Sources of variability in label-free proteomics experiments

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Contents

This is part of the online course Experimental Design and Data-Analysis in Label-Free Quantitative LC/MS Proteomics - A Tutorial with msqrob2 (hupo21)

Click to see libraries that are loaded

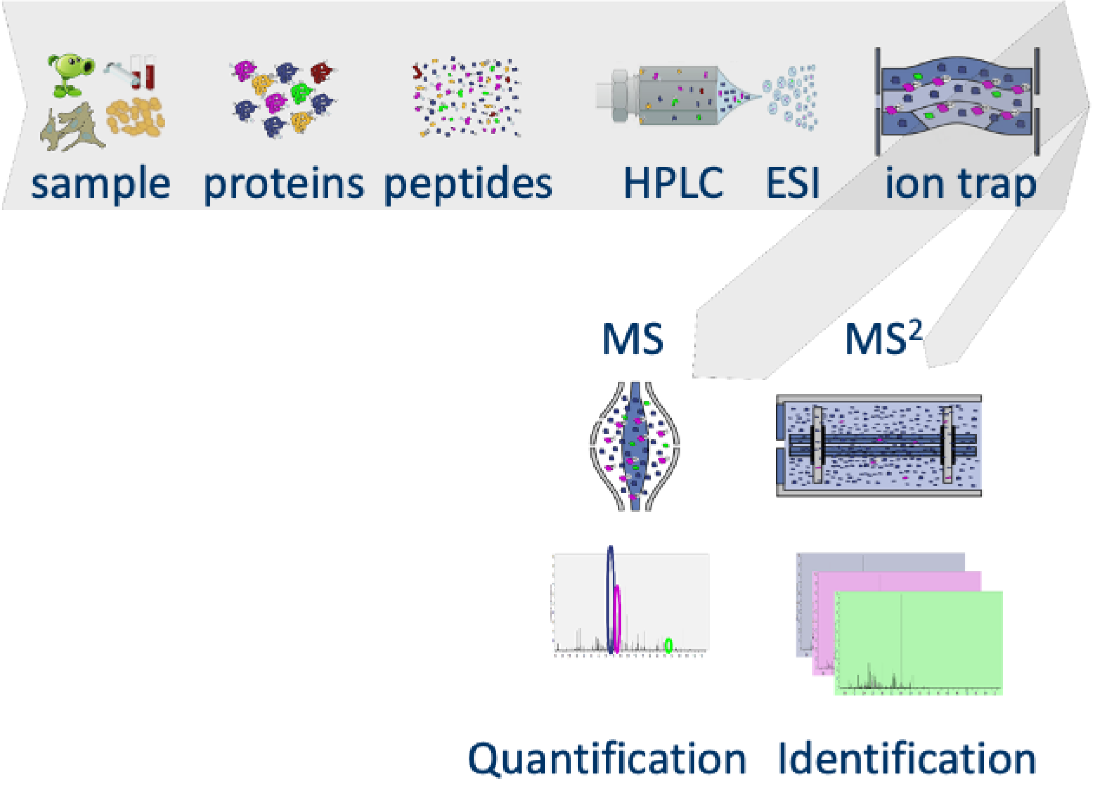
```
library(tidyverse)
library(limma)
library(QFeatures)
library(msqrob2)
library(plotly)
library(gridExtra)
```

1 Intro

1. Background + cptac study
2. Sources of variability
3. Summarization

The summary .

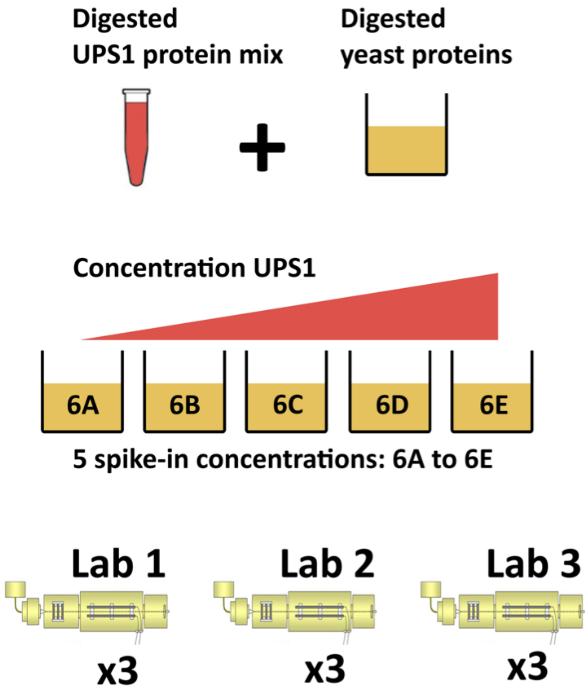
1.1 MS-based workflow



- Peptide Characteristics
  - Modifications
  - Ionisation Efficiency: huge variability
  - Identification
    - Misidentification → outliers
    - MS^2 selection on peptide abundance
    - Context depending missingness
    - Non-random missingness

→ Unbalanced pepide identifications across samples and messy data

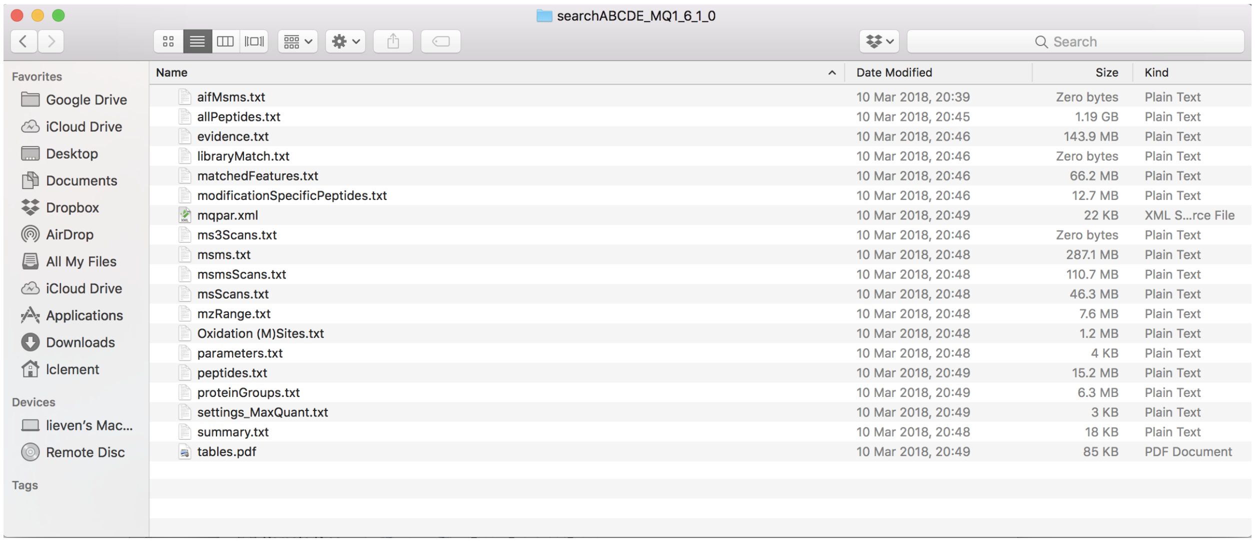
1.2 CPTAC Spike-in Study



- Same trypsin-digested yeast proteome background in each sample
- Trypsin-digested Sigma UPS1 standard: 48 different human proteins spiked in at 5 different concentrations (treatment A-E)
- Samples repeatedly run on different instruments in different labs
- 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

1.2.1 Maxquant output



Name	Date Modified	Size	Kind
aifMsms.txt	10 Mar 2018, 20:39	Zero bytes	Plain Text
allPeptides.txt	10 Mar 2018, 20:45	1.19 GB	Plain Text
evidence.txt	10 Mar 2018, 20:46	143.9 MB	Plain Text
libraryMatch.txt	10 Mar 2018, 20:46	Zero bytes	Plain Text
matchedFeatures.txt	10 Mar 2018, 20:46	66.2 MB	Plain Text
modificationSpecificPeptides.txt	10 Mar 2018, 20:46	12.7 MB	Plain Text
mqpar.xml	10 Mar 2018, 20:49	22 KB	XML S...rce File
ms3Scans.txt	10 Mar 2018, 20:46	Zero bytes	Plain Text
msms.txt	10 Mar 2018, 20:48	287.1 MB	Plain Text
msmsScans.txt	10 Mar 2018, 20:48	110.7 MB	Plain Text
msScans.txt	10 Mar 2018, 20:48	46.3 MB	Plain Text
mzRange.txt	10 Mar 2018, 20:48	7.6 MB	Plain Text
Oxidation (M)Sites.txt	10 Mar 2018, 20:48	1.2 MB	Plain Text
parameters.txt	10 Mar 2018, 20:48	4 KB	Plain Text
peptides.txt	10 Mar 2018, 20:49	15.2 MB	Plain Text
proteinGroups.txt	10 Mar 2018, 20:49	6.3 MB	Plain Text
settings_MaxQuant.txt	10 Mar 2018, 20:49	3 KB	Plain Text
summary.txt	10 Mar 2018, 20:48	18 KB	Plain Text
tables.pdf	10 Mar 2018, 20:49	85 KB	PDF Document

1.2.2 Read data

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/PDA21/data/quantification/fullCptacDatasSe
```

2. Maxquant stores the intensity data for the different samples in columnns that start with Intensity. We can retreive 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")
```

1.2.3 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
```

2 Sources of variation

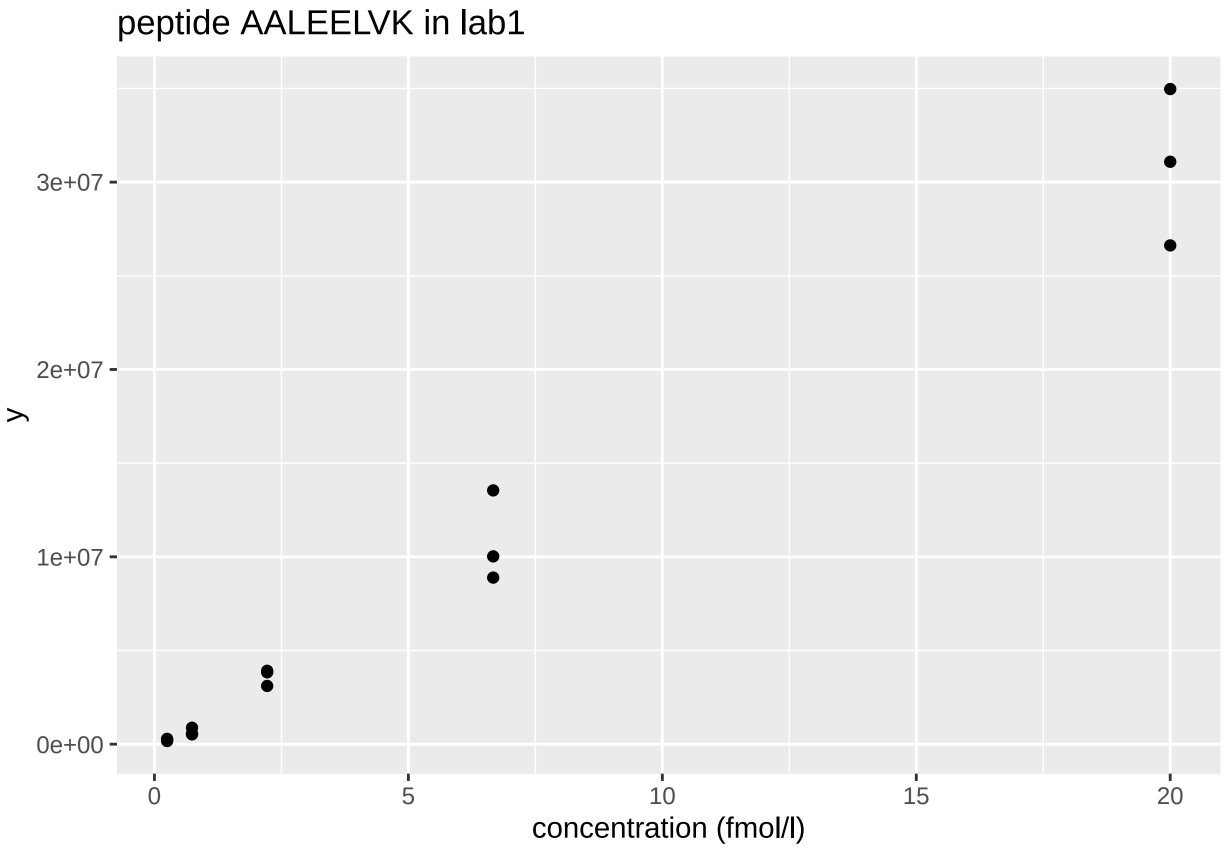
2.1 Intensities of one peptide

Peptide AALEELVK from spiked-in UPS protein P12081. We only show data from lab1.

Click to see code to make plot

```
subset <- pe["AALEELVK",colData(pe)$lab=="lab1"]
plotWhyLog <- data.frame(concentration = colData(subset)$spikeConcentration,
           y = assay(subset[["peptideRaw"]]) %>% c
           ) %>%
  ggplot(aes(concentration, y)) +
  geom_point() +
  xlab("concentration (fmol/l)") +
  ggtitle("peptide AALEELVK in lab1")
```

```
plotWhyLog
```

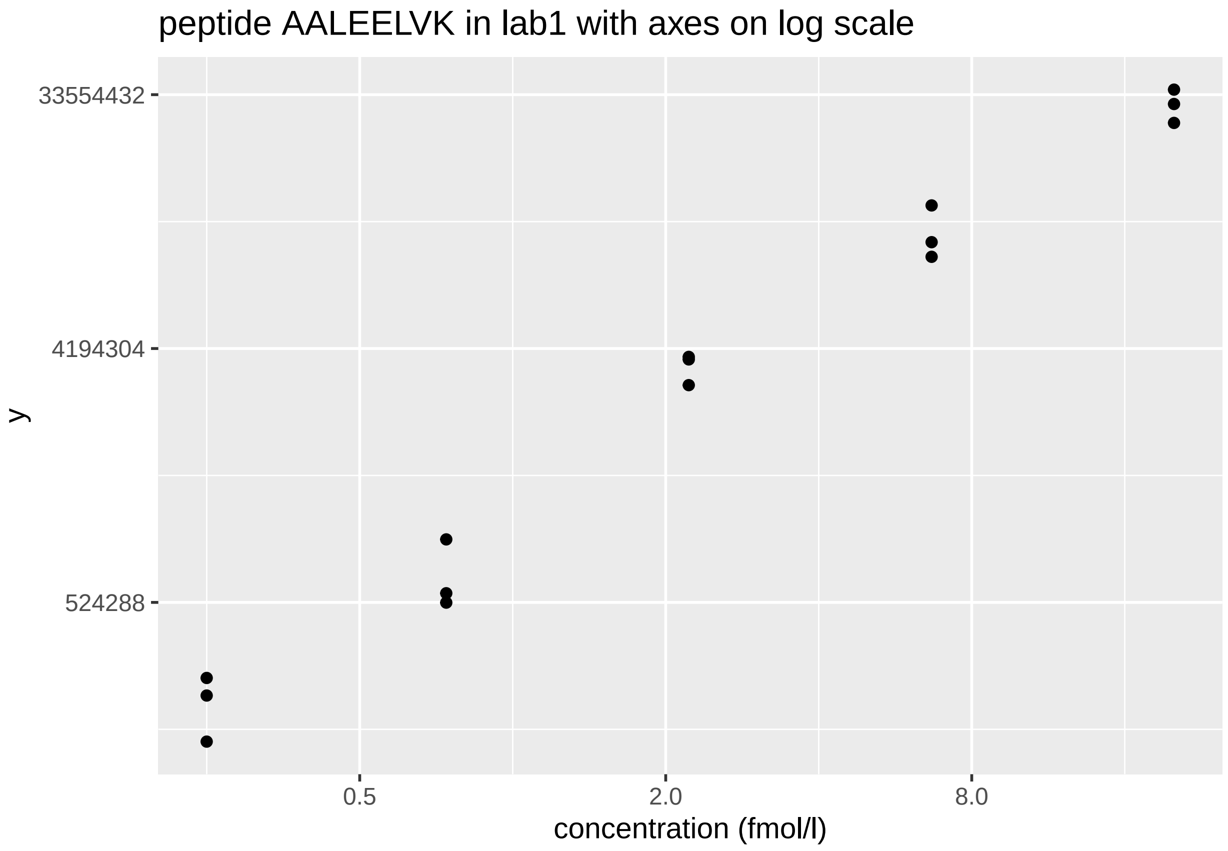


- Variance increases with the mean → Multiplicative error structure

Click to see code to make plot

```
plotLog <- data.frame(concentration = colData(subset)$spikeConcentration,
           y = assay(subset[["peptideRaw"]]) %>% c
           ) %>%
  ggplot(aes(concentration, y)) +
  geom_point() +
  scale_x_continuous(trans='log2') +
  scale_y_continuous(trans='log2') +
  xlab("concentration (fmol/l)") +
  ggtitle("peptide AALEELVK in lab1 with axes on log scale")
```

```
plotLog
```



- Data seems to be homoscedastic on log-scale → log transformation of the intensity data
- In quantitative proteomics analysis on $\log_2$

→ Differences on a $\log_2$ scale: $\log_2$ fold changes

$$\log_2 B - \log_2 A = \log_2 \frac{B}{A} = \log FC_{\text{B - A}}$$

$$log_2 FC = 1 \rightarrow FC = 2^1 = 2$$
$$log_2 FC = 2 \rightarrow FC = 2^2 = 4$$

2.2 Log-transform

Click to see code to log-transfrom 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")
```

2.3 Filtering

Click to see code to filter the data

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[["peptideLog"]] <-
 pe[["peptideLog"]][rowData(pe[["peptideLog"]])$Proteins
 %in% smallestUniqueGroups(rowData(pe[["peptideLog"]])$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[["peptideLog"]] <- pe[["peptideLog"]][rowData(pe[["peptideLog"]])$Reverse != "+", ]
pe[["peptideLog"]] <- pe[["peptideLog"]][rowData(pe[["peptideLog"]])$
    Potential.contaminant != "+", ]
```

3. Drop peptides that were only identified in one sample

We keep peptides that were observed at last twice.

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

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

We keep 10478 peptides upon filtering.

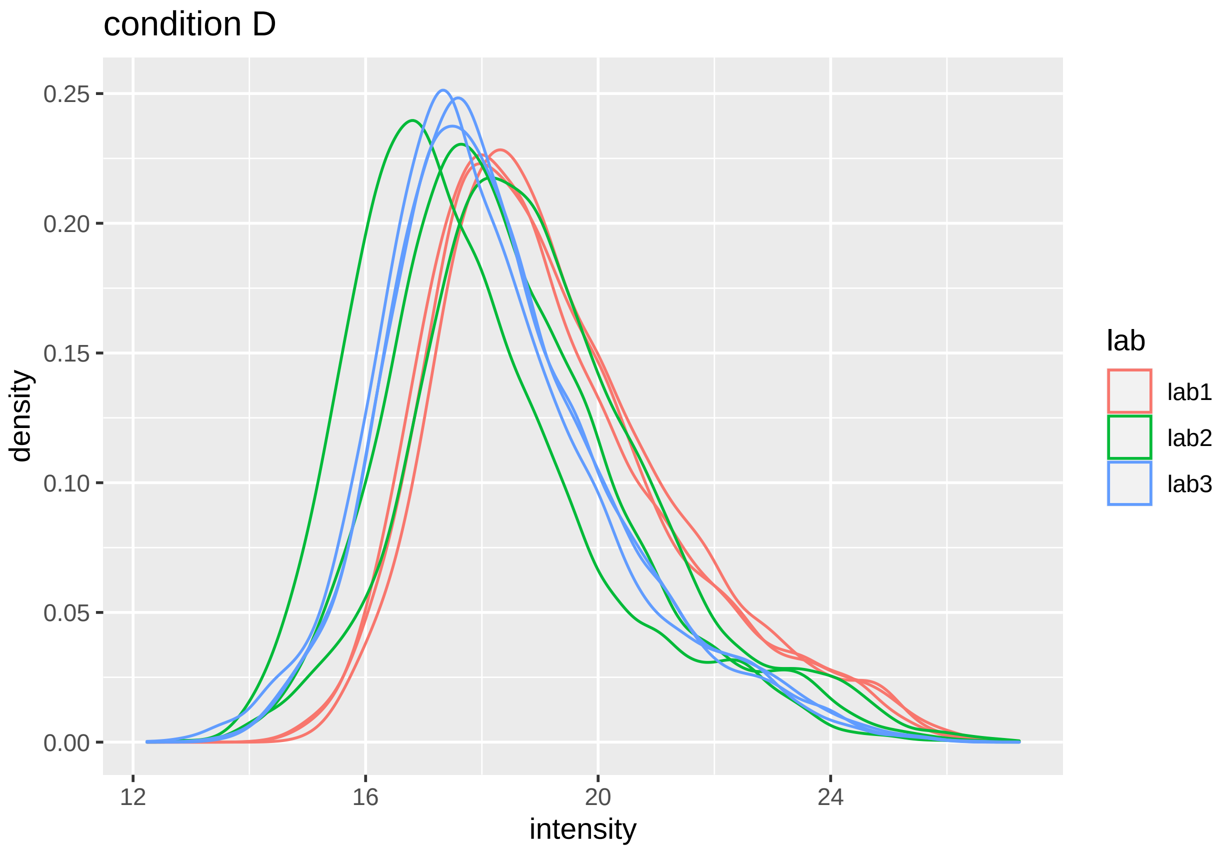
2.4 Technical Variability

```
densityConditionD <- pe[["peptideLog"]][,colData(pe)$condition=="D"] %>%
  assay %>%
  as.data.frame() %>%
  gather(sample, intensity) %>%
  mutate(lab = colData(pe)[sample,"lab"]) %>%
  ggplot(aes(x=intensity,group=sample,color=lab)) +
    geom_density() +
    ggtitle("condition D")

densityLab2 <- pe[["peptideLog"]][,colData(pe)$lab=="lab2"] %>%
  assay %>%
  as.data.frame() %>%
  gather(sample, intensity) %>%
  mutate(condition = colData(pe)[sample,"condition"]) %>%
  ggplot(aes(x=intensity,group=sample,color=condition)) +
    geom_density() +
    ggtitle("lab2")
```

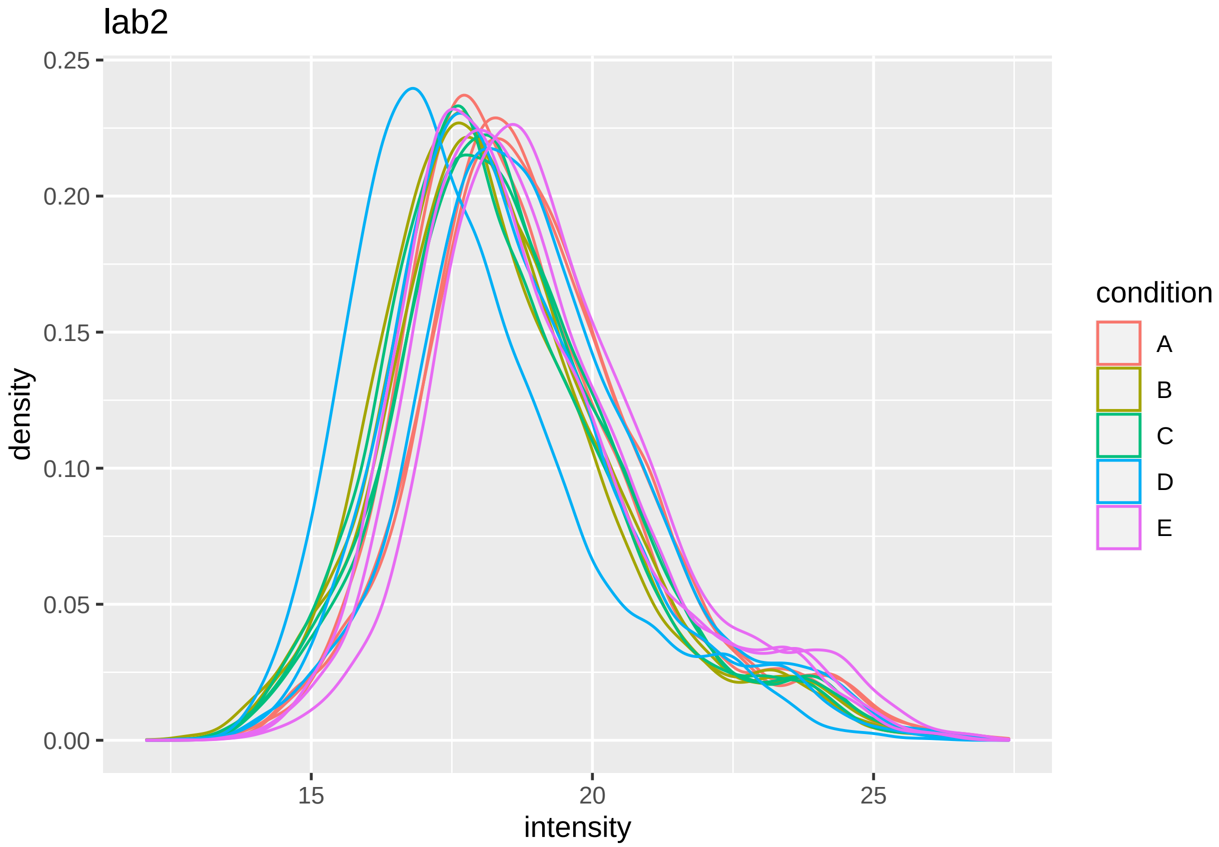
```
densityConditionD
```

```
## Warning: Removed 39179 rows containing non-finite values (stat_density).
```



```
densityLab2
```

```
## Warning: Removed 44480 rows containing non-finite values (stat_density).
```



- Even in very clean synthetic dataset (same background, only 48 UPS proteins can be different) the marginal peptide intensity distribution across samples can be quite distinct
- Considerable effects between and within labs for replicate samples
- Considerable effects between samples with different spike-in concentration

→ Normalization is needed

2.5 Normalization

Normalization of the data by median centering

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

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

Click to see R-code to normalize the data

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

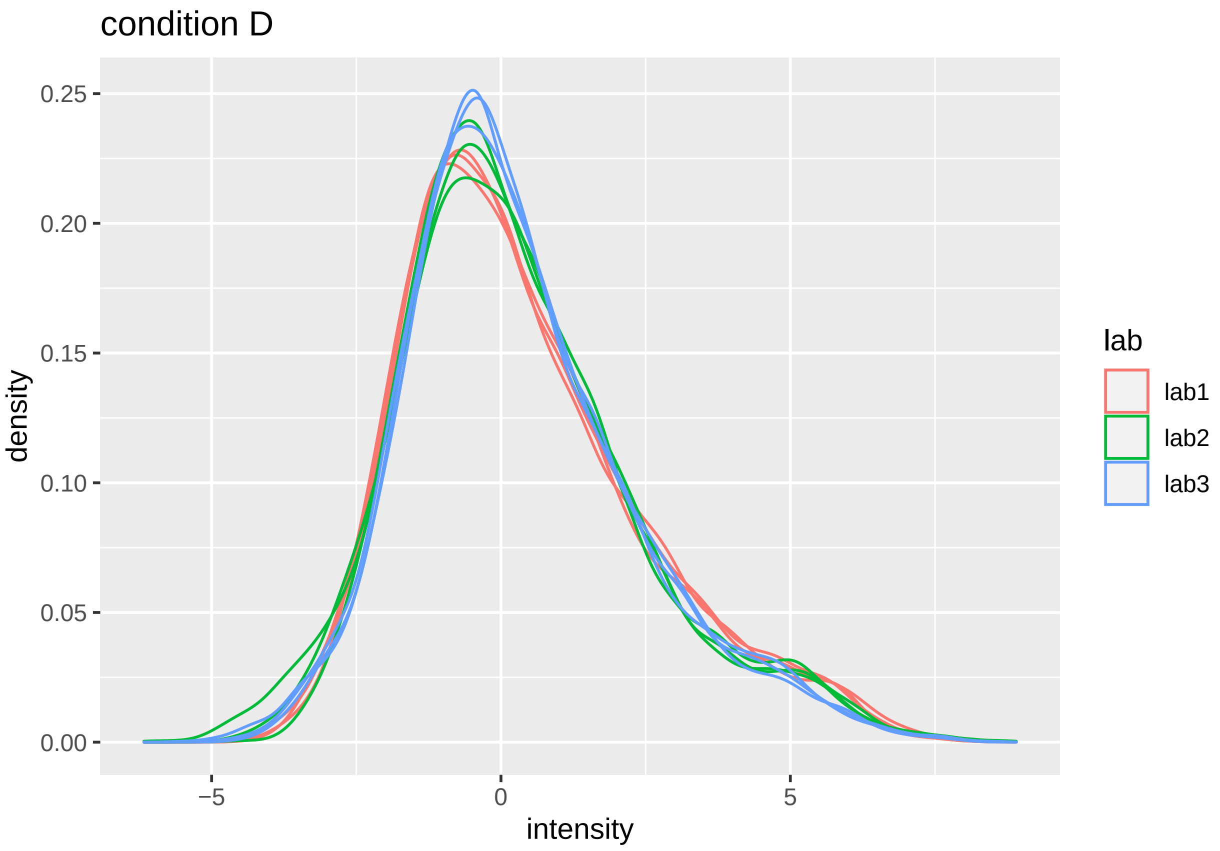
Click to see code to make plot

```
densityConditionDNorm <- pe[["peptideNorm"]][,colData(pe)$condition=="D"] %>%
  assay %>%
  as.data.frame() %>%
  gather(sample, intensity) %>%
  mutate(lab = colData(pe)[sample,"lab"]) %>%
  ggplot(aes(x=intensity,group=sample,color=lab)) +
    geom_density() +
    ggtitle("condition D")

densityLab2Norm <- pe[["peptideNorm"]][,colData(pe)$lab=="lab2"] %>%
  assay %>%
  as.data.frame() %>%
  gather(sample, intensity) %>%
  mutate(condition = colData(pe)[sample,"condition"]) %>%
  ggplot(aes(x=intensity,group=sample,color=condition)) +
    geom_density() +
    ggtitle("lab2")
```

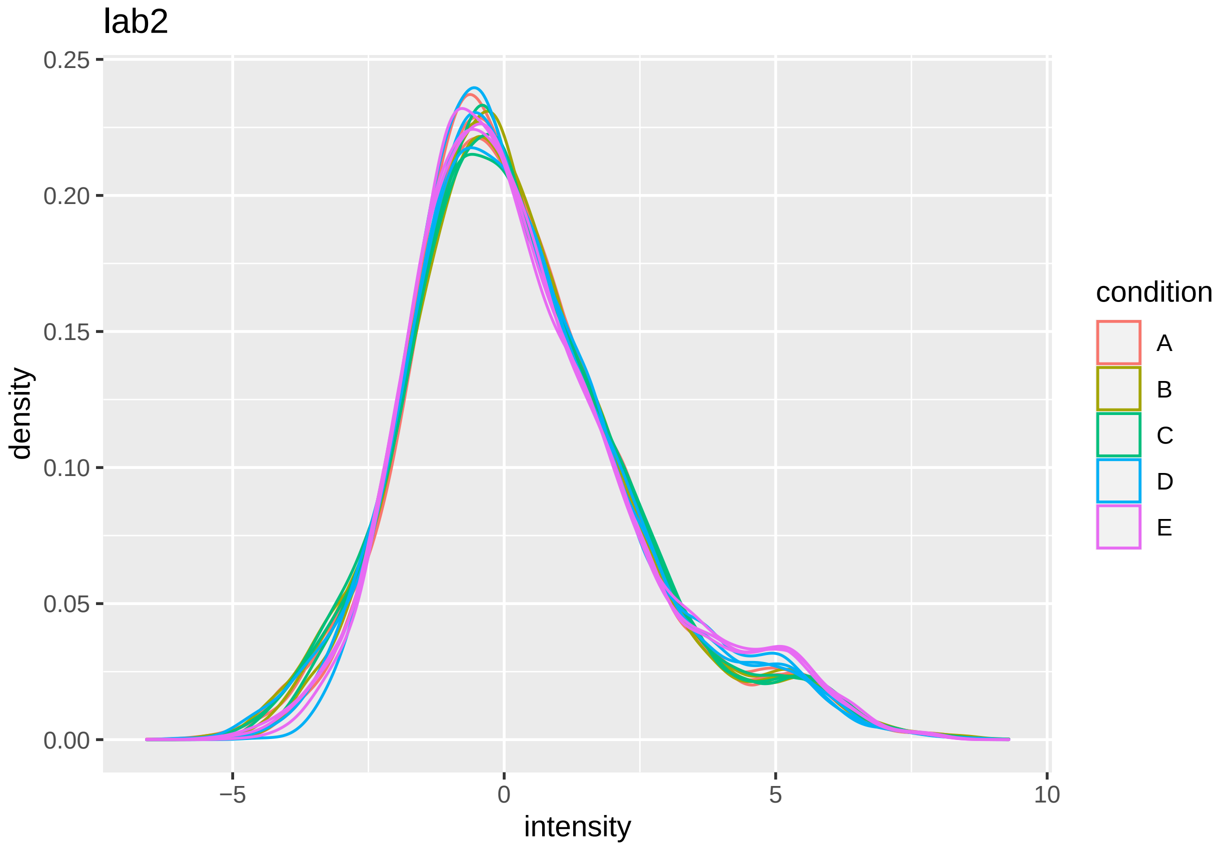
```
densityConditionDNorm
```

```
## Warning: Removed 39179 rows containing non-finite values (stat_density).
```



```
densityLab2Norm
```

```
## Warning: Removed 44480 rows containing non-finite values (stat_density).
```

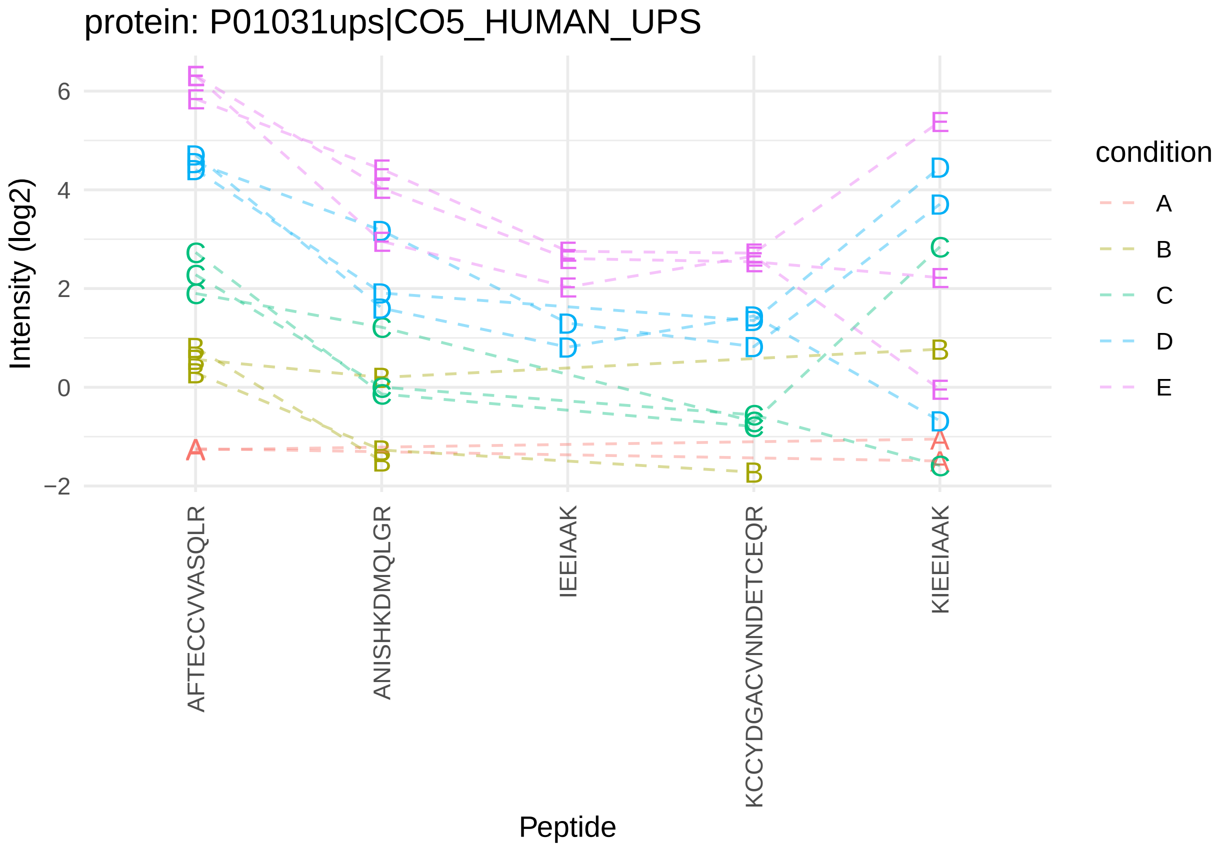


2.6 Pseudo replication

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.leg
  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))
```

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



- Sources of variability in plot:
  - Between treatment variability
  - Between sample variability
  - Between peptide variability
  - within sample variability
- Multiple peptides from same protein in a sample
- Peptide intensities in the same sample are correlated: Pseudo replication

→ Summarization!

- Strong peptide effect
- Unbalanced peptide identification

2.6.1 Illustration on subset of CPTAC study: A vs B comparison in lab 3

2.6.1.1 LFQ Click to see background and code

1. Import data

```
proteinsFile <- "https://raw.githubusercontent.com/statOmics/PDA21/data/quantification/cptacAvsB_lab3/p

ecols <- grep("LFQ\\.intensity\\.", names(read.delim(proteinsFile)))

peLFQ <- readQFeatures(
  table = proteinsFile, fnames = 1, ecol = ecols,
  name = "proteinRaw", sep = "\t"
)

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

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

2. Preprocessing

```
rowData(peLFQ[["proteinRaw"]])$nNonZero <- rowSums(assay(peLFQ[["proteinRaw"]]) > 0)

peLFQ <- zeroIsNA(peLFQ, "proteinRaw") # convert 0 to NA

peLFQ <- logTransform(peLFQ, base = 2, i = "proteinRaw", name = "proteinLog")

peLFQ[["proteinLog"]] <- peLFQ[["proteinLog"]][rowData(peLFQ[["proteinLog"]])$Reverse != "+", ]
peLFQ[["proteinLog"]] <- peLFQ[["proteinLog"]][rowData(peLFQ[["proteinLog"]])$
                                           Potential.contaminant != "+", ]
peLFQ <- normalize(peLFQ,
                i = "proteinLog",
                name = "protein",
                method = "center.median")
```

3. Modeling and Inference

```
peLFQ <- msqrob(object = peLFQ, i = "protein", formula = ~condition)

L <- makeContrast("conditionB=0", parameterNames = c("conditionB"))
peLFQ <- hypothesisTest(object = peLFQ, i = "protein", contrast = L)

volcanoLFQ <- ggplot(rowData(peLFQ[["protein"]])$conditionB,
                 aes(x = logFC, y = -log10(pval), color = adjPval < 0.05)) +
  geom_point(cex = 2.5) +
  scale_color_manual(values = alpha(c("black", "red"), 0.5)) +
  theme_minimal() +
  ggtitle(paste0("maxLFQ: TP = ",sum(rowData(peLFQ[["protein"]])$conditionB$adjPval<0.05&grepl(rownames
```

2.6.1.2 Median & robust summarization Click to see background and code

1. Import Data

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

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

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

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

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

2. Preprocessing

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

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

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

peAB[["peptideLog"]] <-
 peAB[["peptideLog"]][rowData(peAB[["peptideLog"]])$Proteins
 %in% smallestUniqueGroups(rowData(peAB[["peptideLog"]])$Proteins),]

peAB[["peptideLog"]] <- peAB[["peptideLog"]][rowData(peAB[["peptideLog"]])$Reverse != "+", ]
peAB[["peptideLog"]] <- peAB[["peptideLog"]][rowData(peAB[["peptideLog"]])$
    Potential.contaminant != "+", ]

peAB[["peptideLog"]] <- peAB[["peptideLog"]][rowData(peAB[["peptideLog"]])$nNonZero >= 2, ]
nrow(peAB[["peptideLog"]])
```

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

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

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

peAB <- aggregateFeatures(peAB,
  i = "peptideNorm",
  fcol = "Proteins",
  na.rm = TRUE,
  name = "proteinRobust")
```

3. Modeling and inference

```
peAB <- msqrob(object = peAB, i = "proteinMedian", formula = ~condition)
L <- makeContrast("conditionB=0", parameterNames = c("conditionB"))
peAB <- hypothesisTest(object = peAB, i = "proteinMedian", contrast = L)

peAB <- msqrob(object = peAB, i = "proteinRobust", formula = ~condition)
peAB <- hypothesisTest(object = peAB, i = "proteinRobust", contrast = L)

volcanoMedian <- ggplot(rowData(peAB[["proteinMedian"]])$conditionB,
                  aes(x = logFC, y = -log10(pval), color = adjPval < 0.05)) +
  geom_point(cex = 2.5) +
  scale_color_manual(values = alpha(c("black", "red"), 0.5)) +
  theme_minimal() +
  ggtitle(paste0("Median: TP = ",sum(rowData(peAB[["proteinMedian"]])$conditionB$adjPval<0.05&grepl(rown

volcanoRobust<- ggplot(rowData(peAB[["proteinRobust"]])$conditionB,
                  aes(x = logFC, y = -log10(pval), color = adjPval < 0.05)) +
  geom_point(cex = 2.5) +
  scale_color_manual(values = alpha(c("black", "red"), 0.5)) +
  theme_minimal() +
  ggtitle(paste0("Robust: TP = ",sum(rowData(peAB[["proteinRobust"]])$conditionB$adjPval<0.05&grepl(rown
```

```
ylims <- c(0,
           ceiling(max(c(-log10(rowData(peLFQ[["protein"]])$conditionB$pval),
               -log10(rowData(peAB[["proteinMedian"]])$conditionB$pval),
               -log10(rowData(peAB[["proteinRobust"]])$conditionB$pval)),
               na.rm=TRUE))
)

xlims <- max(abs(c(rowData(peLFQ[["protein"]])$conditionB$logFC,
               rowData(peAB[["proteinMedian"]])$conditionB$logFC,
               rowData(peAB[["proteinRobust"]])$conditionB$logFC)),
               na.rm=TRUE) * c(-1,1)
```

```
compBoxPlot <- rbind(rowData(peLFQ[["protein"]])$conditionB %>% mutate(method="maxLFQ") %>% rownames_to_
      rowData(peAB[["proteinMedian"]])$conditionB %>% mutate(method="median")%>% rownames_to_column(var=
      rowData(peAB[["proteinRobust"]])$conditionB%>% mutate(method="robust")%>% rownames_to_column(var=
      mutate(ups= grepl(protein,pattern="UPS")) %>%
    ggplot(aes(x = method, y = logFC, fill = ups)) +
    geom_boxplot() +
    geom_hline(yintercept = log2(0.74 / .25), color = "#00BFC4") +
    geom_hline(yintercept = 0, color = "#F8766D")
```

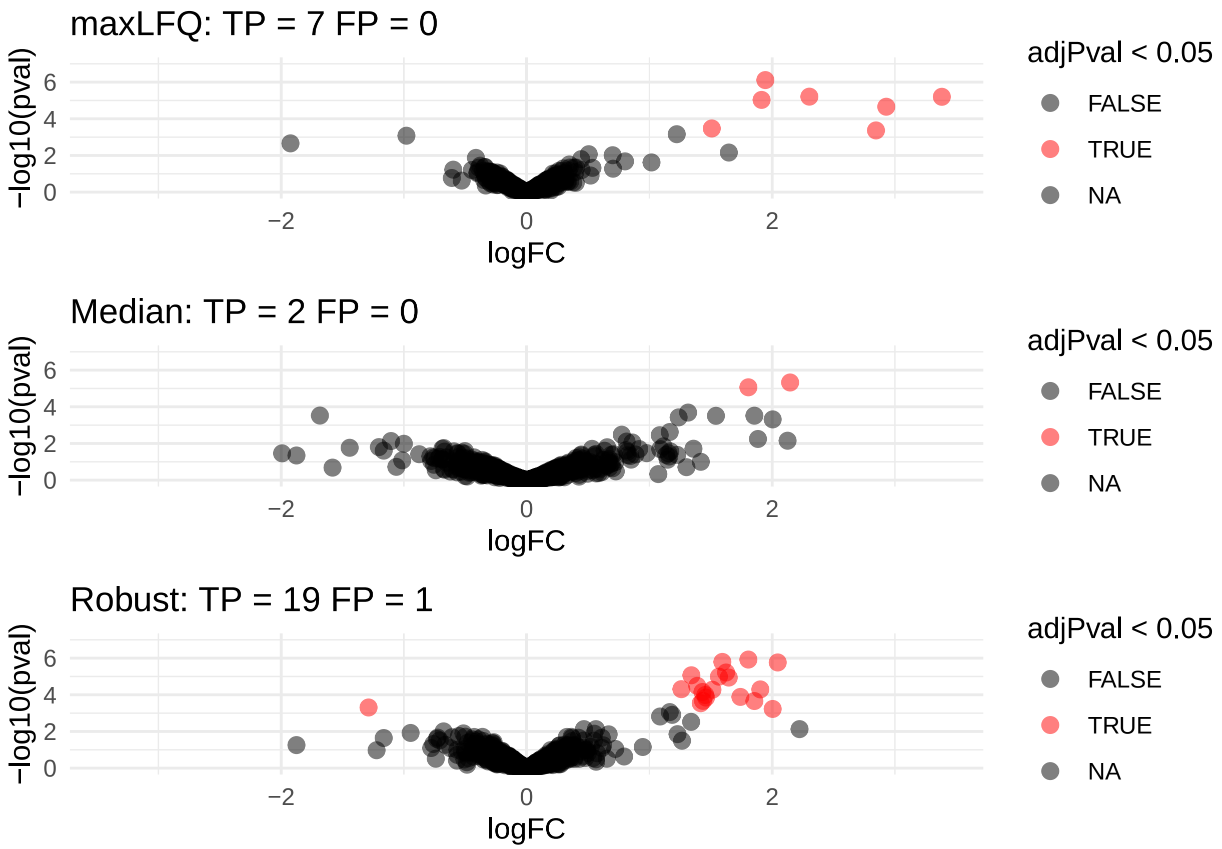
```
grid.arrange(volcanoLFQ + xlim(xlims) + ylim(ylims),
             volcanoMedian + xlim(xlims) + ylim(ylims),
             volcanoRobust + xlim(xlims) + ylim(ylims),
             ncol=1)
```

2.6.1.3 Comparison summarization methods

```
## Warning: Removed 746 rows containing missing values (geom_point).
```

```
## Warning: Removed 166 rows containing missing values (geom_point).
```

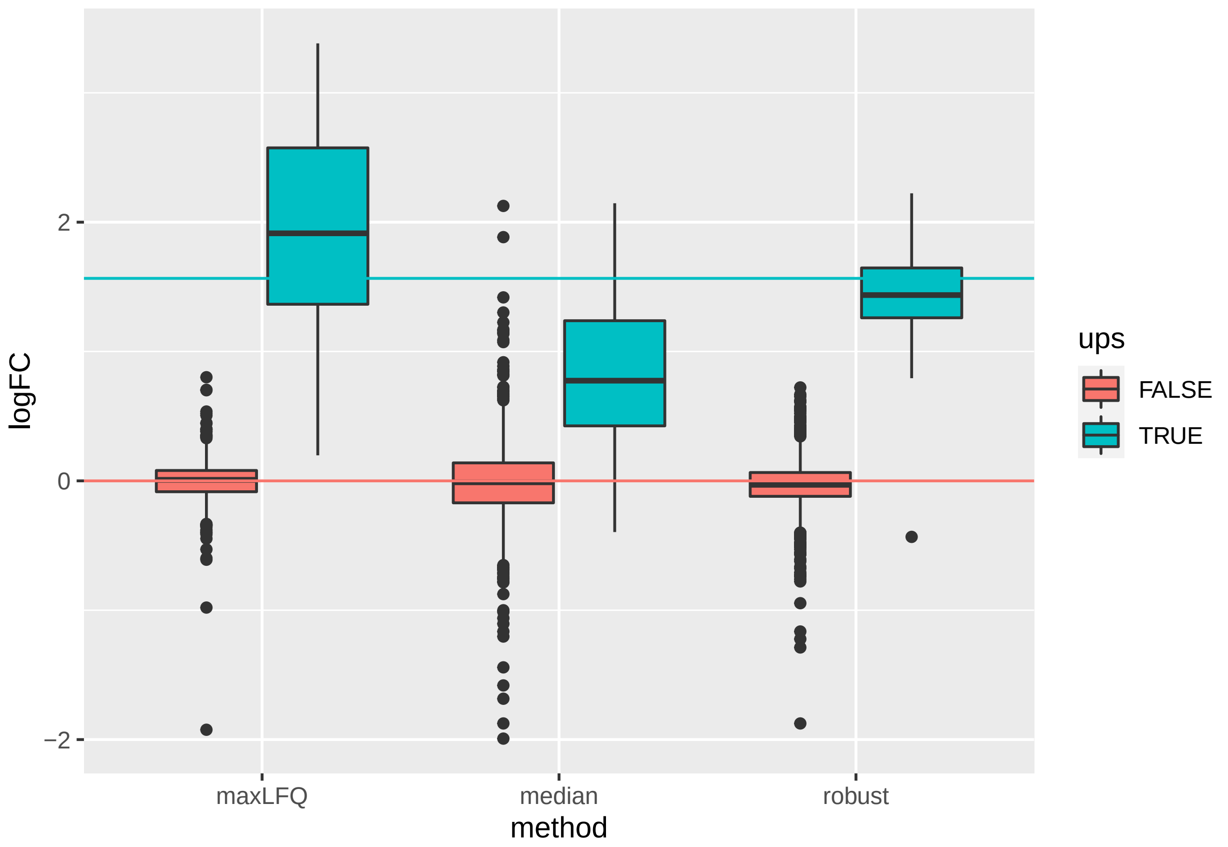
```
## Warning: Removed 167 rows containing missing values (geom_point).
```



- Robust summarization: highest power and still good FDR control: $FDP = \frac{1}{20} = 0.05$.

```
compBoxPlot
```

```
## Warning: Removed 1079 rows containing non-finite values (stat_boxplot).
```



- Median: biased logFC estimates for spike-in proteins
- maxLFQ: more variable logFC estiamtes for spike-in proteins

2.6.2 Median summarization

We first evaluate median summarization for protein P01031ups|CO5_HUMAN_UPS.

Click to see code to make plot

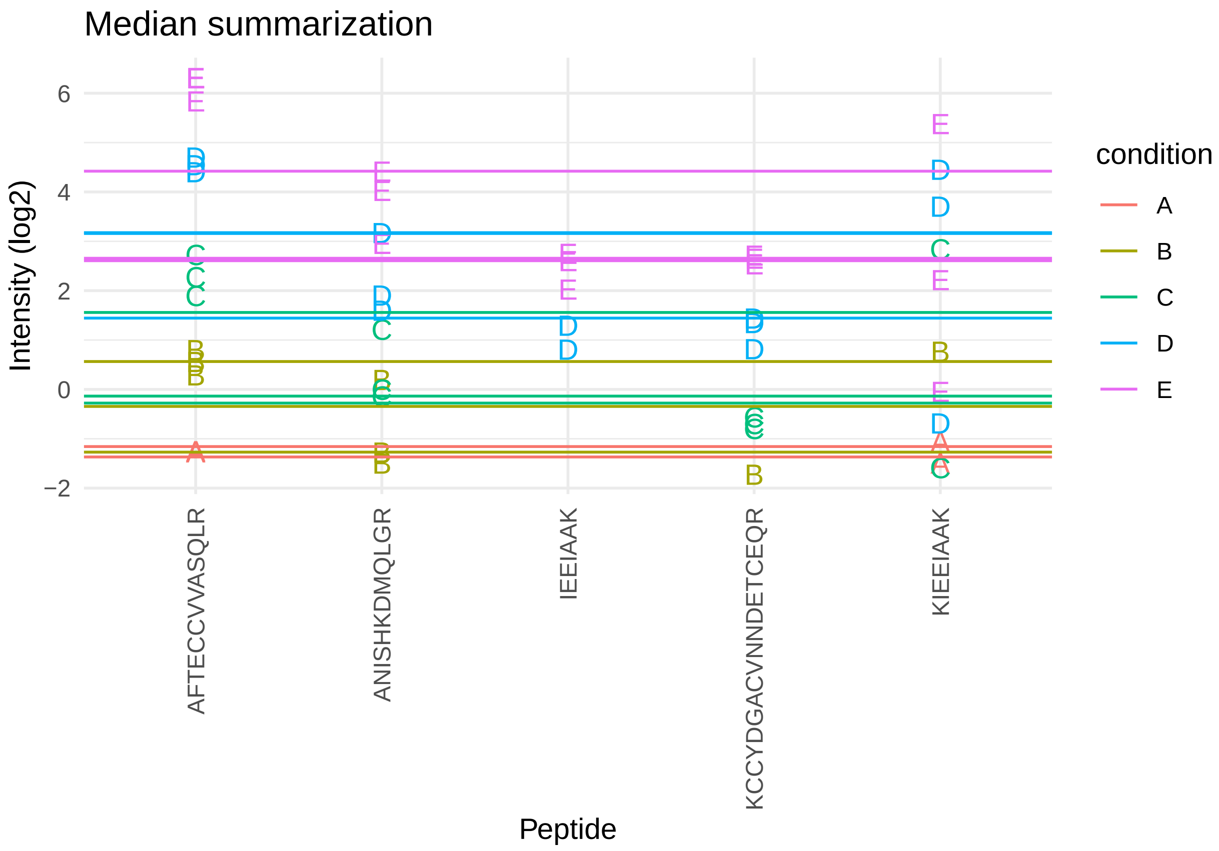
```
dataHlp <- pe[["peptideNorm"]][
    rowData(pe[["peptideNorm"]])$Proteins == prot,
    colData(pe)$lab=="lab3"] %>% assay

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 concencentration and not in samples with low spike-in concentration
- This introduces a bias.

2.6.3 Mean summarization

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

Click to see code to make plot

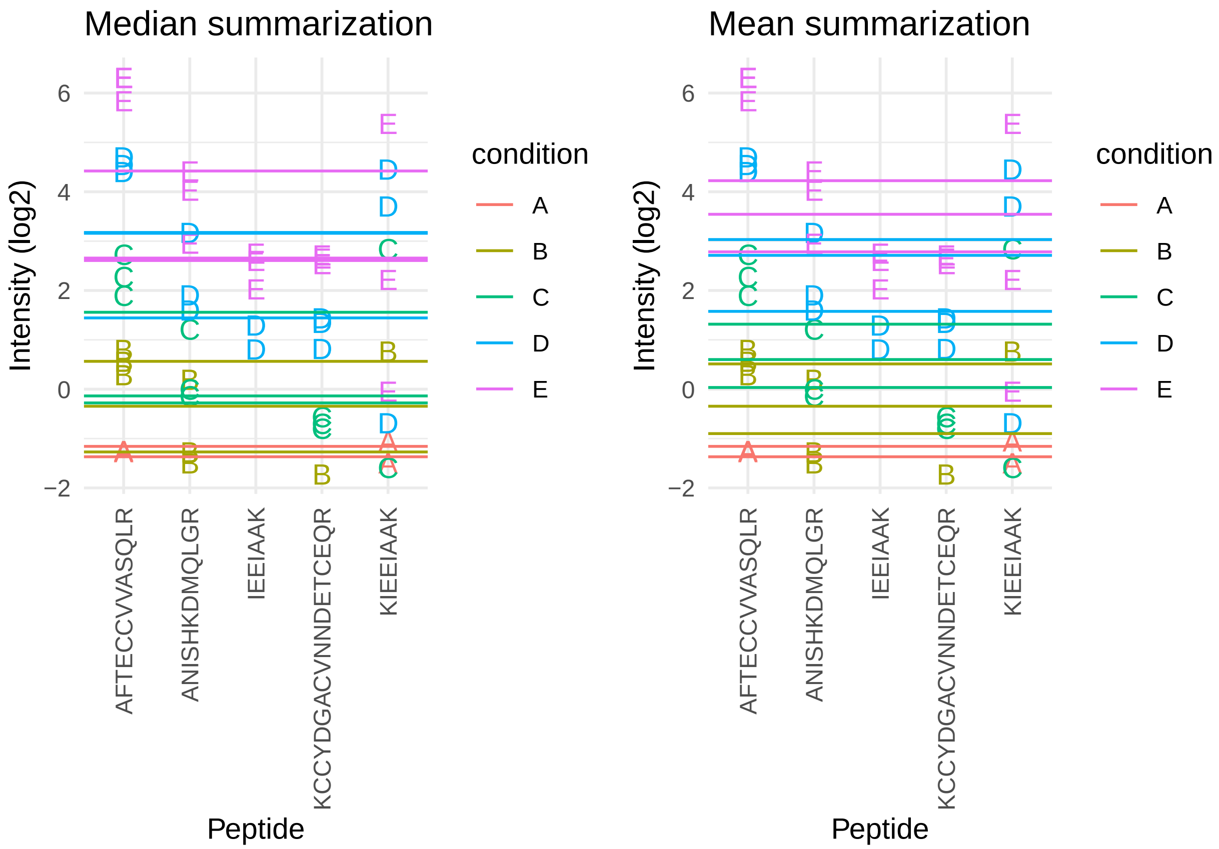
```
sumMeanMod <- lm(intensity ~ -1 + sample,data)

sumMean <- data.frame(
  intensity=sumMeanMod$coef[grep("sample",names(sumMeanMod$coef))],
  condition= names(sumMeanMod$coef)[grep("sample",names(sumMeanMod$coef))] %>% substr(18,18) %>% as.fac
```

```
sumMeanPlot <- sumPlot + geom_hline(
    data = sumMean,
    mapping = aes(yintercept=intensity,color=condition)) +
    ggtitle("Mean summarization")
```

```
grid.arrange(sumMedianPlot, sumMeanPlot, ncol=2)
```

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



2.6.4 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.

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

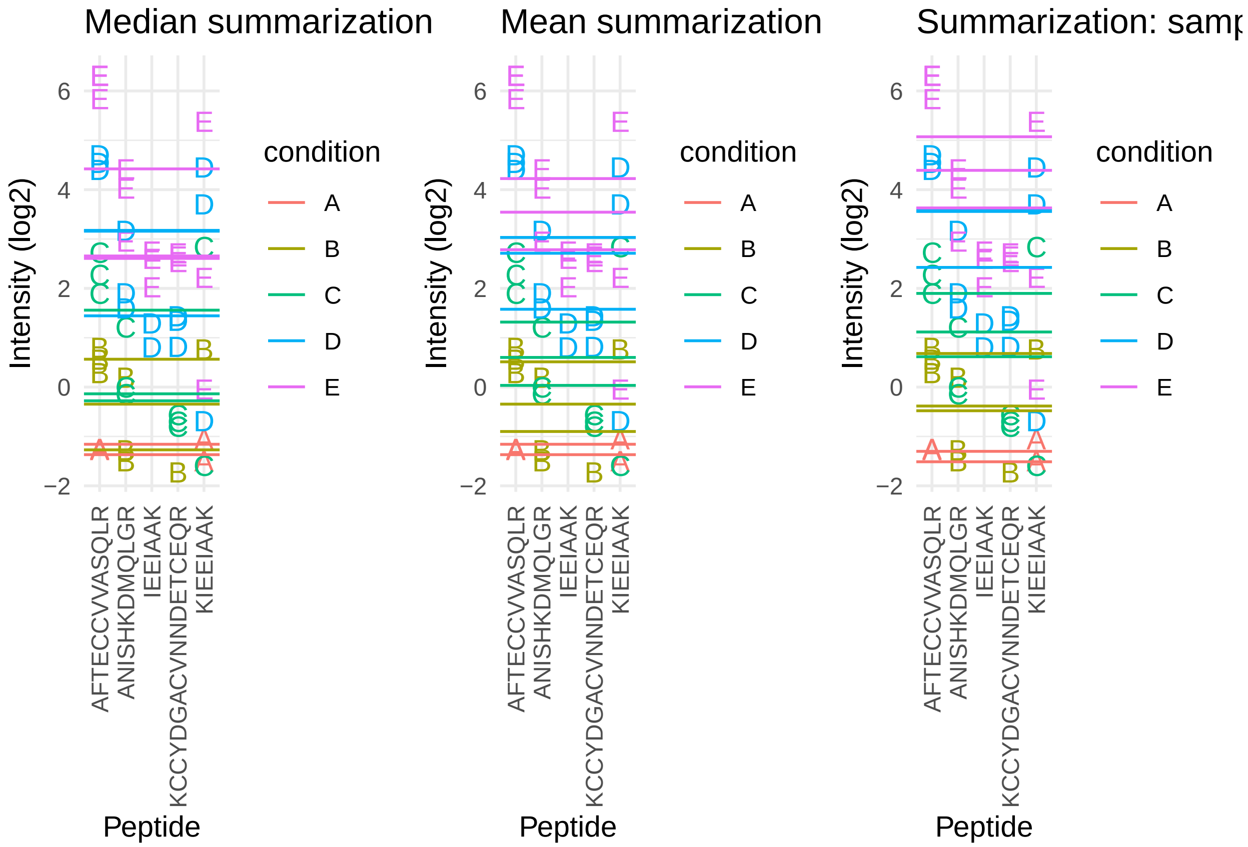
Click to see code to make plot

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

sumMeanPep <- data.frame(
  intensity=sumMeanPepMod$coef[grep("sample",names(sumMeanPepMod$coef))] + mean(data$intensity) - mean(
  condition= names(sumMeanPepMod$coef)[grep("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("Summarization: sample effect")
```

```
grid.arrange(sumMedianPlot, sumMeanPlot, sumLmPlot, nrow=1)
```

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



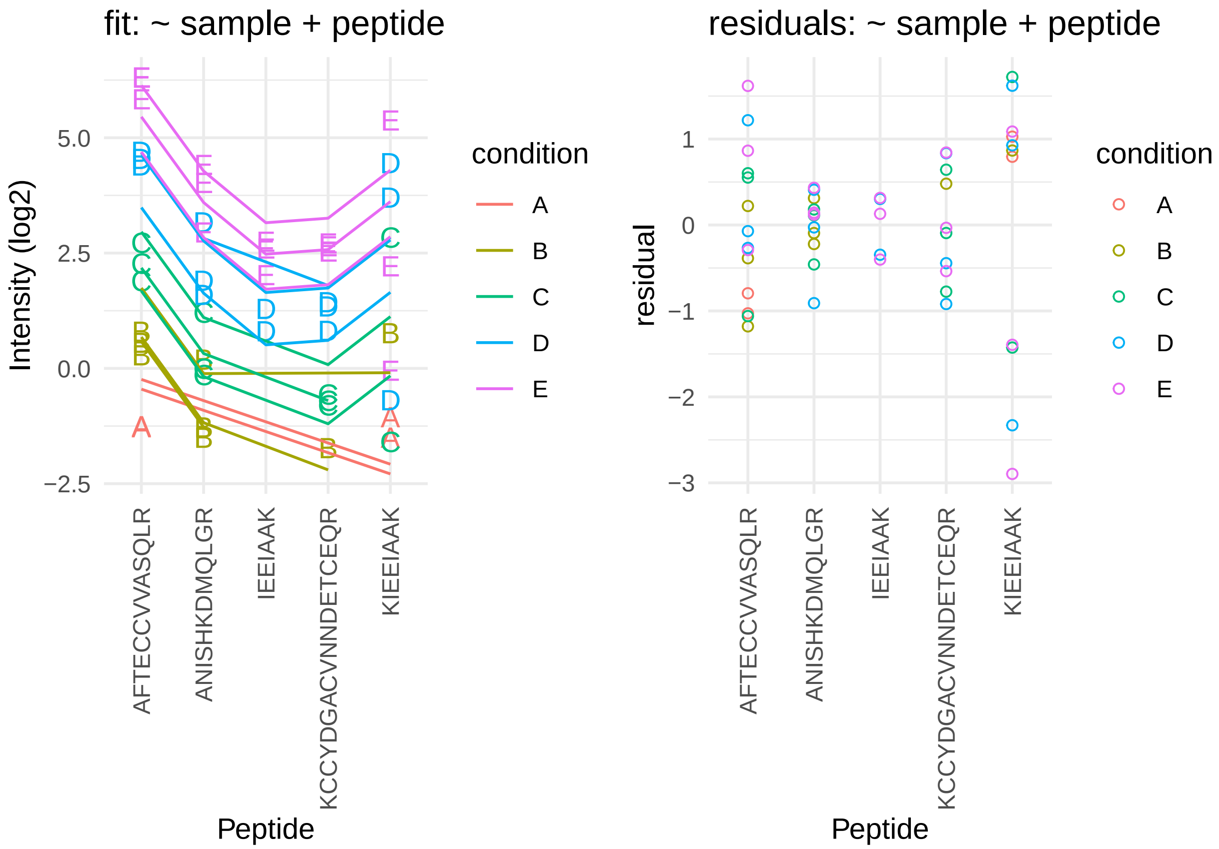
- By correcting for the peptide species the protein expression values are much better separated an better reflect differences in abundance induced by the spike-in condition.
- Indeed, it shows that median and mean summarization that do not account for the peptide effect indeed overestimate the protein 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 that for which the expression values are not well separated according to the spike-in condition.

A residual analysis clearly indicates potential issues:

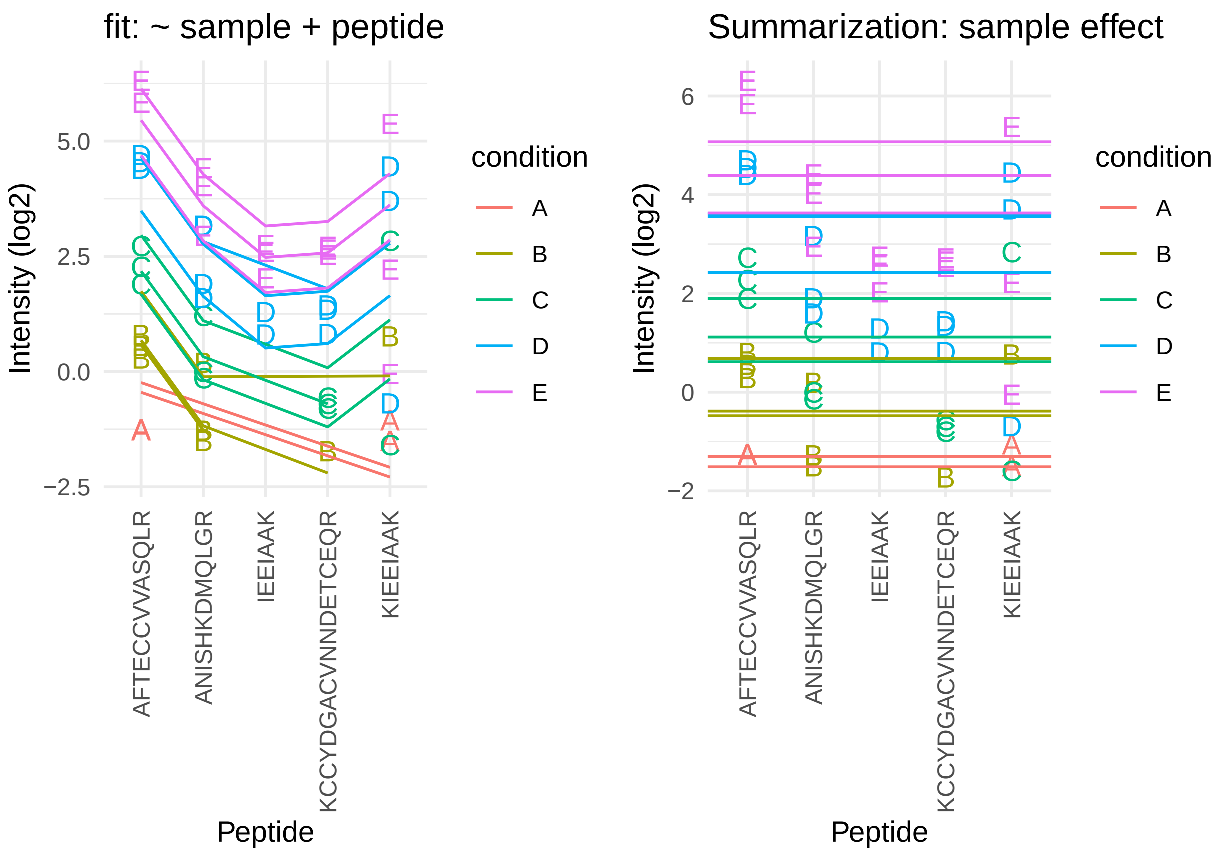
Click to see code to make plot

```
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, resPlot, nrow = 1)
```



```
grid.arrange(fitLmPlot, sumLmPlot, nrow = 1)
```



- 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)

2.6.5 Robust summarization using a peptide-level linear model

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

- 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

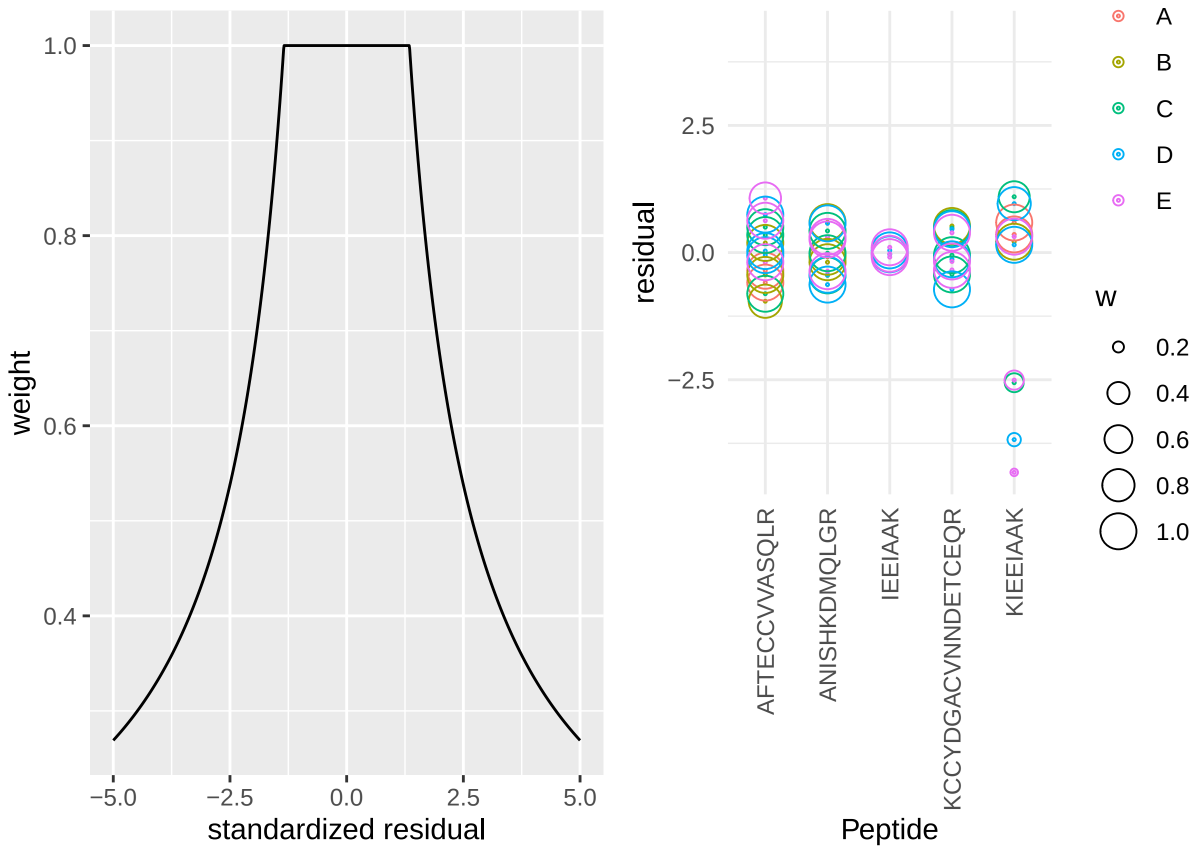
$$\sum_{i,p} w_{ip}\epsilon_{ip}^2 = \sum_{i,p} w_{ip}(y_{ip} - \beta_i^{\text{sample}} - \beta_p^{\text{peptide}})^2$$

- 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

```r
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")
```

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



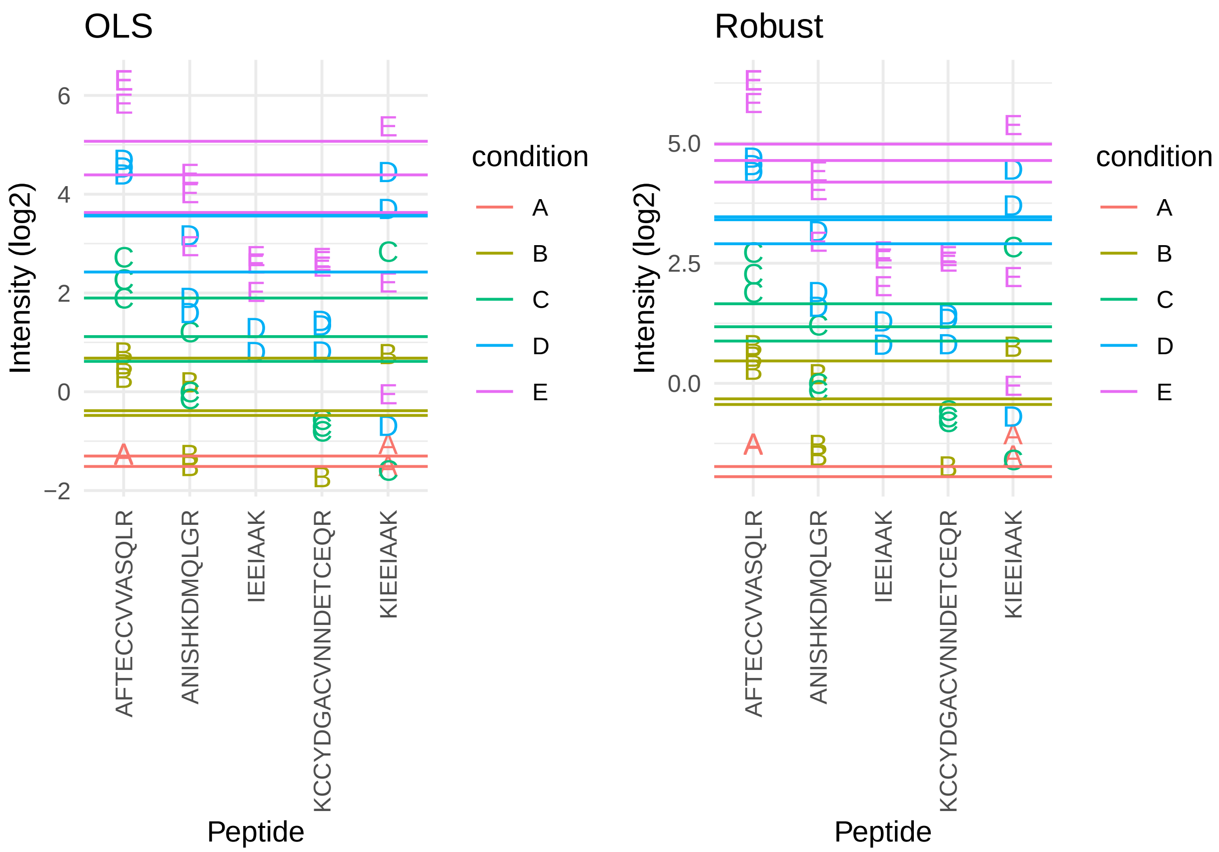
- We clearly see that the weights in the M-estimation procedure will down-weight errors associated with outliers for peptide KIEEIAAK.

Click to see code to make plot

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

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

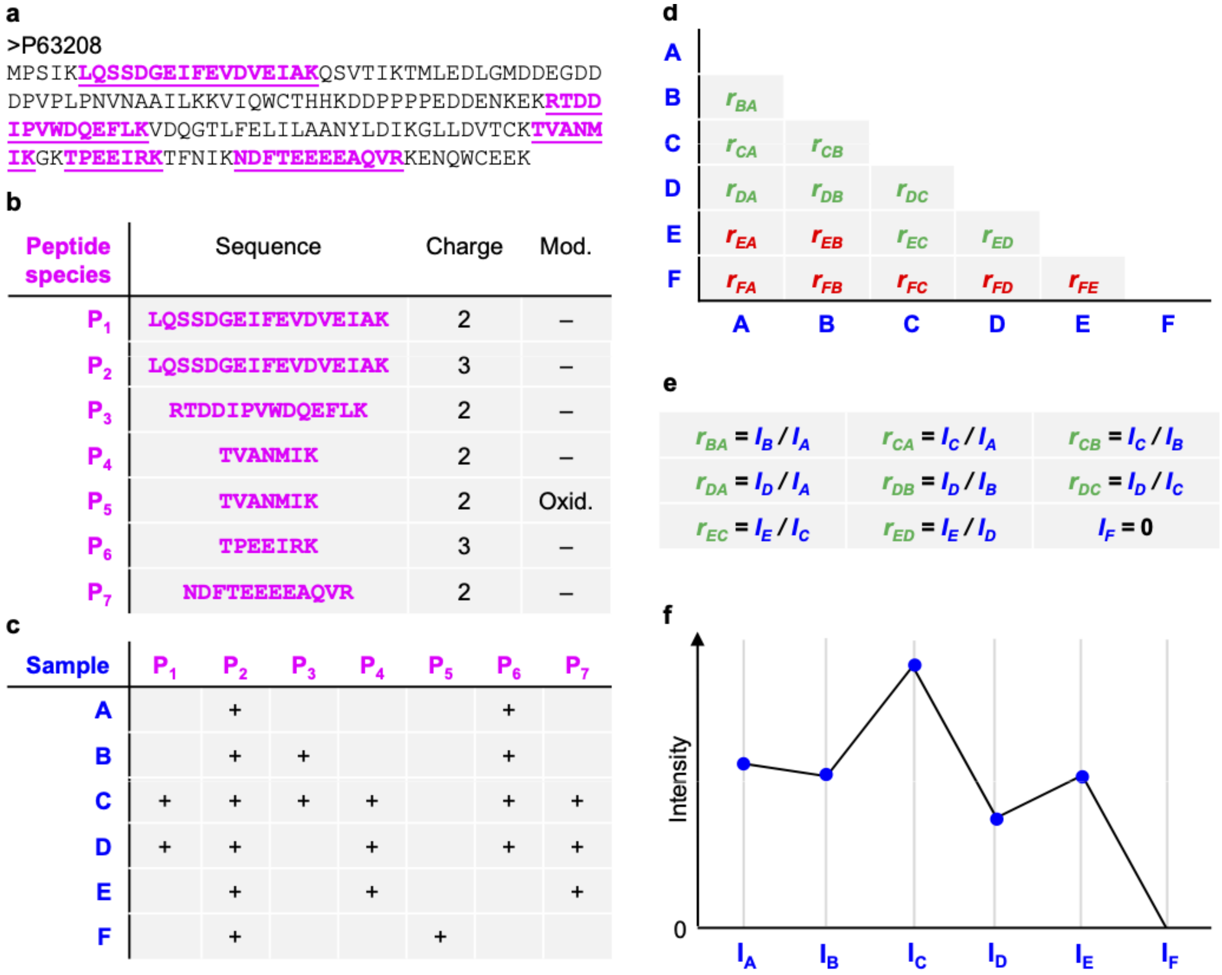
```
grid.arrange(sumLmPlot + ggtitle("OLS"), sumRlmPlot, nrow = 1)
```



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

2.6.6 Comparison summarization methods

- maxLFQ



>P63208
MPSIK**LQSSDGEIFEVDVEIAK**QSVTIKTMLEDLGMDDEGDD
DPVPLPNVNAAILKKVIQWCTHHKDDPPPPEDDENKEK**RTDD**
IPVWDQEFLKVDQGTLFELILAANYLDIKGLLDVTCK**TVANM**
IKGK**TPEEIRK**TFNIK**NDFTEEEEAQVR**KENQWCEEK

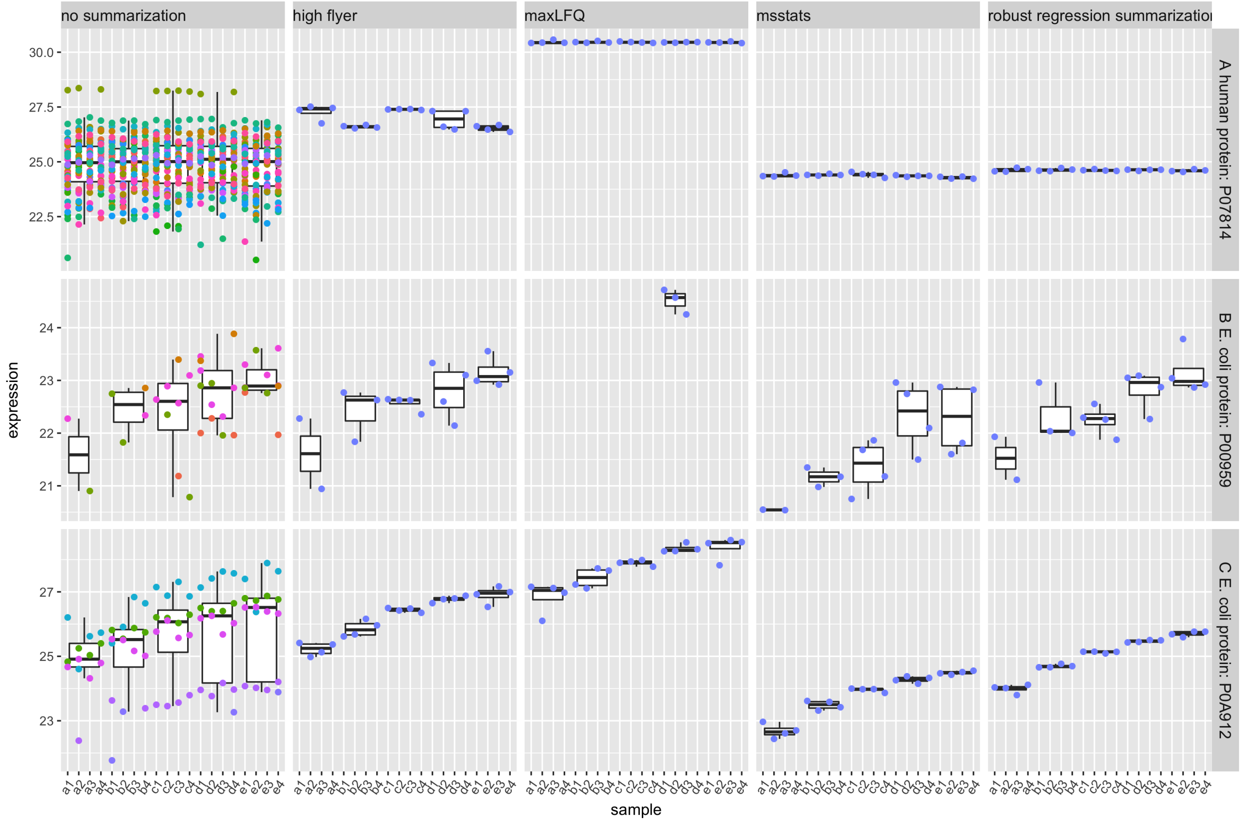
Peptide species	Sequence	Charge	Mod.
P_1	LQSSDGEIFEVDVEIAK	2	–
P_2	LQSSDGEIFEVDVEIAK	3	–
P_3	RTDDIPVWDQEFLK	2	–
P_4	TVANMIK	2	–
P_5	TVANMIK	2	Oxid.
P_6	TPEEIRK	3	–
P_7	NDFTEEEEAQVR	2	–

Sample	P_1	P_2	P_3	P_4	P_5	P_6	P_7
A		+				+	
B		+	+			+	
C	+	+	+	+		+	+
D	+	+		+		+	+
E		+		+			+
F		+			+		

d
A
B
C
D
E
F
r_{BA}
r_{CA} r_{CB}
r_{DA} r_{DB} r_{DC}
r_{EA} r_{EB} r_{EC} r_{ED}
r_{FA} r_{FB} r_{FC} r_{FD} r_{FE}
A
B
C
D
E
F
e

$r_{BA} = I_B / I_A$	$r_{CA} = I_C / I_A$	$r_{CB} = I_C / I_B$
$r_{DA} = I_D / I_A$	$r_{DB} = I_D / I_B$	$r_{DC} = I_D / I_C$
$r_{EC} = I_E / I_C$	$r_{ED} = I_E / I_D$	$I_F = 0$

- MS-stats also uses a robust peptide level model to perform the summarization, however, they typically first impute missing values
- Proteus high-flyer method: mean of three peptides with highest intensity



- (Sticker et al. 2020)
- doi: https://doi.org/10.1074/mcp.RA119.001624

- pdf

References

Sticker, A., L. Goeminne, L. Martens, and L. Clement. 2020. "Robust Summarization and Inference in Proteome-wide Label-free Quantification." *Mol Cell Proteomics* 19 (7): 1209–19.