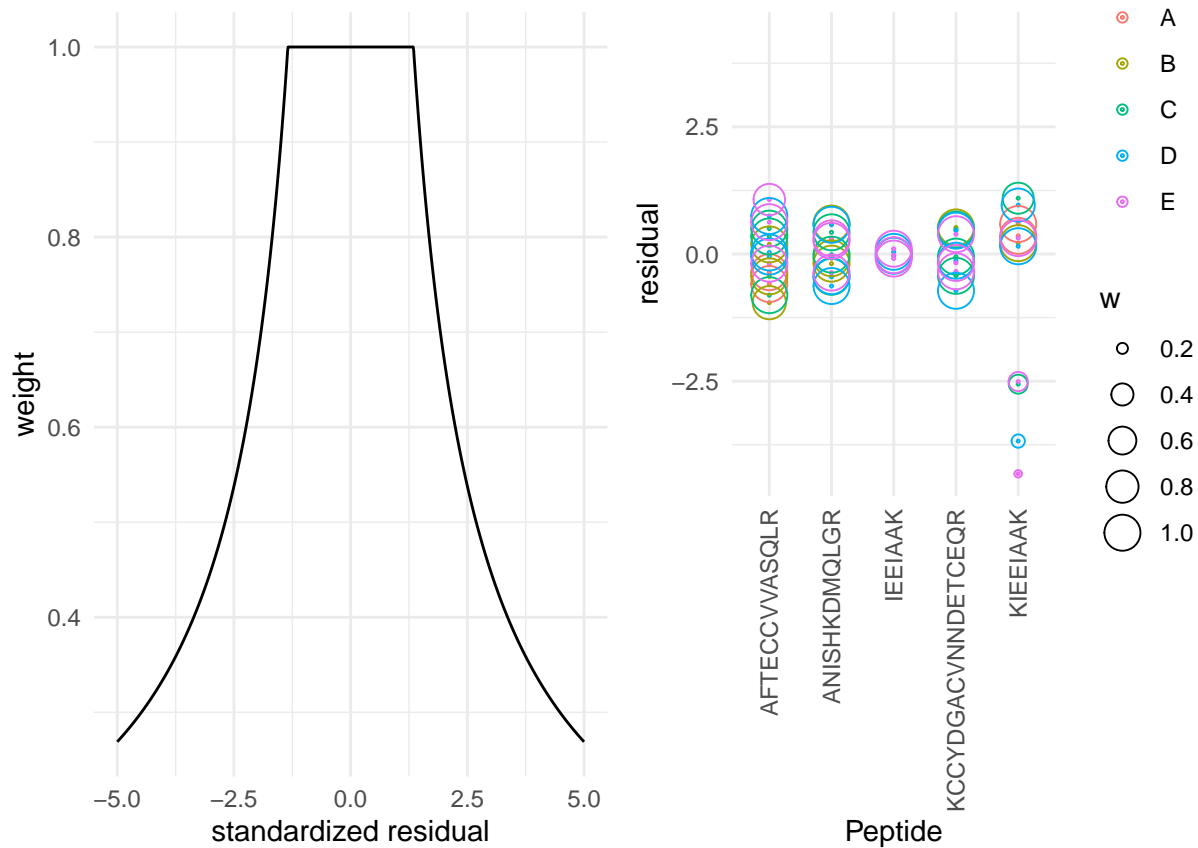
sumMeanPepRobMod <- MASS::rlm(intensity ~ -1 + sample + peptide,data)
resRobPlot <- data %>%
  mutate(res = sumMeanPepRobMod$residuals,
         w = sumMeanPepRobMod$w) %>%
  ggplot(aes(x = peptide, y = res, color = condition, label = condition,size=w), show.legend = FALSE) +
  geom_point(shape=21,size=.2) +
  geom_point(shape=21) +
  theme_minimal() +
  theme(axis.text.x = element_text(angle = 90, vjust = 0.5, hjust = 1)) +
  xlab("Peptide") +
  ylab("residual") +
  ylim(c(-1,1)*max(abs(sumMeanPepRobMod$residuals)))
weightPlot <- qplot(
  seq(-5,5,.01),
  MASS::psi.huber(seq(-5,5,.01)),
  geom="path") +
  xlab("standardized residual") +
  ylab("weight") +
  theme_minimal()

```

```

grid.arrange(weightPlot,resRobPlot,nrow=1)

```



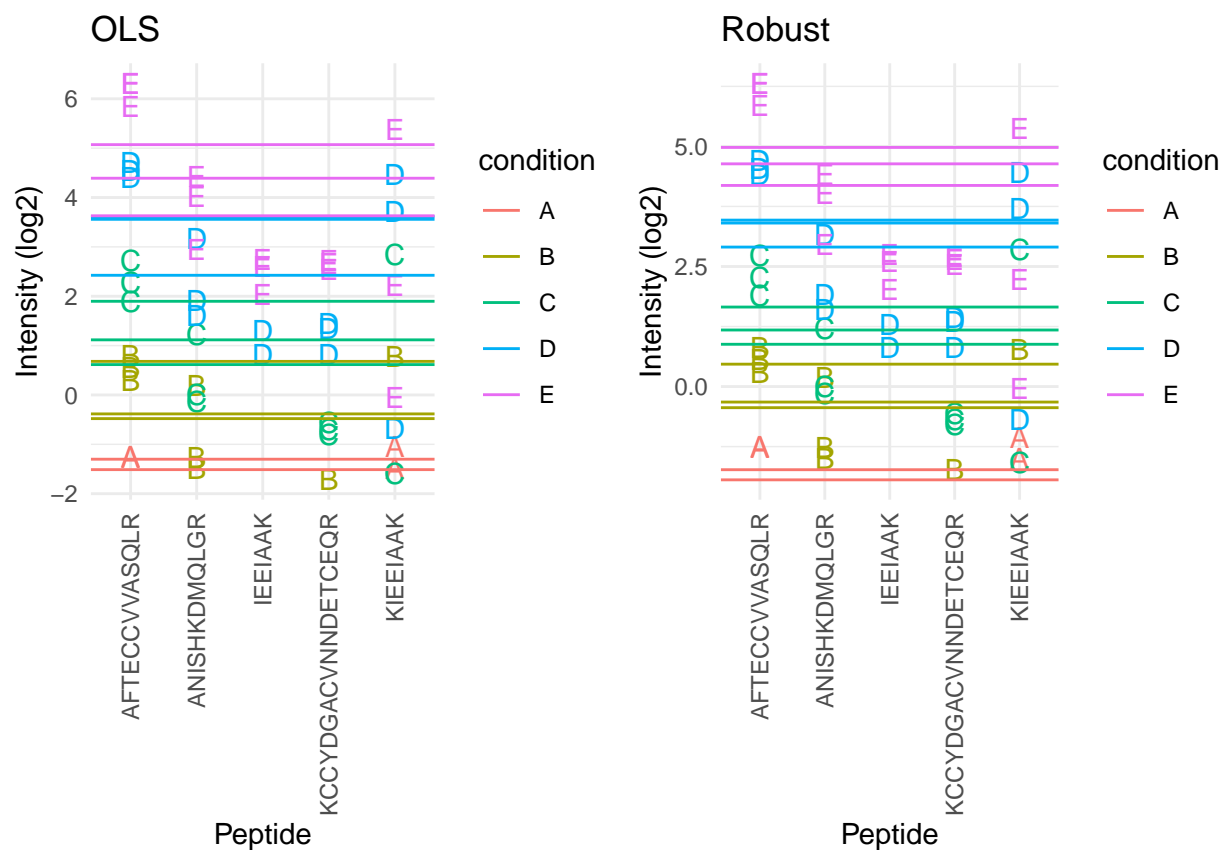
- We clearly see that the weights in the M-estimation procedure will down-weight errors associated with outliers for peptide KIEEIAAK.

Click to see code to make plot

```
sumMeanPepRob <- data.frame(
  intensity=sumMeanPepRobMod$coef[grepl("sample",names(sumMeanPepRobMod$coef))] + mean(data$intensity) -
  condition= names(sumMeanPepRobMod$coef)[grepl("sample",names(sumMeanPepRobMod$coef))] %>% substr(18,18,1)

sumRlmPlot <- sumPlot + geom_hline(
  data=sumMeanPepRob,
  mapping=aes(yintercept=intensity,color=condition)) +
  ggtitle("Robust")

grid.arrange(sumLmPlot + ggtitle("OLS"), sumRlmPlot, nrow = 1)
```



- Robust regression results in a better separation between the protein expression values for the different samples according to their spike-in concentration.

9 Estimation of differential abundance using peptide level model

- Instead of summarising the data we can also directly model the data at the peptide-level.
- But, we will have to address the pseudo-replication.

$$y_{iclp} = \beta_0 + \beta_c^{\text{condition}} + \beta_l^{\text{lab}} + \beta_p^{\text{peptide}} + u_s^{\text{sample}} + \epsilon_{iclp}$$

- protein-level
 - $\beta_c^{\text{condition}}$: spike-in condition $c = b, \dots, e$
 - β_l^{lab} : lab effect $l = l_2 \dots l_3$
 - $u_r^{\text{run}} \sim N(0, \sigma_{\text{run}}^2) \rightarrow$ random effect addresses pseudo-replication
- peptide-level
 - β_p^{peptide} : peptide effect
 - $\epsilon_{rp} \sim N(0, \sigma_\epsilon^2)$ within sample (run) error
- DA estimates:

$$\log_2 FC_{B-A} = \beta_B^{\text{condition}}$$

$$\log_2 FC_{C-B} = \beta_C^{\text{condition}} - \beta_B^{\text{condition}}$$

- Mixed peptide-level models are implemented in msqrob2
- It has the advantages that
 1. it correctly addresses the difference levels of variability in the data
 2. it avoids summarization and therefore also accounts for the difference in the number of peptides that are observed in each sample
 3. more powerful analysis
- It has the disadvantage that
 1. protein summaries are no longer available for plotting
 2. it is difficult to correctly specify the degrees of freedom for the test-statistic leading to inference that is too liberal in experiments with small sample size
 3. sometimes sample level random effect variance are estimated to be zero, then the pseudo-replication is not addressed leading to inference that is too liberal for these specific proteins
 4. they are much more difficult to disseminate to users with limited background in statistics

Hence, for this course we opted to use peptide-level models for summarization, but not for directly inferring on the differential expression at the protein-level.

10 Session Info

With respect to reproducibility, it is highly recommended to include a session info in your script so that readers of your output can see your particular setup of R.

```
sessionInfo()

## R version 4.1.3 (2022-03-10)
## Platform: x86_64-linux-gnu (64-bit)
## Running under: Ubuntu 20.04.5 LTS
##
## Matrix products: default
## BLAS: /usr/lib/x86_64-linux-gnu/blas/libblas.so.3.9.0
## LAPACK: /usr/lib/x86_64-linux-gnu/lapack/liblapack.so.3.9.0
##
```

```

## locale:
## [1] LC_CTYPE=C.UTF-8      LC_NUMERIC=C          LC_TIME=C.UTF-8
## [4] LC_COLLATE=C.UTF-8    LC_MONETARY=C.UTF-8   LC_MESSAGES=C.UTF-8
## [7] LC_PAPER=C.UTF-8      LC_NAME=C             LC_ADDRESS=C
## [10] LC_TELEPHONE=C        LC_MEASUREMENT=C.UTF-8 LC_IDENTIFICATION=C
##
## attached base packages:
## [1] stats4      stats      graphics  grDevices datasets  utils      methods
## [8] base
##
## other attached packages:
## [1] ExploreModelMatrix_1.6.0  gridExtra_2.3
## [3] cowplot_1.1.1             plotly_4.10.0
## [5] msqrob2_1.2.0             QFeatures_1.4.0
## [7] MultiAssayExperiment_1.20.0 SummarizedExperiment_1.24.0
## [9] Biobase_2.54.0            GenomicRanges_1.46.1
## [11] GenomeInfoDb_1.30.1      IRanges_2.28.0
## [13] S4Vectors_0.32.4         BiocGenerics_0.40.0
## [15] MatrixGenerics_1.6.0     matrixStats_0.62.0
## [17] limma_3.50.3             forcats_0.5.2
## [19] stringr_1.4.1            dplyr_1.0.10
## [21] purrr_0.3.4              readr_2.1.2
## [23] tidyr_1.2.0              tibble_3.1.8
## [25] ggplot2_3.3.6            tidyverse_1.3.2
##
## loaded via a namespace (and not attached):
## [1] googledrive_2.0.0        minqa_1.2.4             colorspace_2.0-3
## [4] ellipsis_0.3.2          XVector_0.34.0          fs_1.5.2
## [7] clue_0.3-61             farver_2.1.1            DT_0.23
## [10] fansi_1.0.3             lubridate_1.8.0         xml2_1.3.3
## [13] codetools_0.2-18        splines_4.1.3           cachem_1.0.6
## [16] knitr_1.40              jsonlite_1.8.0          nloptr_2.0.3
## [19] broom_1.0.1            cluster_2.1.2           dbplyr_2.2.1
## [22] shinydashboard_0.7.2    shiny_1.7.2            BiocManager_1.30.18
## [25] compiler_4.1.3          http_1.4.4              backports_1.4.1
## [28] assertthat_0.2.1        Matrix_1.3-4            fastmap_1.1.0
## [31] lazyeval_0.2.2          gargle_1.2.0            cli_3.3.0
## [34] later_1.3.0             htmltools_0.5.3         tools_4.1.3
## [37] igraph_1.3.4            gtable_0.3.1           glue_1.6.2
## [40] GenomeInfoDbData_1.2.7  Rcpp_1.0.8              cellranger_1.1.0
## [43] jquerylib_0.1.4         vctrs_0.4.1            nlme_3.1-153
## [46] rintrojs_0.3.2          xfun_0.32              lme4_1.1-30
## [49] rvest_1.0.3            mime_0.12               lifecycle_1.0.1
## [52] renv_0.15.4             googlesheets4_1.0.1     zlibbioc_1.40.0
## [55] MASS_7.3-54            scales_1.2.1            promises_1.2.0.1
## [58] hms_1.1.2              ProtGenerics_1.26.0     parallel_4.1.3
## [61] AnnotationFilter_1.18.0 yaml_2.3.5              sass_0.4.2
## [64] stringi_1.7.6          highr_0.9               boot_1.3-28
## [67] BiocParallel_1.28.3     rlang_1.0.5             pkgconfig_2.0.3
## [70] bitops_1.0-7           evaluate_0.16           lattice_0.20-45
## [73] labeling_0.4.2         htmlwidgets_1.5.4      tidyselect_1.1.2
## [76] magrittr_2.0.3         R6_2.5.1                generics_0.1.3
## [79] DelayedArray_0.20.0    DBI_1.1.2              pillar_1.8.1
## [82] haven_2.5.1            withr_2.5.0            MsCoreUtils_1.6.2

```


## [85] RCurl_1.98-1.5	modelr_0.1.9	crayon_1.5.1
## [88] utf8_1.2.2	tzdb_0.3.0	rmarkdown_2.16
## [91] grid_4.1.3	readxl_1.4.1	data.table_1.14.2
## [94] reprex_2.0.2	digest_0.6.29	xtable_1.8-4
## [97] httpuv_1.6.5	munsell_0.5.0	viridisLite_0.4.1
## [100] bslib_0.4.0	shinyjs_2.1.0	