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

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This is part of the online course Proteomics Data Analysis 2021 (PDA21)

• Playlist PDA Preprocessing

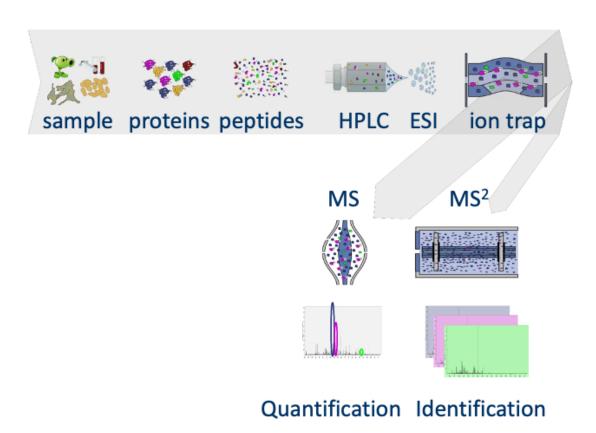
Outline

- 1. Introduction
- 2. Preprocessing
 - $\bullet \quad {\rm Log\text{-}transformation}$
 - 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

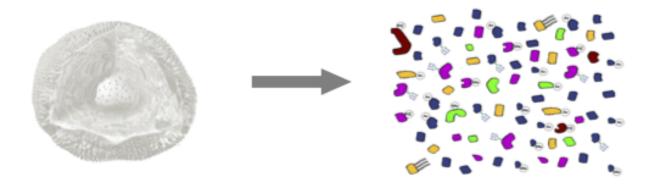
1.1 MS-based workflow



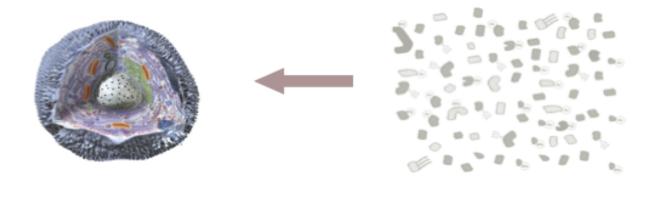
- Peptide Characteristics
 - Modifications
 - Ionisation Efficiency: huge variability
 - Identification
 - * Misidentification \rightarrow outliers
 - * MS 2 selection on peptide abundance
 - * Context depending missingness
 - * Non-random missingness
- \rightarrow Unbalanced pepide identifications across samples and messy data

1.2 Level of quantification

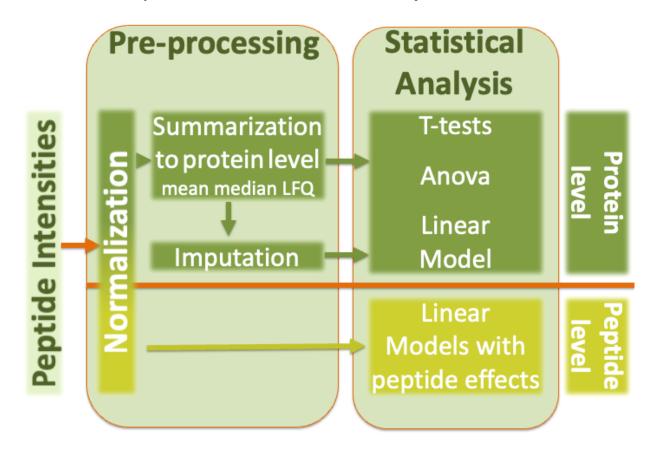
• MS-based proteomics returns peptides: pieces of proteins



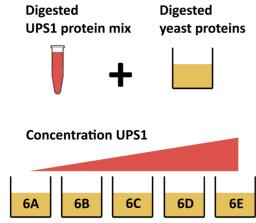
• Quantification commonly required on the protein level



1.3 Label-free Quantitative Proteomics Data Analysis Workflows



1.4 CPTAC Spike-in Study



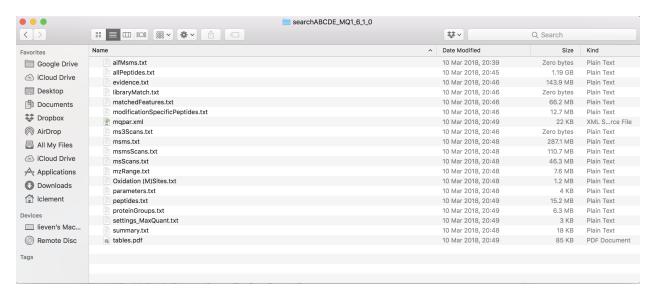
5 spike-in concentrations: 6A to 6E



- 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

 $[\]rightarrow$ vast amount of missingness

1.5 Maxquant output



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

22/06/2021 SE.svg



file: ///Users/lclement/Dropbox/statOmics/PDA21/figures/SE.svg

1/1

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.

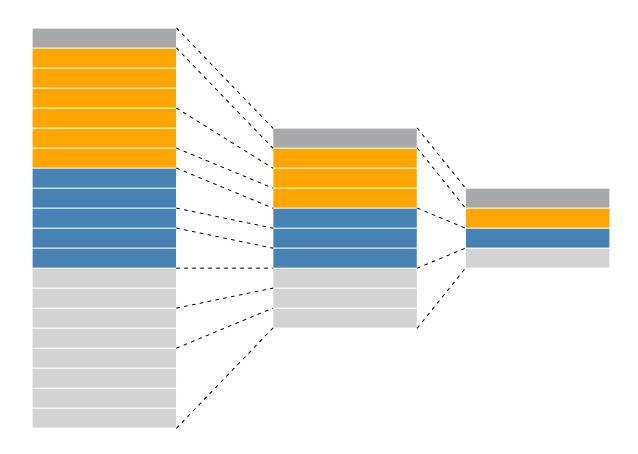


Figure 2: Conceptual representation of a ${\tt QFeatures}$ object and the aggregative relation between different assays.

```
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/fullCptacDatasSe</pre>

2. Maxquant stores the intensity data for the different samples in columnss 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)))</pre>
```

3. Read the data and store it in QFeatures object

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

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
                                               QQLSKAAKAA...
                                                                       AAALAGGKKS...
## AAAALAGGK
## AAAALAGGKK
                          AAAALAGGKK
                                               QQLSKAAKAA...
                                                                       AALAGGKKSK...
## AAADALSDLEIK
                       AAADALSDLE...
                                               MPKETPSKAA...
                                                                       ALSDLEIKDS...
## AAADALSDLEIKDSK
                       AAADALSDLE...
                                               MPKETPSKAA...
                                                                       DLEIKDSKSN...
## ...
## YYSIYDLGNNAVGLAK
                       YYSIYDLGNN...
                                               VGDAFLRKYY...
                                                                       NNAVGLAKAI...
## YYTFNGPNYNENETIR
                       YYTFNGPNYN...
                                               FKDGSYPKYY...
                                                                       YNENETIRHI...
```

	YYTITEVATR	YYTITEV		-	ERYY	TITEVATRAK		
	YYTVFDRDNNR	YYTVFDRDNN		LGDVFIGRYY			VFDRDNNRVG	
	YYTVFDRDNNRVGFAEAAR			LGDVFIGRYY		VGFAEAARL		
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	AAAALAGGKK		K K		A		A	
	AAADALSDLEIK		K K		A		A	
	AAADALSDLEIKDSK		K K		A		A	
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	YYSIYDLGNNAVGLAK				 Ү		 Ү	
	YYTFNGPNYNENETIR		K		Y		Y	
	YYTITEVATR		R		Y		Y	
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	YYTVFDRDNNRVGFAEAAR		R		Y		Y	
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##	AAAALAGGK			G	K		K	
##	AAAALAGGKK			K	K		S	
##	AAADALSDLEIK			I	K		D	
##	AAADALSDLEIKDSK			S	K		S	
##								
##	YYSIYDLGNNAVGLAK			Α	K		Α	
##	YYTFNGPNYNENETIR			I	R		H	
##	YYTITEVATR			T	R		Α	
##	YYTVFDRDNNR			N	R		V	
##	${\tt YYTVFDRDNNRVGFAEAAR}$			Α	R		L	
##		A.Count	R.Count	${\tt N.Count}$	D.Count	C.Count	$Q.\mathtt{Count}$	
##		<integer></integer>	_	<integer></integer>	<integer></integer>	<integer></integer>	<integer></integer>	
	AAAAGAGGAGDSGDAVTK	7	0	0	2	0	0	
	AAAALAGGK	5	0		0	0	0	
	AAAALAGGKK	5	0		0	0	0	
	AAADALSDLEIK	4	0		2	0	0	
	AAADALSDLEIKDSK	4	0	0	3	0	0	
	YYSIYDLGNNAVGLAK							
	YYTFNGPNYNENETIR	2	0		1 0	0	0	
	YYTITEVATR		1		0	0	0	
	YYTVFDRDNNR	1	2		2	0	0	
	YYTVFDRDNNRVGFAEAAR	3	3		2	0	0	
##	TITVIDICUMNICOGIALAAN	E.Count	G.Count	_	I.Count	L.Count	K.Count	
##				<pre>integer></pre>				
	AAAAGAGGAGDSGDAVTK	0	5	_	0	0	1	
	AAAALAGGK	0	2		0	1	1	
##	AAAALAGGKK	0	2		0	1	2	
	AAADALSDLEIK	1	0		1	2	1	
	AAADALSDLEIKDSK	1	0	0	1	2	2	
##	YYSIYDLGNNAVGLAK	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
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                                               P.Count
                                                          S.Count
                                                                     T. Count.
                                                                                W.Count
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## AAAALAGGK
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## AAAALAGGKK
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## AAADALSDLEIK
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                                           0
                                                      0
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## AAADALSDLEIKDSK
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                                                                 2
                                                                           0
                                           0
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## ...
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## YYSIYDLGNNAVGLAK
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                                           0
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                                                                 1
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## YYTFNGPNYNENETIR
                                 0
                                           1
                                                      1
                                                                 0
                                                                           2
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## YYTITEVATR
                                0
                                                      0
                                                                 0
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                                                                                      0
                                           0
## YYTVFDRDNNR
                                0
                                                      0
                                                                 0
                                           1
                                                                           1
                                                                                      0
## YYTVFDRDNNRVGFAEAAR
                                 0
                                           2
                                                      0
                                                                 0
                                                                           1
                                                           Length Missed.cleavages
                          Y.Count
                                     V.Count
                                                U.Count
##
                        <integer> <integer> <integer> <integer>
                                                                          <integer>
## AAAAGAGGAGDSGDAVTK
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                                 0
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## AAAALAGGK
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## AAAALAGGKK
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                                                                10
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## AAADALSDLEIK
                                 0
                                           0
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## AAADALSDLEIKDSK
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## YYSIYDLGNNAVGLAK
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                                                                16
                                           1
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## YYTFNGPNYNENETIR
                                 3
                                           0
                                                      0
                                                                16
                                                                                   0
                                2
## YYTITEVATR
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                                                      0
                                                                10
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## YYTVFDRDNNR
                                2
                                           1
                                                      0
                                                                11
                                                                                   1
## YYTVFDRDNNRVGFAEAAR
                                2
                                           2
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                                                                19
                                                                                   2
                                        Proteins Leading.razor.protein
##
                             Mass
##
                        <numeric>
                                                            <character>
                                     <character>
## AAAAGAGGAGDSGDAVTK
                         1445.675 sp|P38915|...
                                                          sp|P38915|...
## AAAALAGGK
                          728.418 sp|Q3E792|...
                                                          sp|Q3E792|...
## AAAALAGGKK
                          856.513 sp|Q3E792|...
                                                          sp|Q3E792|...
                         1215.635 sp|P09938|...
## AAADALSDLEIK
                                                          sp|P09938|...
## AAADALSDLEIKDSK
                         1545.789 sp|P09938|...
                                                          sp|P09938|...
                              . . .
## YYSIYDLGNNAVGLAK
                          1759.88 sp|P07267|...
                                                          sp|P07267|...
## YYTFNGPNYNENETIR
                          1993.88 sp|Q00955|...
                                                          sp|Q00955|...
## YYTITEVATR
                          1215.61 sp|P38891|...
                                                          sp|P38891|...
                          1461.66 P07339ups|...
## YYTVFDRDNNR
                                                          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
##
                                    . . .
                                                  . . .
                                                                   . . .
## YYSIYDLGNNAVGLAK
                                    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
                                                    2 1.1843e-05
                                                                     82.942
                                      yes
## AAAALAGGK
                                       no
                                                    2 7.4562e-06
                                                                    134.810
## AAAALAGGKK
                                                    2 3.3094e-09
                                                                    143.730
                                       no
## AAADALSDLEIK
                                      yes
                                                     2 9.1593e-23
                                                                    182.230
## AAADALSDLEIKDSK
                                                    3 1.5319e-04
                                                                     73.927
                                      yes
                                                         . . .
## ...
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## YYSIYDLGNNAVGLAK
                                      yes
                                                    2 7.7415e-37
                                                                    174.240
## YYTFNGPNYNENETIR
                                                    2 4.2208e-21
                                                                    147.750
                                      yes
## YYTITEVATR
                                      yes
                                                    2 1.3566e-04
                                                                    109.160
## YYTVFDRDNNR
                                                     2 6.1425e-04
                                                                    110.930
                                      yes
## YYTVFDRDNNRVGFAEAAR
                                                     3 8.9859e-04
                                      yes
                                                                     59.728
                       Identification.type.6A_1 Identification.type.6A_2
##
                                     <character>
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## AAAAGAGGAGDSGDAVTK
                                   By matchin...
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## AAAALAGGK
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## AAAALAGGKK
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## AAADALSDLEIK
## AAADALSDLEIKDSK
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## YYSIYDLGNNAVGLAK
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## YYTITEVATR
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## YYTVFDRDNNR
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## YYTVFDRDNNRVGFAEAAR
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##
                                     <character>
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## AAAAGAGGAGDSGDAVTK
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##
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## AAAAGAGGAGDSGDAVTK
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## AAADALSDLEIK
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## YYTVFDRDNNR
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## YYTVFDRDNNRVGFAEAAR
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                       Identification.type.6A_7 Identification.type.6A_8
##
                                     <character>
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## AAAAGAGGAGDSGDAVTK
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```
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## YYTVFDRDNNRVGFAEAAR
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## AAADALSDLEIKDSK
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## YYSIYDLGNNAVGLAK
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## YYTFNGPNYNENETIR
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## YYTITEVATR
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## YYTVFDRDNNR
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## YYTVFDRDNNRVGFAEAAR
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                        Identification.type.6B_6 Identification.type.6B_7
                                     <character>
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## AAAAGAGGAGDSGDAVTK
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## AAAALAGGK
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## AAADALSDLEIKDSK
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## AAADALSDLEIKDSK
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## YYSIYDLGNNAVGLAK
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## AAADALSDLEIKDSK
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## AAADALSDLEIKDSK
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## AAADALSDLEIKDSK
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## ...
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## YYTFNGPNYNENETIR
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## YYTITEVATR
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## AAADALSDLEIKDSK
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## YYTITEVATR
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## AAADALSDLEIKDSK
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## AAADALSDLEIK
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## AAADALSDLEIKDSK
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## YYTITEVATR
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## YYTVFDRDNNRVGFAEAAR
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	YYTFNGPNYNENETIR	1	NA NA	NA NA
	YYTITEVATR	NA	1	1
	YYTVFDRDNNR	NA NA	NA	NA
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## ## ## ## ## ##	AAAALAGGKK AAADALSDLEIK AAADALSDLEIKDSK YYSIYDLGNNAVGLAK YYTFNGPNYNENETIR YYTITEVATR	1 1 NA NA NA 1 NA	1 1 1 1 1 1 NA	NA 1 1 1 1 1 NA NA
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# # # # # # # # # # # # # # # # # # #	AAAALAGGKK AAADALSDLEIK AAADALSDLEIKDSK YYSIYDLGNNAVGLAK YYTFNGPNYNENETIR YYTITEVATR YYTVFDRDNNR YYTVFDRDNNRVGFAEAAR AAAAGAGGGAGDSGDAVTK AAAALAGGK AAAALAGGK AAADALSDLEIK AAADALSDLEIK YYSIYDLGNNAVGLAK	1 1 NA NA NA 1 NA NA 1 NA NA Experiment.6B_6 <integer> 1 NA NA 1 1 1</integer>	1 1 1 1 1 1 1 1 1 1 NA NA NA Experiment.6B_7 <integer> NA 2 1 1 1 1 NA</integer>	NA 1 1 1 1 1 1 1 1 NA NA Experiment.6B_8 <integer> 1 1 1 1 1 1 NA NA</integer>
######################################	AAAALAGGKK AAADALSDLEIK AAADALSDLEIKDSK YYSIYDLGNNAVGLAK YYTFNGPNYNENETIR YYTITEVATR YYTVFDRDNNR YYTVFDRDNNRVGFAEAAR AAAAGAGGAGDSGDAVTK AAAALAGGK AAAALAGGK AAADALSDLEIK AAADALSDLEIKDSK YYSIYDLGNNAVGLAK YYTFNGPNYNENETIR	1 1 1 NA NA NA 1 NA 1 NA Experiment.6B_6 <integer> 1 NA NA 1 1 1 1 1 1</integer>	1 1 1 1 1 1 1 1 NA NA Experiment.6B_7 <integer> NA 2 1 1 1 NA 1</integer>	NA 1 1 1 1 1 1 NA NA Experiment.6B_8 <integer> 1 1 1 1 1 NA NA NA NA</integer>
########################	AAAALAGGKK AAADALSDLEIK AAADALSDLEIKDSK YYSIYDLGNNAVGLAK YYTFNGPNYNENETIR YYTITEVATR YYTVFDRDNNR YYTVFDRDNNRVGFAEAAR AAAAGAGGAGDSGDAVTK AAAALAGGK AAAALAGGK AAADALSDLEIK AAADALSDLEIK YYSIYDLGNNAVGLAK YYTFNGPNYNENETIR YYTITEVATR	1 1 1 NA NA NA 1 NA 1 NA Experiment.6B_6 <integer> 1 NA NA 1 1 1 1 1 1 1</integer>	1 1 1 1 1 1 1 1 1 1 NA NA Experiment.6B_7 <integer> NA 2 1 1 1 1 NA 1 NA</integer>	NA 1 1 1 1 NA NA Experiment.6B_8 <integer> 1 1 1 1 NA NA NA NA NA NA NA</integer>
##########################	AAAALAGGKK AAADALSDLEIK AAADALSDLEIKDSK YYSIYDLGNNAVGLAK YYTFNGPNYNENETIR YYTUTEVATR YYTVFDRDNNR YYTVFDRDNNRVGFAEAAR AAAAGAGGAGDSGDAVTK AAAALAGGK AAAALAGGK AAADALSDLEIK AAADALSDLEIK YYSIYDLGNNAVGLAK YYTFNGPNYNENETIR YYTTTEVATR YYTVFDRDNNR	1 1 1 NA NA NA 1 NA 1 NA Experiment.6B_6 <integer> 1 NA NA 1 1 1 1 1 1 1 1</integer>	1 1 1 1 1 1 1 1 1 1 1 1 1 NA NA Experiment.6B_7 <integer> NA 2 1 1 1 1 1 NA NA</integer>	NA 1 1 1 1 NA NA Experiment.6B_8 <integer> 1 1 1 NA NA NA Experiment.6B_N I NA NA NA NA NA NA NA NA NA</integer>
#######################	AAAALAGGKK AAADALSDLEIK AAADALSDLEIKDSK YYSIYDLGNNAVGLAK YYTFNGPNYNENETIR YYTITEVATR YYTVFDRDNNR YYTVFDRDNNRVGFAEAAR AAAAGAGGAGDSGDAVTK AAAALAGGK AAAALAGGK AAADALSDLEIK AAADALSDLEIK YYSIYDLGNNAVGLAK YYTFNGPNYNENETIR YYTITEVATR	1 1 1 NA NA NA 1 NA Experiment.6B_6 <integer> 1 NA NA 1 1 1 1 1 1 NA NA</integer>	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 NA NA Experiment.6B_7 <integer> NA 2 1 1 1 1 1 1 NA NA NA NA</integer>	NA 1 1 1 1 1 1 1 1 1 NA NA Experiment.6B_8 <integer> 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1</integer>
##########################	AAAALAGGKK AAADALSDLEIK AAADALSDLEIKDSK YYSIYDLGNNAVGLAK YYTFNGPNYNENETIR YYTUTEVATR YYTVFDRDNNR YYTVFDRDNNRVGFAEAAR AAAAGAGGAGDSGDAVTK AAAALAGGK AAAALAGGK AAADALSDLEIK AAADALSDLEIK YYSIYDLGNNAVGLAK YYTFNGPNYNENETIR YYTTTEVATR YYTVFDRDNNR	1 1 1 NA NA NA 1 NA Experiment.6B_6 <integer> 1 NA NA 1 1 1 1 1 1 NA NA</integer>	1 1 1 1 1 1 1 1 1 1 1 1 1 NA NA Experiment.6B_7 <integer> NA 2 1 1 1 1 1 NA NA</integer>	NA 1 1 1 1 1 1 1 1 1 NA NA Experiment.6B_8 <integer> 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1</integer>

		4	NT A	NT A
	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
##	${\tt YYTVFDRDNNRVGFAEAAR}$	NA	NA	NA
##		Experiment.6C_3	Experiment.6C_4	Experiment.6C_5
##		<integer></integer>	<integer></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_7	
##		<pre><integer></integer></pre>	<pre><integer></integer></pre>	<pre><integer></integer></pre>
	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.6D_2
##		<pre><integer></integer></pre>	<pre><integer></integer></pre>	<pre><integer></integer></pre>
	AAAAGAGGAGDSGDAVTK	1	NA	NA
	AAAALAGGK	1	NA	1
	AAAALAGGKK	1	NA	NA
	AAADALSDLEIK	1	1	1
	AAADALSDLEIKDSK	1	1	1
##				<u>-</u>
	YYSIYDLGNNAVGLAK	NA	NA	NA
	YYTFNGPNYNENETIR	1	NA	NA
	YYTITEVATR	1	NA NA	1
	YYTVFDRDNNR	NA	NA NA	NA NA
	YYTVFDRDNNRVGFAEAAR	NA NA	NA NA	NA NA
##	DIVERNITY OF HEADING		Experiment.6D_4	
##		<pre><integer></integer></pre>	<pre><integer></integer></pre>	<pre><integer></integer></pre>
	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_8
##		<integer></integer>	<integer></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.6E_2
##		<integer></integer>	<integer></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_5
##		<integer></integer>	<integer></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_8
##		<integer></integer>	<integer></integer>	<integer></integer>
	AAAAGAGGAGDSGDAVTK	1	NA	NA
	AAAALAGGK	NA	2	2
	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	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			Potenti	al.contamina	
##		_		<character></character>		<characte< th=""><th>r></th></characte<>	r>
	AAAAGAGGAGDSGDAVTK	NA					
	AAAALAGGK		280990000				
	AAAALAGGKK	1					
	AAADALSDLEIK	1					
##	AAADALSDLEIKDSK	1	18910000				
##	• • •		• • •			•	
	YYSIYDLGNNAVGLAK	NA					
##	YYTFNGPNYNENETIR	1	5608800				
	YYTITEVATR	NA					
	YYTVFDRDNNR	1					
##	YYTVFDRDNNRVGFAEAAR	1					
##		id Prote	in.group.ID	s Modpept:	ide.IDs	Evidence.ID	S
##		<integer></integer>	<character< th=""><th>> <char< th=""><th>racter></th><th><character< th=""><th></th></character<></th></char<></th></character<>	> <char< th=""><th>racter></th><th><character< th=""><th></th></character<></th></char<>	racter>	<character< th=""><th></th></character<>	
##	AAAAGAGGAGDSGDAVTK	0	85			0;1;2;3;4;	
##	AAAALAGGK	1	23	0		24;25;26;2	
##	AAAALAGGKK	2	23	0	2	74;75;76;7	
	AAADALSDLEIK	3	22			99;100;101	
##	AAADALSDLEIKDSK	4	22	9	4	144;145;14	•
##	• • •	• • •		•			
##	YYSIYDLGNNAVGLAK	11461	19	6		331367;331	
##	YYTFNGPNYNENETIR	11462	125	4		331384;331	
##	YYTITEVATR	11463	85			331411;331	
	YYTVFDRDNNR	11464	3	4		331439;331	
##	YYTVFDRDNNRVGFAEAAR	11465	3			331455;331	
##		MS.MS.IDs B	est.MS.MS O	xidationM	site.I	Ds MS.MS.Cou	
##			<integer></integer>	<	characte	r> <intege< th=""><th>r></th></intege<>	r>
	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

 $\bullet\,$ 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)</pre>
```

• We explore the colData again

colData(pe)

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

3 Preprocessing

3.1 Log-transformation

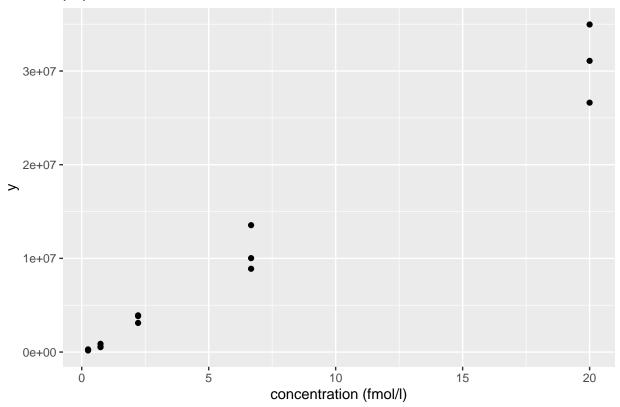
3.1.1 Explore the data with plots

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

Click to see code to make plot

plotWhyLog

peptide AALEELVK in lab1

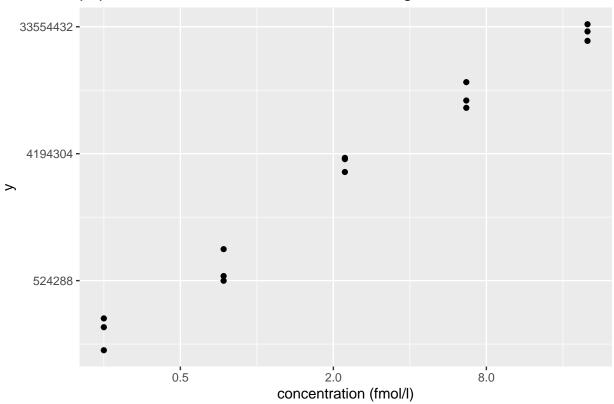


• Variance increases with the mean \rightarrow Multiplicative error structure

Click to see code to make plot

plotLog

peptide AALEELVK in lab1 with axes on log scale



- Data seems to be homoscedastic on log-scale \rightarrow log transformation of the intensity data
- In quantitative proteomics analysis on log₂
- \rightarrow Differences on a \log_2 scale: \log_2 fold changes

$$\begin{split} \log_2 B - \log_2 A &= \log_2 \frac{B}{A} = \log F C_{\text{B - A}} \\ log_2 F C &= 1 \rightarrow F C = 2^1 = 2 \\ log_2 F C &= 2 \rightarrow F C = 2^2 = 4 \end{split}$$

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.

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")</pre>
```

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

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 != "+")</pre>
```

3. Drop peptides that were only identified in one sample

We keep peptides that were observed at last twice.

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

[1] 10478

We keep 10478 peptides upon filtering.

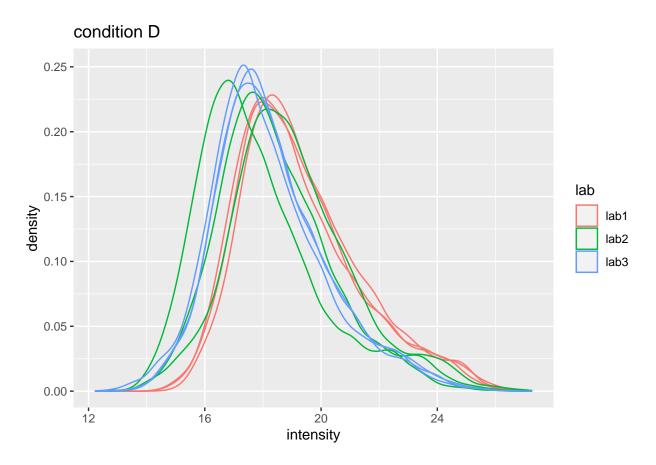
3.3 Normalization

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).



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

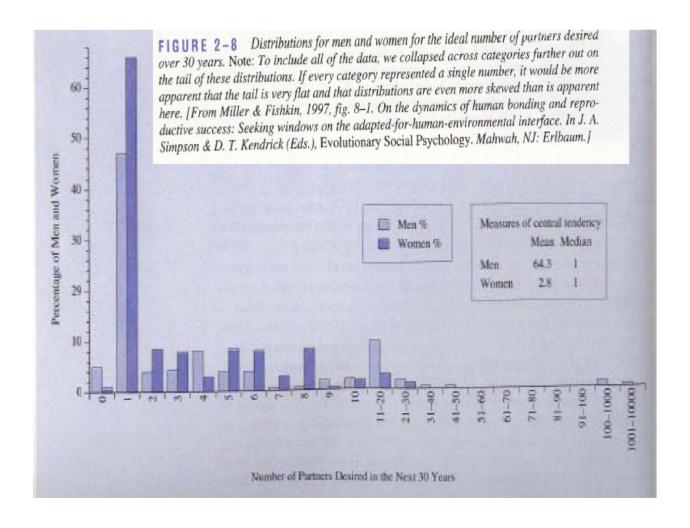


- Even in very clean synthetic dataset (same background, only 48 UPS proteins can be different) the marginal peptide intensity distribution across samples can be quite distinct
 - Considerable effects between and within labs for replicate samples
 - Considerable effects between samples with different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

3.3.3 Plots of normalized data

Click to see code to make plot

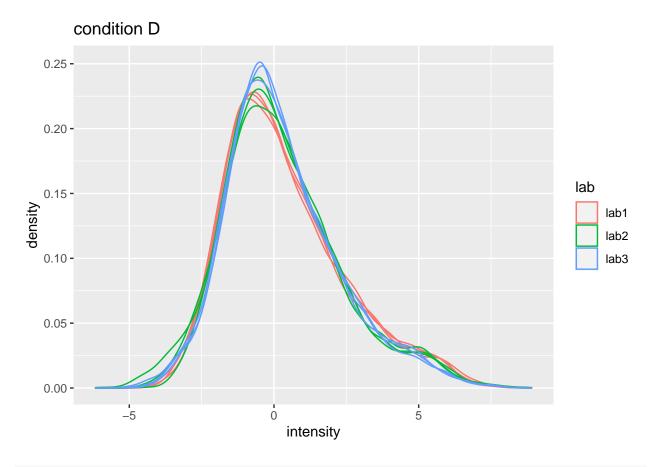
```
densityConditionDNorm <- pe[["peptideNorm"]][,colData(pe)$condition=="D"] %>%
  assay %>%
  as.data.frame() %>%
```

```
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.

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

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).

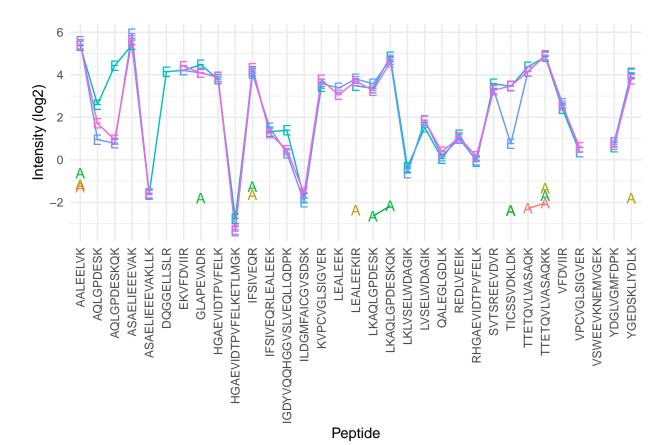
Click to see code to make plot

```
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.legengeom_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 more alike than peptide intensities from a particular protein between samples.
- \rightarrow 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 sumarization in aggregateFeatures, which is robust model based summarization.

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

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 fits 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 (L. J. Goeminne, Gevaert, and Clement 2016), (L. J. E. 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

References

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.

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