

Statistical Methods for Quantitative MS-based Proteomics: Part I. Preprocessing

Lieven Clement

June 30, 2021

Contents

Outline	2
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
1.4 CPTAC Spike-in Study	5
1.5 Maxquant output	6
2 Import the data in R	6
2.1 Data infrastructure	6
2.2 Import data in R	6
3 Preprocessing	22
3.1 Log-transformation	22
3.2 Filtering	25
3.3 Normalization	26
3.4 Summarization	30
4 Exercise	32
5 Software & code	33
5.1 Code	33
5.2 Data analysis with the GUI/shinyApp msqrob2gui	33
• Playlist PDA Preprocessing	

Outline

1. Introduction
2. Preprocessing
 - Log-transformation
 - Filtering
 - Normalization
 - Summarization

Note, that the R-code is included for learners who are aiming to develop R/markdown scripts to automate their quantitative proteomics data analyses. According to the target audience of the course we either work with a graphical user interface (GUI) in a R/shiny App msqrob2gui (e.g. Proteomics Bioinformatics course of the EBI and the Proteomics Data Analysis course at the Gulbenkian institute) or with R/markdowns scripts (e.g. Bioinformatics Summer School at UCLouvain or the Statistical Genomics Course at Ghent University).

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



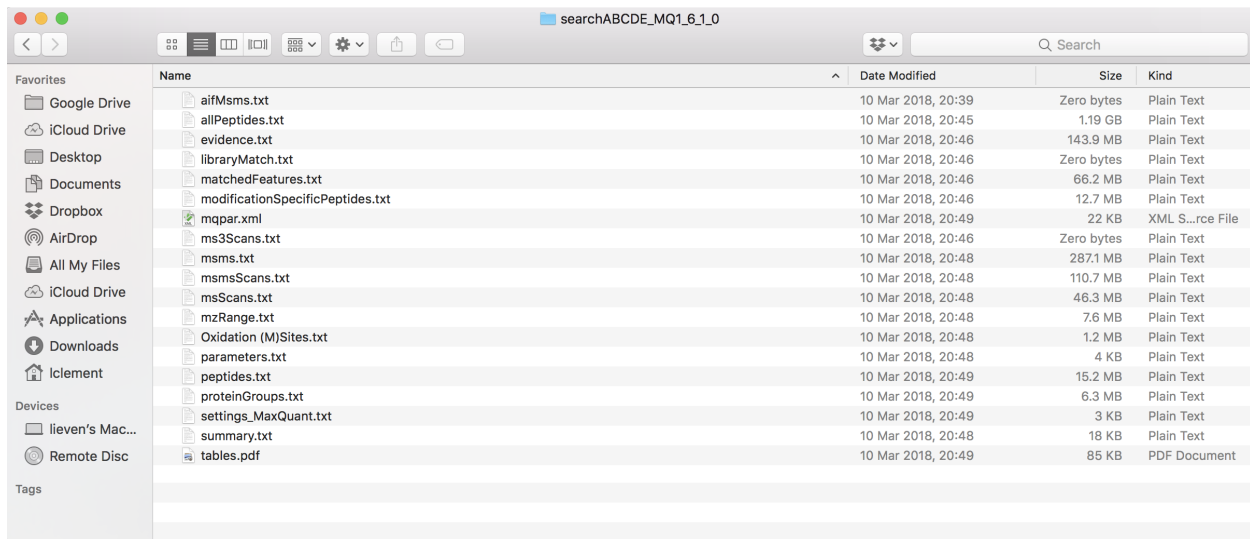
1.4 CPTAC Spike-in Study



- 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

1.5 Maxquant output



Name	Date Modified	Size	Kind
aifMsm.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
mmpar.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

2 Import the data in R

2.1 Data infrastructure

Click to see background on data infrastructure used in R to store proteomics data

- 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

2.2 Import data in R

2.2.1 Load libraries

Click to see code



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.


```
library(tidyverse)
library(limma)
library(QFeatures)
library(msqrob2)
library(plotly)
library(ggplot2)
```

2.2.2 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/fullCptacDataSe"
```

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")
```

2.2.3 Explore object

Click to see background and code

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

```
rowData(pe[["peptideRaw"]])
```

```
## DataFrame with 11466 rows and 143 columns
##               Sequence N.term.cleavage.window C.term.cleavage.window
##               <character>                <character>                <character>
## AAAAGAGGAGDSGDAVTK AAAAGAGGAG...      EHQHDEQKAA...      DSGDAVTKIG...
## AAAALAGGK          AAAALAGGK          QQLSKAAKAA...      AAALAGGKKS...
## AAAALAGGKK          AAAALAGGKK          QQLSKAAKAA...      AALAGGKKSK...
## AAADALSDLEIK        AAADALSDLE...      MPKETPSKAA...      ALSDLEIKDS...
## AAADALSDLEIKDSK     AAADALSDLE...      MPKETPSKAA...      DLEIKDSKSN...
## ...                ...                ...                ...
## YYSIYDLGNNAVLAK     YYSIYDLGNN...      VGDAFLRKYY...      NNAVLAKAI...
## YYTFNGPNYNENETIR    YYTFNGPNYN...      FKDGSPKYY...      YNENETIRHI...
```

##	YYTITEVATR	YYTITEVATR	QEWDIRERY...	TITEVATRAK...			
##	YYTVFDRDNNR	YYTVFDRDNN...	LGDVFIGRYY...	VFDRDNNRVG...			
##	YYTVFDRDNNRVGFAEAAR	YYTVFDRDNN...	LGDVFIGRYY...	VGFAEAARL_...			
##		Amino.acid.before	First.amino.acid	Second.amino.acid			
##		<character>	<character>	<character>			
##	AAAAGAGGAGDSGDAVTK	K	A	A			
##	AAAALAGGK	K	A	A			
##	AAAALAGGKK	K	A	A			
##	AAADALSDLEIK	K	A	A			
##	AAADALSDLEIKDSK	K	A	A			
##			
##	YYSIYDLGNNNAVGLAK	K	Y	Y			
##	YYTFNGPNYNENETIR	K	Y	Y			
##	YYTITEVATR	R	Y	Y			
##	YYTVFDRDNNR	R	Y	Y			
##	YYTVFDRDNNRVGFAEAAR	R	Y	Y			
##		Second.last.amino.acid	Last.amino.acid	Amino.acid.after			
##		<character>	<character>	<character>			
##	AAAAGAGGAGDSGDAVTK	T	K	I			
##	AAAALAGGK	G	K	K			
##	AAAALAGGKK	K	K	S			
##	AAADALSDLEIK	I	K	D			
##	AAADALSDLEIKDSK	S	K	S			
##			
##	YYSIYDLGNNNAVGLAK	A	K	A			
##	YYTFNGPNYNENETIR	I	R	H			
##	YYTITEVATR	T	R	A			
##	YYTVFDRDNNR	N	R	V			
##	YYTVFDRDNNRVGFAEAAR	A	R	L			
##		A.Count	R.Count	N.Count	D.Count	C.Count	Q.Count
##		<integer>	<integer>	<integer>	<integer>	<integer>	<integer>
##	AAAAGAGGAGDSGDAVTK	7	0	0	2	0	0
##	AAAALAGGK	5	0	0	0	0	0
##	AAAALAGGKK	5	0	0	0	0	0
##	AAADALSDLEIK	4	0	0	2	0	0
##	AAADALSDLEIKDSK	4	0	0	3	0	0
##
##	YYSIYDLGNNNAVGLAK	2	0	2	1	0	0
##	YYTFNGPNYNENETIR	0	1	4	0	0	0
##	YYTITEVATR	1	1	0	0	0	0
##	YYTVFDRDNNR	0	2	2	2	0	0
##	YYTVFDRDNNRVGFAEAAR	3	3	2	2	0	0
##		E.Count	G.Count	H.Count	I.Count	L.Count	K.Count
##		<integer>	<integer>	<integer>	<integer>	<integer>	<integer>
##	AAAAGAGGAGDSGDAVTK	0	5	0	0	0	1
##	AAAALAGGK	0	2	0	0	1	1
##	AAAALAGGKK	0	2	0	0	1	2
##	AAADALSDLEIK	1	0	0	1	2	1
##	AAADALSDLEIKDSK	1	0	0	1	2	2
##
##	YYSIYDLGNNNAVGLAK	0	2	0	1	2	1
##	YYTFNGPNYNENETIR	2	1	0	1	0	0
##	YYTITEVATR	1	0	0	1	0	0
##	YYTVFDRDNNR	0	0	0	0	0	0

##	YYTVFDRDNNRVGFAEAAR	1	1	0	0	0	0
##		M.Count	F.Count	P.Count	S.Count	T.Count	W.Count
##		<integer>	<integer>	<integer>	<integer>	<integer>	<integer>
##	AAAAGAGGAGDSGDAVTK	0	0	0	1	1	0
##	AAAALAGGK	0	0	0	0	0	0
##	AAAALAGGKK	0	0	0	0	0	0
##	AAADALSDLEIK	0	0	0	1	0	0
##	AAADALSDLEIKDSK	0	0	0	2	0	0
##
##	YYSIYDLGNNAVLAK	0	0	0	1	0	0
##	YYTFNGPNYNENETIR	0	1	1	0	2	0
##	YYTITEVATR	0	0	0	0	3	0
##	YYTVFDRDNNR	0	1	0	0	1	0
##	YYTVFDRDNNRVGFAEAAR	0	2	0	0	1	0
##		Y.Count	V.Count	U.Count	Length	Missed.cleavages	
##		<integer>	<integer>	<integer>	<integer>	<integer>	
##	AAAAGAGGAGDSGDAVTK	0	1	0	18		0
##	AAAALAGGK	0	0	0	9		0
##	AAAALAGGKK	0	0	0	10		1
##	AAADALSDLEIK	0	0	0	12		0
##	AAADALSDLEIKDSK	0	0	0	15		1
##
##	YYSIYDLGNNAVLAK	3	1	0	16		0
##	YYTFNGPNYNENETIR	3	0	0	16		0
##	YYTITEVATR	2	1	0	10		0
##	YYTVFDRDNNR	2	1	0	11		1
##	YYTVFDRDNNRVGFAEAAR	2	2	0	19		2
##		Mass	Proteins	Leading.razor.protein			
##		<numeric>	<character>	<character>			
##	AAAAGAGGAGDSGDAVTK	1445.675	sp P38915 ...	sp P38915 ...			
##	AAAALAGGK	728.418	sp Q3E792 ...	sp Q3E792 ...			
##	AAAALAGGKK	856.513	sp Q3E792 ...	sp Q3E792 ...			
##	AAADALSDLEIK	1215.635	sp P09938 ...	sp P09938 ...			
##	AAADALSDLEIKDSK	1545.789	sp P09938 ...	sp P09938 ...			
##			
##	YYSIYDLGNNAVLAK	1759.88	sp P07267 ...	sp P07267 ...			
##	YYTFNGPNYNENETIR	1993.88	sp Q00955 ...	sp Q00955 ...			
##	YYTITEVATR	1215.61	sp P38891 ...	sp P38891 ...			
##	YYTVFDRDNNR	1461.66	P07339ups ...	P07339ups ...			
##	YYTVFDRDNNRVGFAEAAR	2263.08	P07339ups ...	P07339ups ...			
##		Start.position	End.position	Unique..Groups.			
##		<integer>	<integer>	<character>			
##	AAAAGAGGAGDSGDAVTK	97	114	yes			
##	AAAALAGGK	13	21	yes			
##	AAAALAGGKK	13	22	yes			
##	AAADALSDLEIK	9	20	yes			
##	AAADALSDLEIKDSK	9	23	yes			
##			
##	YYSIYDLGNNAVLAK	388	403	yes			
##	YYTFNGPNYNENETIR	1275	1290	yes			
##	YYTITEVATR	311	320	yes			
##	YYTVFDRDNNR	225	235	yes			
##	YYTVFDRDNNRVGFAEAAR	225	243	yes			
##		Unique..Proteins.	Charges	PEP	Score		

##	<character>	<character>	<numeric>	<numeric>
## AAAAGAGGAGDSGDAVTK	yes		2 1.1843e-05	82.942
## AAAALAGGK	no		2 7.4562e-06	134.810
## AAAALAGGKK	no		2 3.3094e-09	143.730
## AAADALSDLEIK	yes		2 9.1593e-23	182.230
## AAADALSDLEIKDSK	yes		3 1.5319e-04	73.927
##
## YYSIYDLGNNAVGLAK	yes		2 7.7415e-37	174.240
## YYTFNGPNYNENETIR	yes		2 4.2208e-21	147.750
## YYTITEVATR	yes		2 1.3566e-04	109.160
## YYTVFDRDNNR	yes		2 6.1425e-04	110.930
## YYTVFDRDNNRVGFAEAAR	yes		3 8.9859e-04	59.728
##	Identification.type.6A_1	Identification.type.6A_2		
##	<character>	<character>		
## AAAAGAGGAGDSGDAVTK	By matchin...	By MS/MS		
## AAAALAGGK	By matchin...	By matchin...		
## AAAALAGGKK	By matchin...	By matchin...		
## AAADALSDLEIK	By MS/MS	By MS/MS		
## AAADALSDLEIKDSK	By matchin...	By matchin...		
##		
## YYSIYDLGNNAVGLAK	By matchin...	By matchin...		
## YYTFNGPNYNENETIR	By matchin...	By matchin...		
## YYTITEVATR	By MS/MS	By matchin...		
## YYTVFDRDNNR	By matchin...	By matchin...		
## YYTVFDRDNNRVGFAEAAR	By matchin...	By matchin...		
##	Identification.type.6A_3	Identification.type.6A_4		
##	<character>	<character>		
## AAAAGAGGAGDSGDAVTK	By matchin...	By MS/MS		
## AAAALAGGK	By matchin...	By MS/MS		
## AAAALAGGKK	By matchin...	By MS/MS		
## AAADALSDLEIK	By matchin...	By MS/MS		
## AAADALSDLEIKDSK	By matchin...	By MS/MS		
##		
## YYSIYDLGNNAVGLAK	By matchin...	By MS/MS		
## YYTFNGPNYNENETIR	By matchin...	By MS/MS		
## YYTITEVATR	By matchin...	By matchin...		
## YYTVFDRDNNR	By matchin...	By matchin...		
## YYTVFDRDNNRVGFAEAAR	By matchin...	By matchin...		
##	Identification.type.6A_5	Identification.type.6A_6		
##	<character>	<character>		
## AAAAGAGGAGDSGDAVTK	By matchin...	By matchin...		
## AAAALAGGK	By matchin...	By matchin...		
## AAAALAGGKK	By matchin...	By matchin...		
## AAADALSDLEIK	By MS/MS	By MS/MS		
## AAADALSDLEIKDSK	By MS/MS	By MS/MS		
##		
## YYSIYDLGNNAVGLAK	By MS/MS	By MS/MS		
## YYTFNGPNYNENETIR	By MS/MS	By MS/MS		
## YYTITEVATR	By matchin...	By matchin...		
## YYTVFDRDNNR	By matchin...	By matchin...		
## YYTVFDRDNNRVGFAEAAR	By matchin...	By matchin...		
##	Identification.type.6A_7	Identification.type.6A_8		
##	<character>	<character>		
## AAAAGAGGAGDSGDAVTK	By MS/MS	By MS/MS		

## AAAALAGGK	By MS/MS	By MS/MS
## AAAALAGGKK	By MS/MS	By MS/MS
## AAADALSDLEIK	By MS/MS	By matchin...
## AAADALSDLEIKDSK	By MS/MS	By MS/MS
##
## YYSIYDLGNNAVGLAK	By matchin...	By matchin...
## YYTFNGPNYNENETIR	By matchin...	By matchin...
## YYTITEVATR	By MS/MS	By matchin...
## YYTVFDRDNNR	By matchin...	By matchin...
## YYTVFDRDNNRVGFAEAAR	By matchin...	By matchin...
##	Identification.type.6A_9	Identification.type.6B_1
##	<character>	<character>
## AAAAGAGGAGDSGDAVTK	By MS/MS	By matchin...
## AAAALAGGK	By MS/MS	By MS/MS
## AAAALAGGKK	By MS/MS	By matchin...
## AAADALSDLEIK	By MS/MS	By MS/MS
## AAADALSDLEIKDSK	By MS/MS	By matchin...
##
## YYSIYDLGNNAVGLAK	By matchin...	By matchin...
## YYTFNGPNYNENETIR	By matchin...	By matchin...
## YYTITEVATR	By matchin...	By MS/MS
## YYTVFDRDNNR	By matchin...	By matchin...
## YYTVFDRDNNRVGFAEAAR	By matchin...	By matchin...
##	Identification.type.6B_2	Identification.type.6B_3
##	<character>	<character>
## AAAAGAGGAGDSGDAVTK	By matchin...	By matchin...
## AAAALAGGK	By matchin...	By matchin...
## AAAALAGGKK	By MS/MS	By MS/MS
## AAADALSDLEIK	By MS/MS	By matchin...
## AAADALSDLEIKDSK	By matchin...	By matchin...
##
## YYSIYDLGNNAVGLAK	By matchin...	By matchin...
## YYTFNGPNYNENETIR	By matchin...	By matchin...
## YYTITEVATR	By matchin...	By matchin...
## YYTVFDRDNNR	By matchin...	By matchin...
## YYTVFDRDNNRVGFAEAAR	By matchin...	By matchin...
##	Identification.type.6B_4	Identification.type.6B_5
##	<character>	<character>
## AAAAGAGGAGDSGDAVTK	By matchin...	By matchin...
## AAAALAGGK	By matchin...	By matchin...
## AAAALAGGKK	By matchin...	By matchin...
## AAADALSDLEIK	By MS/MS	By MS/MS
## AAADALSDLEIKDSK	By MS/MS	By MS/MS
##
## YYSIYDLGNNAVGLAK	By MS/MS	By matchin...
## YYTFNGPNYNENETIR	By MS/MS	By MS/MS
## YYTITEVATR	By MS/MS	By MS/MS
## YYTVFDRDNNR	By matchin...	By matchin...
## YYTVFDRDNNRVGFAEAAR	By matchin...	By matchin...
##	Identification.type.6B_6	Identification.type.6B_7
##	<character>	<character>
## AAAAGAGGAGDSGDAVTK	By matchin...	By matchin...
## AAAALAGGK	By matchin...	By MS/MS
## AAAALAGGKK	By matchin...	By MS/MS

## AAADALSDLEIK	By MS/MS	By MS/MS
## AAADALSDLEIKDSK	By MS/MS	By MS/MS
##
## YYSIYDLGNNNAVGLAK	By matchin...	By matchin...
## YYTFNGPNYNENETIR	By MS/MS	By matchin...
## YYTITEVATR	By matchin...	By matchin...
## YYTVFDRDNNR	By matchin...	By matchin...
## YYTVFDRDNNRVGFAEAAR	By matchin...	By matchin...
##	Identification.type.6B_8	Identification.type.6B_9
##	<character>	<character>
## AAAAGAGGAGDSGDAVTK	By MS/MS	By MS/MS
## AAAALAGGK	By MS/MS	By MS/MS
## AAAALAGGKK	By MS/MS	By MS/MS
## AAADALSDLEIK	By matchin...	By matchin...
## AAADALSDLEIKDSK	By MS/MS	By MS/MS
##
## YYSIYDLGNNNAVGLAK	By matchin...	By matchin...
## YYTFNGPNYNENETIR	By matchin...	By matchin...
## YYTITEVATR	By MS/MS	By matchin...
## YYTVFDRDNNR	By matchin...	By matchin...
## YYTVFDRDNNRVGFAEAAR	By matchin...	By matchin...
##	Identification.type.6C_1	Identification.type.6C_2
##	<character>	<character>
## AAAAGAGGAGDSGDAVTK	By matchin...	By matchin...
## AAAALAGGK	By matchin...	By MS/MS
## AAAALAGGKK	By matchin...	By MS/MS
## AAADALSDLEIK	By MS/MS	By matchin...
## AAADALSDLEIKDSK	By matchin...	By matchin...
##
## YYSIYDLGNNNAVGLAK	By matchin...	By matchin...
## YYTFNGPNYNENETIR	By matchin...	By matchin...
## YYTITEVATR	By matchin...	By matchin...
## YYTVFDRDNNR	By matchin...	By matchin...
## YYTVFDRDNNRVGFAEAAR	By matchin...	By matchin...
##	Identification.type.6C_3	Identification.type.6C_4
##	<character>	<character>
## AAAAGAGGAGDSGDAVTK	By matchin...	By matchin...
## AAAALAGGK	By matchin...	By MS/MS
## AAAALAGGKK	By matchin...	By MS/MS
## AAADALSDLEIK	By MS/MS	By MS/MS
## AAADALSDLEIKDSK	By matchin...	By MS/MS
##
## YYSIYDLGNNNAVGLAK	By matchin...	By MS/MS
## YYTFNGPNYNENETIR	By matchin...	By MS/MS
## YYTITEVATR	By MS/MS	By matchin...
## YYTVFDRDNNR	By matchin...	By matchin...
## YYTVFDRDNNRVGFAEAAR	By matchin...	By matchin...
##	Identification.type.6C_5	Identification.type.6C_6
##	<character>	<character>
## AAAAGAGGAGDSGDAVTK	By MS/MS	By matchin...
## AAAALAGGK	By matchin...	By matchin...
## AAAALAGGKK	By matchin...	By matchin...
## AAADALSDLEIK	By MS/MS	By MS/MS
## AAADALSDLEIKDSK	By MS/MS	By MS/MS

```

## ...
## YYSIYDLGNNAVGLAK By MS/MS By MS/MS
## YYTFNGPNYNENETIR By matchin... By matchin...
## YYTITEVATR By matchin... By matchin...
## YYTVFDRDNNR By matchin... By matchin...
## YYTVFDRDNNRVGFAEAAR By matchin... By matchin...
## Identification.type.6C_7 Identification.type.6C_8
## <character> <character>
## AAAAGAGGAGDSGDAVTK By MS/MS By matchin...
## AAAALAGGK By MS/MS By MS/MS
## AAAALAGGKK By MS/MS By MS/MS
## AAADALSDLEIK By matchin... By MS/MS
## AAADALSDLEIKDSK By MS/MS By MS/MS
## ...
## YYSIYDLGNNAVGLAK By matchin... By matchin...
## YYTFNGPNYNENETIR By matchin... By matchin...
## YYTITEVATR By matchin... By MS/MS
## YYTVFDRDNNR By matchin... By matchin...
## YYTVFDRDNNRVGFAEAAR By matchin... By matchin...
## Identification.type.6C_9 Identification.type.6D_1
## <character> <character>
## AAAAGAGGAGDSGDAVTK By matchin... By matchin...
## AAAALAGGK By MS/MS By matchin...
## AAAALAGGKK By MS/MS By matchin...
## AAADALSDLEIK By MS/MS By MS/MS
## AAADALSDLEIKDSK By MS/MS By MS/MS
## ...
## YYSIYDLGNNAVGLAK By matchin... By matchin...
## YYTFNGPNYNENETIR By matchin... By matchin...
## YYTITEVATR By MS/MS By matchin...
## YYTVFDRDNNR By matchin... By matchin...
## YYTVFDRDNNRVGFAEAAR By matchin... By matchin...
## Identification.type.6D_2 Identification.type.6D_3
## <character> <character>
## AAAAGAGGAGDSGDAVTK By matchin... By matchin...
## AAAALAGGK By matchin... By matchin...
## AAAALAGGKK By matchin... By matchin...
## AAADALSDLEIK By matchin... By matchin...
## AAADALSDLEIKDSK By MS/MS By matchin...
## ...
## YYSIYDLGNNAVGLAK By matchin... By matchin...
## YYTFNGPNYNENETIR By matchin... By matchin...
## YYTITEVATR By MS/MS By MS/MS
## YYTVFDRDNNR By matchin... By matchin...
## YYTVFDRDNNRVGFAEAAR By matchin... By matchin...
## Identification.type.6D_4 Identification.type.6D_5
## <character> <character>
## AAAAGAGGAGDSGDAVTK By matchin... By matchin...
## AAAALAGGK By matchin... By matchin...
## AAAALAGGKK By MS/MS By matchin...
## AAADALSDLEIK By MS/MS By MS/MS
## AAADALSDLEIKDSK By MS/MS By MS/MS
## ...
## YYSIYDLGNNAVGLAK By MS/MS By MS/MS

```

## YYTFNGPNYNENETIR	By MS/MS	By MS/MS
## YYTITEVATR	By matchin...	By matchin...
## YYTVFDRDNNR	By matchin...	By matchin...
## YYTVFDRDNNRVGFAEAAR	By matchin...	By matchin...
##	Identification.type.6D_6	Identification.type.6D_7
##	<character>	<character>
## AAAAGAGGAGDSGDAVTK	By MS/MS	By matchin...
## AAAALAGGK	By matchin...	By MS/MS
## AAAALAGGKK	By matchin...	By MS/MS
## AAADALSDLEIK	By MS/MS	By matchin...
## AAADALSDLEIKDSK	By matchin...	By MS/MS
##
## YYSIYDLGNNAVGLAK	By MS/MS	By matchin...
## YYTFNGPNYNENETIR	By MS/MS	By matchin...
## YYTITEVATR	By matchin...	By matchin...
## YYTVFDRDNNR	By matchin...	By MS/MS
## YYTVFDRDNNRVGFAEAAR	By matchin...	By matchin...
##	Identification.type.6D_8	Identification.type.6D_9
##	<character>	<character>
## AAAAGAGGAGDSGDAVTK	By matchin...	By matchin...
## AAAALAGGK	By MS/MS	By MS/MS
## AAAALAGGKK	By MS/MS	By MS/MS
## AAADALSDLEIK	By MS/MS	By MS/MS
## AAADALSDLEIKDSK	By MS/MS	By MS/MS
##
## YYSIYDLGNNAVGLAK	By matchin...	By matchin...
## YYTFNGPNYNENETIR	By matchin...	By matchin...
## YYTITEVATR	By MS/MS	By matchin...
## YYTVFDRDNNR	By MS/MS	By matchin...
## YYTVFDRDNNRVGFAEAAR	By matchin...	By matchin...
##	Identification.type.6E_1	Identification.type.6E_2
##	<character>	<character>
## AAAAGAGGAGDSGDAVTK	By matchin...	By matchin...
## AAAALAGGK	By matchin...	By matchin...
## AAAALAGGKK	By matchin...	By matchin...
## AAADALSDLEIK	By MS/MS	By MS/MS
## AAADALSDLEIKDSK	By MS/MS	By MS/MS
##
## YYSIYDLGNNAVGLAK	By matchin...	By matchin...
## YYTFNGPNYNENETIR	By matchin...	By matchin...
## YYTITEVATR	By matchin...	By matchin...
## YYTVFDRDNNR	By matchin...	By matchin...
## YYTVFDRDNNRVGFAEAAR	By matchin...	By matchin...
##	Identification.type.6E_3	Identification.type.6E_4
##	<character>	<character>
## AAAAGAGGAGDSGDAVTK	By matchin...	By matchin...
## AAAALAGGK	By matchin...	By MS/MS
## AAAALAGGKK	By matchin...	By matchin...
## AAADALSDLEIK	By matchin...	By MS/MS
## AAADALSDLEIKDSK	By MS/MS	By matchin...
##
## YYSIYDLGNNAVGLAK	By matchin...	By MS/MS
## YYTFNGPNYNENETIR	By matchin...	By MS/MS
## YYTITEVATR	By matchin...	By matchin...

## YYTVFDRDNNR	By matchin...	By MS/MS	
## YYTVFDRDNNRVGFAEAAR	By matchin...	By matchin...	
##	Identification.type.6E_5	Identification.type.6E_6	
##	<character>	<character>	
## AAAAGAGGAGDSGDAVTK	By matchin...	By matchin...	
## AAAALAGGK	By matchin...	By matchin...	
## AAAALAGGKK	By matchin...	By matchin...	
## AAADALSDLEIK	By MS/MS	By matchin...	
## AAADALSDLEIKDSK	By MS/MS	By MS/MS	
##	
## YYSIYDLGNNAVGLAK	By MS/MS	By MS/MS	
## YYTFNGPNYNENETIR	By MS/MS	By MS/MS	
## YYTITEVATR	By matchin...	By matchin...	
## YYTVFDRDNNR	By MS/MS	By matchin...	
## YYTVFDRDNNRVGFAEAAR	By matchin...	By MS/MS	
##	Identification.type.6E_7	Identification.type.6E_8	
##	<character>	<character>	
## AAAAGAGGAGDSGDAVTK	By matchin...	By matchin...	
## AAAALAGGK	By MS/MS	By MS/MS	
## AAAALAGGKK	By MS/MS	By MS/MS	
## AAADALSDLEIK	By MS/MS	By MS/MS	
## AAADALSDLEIKDSK	By matchin...	By MS/MS	
##	
## YYSIYDLGNNAVGLAK	By matchin...	By matchin...	
## YYTFNGPNYNENETIR	By matchin...	By matchin...	
## YYTITEVATR	By matchin...	By matchin...	
## YYTVFDRDNNR	By MS/MS	By MS/MS	
## YYTVFDRDNNRVGFAEAAR	By matchin...	By matchin...	
##	Identification.type.6E_9	Experiment.6A_1	Experiment.6A_2
##	<character>	<integer>	<integer>
## AAAAGAGGAGDSGDAVTK	By matchin...	NA	1
## AAAALAGGK	By MS/MS	NA	1
## AAAALAGGKK	By MS/MS	NA	1
## AAADALSDLEIK	By MS/MS	1	1
## AAADALSDLEIKDSK	By MS/MS	1	1
##
## YYSIYDLGNNAVGLAK	By matchin...	NA	NA
## YYTFNGPNYNENETIR	By MS/MS	NA	NA
## YYTITEVATR	By matchin...	1	NA
## YYTVFDRDNNR	By MS/MS	NA	NA
## YYTVFDRDNNRVGFAEAAR	By matchin...	NA	NA
##	Experiment.6A_3	Experiment.6A_4	Experiment.6A_5
##	<integer>	<integer>	<integer>
## AAAAGAGGAGDSGDAVTK	NA	1	1
## AAAALAGGK	2	1	1
## AAAALAGGKK	NA	1	NA
## AAADALSDLEIK	1	1	1
## AAADALSDLEIKDSK	NA	1	1
##
## YYSIYDLGNNAVGLAK	NA	1	1
## YYTFNGPNYNENETIR	NA	1	1
## YYTITEVATR	1	NA	NA
## YYTVFDRDNNR	NA	NA	NA
## YYTVFDRDNNRVGFAEAAR	NA	NA	NA

##	Experiment.6A_6	Experiment.6A_7	Experiment.6A_8
##	<integer>	<integer>	<integer>
##	AAAAGAGGAGDSGDAVTK	1	1
##	AAAALAGGK	1	2
##	AAAALAGGKK	1	1
##	AAADALSDLEIK	1	1
##	AAADALSDLEIKDSK	1	1
##
##	YYSIYDLGNNAVGLAK	1	NA
##	YYTFNGPNYNENETIR	1	1
##	YYTITEVATR	1	1
##	YYTVFDRDNNR	NA	NA
##	YYTVFDRDNNRVGFAEAAR	NA	NA
##	Experiment.6A_9	Experiment.6B_1	Experiment.6B_2
##	<integer>	<integer>	<integer>
##	AAAAGAGGAGDSGDAVTK	1	NA
##	AAAALAGGK	1	1
##	AAAALAGGKK	1	NA
##	AAADALSDLEIK	1	1
##	AAADALSDLEIKDSK	1	NA
##
##	YYSIYDLGNNAVGLAK	NA	NA
##	YYTFNGPNYNENETIR	1	NA
##	YYTITEVATR	NA	1
##	YYTVFDRDNNR	NA	NA
##	YYTVFDRDNNRVGFAEAAR	NA	NA
##	Experiment.6B_3	Experiment.6B_4	Experiment.6B_5
##	<integer>	<integer>	<integer>
##	AAAAGAGGAGDSGDAVTK	NA	NA
##	AAAALAGGK	1	2
##	AAAALAGGKK	1	1
##	AAADALSDLEIK	1	1
##	AAADALSDLEIKDSK	NA	1
##
##	YYSIYDLGNNAVGLAK	NA	1
##	YYTFNGPNYNENETIR	NA	1
##	YYTITEVATR	1	1
##	YYTVFDRDNNR	NA	NA
##	YYTVFDRDNNRVGFAEAAR	NA	NA
##	Experiment.6B_6	Experiment.6B_7	Experiment.6B_8
##	<integer>	<integer>	<integer>
##	AAAAGAGGAGDSGDAVTK	1	NA
##	AAAALAGGK	NA	2
##	AAAALAGGKK	NA	1
##	AAADALSDLEIK	1	1
##	AAADALSDLEIKDSK	1	1
##
##	YYSIYDLGNNAVGLAK	1	NA
##	YYTFNGPNYNENETIR	1	1
##	YYTITEVATR	1	NA
##	YYTVFDRDNNR	NA	NA
##	YYTVFDRDNNRVGFAEAAR	NA	NA
##	Experiment.6B_9	Experiment.6C_1	Experiment.6C_2
##	<integer>	<integer>	<integer>

## AAAAGAGGAGDSGDAVTK	1	NA	NA
## AAAALAGGK	2	NA	1
## AAAALAGGKK	1	NA	1
## AAADALSDLEIK	1	1	1
## AAADALSDLEIKDSK	1	1	1
##
## YYSIYDLGNNAVGLAK	NA	NA	NA
## YYTFNGPNYNENETIR	NA	NA	NA
## YYTITEVATR	NA	1	1
## YYTVFDRDNNR	NA	NA	NA
## YYTVFDRDNNRVGFAEAAR	NA	NA	NA
##	Experiment.6C_3	Experiment.6C_4	Experiment.6C_5
##	<integer>	<integer>	<integer>
## AAAAGAGGAGDSGDAVTK	NA	1	1
## AAAALAGGK	2	2	NA
## AAAALAGGKK	NA	1	NA
## AAADALSDLEIK	1	1	1
## AAADALSDLEIKDSK	1	1	1
##
## YYSIYDLGNNAVGLAK	NA	1	1
## YYTFNGPNYNENETIR	NA	1	1
## YYTITEVATR	1	1	NA
## YYTVFDRDNNR	NA	NA	NA
## YYTVFDRDNNRVGFAEAAR	NA	NA	NA
##	Experiment.6C_6	Experiment.6C_7	Experiment.6C_8
##	<integer>	<integer>	<integer>
## AAAAGAGGAGDSGDAVTK	1	1	1
## AAAALAGGK	NA	2	1
## AAAALAGGKK	NA	1	1
## AAADALSDLEIK	1	1	1
## AAADALSDLEIKDSK	1	1	1
##
## YYSIYDLGNNAVGLAK	1	NA	NA
## YYTFNGPNYNENETIR	1	1	1
## YYTITEVATR	1	NA	1
## YYTVFDRDNNR	1	NA	1
## YYTVFDRDNNRVGFAEAAR	NA	NA	NA
##	Experiment.6C_9	Experiment.6D_1	Experiment.6D_2
##	<integer>	<integer>	<integer>
## AAAAGAGGAGDSGDAVTK	1	NA	NA
## AAAALAGGK	1	NA	1
## AAAALAGGKK	1	NA	NA
## AAADALSDLEIK	1	1	1
## AAADALSDLEIKDSK	1	1	1
##
## YYSIYDLGNNAVGLAK	NA	NA	NA
## YYTFNGPNYNENETIR	1	NA	NA
## YYTITEVATR	1	NA	1
## YYTVFDRDNNR	NA	NA	NA
## YYTVFDRDNNRVGFAEAAR	NA	NA	NA
##	Experiment.6D_3	Experiment.6D_4	Experiment.6D_5
##	<integer>	<integer>	<integer>
## AAAAGAGGAGDSGDAVTK	NA	1	1
## AAAALAGGK	1	1	1

##	AAAALAGGKK	NA	1	NA
##	AAADALSDLEIK	1	1	1
##	AAADALSDLEIKDSK	1	1	1
##
##	YYSIYDLGNNAVGLAK	NA	1	1
##	YYTFNGPNYNENETIR	NA	1	1
##	YYTITEVATR	1	1	1
##	YYTVFDRDNNR	NA	1	1
##	YYTVFDRDNNRVGFAEAAR	NA	1	NA
##		Experiment.6D_6	Experiment.6D_7	Experiment.6D_8
##		<integer>	<integer>	<integer>
##	AAAAGAGGAGDSGDAVTK	1	1	NA
##	AAAALAGGK	NA	2	1
##	AAAALAGGKK	NA	1	1
##	AAADALSDLEIK	1	1	1
##	AAADALSDLEIKDSK	1	1	1
##
##	YYSIYDLGNNAVGLAK	1	1	NA
##	YYTFNGPNYNENETIR	1	1	1
##	YYTITEVATR	1	NA	1
##	YYTVFDRDNNR	1	1	1
##	YYTVFDRDNNRVGFAEAAR	NA	NA	NA
##		Experiment.6D_9	Experiment.6E_1	Experiment.6E_2
##		<integer>	<integer>	<integer>
##	AAAAGAGGAGDSGDAVTK	NA	NA	1
##	AAAALAGGK	2	NA	1
##	AAAALAGGKK	1	NA	NA
##	AAADALSDLEIK	1	1	1
##	AAADALSDLEIKDSK	1	1	1
##
##	YYSIYDLGNNAVGLAK	NA	NA	NA
##	YYTFNGPNYNENETIR	1	NA	NA
##	YYTITEVATR	NA	NA	1
##	YYTVFDRDNNR	1	1	NA
##	YYTVFDRDNNRVGFAEAAR	NA	NA	NA
##		Experiment.6E_3	Experiment.6E_4	Experiment.6E_5
##		<integer>	<integer>	<integer>
##	AAAAGAGGAGDSGDAVTK	NA	NA	1
##	AAAALAGGK	2	2	1
##	AAAALAGGKK	NA	1	NA
##	AAADALSDLEIK	1	1	1
##	AAADALSDLEIKDSK	1	1	1
##
##	YYSIYDLGNNAVGLAK	1	1	1
##	YYTFNGPNYNENETIR	NA	1	1
##	YYTITEVATR	1	1	1
##	YYTVFDRDNNR	1	1	1
##	YYTVFDRDNNRVGFAEAAR	NA	1	1
##		Experiment.6E_6	Experiment.6E_7	Experiment.6E_8
##		<integer>	<integer>	<integer>
##	AAAAGAGGAGDSGDAVTK	1	NA	NA
##	AAAALAGGK	NA	2	2
##	AAAALAGGKK	NA	1	1
##	AAADALSDLEIK	1	1	1

```

## AAADALSDLEIKDSK      1      NA      1
## ...      ...      ...      ...
## YYSIYDLGNNAVGLAK      1      NA      NA
## YYTFNGPNYNENETIR      1      1      1
## YYTITEVATR      NA      NA      NA
## YYTVFDRDNNR      1      1      1
## YYTVFDRDNNRVGFAEAAR      1      1      1
##      Experiment.6E_9 Intensity      Reverse Potential.contaminant
##      <integer> <numeric> <character>      <character>
## AAAAGAGGAGDSGDAVTK      NA      1190800
## AAAALAGGK      1      280990000
## AAAALAGGKK      1      33360000
## AAADALSDLEIK      1      54622000
## AAADALSDLEIKDSK      1      18910000
## ...      ...      ...      ...
## YYSIYDLGNNAVGLAK      NA      2145900
## YYTFNGPNYNENETIR      1      5608800
## YYTITEVATR      NA      13034000
## YYTVFDRDNNR      1      8702500
## YYTVFDRDNNRVGFAEAAR      1      2391100
##      id Protein.group.IDs Mod..peptide.IDs Evidence.IDs
##      <integer>      <character>      <character>      <character>
## AAAAGAGGAGDSGDAVTK      0      859      0 0;1;2;3;4;...
## AAAALAGGK      1      230      1 24;25;26;2...
## AAAALAGGKK      2      230      2 74;75;76;7...
## AAADALSDLEIK      3      229      3 99;100;101...
## AAADALSDLEIKDSK      4      229      4 144;145;14...
## ...      ...      ...      ...
## YYSIYDLGNNAVGLAK      11461      196      12240 331367;331...
## YYTFNGPNYNENETIR      11462      1254      12241 331384;331...
## YYTITEVATR      11463      854      12242 331411;331...
## YYTVFDRDNNR      11464      34      12243 331439;331...
## YYTVFDRDNNRVGFAEAAR      11465      34      12244 331455;331...
##      MS.MS.IDs Best.MS.MS Oxidation..M..site.IDs MS.MS.Count
##      <character> <integer>      <character>      <integer>
## AAAAGAGGAGDSGDAVTK 0;1;2;3;4;...      0      10
## AAAALAGGK      10;11;12;1...      21      18
## AAAALAGGKK      30;31;32;3...      31      21
## AAADALSDLEIK      51;52;53;5...      72      29
## AAADALSDLEIKDSK      85;86;87;8...      94      32
## ...      ...      ...      ...
## YYSIYDLGNNAVGLAK      169138;169...      169147      13
## YYTFNGPNYNENETIR      169151;169...      169159      14
## YYTITEVATR      169165;169...      169173      12
## YYTVFDRDNNR      169177;169...      169180      7
## YYTVFDRDNNRVGFAEAAR      169184      169184      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
```

3 Preprocessing

3.1 Log-transformation

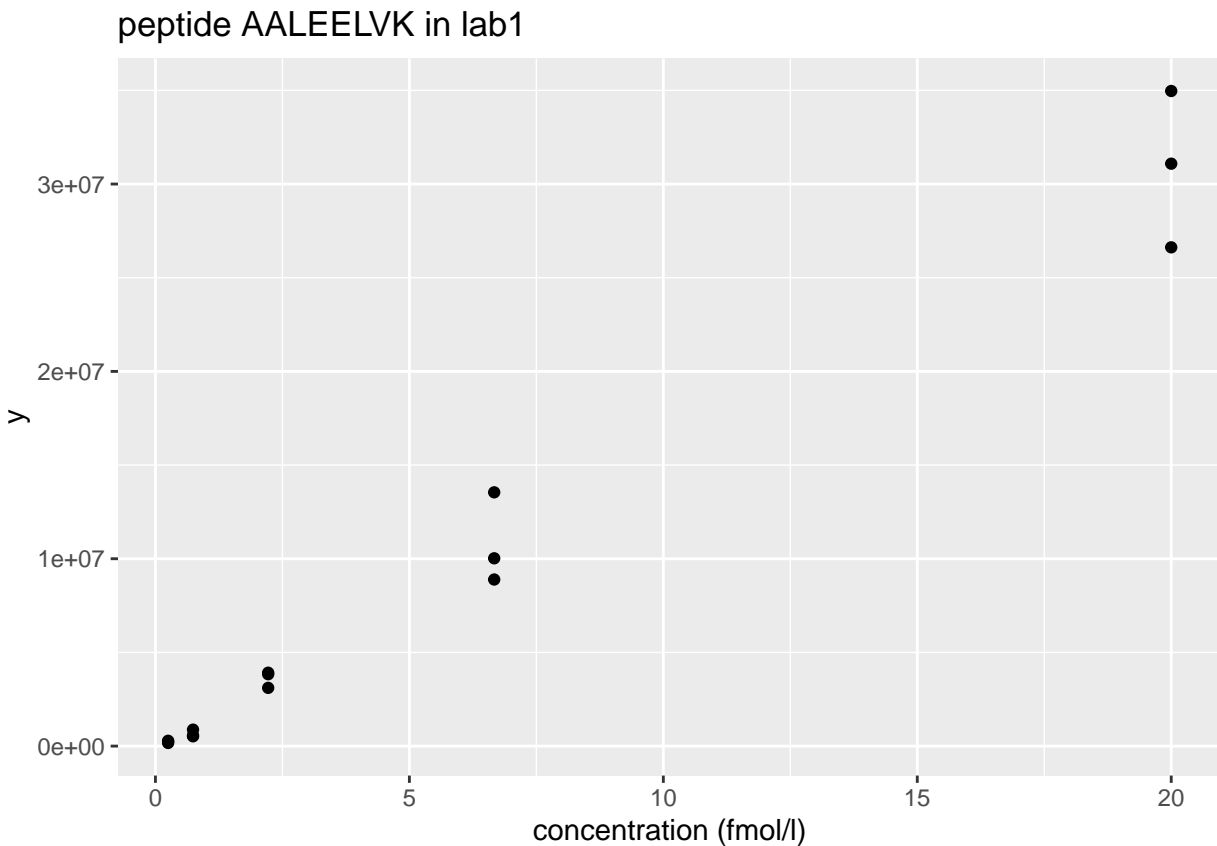
3.1.1 Explore the data with plots

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

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

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

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

3.1.2 log-transformation of the data

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")
```

3.2 Filtering

- Reverse sequences
- Only identified by modification site (only modified peptides detected)
- Razor peptides: non-unique peptides assigned to the protein group with the most other peptides
- Contaminants
- Peptides few identifications
- Proteins that are only identified with one or a few peptides

Filtering does not induce bias if the criterion is independent from the downstream data analysis!

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 least twice.

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

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

We keep 10478 peptides upon filtering.

3.3 Normalization

Click to see code to make plot

```
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

3.3.1 Mean or median?

- Miller and Fishkin (1997) reported that over a period of 30 years males would like to have on average 64.3 partners and females 2.8.
- Miller and Fishkin (1997) reported that the median number of partners someone would like to have over a period of 30 years males is 1 for both males and females.

Mean is very sensitive to outliers!



3.3.2 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")
```

3.3.3 Plots of normalized data

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).
```



- Upon normalization the marginal distributions of the peptide intensities across samples are much more comparable
- We still see deviations
- This can be due to technical variability
- In micro-array literature, quantile normalisation is used to force the median and all other quantiles to be equal across samples
- In proteomics quantile normalisation often introduces artifacts due to a difference in missing peptides across samples
- More advanced methods should be developed for normalizing proteomics data
- If there are differences in the width of the marginal distributions of the data across samples. They can also be standardized by using a robust estimator for location and scale, i.e.

$$y_{ip}^{\text{norm}} = \frac{y_{ip} - \mu_i}{s_i}$$

3.4 Summarization

- We illustrate summarization issues using a subset of the cptac study (Lab 2, condition A and E) for a spiked protein (UPS P12081).

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

```
summaryPlot <- pe[["peptideNorm"]][
  rowData(pe[["peptideNorm"]])$Proteins == "P12081ups|SYHC_HUMAN_UPS",
  colData(pe)$lab=="lab2"&colData(pe)$condition %in% c("A","E")] %>%
  assay %>%
  as.data.frame %>%
  rownames_to_column(var = "peptide") %>%
  gather(sample, intensity, -peptide) %>%
  mutate(condition = colData(pe)[sample,"condition"]) %>%
  ggplot(aes(x = peptide, y = intensity, color = sample, group = sample, label = condition), show.legend = FALSE) +
  geom_line(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)")
```

```
summaryPlot
```

```
## Warning: Removed 10 row(s) containing missing values (geom_path).
```

```
## Warning: Removed 90 rows containing missing values (geom_text).
```



We observe:

- intensities from multiple peptides for each protein in a sample

- Strong peptide effect -Unbalanced peptide identification
- Pseudo-replication: peptide intensities from a particular protein in the same sample are correlated, i.e. they are more alike than peptide intensities from a particular protein between samples.

→ Summarize all peptide intensities from the same protein in a sample into a single protein expression value
Commonly used methods are

- Mean summarization

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

- Median summarization
- Maxquant's maxLFQ summarization (in protein groups file)
- Model based summarization:

$$y_{ip} = \beta_i^{\text{samp}} + \beta_p^{\text{pep}} + \epsilon_{ip}$$

Click to see R-code to normalize the data

We use the standard summarization in `aggregateFeatures`, which is robust model based summarization.

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

```
## 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.
```

Other summarization methods can be implemented by using the `fun` argument in the `aggregateFeatures` function.

- `fun = MsCoreUtils::medianPolish()` to fit an additive model (two way decomposition) using Tukey's median polish procedure using `stats::medpolish()`
- `fun = MsCoreUtils::robustSummary()` to calculate a robust aggregation using `MASS::rlm()` (default)
- `fun = base::colMeans()` to use the mean of each column
- `fun = matrixStats::colMedians()` to use the median of each column
- `fun = base::colSums()` to use the sum of each column

4 Exercise

1. We will evaluate different summarization methods (Maxquant maxLFQ, median and robust model based) in the tutorial session before discussing on their advantages/disadvantages.
 2. Can you anticipate on potential problems related to the summarization?
-

5 Software & code

- Our R/Bioconductor package [msqrob2](#) can be used in R markdown scripts or with a GUI/shinyApp in the [msqrob2gui](#) package.
- The GUI is intended as a introduction to the key concepts of proteomics data analysis for users who have no experience in R.
- However, learning how to code data analyses in R markdown scripts is key for open en reproducible science and for reporting your proteomics data analyses and interpretation in a reproducible way.
- More information on our tools can be found in our papers (Goeminne, Gevaert, and Clement 2016), (Goeminne et al. 2020) and (Sticker et al. 2020). Please refer to our work when using our tools.

5.1 Code

1. Data infrastructure
2. Import proteomics data
3. Preprocessing
 - Log-transformation
 - Filtering
 - Normalisation
 - Summarization

5.2 Data analysis with the GUI/shinyApp msqrob2gui

Goeminne, L. J. E., A. Sticker, L. Martens, K. Gevaert, and L. Clement. 2020. “MSqRob Takes the Missing Hurdle: Uniting Intensity- and Count-Based Proteomics.” *Anal Chem* 92 (9): 6278–87.

Goeminne, L. J., K. Gevaert, and L. Clement. 2016. “Peptide-level Robust Ridge Regression Improves Estimation, Sensitivity, and Specificity in Data-dependent Quantitative Label-free Shotgun Proteomics.” *Mol Cell Proteomics* 15 (2): 657–68.

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.