

# 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
  - 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).

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# 1 Intro: Challenges in Label-Free Quantitative Proteomics

## 1.1 MS-based workflow



- Peptide Characteristics
  - Modifications

- Ionisation Efficiency: huge variability
- Identification
  - \* Misidentification → outliers
  - \* MS<sup>2</sup> selection on peptide abundance
  - \* Context depending missingness
  - \* Non-random missingness

→ Unbalanced peptide identifications across samples and messy data

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## 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



## 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

```
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





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.

```
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...
## ...                 ...                 ...                 ...
## YYSIYDLGNNAVGLAK    YYSIYDLGNN...      VGDAFLRKYY...      NNAVGLAKAI...
## YYTFNGPNYNENETIR    YYTFNGPNYN...      FKDGSPKYY...      YNENETIRHI...
## YTTITEVATR           YTTITEVATR           QEWDINERY...      TITEVATR...
## YTTVFDRDNNR         YTTVFDRDNN...      LGDVFIGRYY...      VFDRDNNRVG...
## YTTVFDRDNNRVGFAEAAR YTTVFDRDNN...      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
## ...                 ...                 ...                 ...
## YYSIYDLGNNAVGLAK           K           Y           Y
## YYTFNGPNYNENETIR          K           Y           Y
## YTTITEVATR           R           Y           Y
## YTTVFDRDNNR           R           Y           Y
## YTTVFDRDNNRVGFAEAAR         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
## ...                 ...                 ...                 ...
## YYSIYDLGNNAVGLAK           A           K           A
## YYTFNGPNYNENETIR          I           R           H
## YTTITEVATR           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         |   |
| ## | AAADALSLEIK         | 4         | 0         | 0         | 2         | 0                | 0         |   |
| ## | AAADALSLEIKDSK      | 4         | 0         | 0         | 3         | 0                | 0         |   |
| ## | ...                 | ...       | ...       | ...       | ...       | ...              | ...       |   |
| ## | YYSIYDLGNNAVLAK     | 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         |   |
| ## | AAADALSLEIK         | 1         | 0         | 0         | 1         | 2                | 1         |   |
| ## | AAADALSLEIKDSK      | 1         | 0         | 0         | 1         | 2                | 2         |   |
| ## | ...                 | ...       | ...       | ...       | ...       | ...              | ...       |   |
| ## | YYSIYDLGNNAVLAK     | 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         |   |
| ## | AAADALSLEIK         | 0         | 0         | 0         | 1         | 0                | 0         |   |
| ## | AAADALSLEIKDSK      | 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                |           |   |
| ## | AAADALSLEIK         | 0         | 0         | 0         | 12        | 0                |           |   |
| ## | AAADALSLEIKDSK      | 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 ...         |           |
| ## ...                 | ...                      | ...                      | ...                   |           |
| ## YYSIYDLGNNAVGLAK    | 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                   |           |
| ## ...                 | ...                      | ...                      | ...                   |           |
| ## 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  | 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>              |                       |           |

|                        |                          |                          |
|------------------------|--------------------------|--------------------------|
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| ## AAAALAGGK           | By matchin...            | By MS/MS                 |
| ## AAAALAGGKK          | By matchin...            | By MS/MS                 |
| ## AAADALSDLEIK        | By matchin...            | By MS/MS                 |
| ## AAADALSDLEIKDSK     | By matchin...            | By MS/MS                 |
| ## ...                 | ...                      | ...                      |
| ## YYSIYDLGNNNAVGLAK   | 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...            |
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| ## AAAALAGGKK          | By matchin...            | By matchin...            |
| ## AAADALSDLEIK        | By MS/MS                 | By MS/MS                 |
| ## AAADALSDLEIKDSK     | By MS/MS                 | By MS/MS                 |
| ## ...                 | ...                      | ...                      |
| ## YYSIYDLGNNNAVGLAK   | 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                 |
| ## ...                 | ...                      | ...                      |
| ## 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...            |
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| ## 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...            |
| ## ...                 | ...                      | ...                      |
| ## YYSIYDLGNNNAVGLAK   | 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...            |
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| ## 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...            |
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| ## 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                 |
| ## ...                 | ...                      | ...                      |
| ## YYSIYDLGNNAVGLAK    | 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                 |
| ## ...                 | ...                      | ...                      |
| ## 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.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                 |
| ## ...                 | ...                      | ...                      |
| ## YYSIYDLGNNNAVGLAK   | 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                 |
| ## ...                 | ...                      | ...                      |
| ## YYSIYDLGNNNAVGLAK   | 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                 |

|    |                      |   |                                 |
|----|----------------------|---|---------------------------------|
| ## | YYTVFDRDNNRVGFAEAAAR | 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                           |
| ## | YYTVFDRDNNRVGFAEAAAR | 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  |                                 |
| ## | YYTVFDRDNNRVGFAEAAAR | NA NA NA  |                                 |
| ## |                      | Experiment.6A_6 Experiment.6A_7 Experiment.6A_8 |                                 |
| ## |                      | <integer> <integer> <integer>                   |                                 |
| ## | AAAAGAGGAGDSGDAVTK   | 1 1 1   |                                 |
| ## | AAAALAGGK            | 1 2 1   |                                 |
| ## | AAAALAGGKK           | 1 1 1   |                                 |
| ## | AAADALSDLEIK         | 1 1 1   |                                 |
| ## | AAADALSDLEIKDSK      | 1 1 1   |                                 |
| ## | ...                  | ...   |                                 |
| ## | YYSIYDLGNNAVGLAK     | 1 NA NA   |                                 |
| ## | YYTFNGPNYNENETIR     | 1 1 NA  |                                 |
| ## | YYTITEVATR           | 1 1 NA  |                                 |
| ## | YYTVFDRDNNR          | NA NA NA  |                                 |
| ## | YYTVFDRDNNRVGFAEAAAR | NA NA NA  |                                 |
| ## |                      | Experiment.6A_9 Experiment.6B_1 Experiment.6B_2 |                                 |
| ## |                      | <integer> <integer> <integer>                   |                                 |
| ## | AAAAGAGGAGDSGDAVTK   | 1 NA NA   |                                 |
| ## | AAAALAGGK            | 1 1 1   |                                 |
| ## | AAAALAGGKK           | 1 NA 1  |                                 |
| ## | AAADALSDLEIK         | 1 1 1   |                                 |
| ## | AAADALSDLEIKDSK      | 1 NA 1  |                                 |
| ## | ...                  | ...   |                                 |
| ## | YYSIYDLGNNAVGLAK     | NA NA NA  |                                 |
| ## | YYTFNGPNYNENETIR     | 1 NA NA   |                                 |
| ## | YYTITEVATR           | NA 1 1  |                                 |
| ## | YYTVFDRDNNR          | NA NA NA  |                                 |
| ## | YYTVFDRDNNRVGFAEAAAR | NA NA NA  |                                 |
| ## |                      | Experiment.6B_3 Experiment.6B_4 Experiment.6B_5 |                                 |

|                        |                 |                 |                 |
|------------------------|-----------------|-----------------|-----------------|
| ##                     | <integer>       | <integer>       | <integer>       |
| ## AAAAGAGGAGDSGDAVTK  | NA              | NA              | 1               |
| ## AAAALAGGK           | 1               | 2               | 1               |
| ## AAAALAGGKK          | 1               | 1               | NA              |
| ## AAADALSDLEIK        | 1               | 1               | 1               |
| ## AAADALSDLEIKDSK     | NA              | 1               | 1               |
| ## ...                 | ...             | ...             | ...             |
| ## YYSIYDLGNNAVGLAK    | NA              | 1               | 1               |
| ## YYTFNGPNYNENETIR    | NA              | 1               | 1               |
| ## YYTITEVATR          | 1               | 1               | 1               |
| ## YYTVFDRDNNR         | NA              | NA              | NA              |
| ## YYTVFDRDNNRVGFAEAAR | NA              | NA              | NA              |
| ##                     | Experiment.6B_6 | Experiment.6B_7 | Experiment.6B_8 |
| ##                     | <integer>       | <integer>       | <integer>       |
| ## AAAAGAGGAGDSGDAVTK  | 1               | NA              | 1               |
| ## AAAALAGGK           | NA              | 2               | 1               |
| ## AAAALAGGKK          | NA              | 1               | 1               |
| ## AAADALSDLEIK        | 1               | 1               | 1               |
| ## AAADALSDLEIKDSK     | 1               | 1               | 1               |
| ## ...                 | ...             | ...             | ...             |
| ## YYSIYDLGNNAVGLAK    | 1               | NA              | NA              |
| ## YYTFNGPNYNENETIR    | 1               | 1               | NA              |
| ## YYTITEVATR          | 1               | NA              | 1               |
| ## YYTVFDRDNNR         | NA              | NA              | NA              |
| ## YYTVFDRDNNRVGFAEAAR | NA              | 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>                   |
| ## | 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              |
| ## |                     | <integer>       | <character>       | Evidence.IDs                  |
| ## | 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
## AAADALSDEIK      51;52;53;5...      72      29
## AAADALSDEIKDSK      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")
```





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

2. Remove reverse sequences (decoys) and contaminants

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

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

3. Drop peptides that were only identified in one sample

We keep peptides that were observed at least twice.

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

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

We keep 10478 peptides upon filtering.

### 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

## 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.
- 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.