Introduction to proteomics data analysis: robust summarization

Lieven Clement

statOmics, Ghent University (https://statomics.github.io)

Contents

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

1 Background

This case-study is a subset of the data of the 6th study of the Clinical Proteomic Technology Assessment for Cancer (CPTAC). In this experiment, the authors spiked the Sigma Universal Protein Standard mixture 1 (UPS1) containing 48 different human proteins in a protein background of 60 ng/μL Saccharomyces cerevisiae strain BY4741. Two different spike-in concentrations were used: 6A (0.25 fmol UPS1 proteins/μL) and 6B (0.74 fmol UPS1 proteins/μL) [5]. We limited ourselves to the data of LTQ-Orbitrap W at site 56. The data were searched with MaxQuant version 1.5.2.8, and detailed search settings were described in Goeminne et al. (2016) [1]. Three replicates are available for each concentration.

2 Data

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

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

1. Load libraries

```
library(tidyverse)
library(limma)
library(QFeatures)
library(msqrob2)
library(plotly)
library(data.table)
library(pcaMethods)
```

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

```
peptidesTable <- fread("https://raw.githubusercontent.com/statOmics/SGA2020/data/quantification/cptacAva
int64 <- which(sapply(peptidesTable,class) == "integer64")
for (j in int64) peptidesTable[[j]] <- as.numeric(peptidesTable[[j]])
```

3. Maxquant stores the intensity data for the different samples in columnns that start with Intensity. We can retreive the column names with the intensity data with the code below:

```
quantCols <- grep("Intensity ", names(peptidesTable))
```

4. Read the data and store it in QFeatures object, the first column of the peptides file contains the peptide sequence, which is a unique identifier for the peptide. We will use this column as the rownames of the assay data `fnames = 1`.

```
pe <- readQFeatures(
  assayData = peptidesTable,
  fnames = 1,
  quantCols =  quantCols,
  name = "peptideRaw")
```

```
## Checking arguments.

## Loading data as a 'SummarizedExperiment' object.

## Formatting sample annotations (colData).

## Formatting data as a 'QFeatures' object.

## Setting assay rownames.
```

```
rm(peptidesTable)
gc()
```

```
##            used  (Mb) gc trigger  (Mb) limit (Mb) max used  (Mb)
## Ncells  8276612 442.1   16546598 883.7         NA 16546598 883.7
## Vcells 16021835 122.3   33213111 253.4      16384 33213111 253.4
```

```
gc()
```

```
##            used  (Mb) gc trigger  (Mb) limit (Mb) max used  (Mb)
## Ncells  8276073 442.0   16546598 883.7         NA 16546598 883.7
## Vcells 16014514 122.2   33213111 253.4      16384 33213111 253.4
```

```
colnames(pe)
```

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

```
## [["peptideRaw"]] Intensity 6A_7 Intensity 6A_8 ... Intensity 6B_9
```

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

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

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

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
```

2.1 Data exploration

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

3 Preprocessing

This section preforms preprocessing for the peptide data. This include

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

3.1 Log transform the data

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

3.2 Filtering

1. Handling overlapping protein groups

We remove peptides that could not be mapped to a protein or that map to multiple proteins (the protein identifier contains multiple identifiers separated by a ;).

```
pe <- filterFeatures(
    pe, ~ Proteins != "" & ## Remove failed protein inference
        !grepl(";", Proteins)) ## Remove protein groups
```

```
## 'Proteins' found in 2 out of 2 assay(s).
```

2. Remove reverse sequences (decoys) and contaminants

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

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

```
## 'Reverse' found in 2 out of 2 assay(s).
```

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

```
## 'Potential.contaminant' found in 2 out of 2 assay(s).
```

3. Drop peptides that were identified in less than three sample.

We tolerate the following proportion of NAs: pNA = (n-3)/n.

```
nObs <- 3
n <- ncol(pe[["peptideLog"]])
pNA <- (n-nObs)/n
pe <- filterNA(pe, pNA = pNA, i = "peptideLog")
nrow(pe[["peptideLog"]])
```

```
## [1] 5910
```

We keep 5910 peptides upon filtering.

3.3 Normalize the data using median centering

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

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

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

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

3.4 Explore normalized data

Upon the normalisation the density curves are nicely registered

```
pe[["peptideNorm"]] |>
  assay() |>
  as.data.frame() |>
  gather(sample, intensity) |>
  mutate(condition = colData(pe)[sample,"condition"]) |>
  ggplot(aes(x = intensity,group = sample,color = condition)) +
  geom_density() +
  theme_minimal()
```

```
## Warning: Removed 4568 rows containing non-finite outside the scale range
## (`stat_density()`).
```
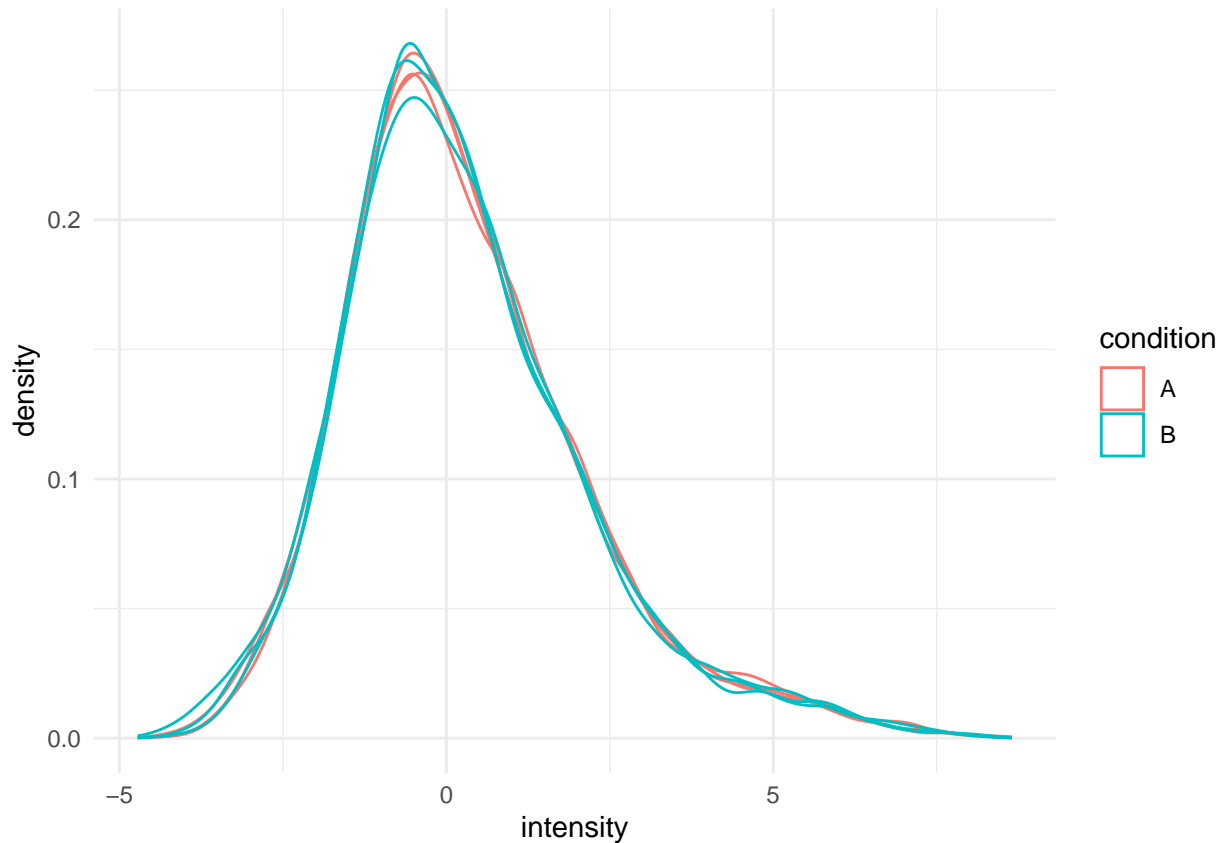
We can visualize our data using a PCA plot eg. as provided by Nipals from the `pcaMethods` package, that can handle missing data.

```r
plotPCA <- function (pe, assayName, varName=NULL)
{
    require(pcaMethods)
    if (!is.null(varName)) varName <- as.character(varName)
    #extract assayData and transpose
    dat <- pe[[assayName]] |>
      assay() |>
      t()
    # Replace NaN values to NA (often occur upon median summarisation)
    dat[is.nan(dat)] <- NA
    # Run Nipals
    pc <- pca(dat, method = "nipals")
    # Make data frame for plotting
    df <- merge(scores(pc), colData(pe), by = 0)
    if (!is.null(varName)) return(
      ggplot(df, aes(PC1, PC2, col = !!sym(varName))) +
      geom_point() +
      xlab(paste0("PC1 (", round(pc@R2[1] * 100, 1), "%)")) +
      ylab(paste0("PC2 (", round(pc@R2[2] * 100, 1), "%)")) +
      theme_minimal()
    ) else return(
      ggplot(df, aes(PC1, PC2)) +
      geom_point() +
      xlab(paste0("PC1 (", round(pc@R2[1] * 100, 1), "%)")) +
```

```
      ylab(paste0("PC2 (", round(pc@R2[2] * 100, 1), "%)")) +
      theme_minimal()
    )
}
plotPCA(pe, "peptideNorm", "condition")
```
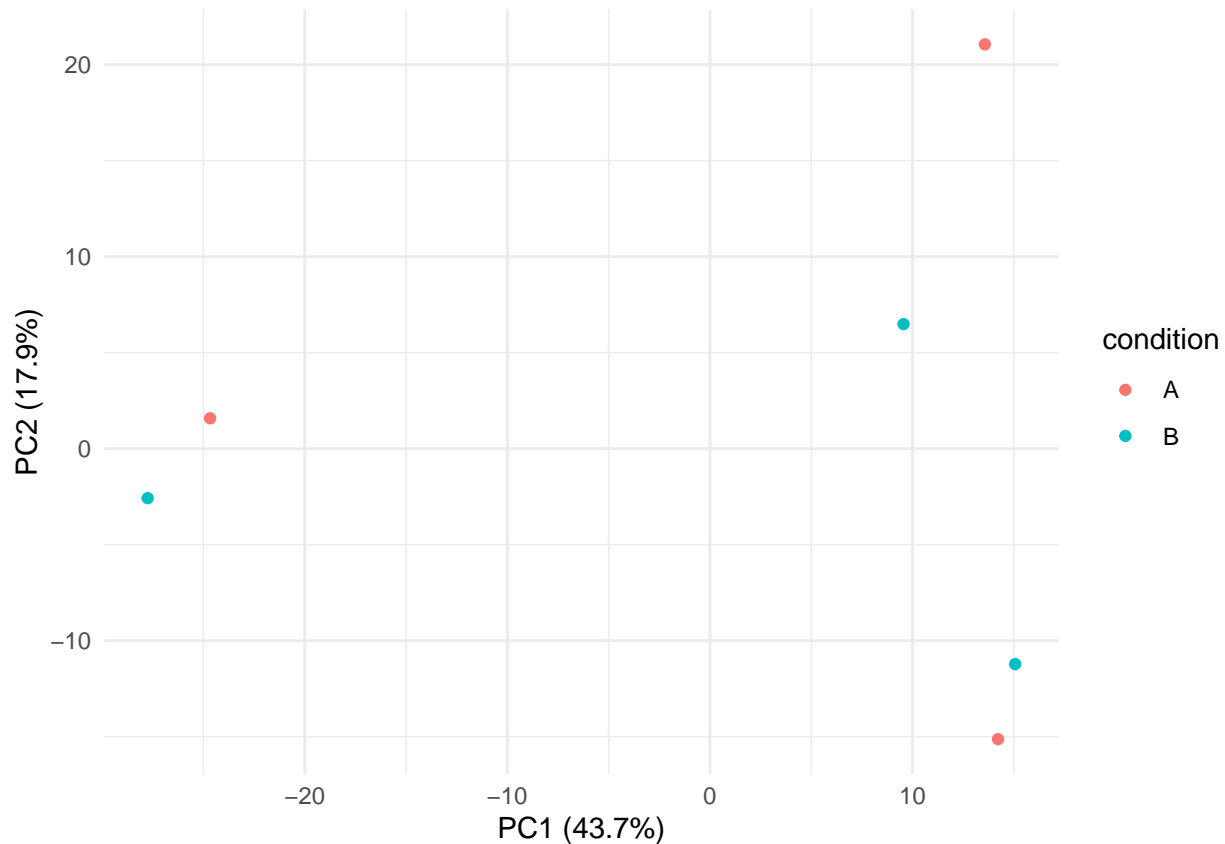

The first axis in the plot is showing the leading variability (log fold changes, differences on a log scale) between the samples.

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

3.5   Summarization to protein level

- By default robust summarization is used: `fun = MsCoreUtils::robustSummary()`

```
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.
```

```
## Aggregated: 1/1
```

3.6 Filter proteins that contain many missing values

We want to have at least 4 observed proteins so that most proteins have at least 2 observations in each group. So we tolerate a proportion of (n-4)/n NAs.

```
nObs <- 4
n <- ncol(pe[["protein"]])
pNA <- (n-nObs)/n
pe <- filterNA(pe, pNA = pNA, i = "protein")
```

```
plotPCA(pe, "protein", "condition")
```
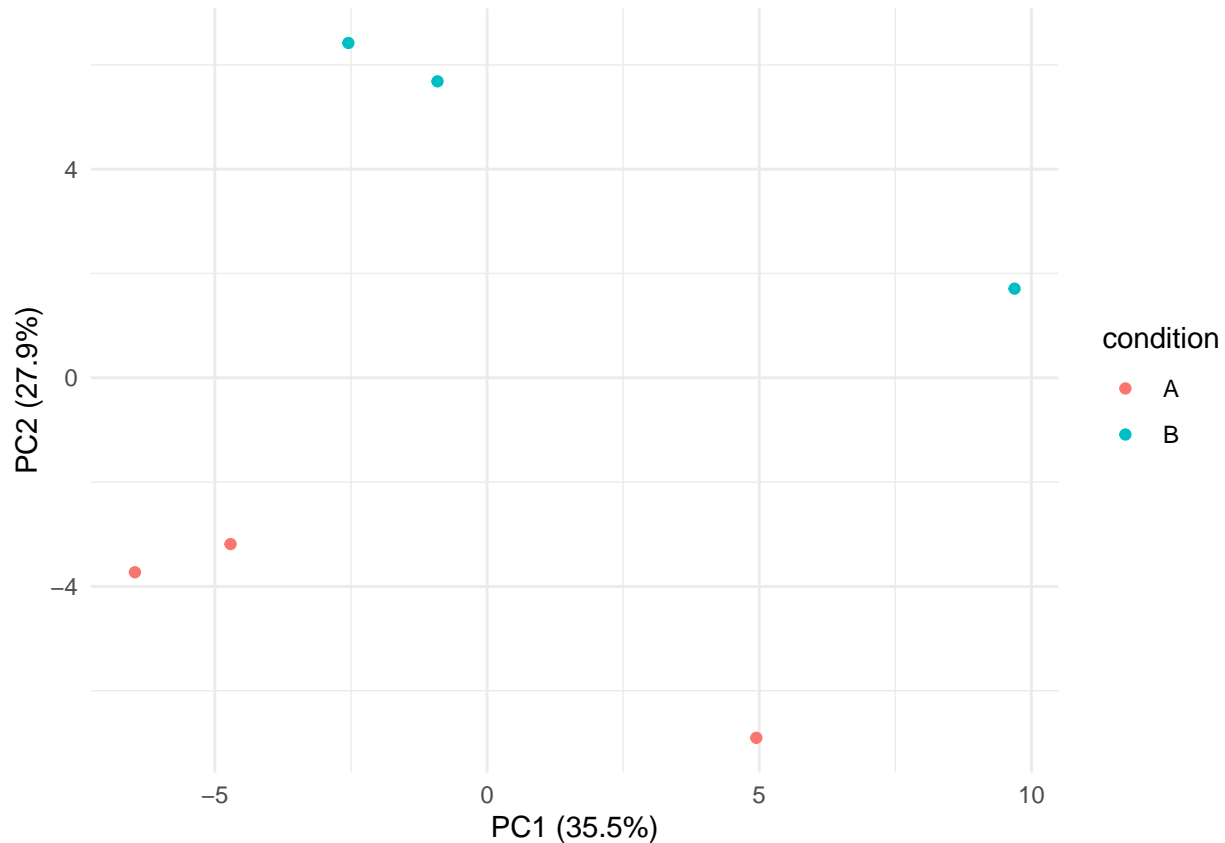

Note that the samples upon robust summarisation show a clear separation according to the spike-in condition in the second dimension of the MDS plot.

4 Data Analysis

4.1 Estimation

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

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

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

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

4.2 Inference

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

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

```
## (Intercept)   conditionB
##   -2.659614     1.502650
```

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

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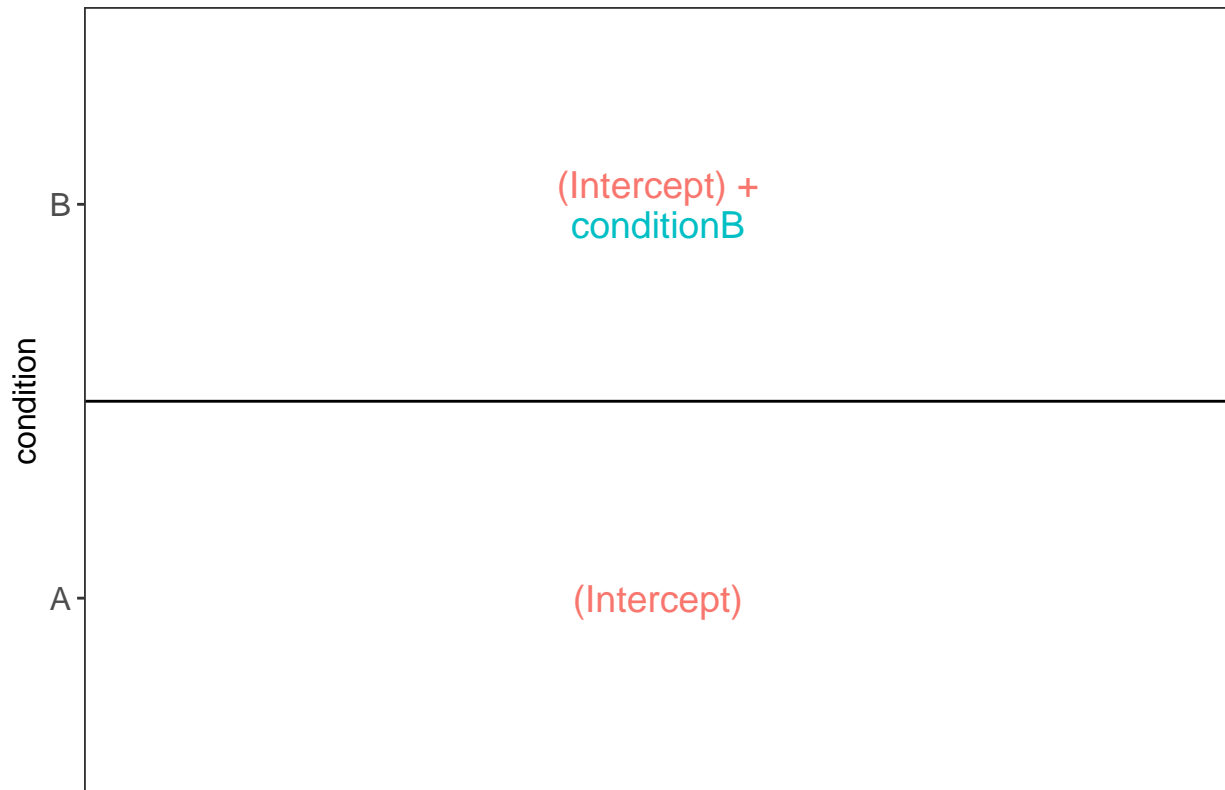

Spike-in condition `A` is the reference class. So the mean log2 expression for samples from condition A is '(Intercept). The mean log2 expression for samples from condition B is'(Intercept)+conditionB'. Hence, the average log2 fold change between condition b and condition a is modelled using the parameter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

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

4.3 Plots

4.3.1 Volcano-plot

```
volcano <- ggplot(rowData(pe[["protein"]])$conditionB,
                  aes(x = logFC, y = -log10(pval), color = adjPval < 0.05)) +
  geom_point(cex = 2.5) +
  scale_color_manual(values = alpha(c("black", "red"), 0.5)) + theme_minimal()
volcano
```
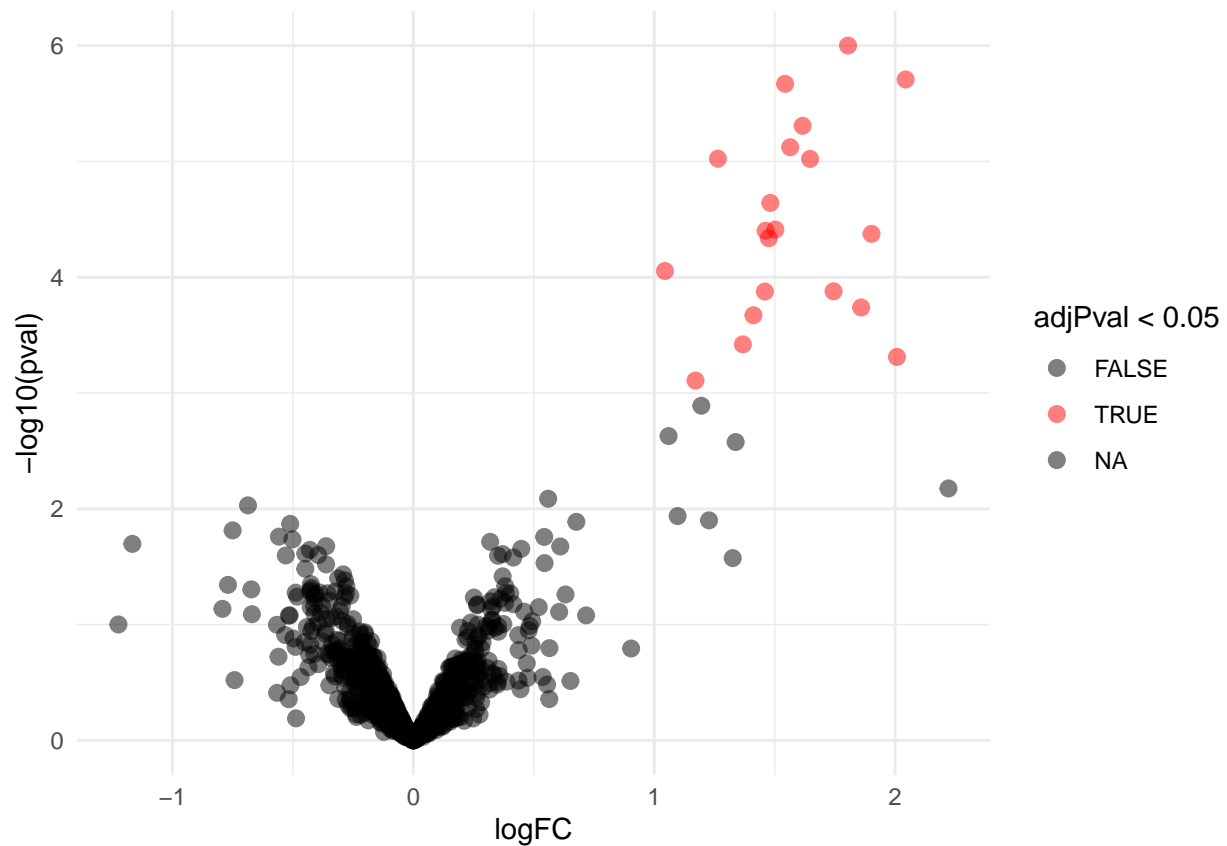

Note, that 20 proteins are found to be differentially abundant.

4.3.2 Heatmap

We first select the names of the proteins that were declared signficant.

```
sigNames <- rowData(pe[["protein"]])$conditionB %>%
  rownames_to_column("protein") %>%
  filter(adjPval<0.05) %>%
  pull(protein)
heatmap(assay(pe[["protein"]])[sigNames, ])
```
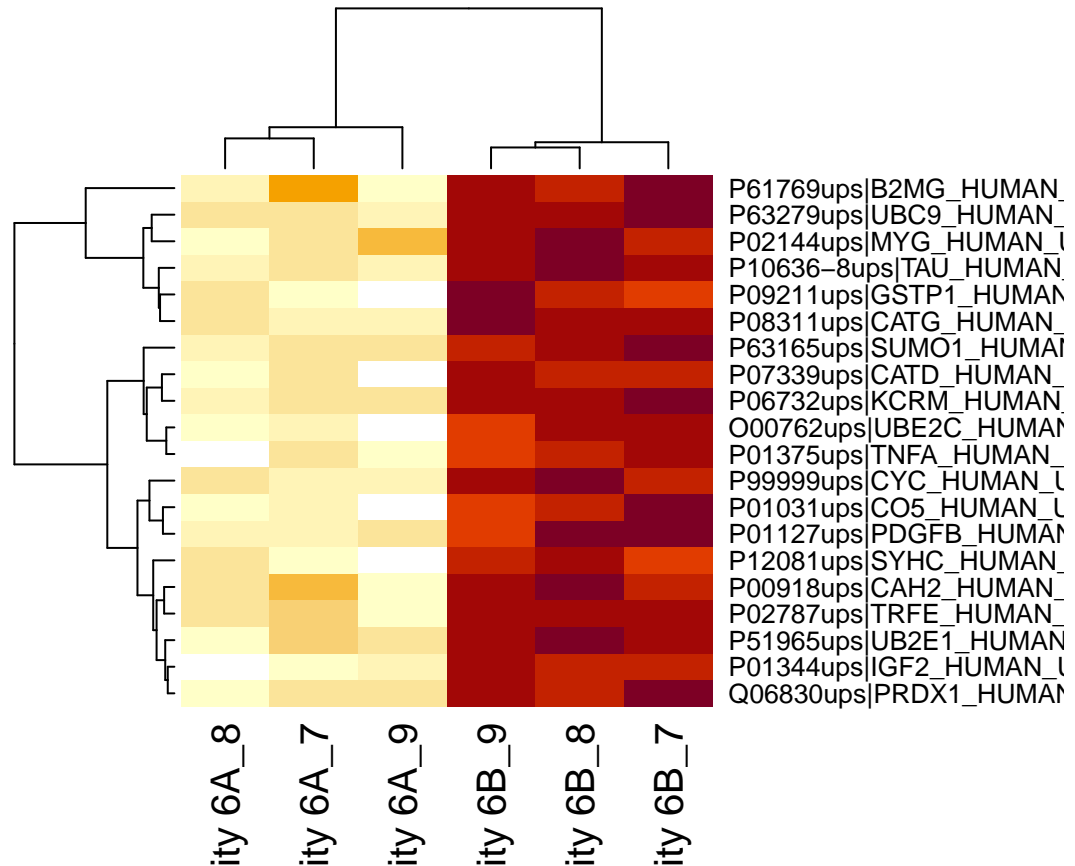
The majority of the proteins are indeed UPS proteins. 1 yeast protein is returned. Note, that the yeast protein indeed shows evidence for differential abundance.

4.3.3 Boxplots

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

```
rowData(pe[["protein"]])$conditionB %>%
  rownames_to_column(var = "protein") %>%
  ggplot(aes(x=grepl("UPS",protein),y=logFC)) +
  geom_boxplot() +
  xlab("UPS") +
  geom_segment(
    x = 1.5,
    xend = 2.5,
    y = log2(0.74/0.25),
    yend = log2(0.74/0.25),
    colour="red") +
  geom_segment(
    x = 0.5,
    xend = 1.5,
    y = 0,
    yend = 0,
    colour="red") +
  annotate(
    "text",
    x = c(1,2),
```

```
    y = c(0,log2(0.74/0.25))+.1,
    label = c(
      "log2 FC Ecoli = 0",
      paste0("log2 FC UPS = ",round(log2(0.74/0.25),2))
      ),
    colour = "red")
```

```
## Warning: Removed 30 rows containing non-finite outside the scale range
## (`stat_boxplot()`).
```
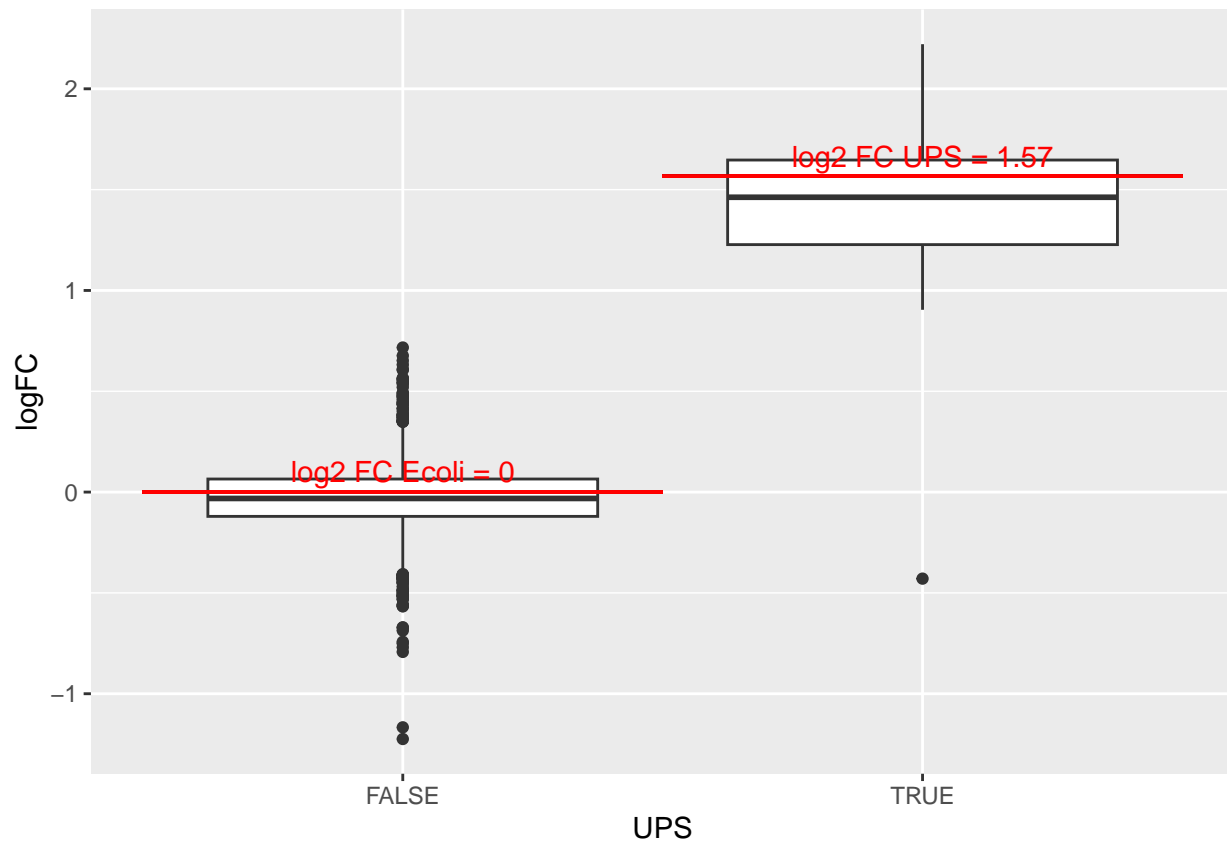

What do you observe?

4.3.4   Detail plots

We first extract the normalized peptideRaw expression values for a particular protein.

```
for (protName in sigNames)
{
pePlot <- pe[protName, , c("peptideNorm","protein")]
pePlotDf <- data.frame(longForm(pePlot))
pePlotDf$assay <- factor(pePlotDf$assay,
                      levels = c("peptideNorm", "protein"))
pePlotDf$condition <- as.factor(colData(pePlot)[pePlotDf$colname, "condition"])

# plotting
p1 <- ggplot(data = pePlotDf,
      aes(x = colname, y = value, group = rowname)) +
```

```r
    geom_line() +
    geom_point() +
    theme(axis.text.x = element_text(angle = 70, hjust = 1, vjust = 0.5)) +
    facet_grid(~assay) +
    ggtitle(protName)
print(p1)

# plotting 2
p2 <- ggplot(pePlotDf, aes(x = colname, y = value, fill = condition)) +
  geom_boxplot(outlier.shape = NA) +
  geom_point(
    position = position_jitter(width = .1),
    aes(shape = rowname)) +
  scale_shape_manual(values = 1:nrow(pePlotDf)) +
  labs(title = protName, x = "sample", y = "peptide intensity (log2)") +
  theme(axis.text.x = element_text(angle = 70, hjust = 1, vjust = 0.5)) +
  facet_grid(~assay)
print(p2)
}
```

O00762ups|UBE2C_HUMAN_UPS
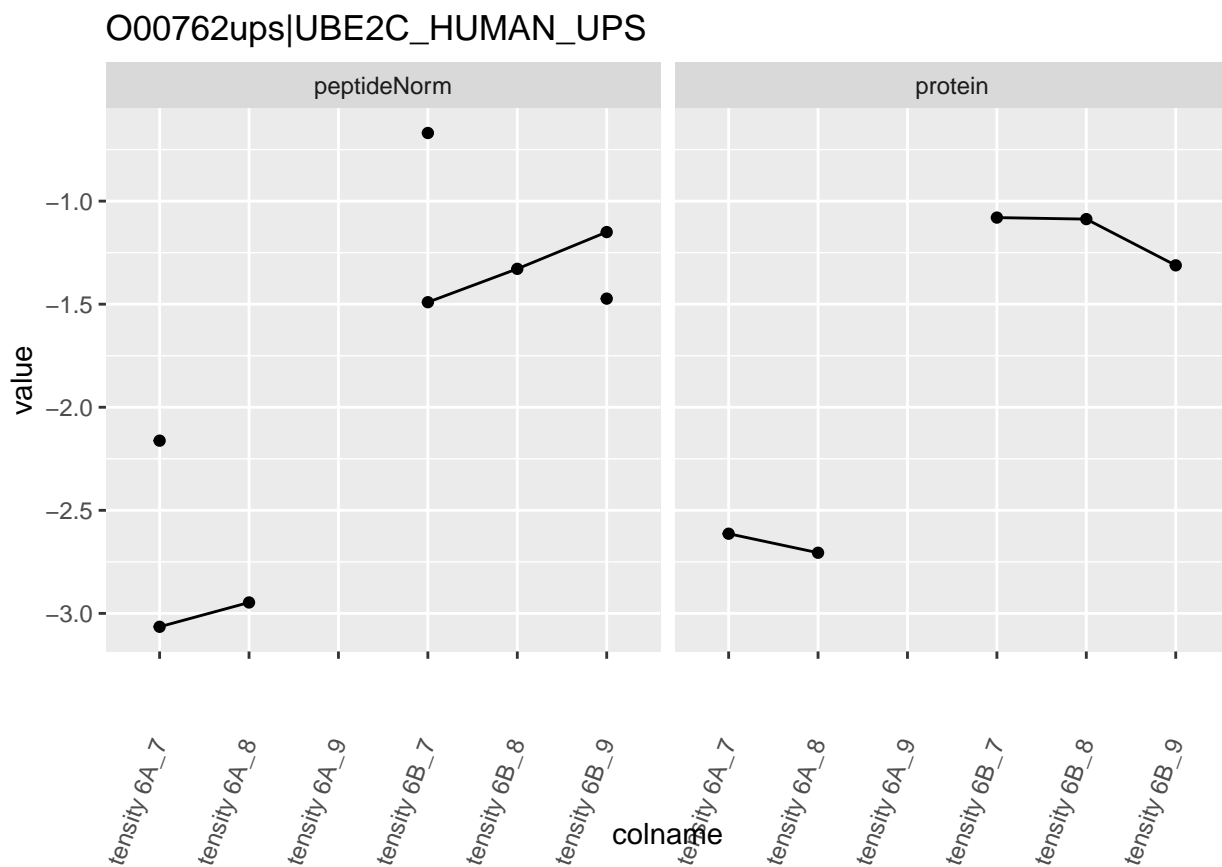
O00762ups|UBE2C_HUMAN_UPS

P00918ups|CAH2_HUMAN_UPS

P00918ups|CAH2_HUMAN_UPS

P01031ups|CO5_HUMAN_UPS

P01031ups|CO5_HUMAN_UPS

P01127ups|PDGFB_HUMAN_UPS

P01127ups|PDGFB_HUMAN_UPS

P01344ups|IGF2_HUMAN_UPS

P01344ups|IGF2_HUMAN_UPS

P01375ups|TNFA_HUMAN_UPS

P01375ups|TNFA_HUMAN_UPS

P02144ups|MYG_HUMAN_UPS

P02144ups|MYG_HUMAN_UPS

P02787ups|TRFE_HUMAN_UPS

P02787ups|TRFE_HUMAN_UPS

Legend:

- ○ APNHAVVTR
- △ CSTSSLLEACTFR
- + FDEFFSEGCAPGSKK
- × HQTVPQNTGGK
- ◇ IECVSAETTEDCIAK
- ▽ KASYLDCIR
- ⊠ KPVEEYANCHLAR
- ✳ NLREGTCPEAPTDECKPVK
- ⬦ P02787ups|TRFE_HUMAN_UPS
- ⊕ SASDLTWDNLK
- ✪ SCHTAVGR
- ⊞ SETKDLLFRDDTVCLAK
- ⊗ YLGEEYVK

condition

- A
- B

P06732ups|KCRM_HUMAN_UPS

P06732ups|KCRM_HUMAN_UPS
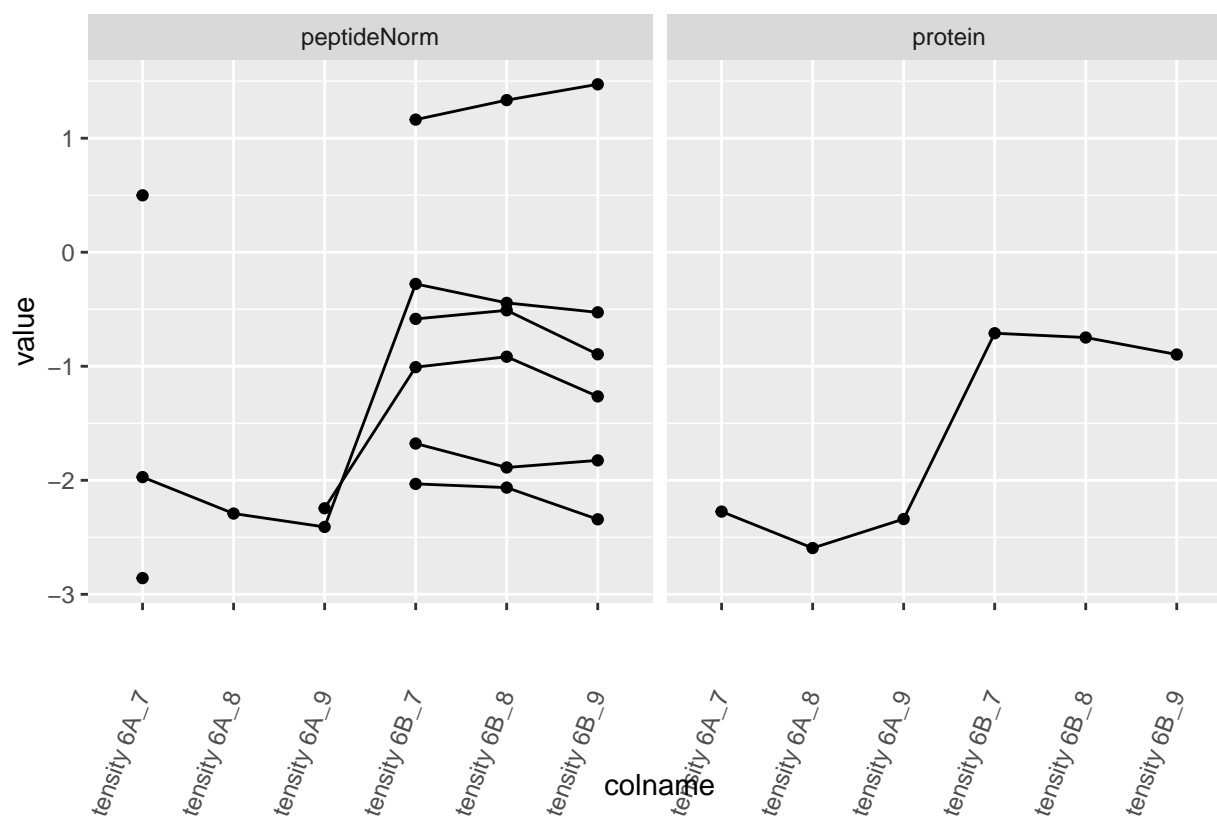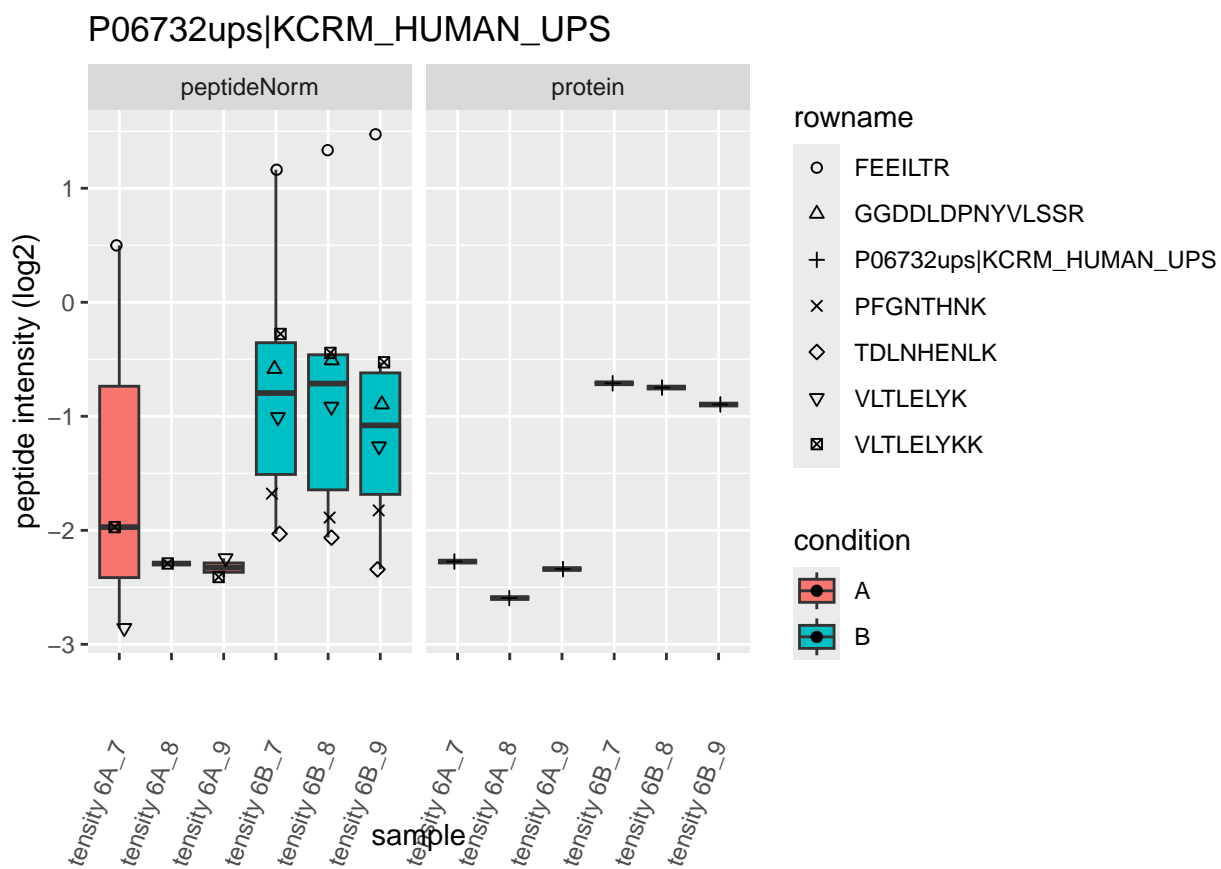
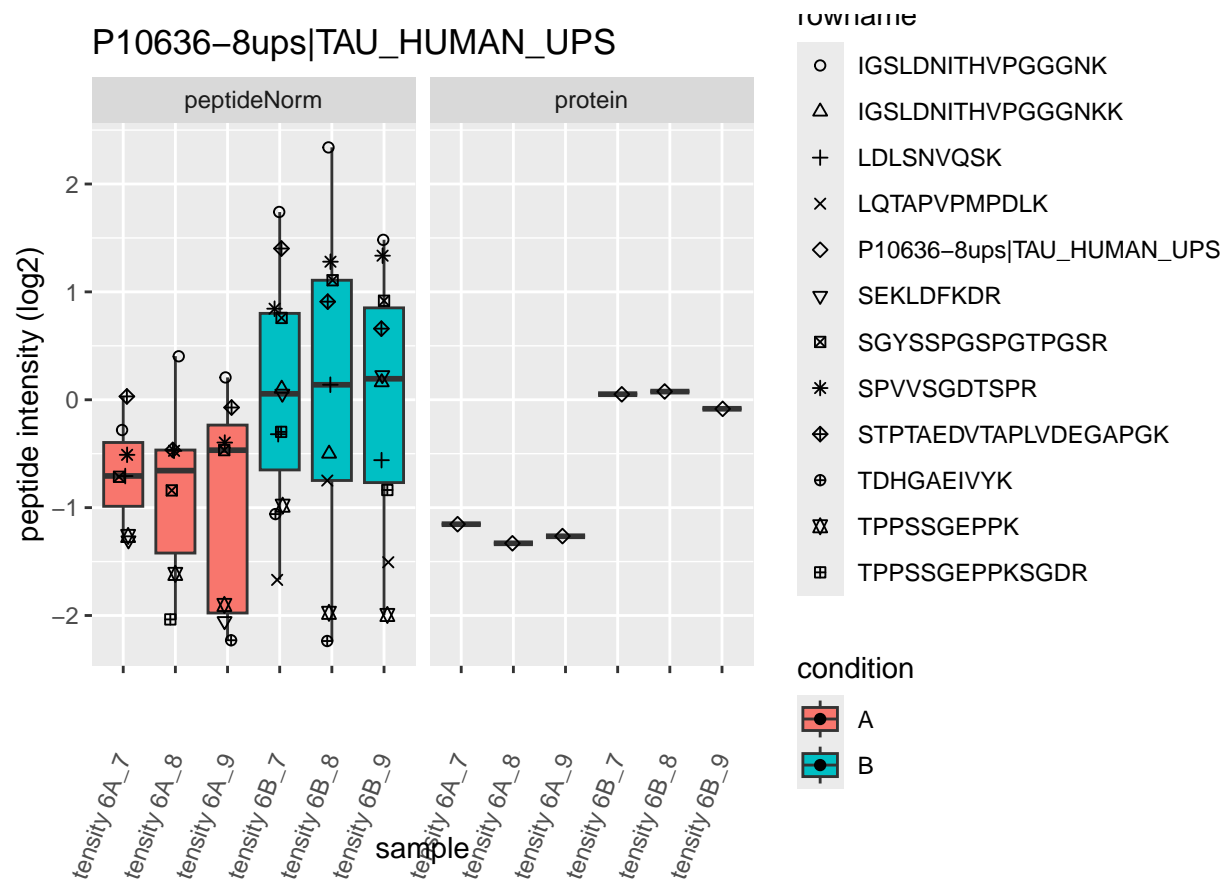# P07339ups|CATD_HUMAN_UPS

P07339ups|CATD_HUMAN_UPS

P08311ups|CATG_HUMAN_UPS

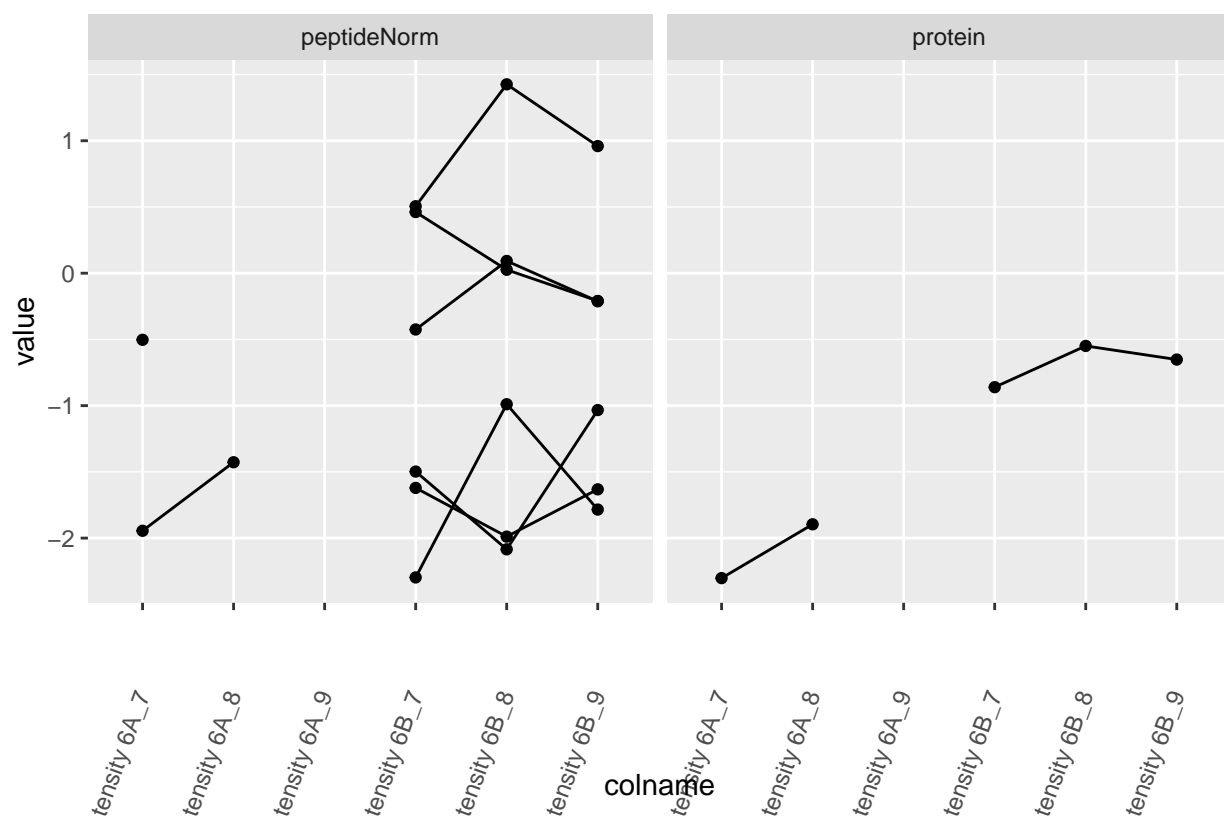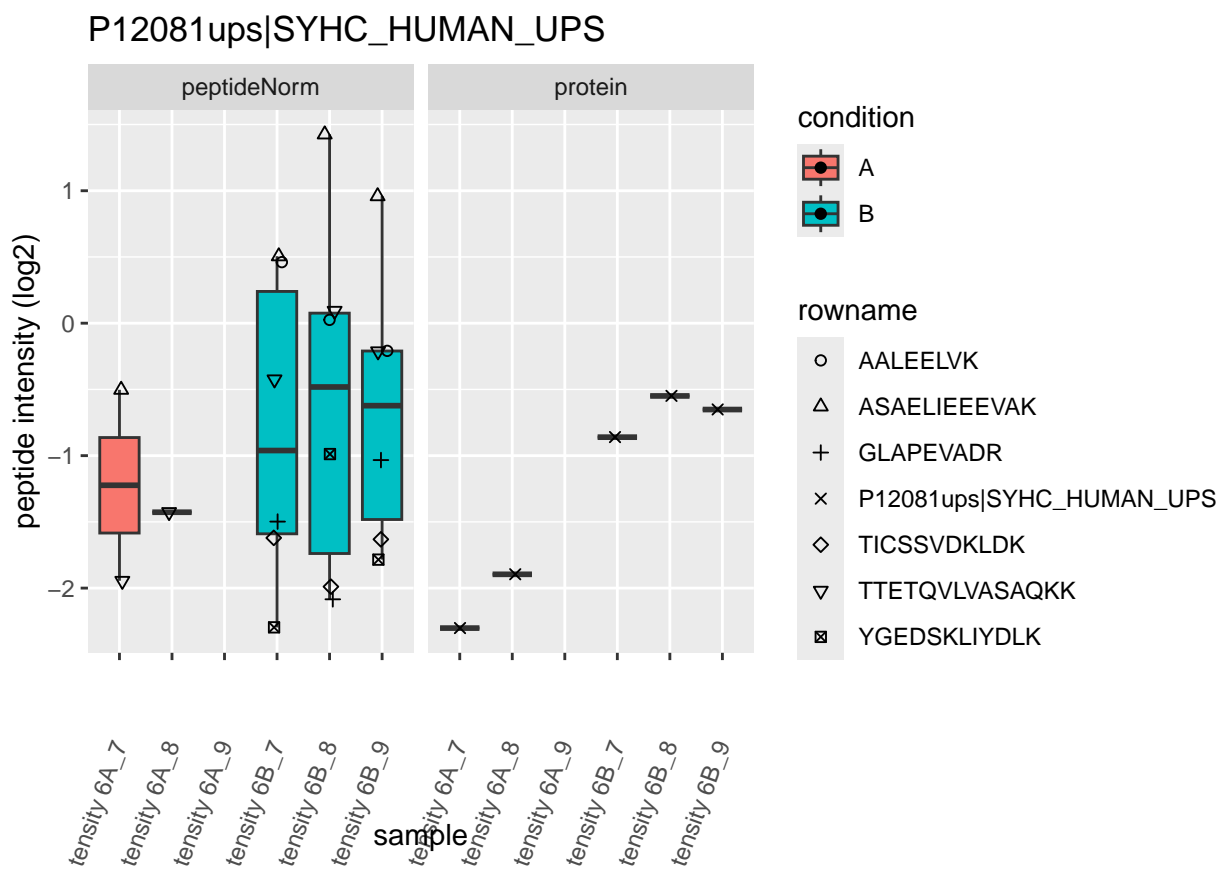P08311ups|CATG_HUMAN_UPS

P09211ups|GSTP1_HUMAN_UPS

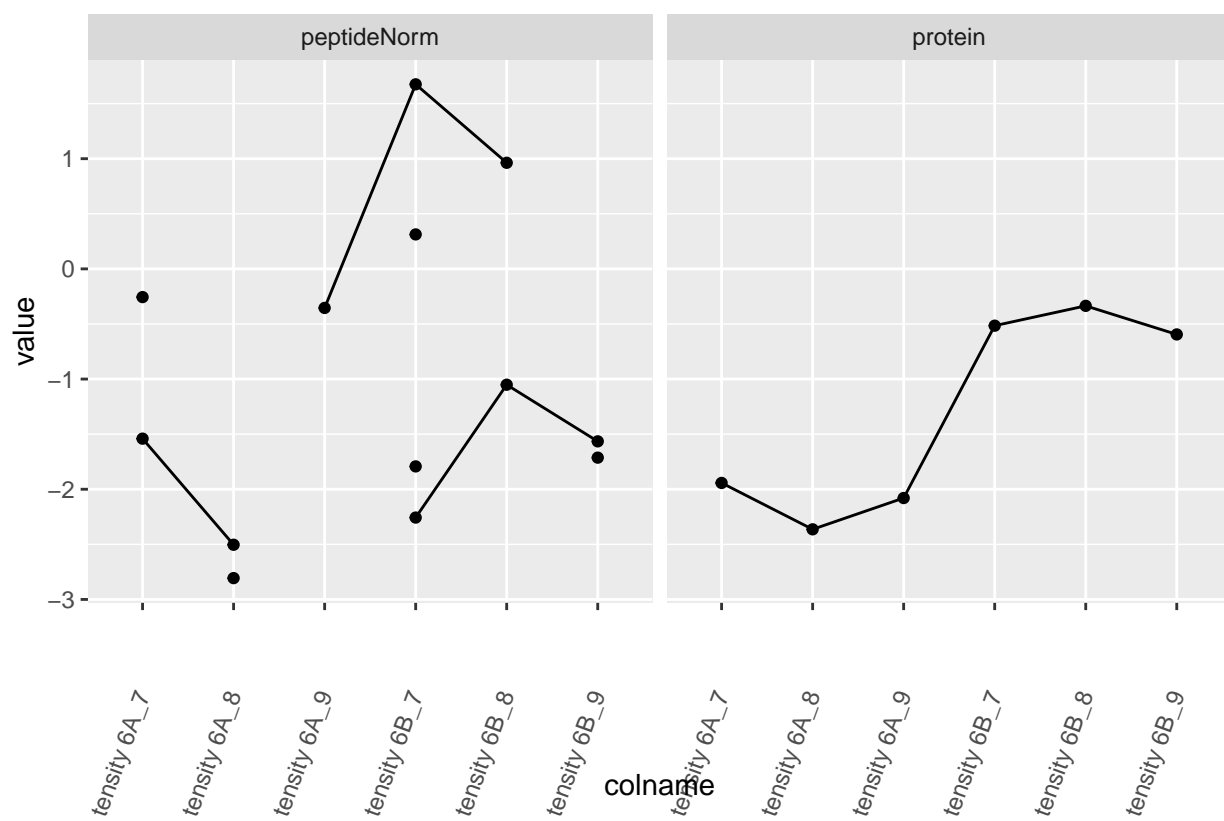P10636−8ups|TAU_HUMAN_UPS
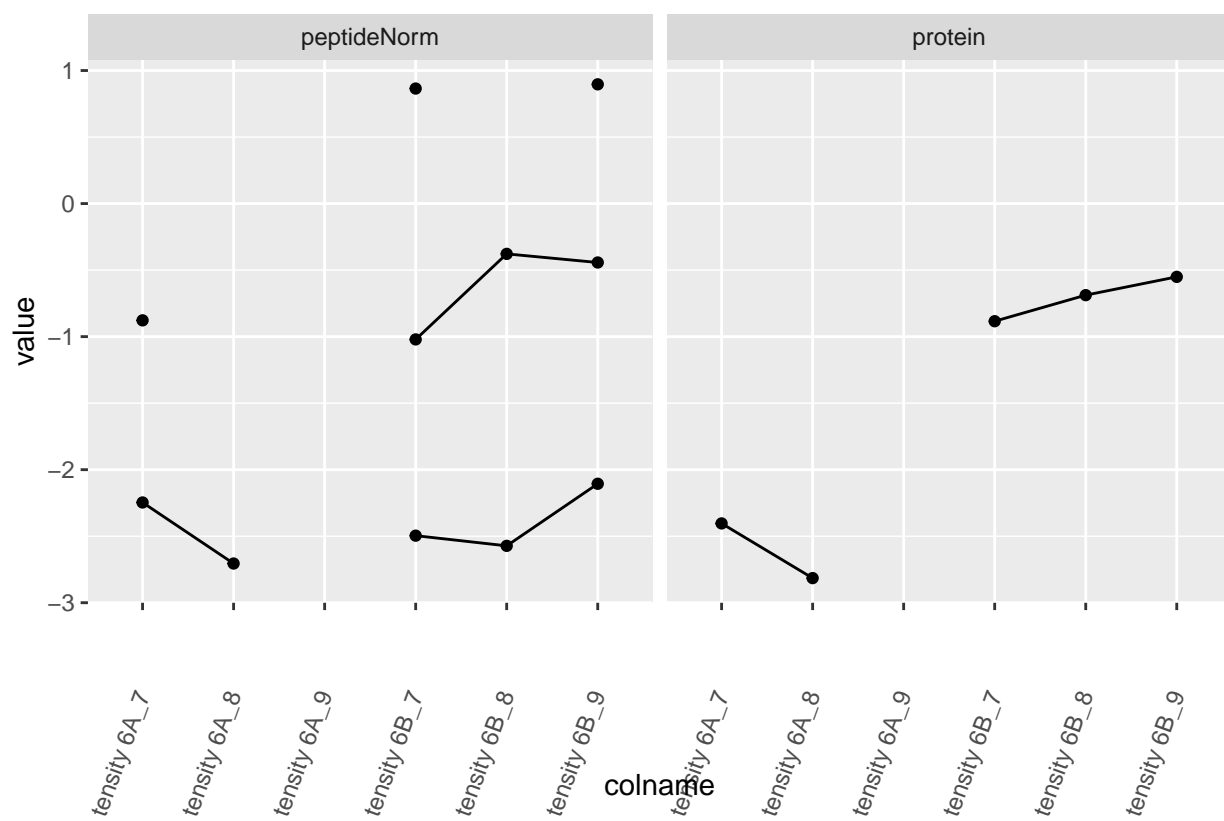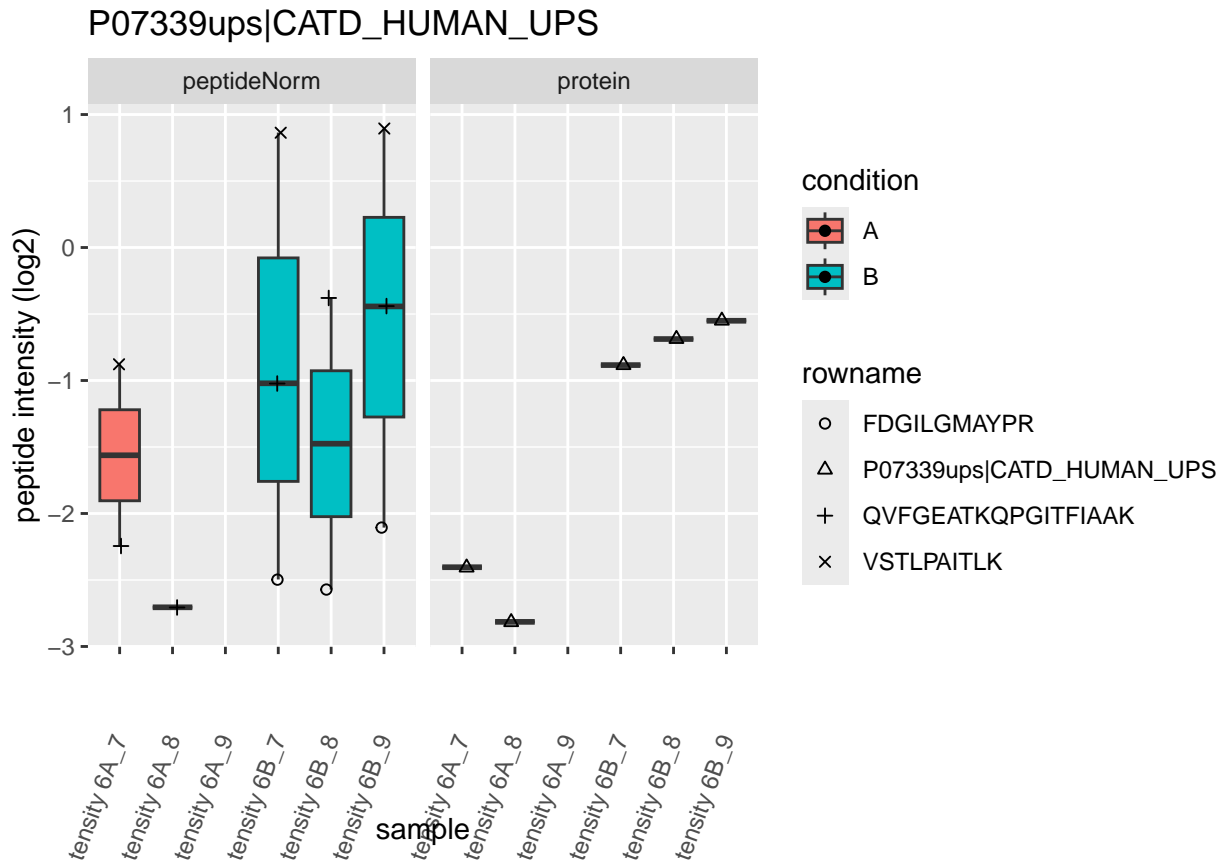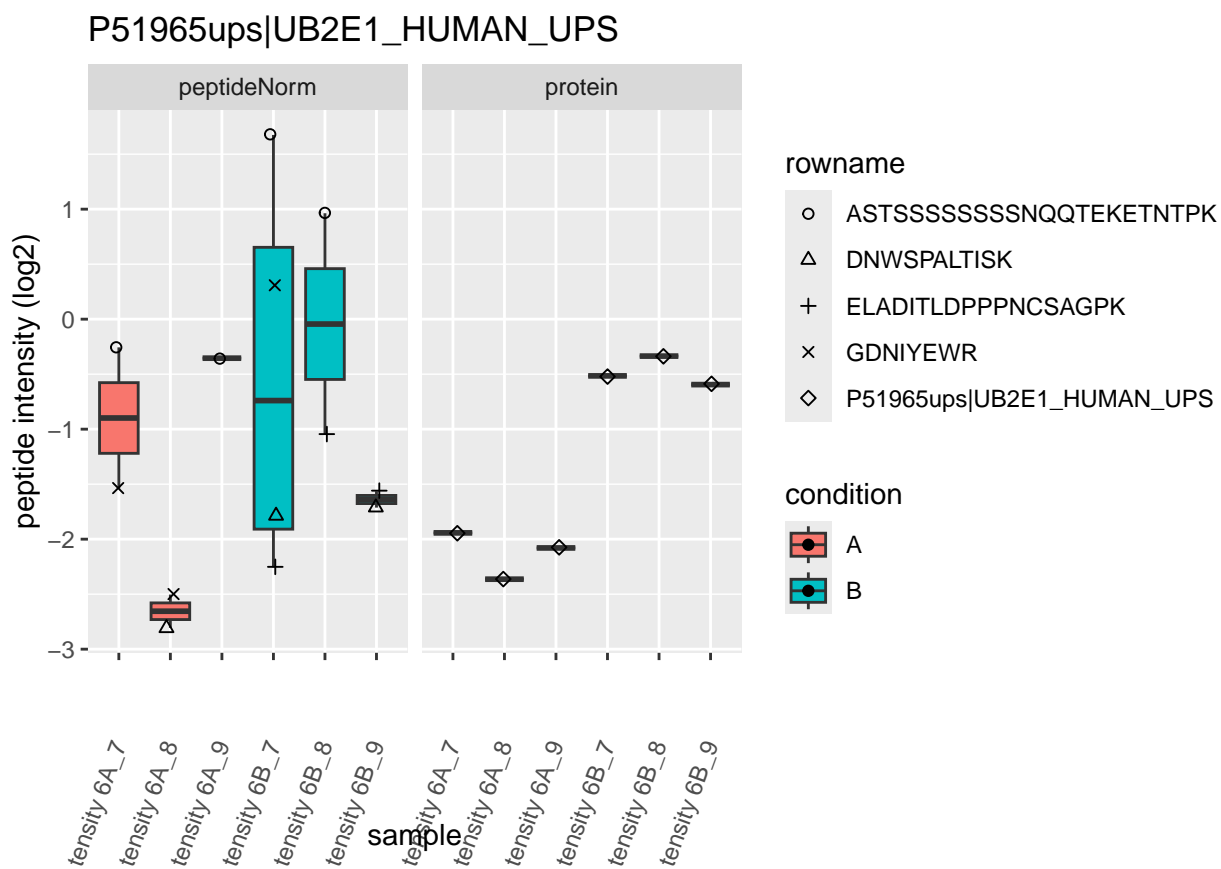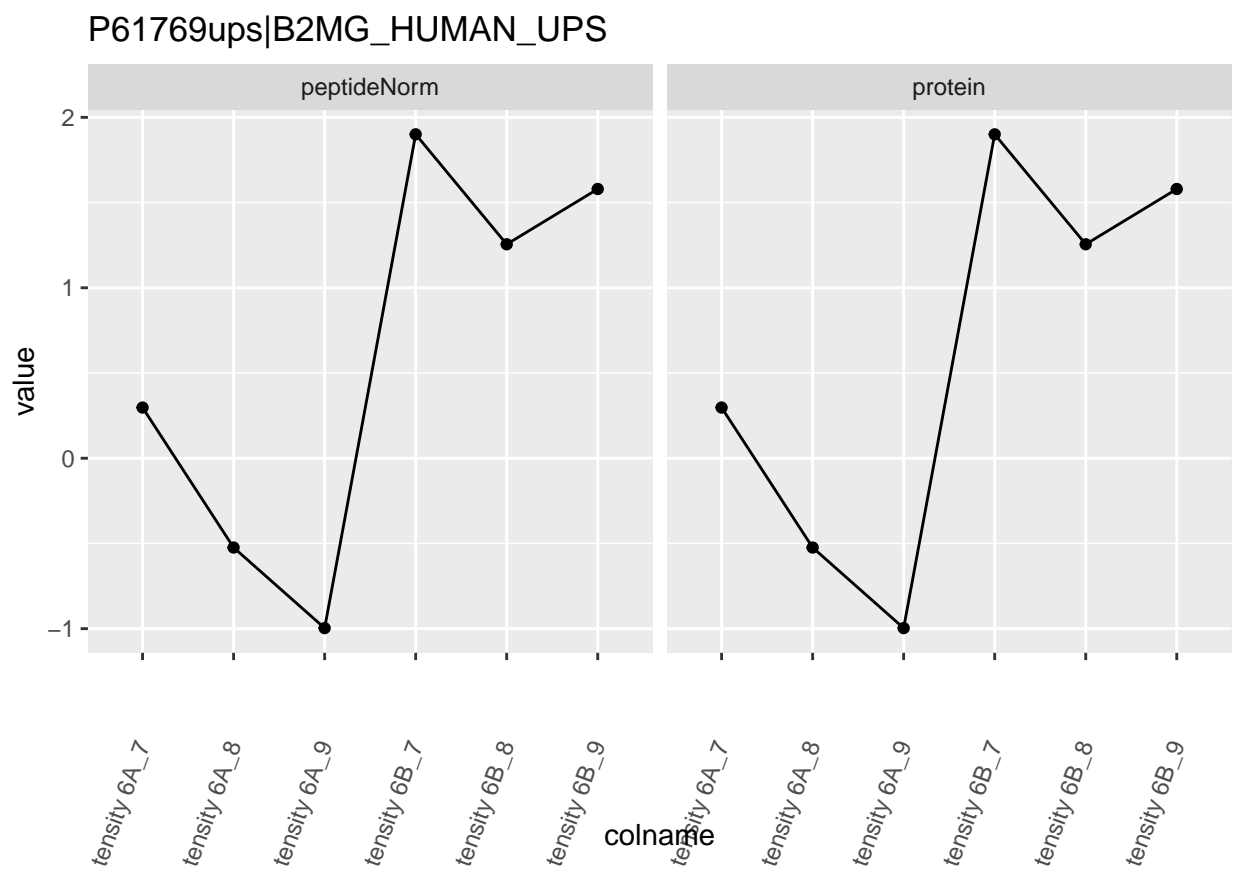
P10636−8ups|TAU_HUMAN_UPS
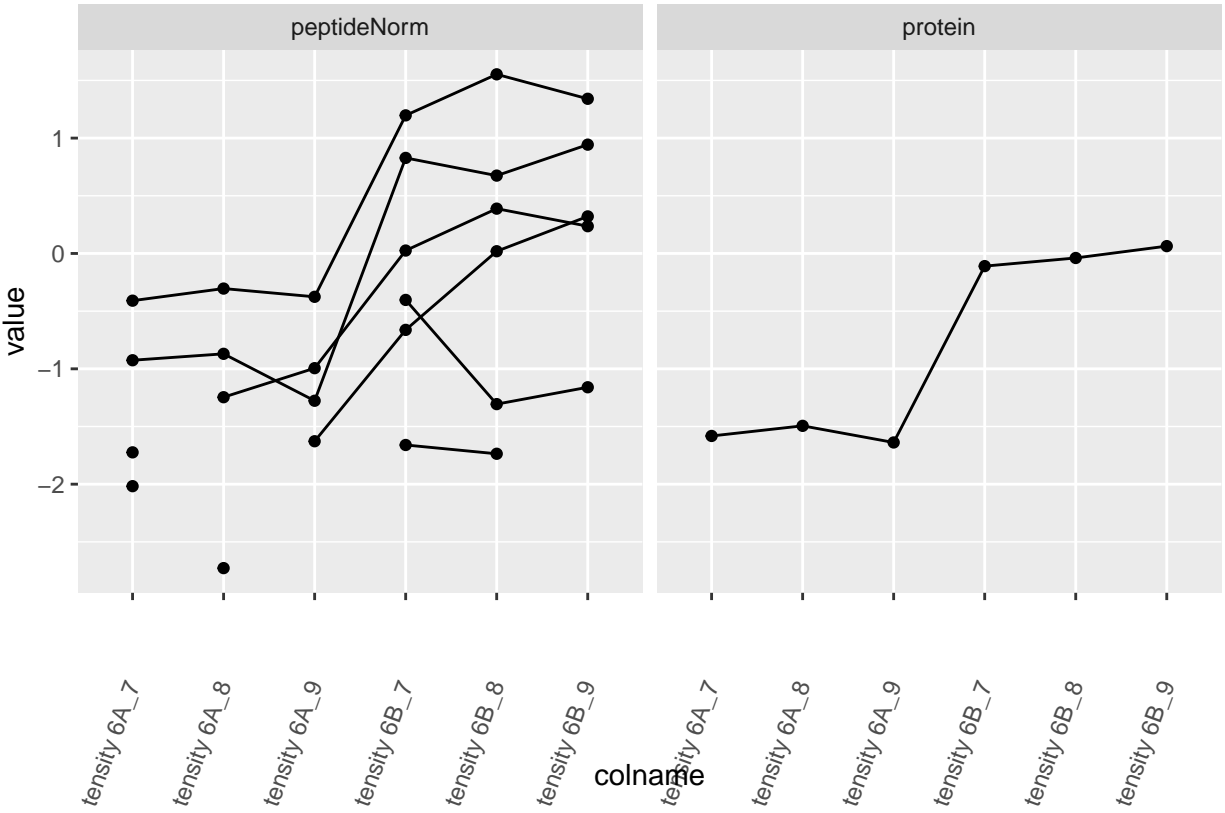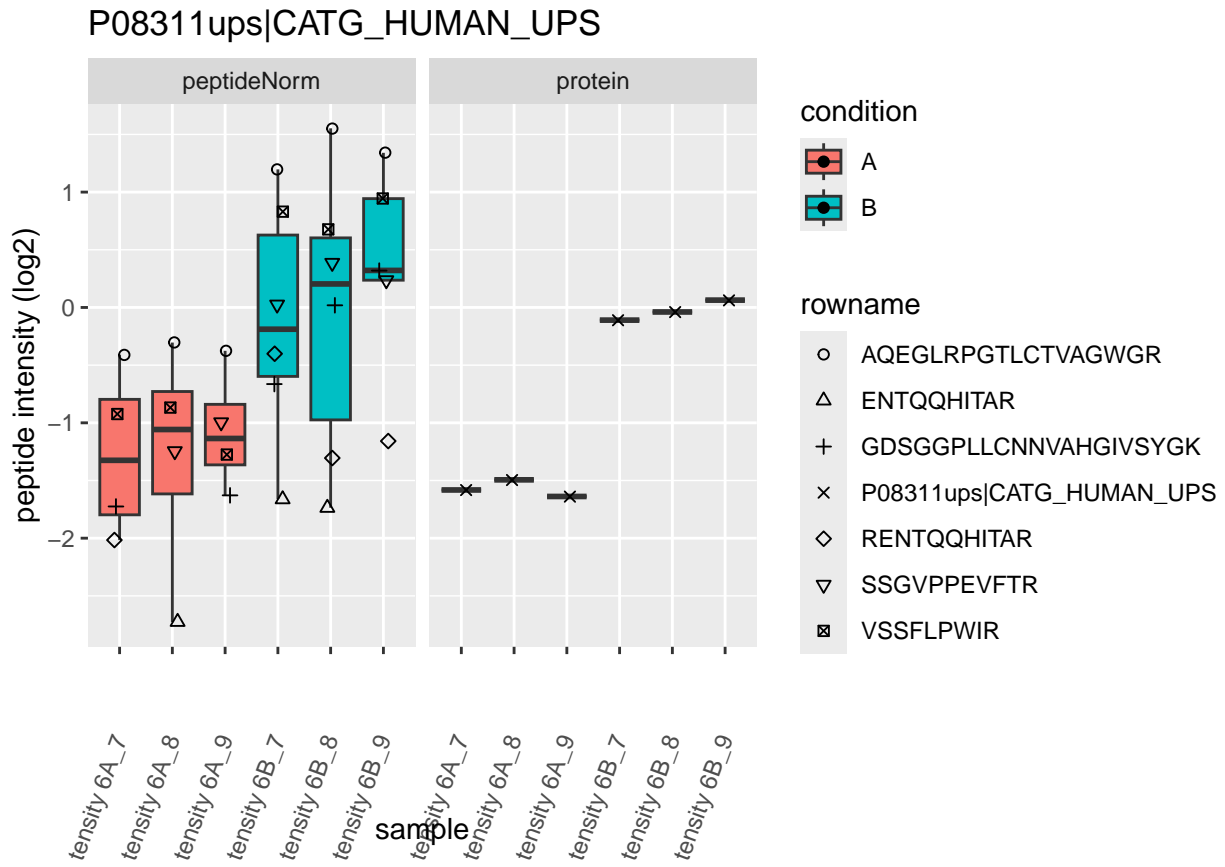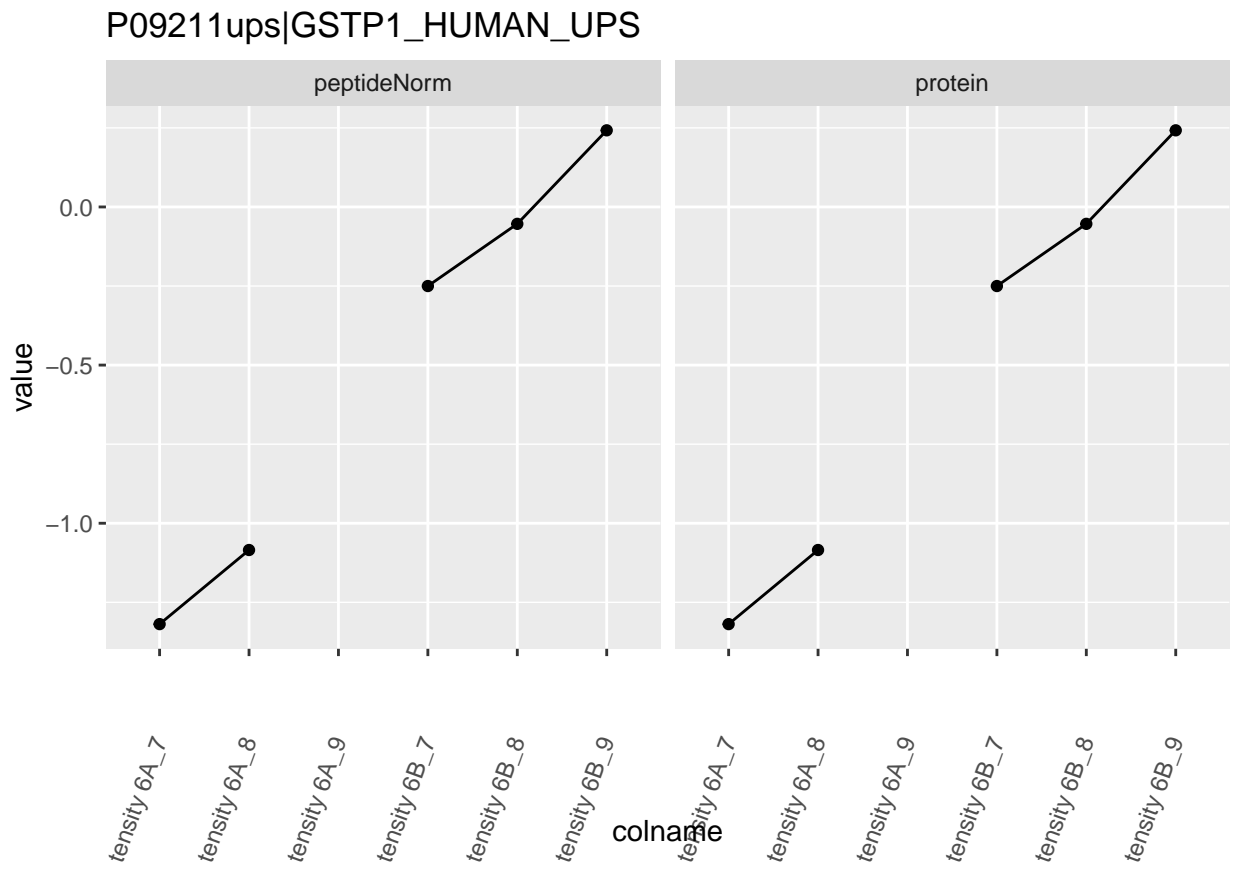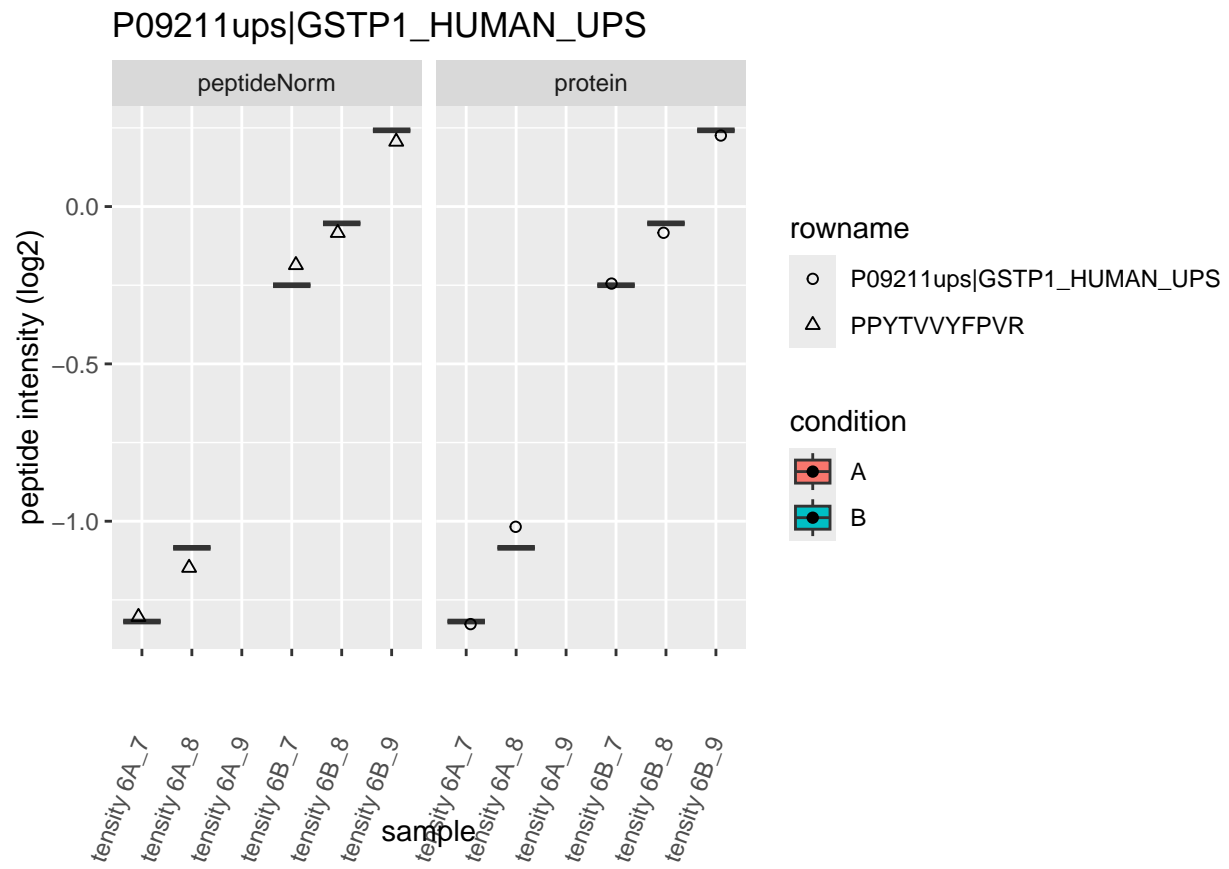
P12081ups|SYHC_HUMAN_UPS
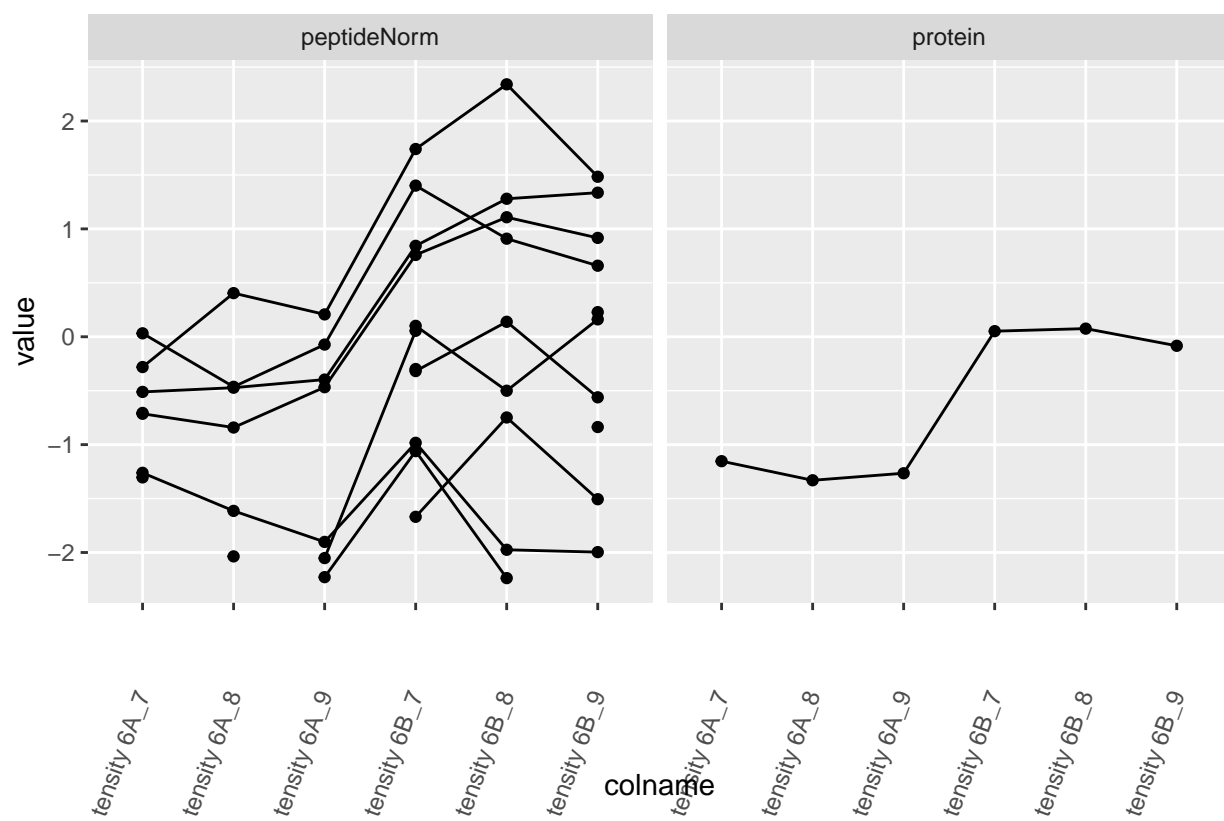
P12081ups|SYHC_HUMAN_UPS

P51965ups|UB2E1_HUMAN_UPS

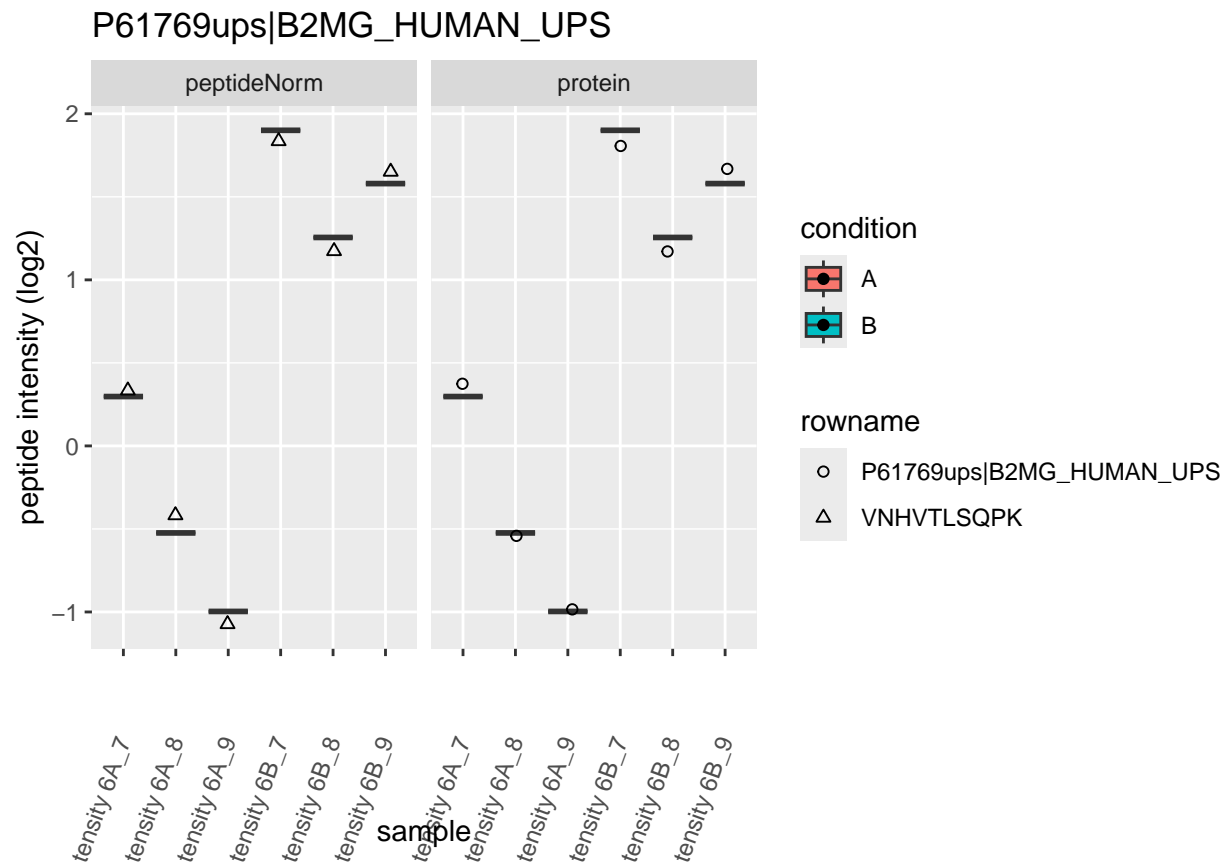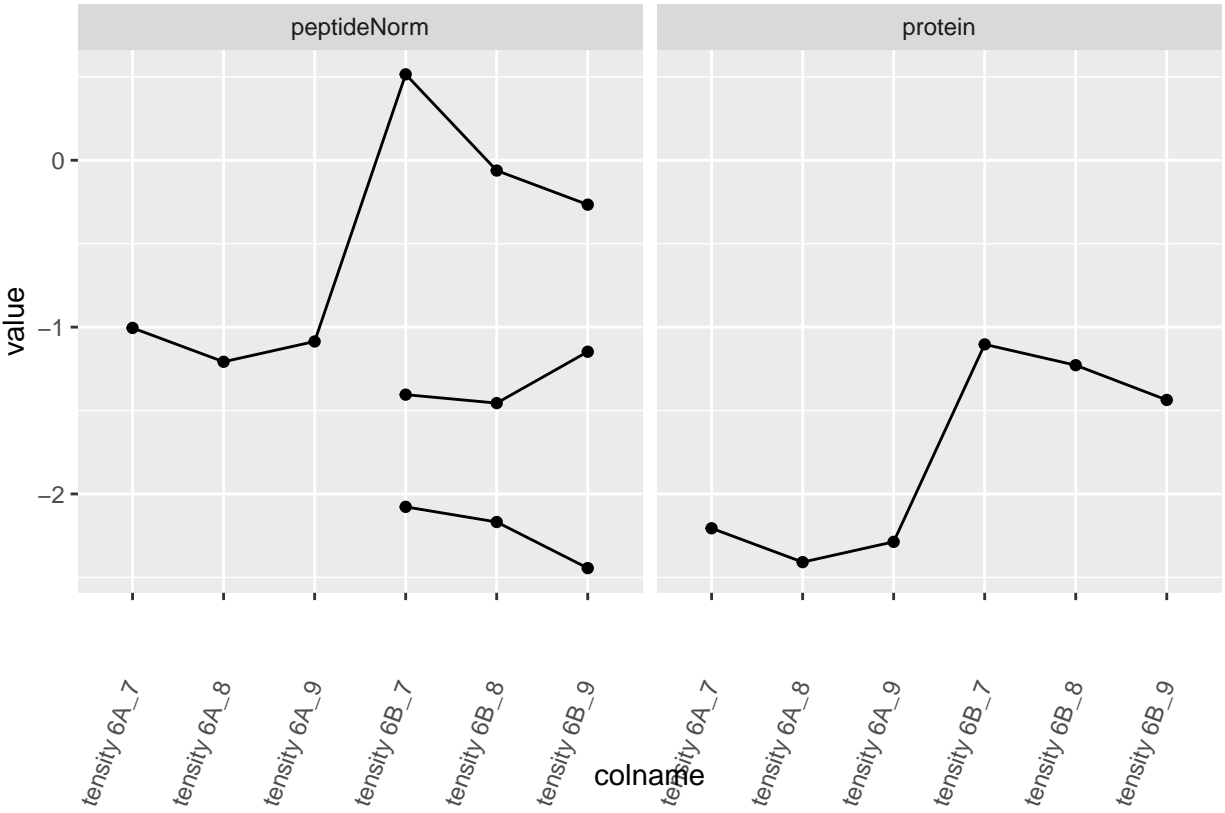# P51965ups|UB2E1_HUMAN_UPS

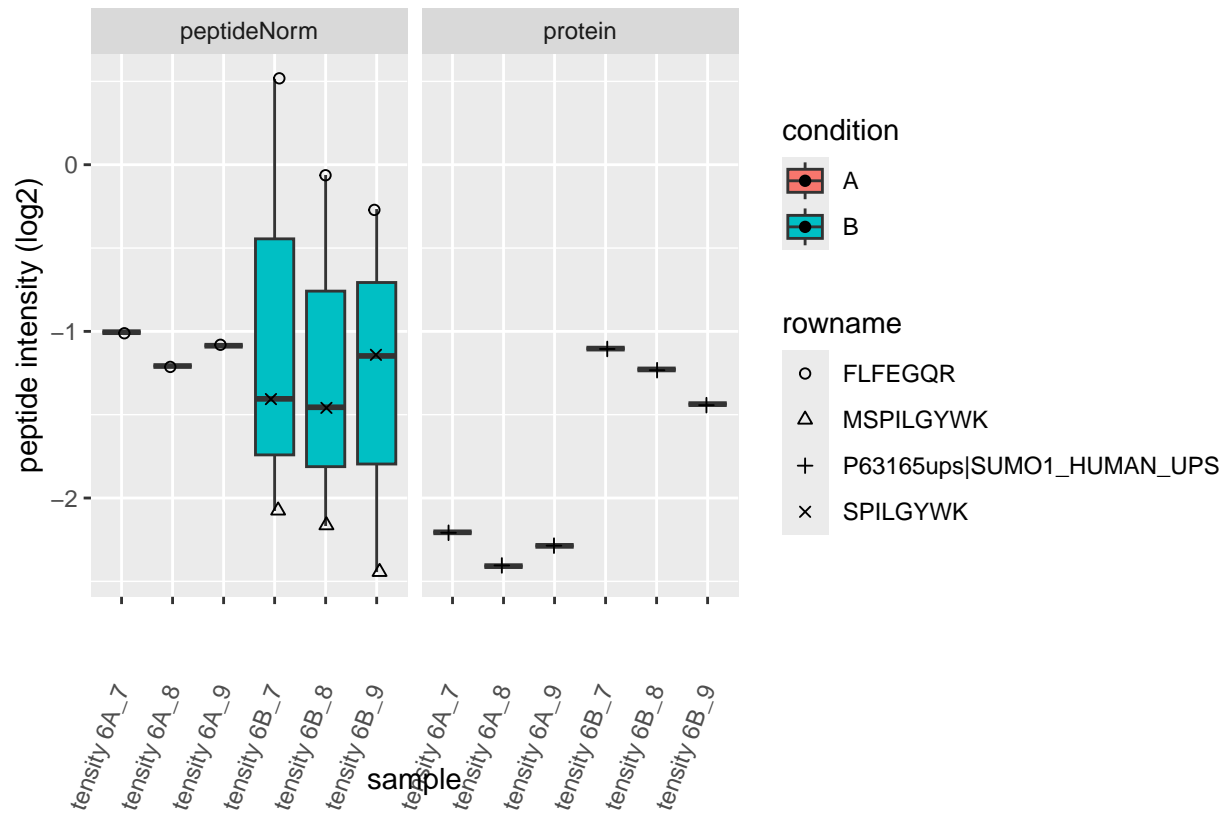# P61769ups|B2MG_HUMAN_UPS

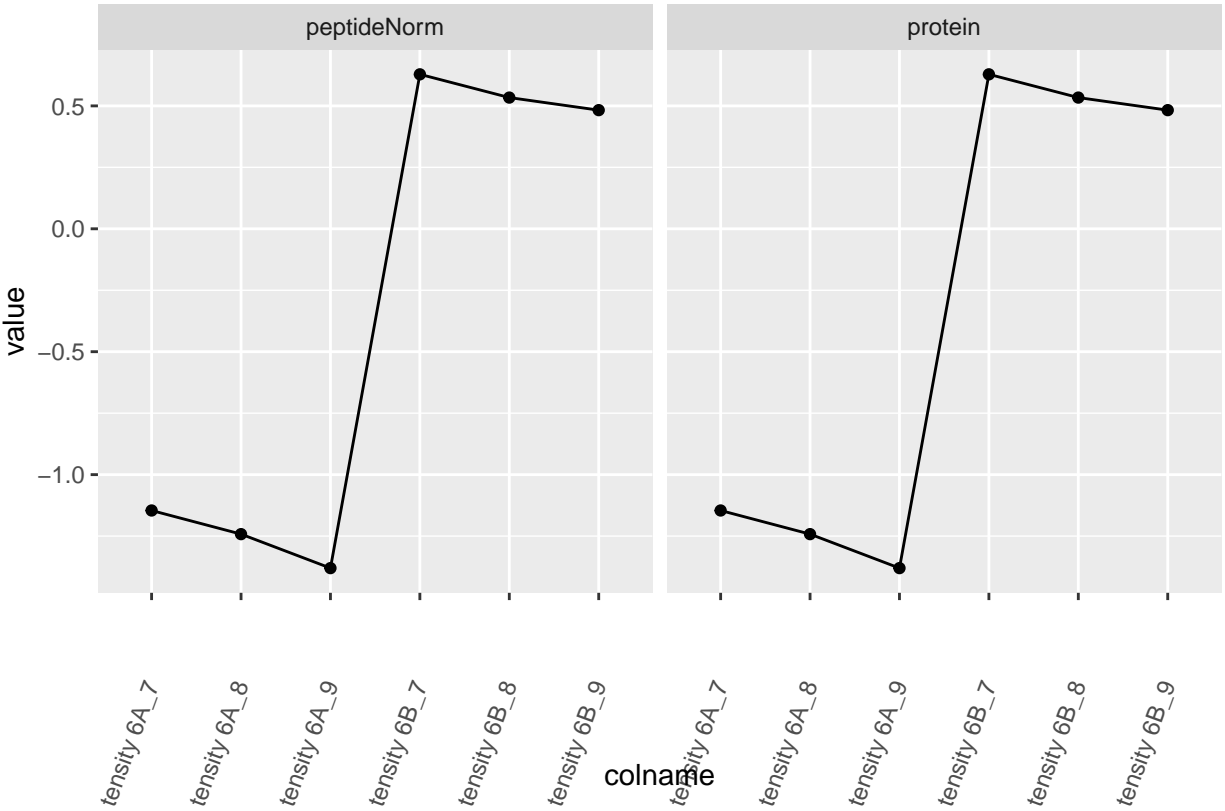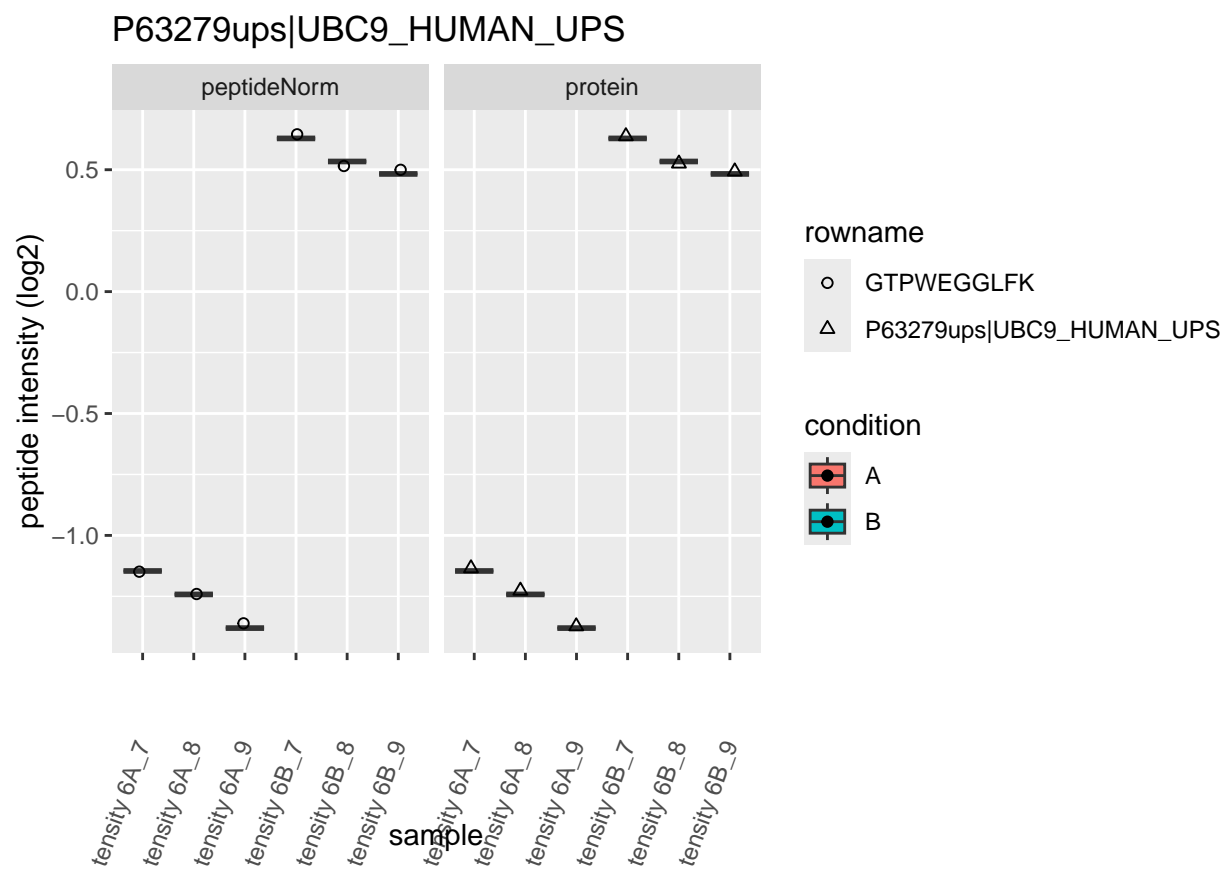# P61769ups|B2MG_HUMAN_UPS

P63165ups|SUMO1_HUMAN_UPS

P63165ups|SUMO1_HUMAN_UPS

P63279ups|UBC9_HUMAN_UPS
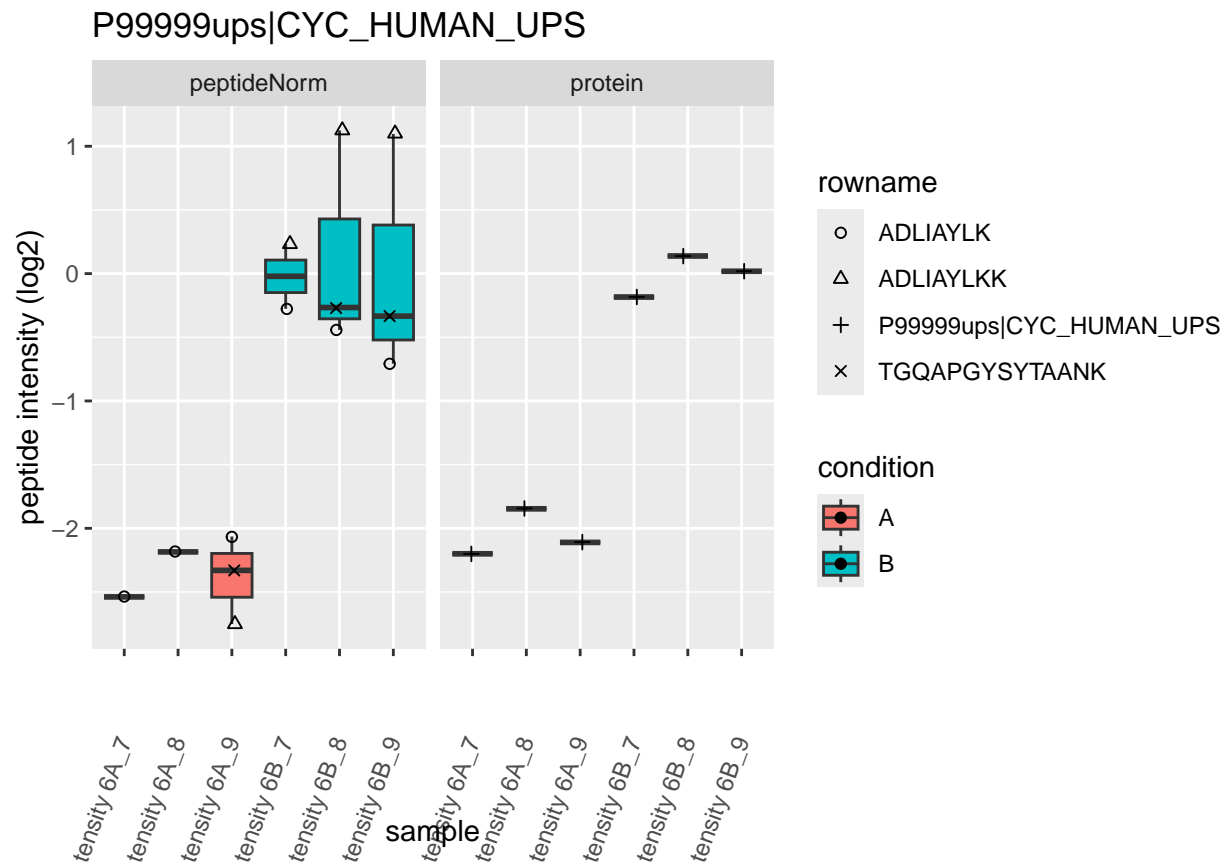
P63279ups|UBC9_HUMAN_UPS

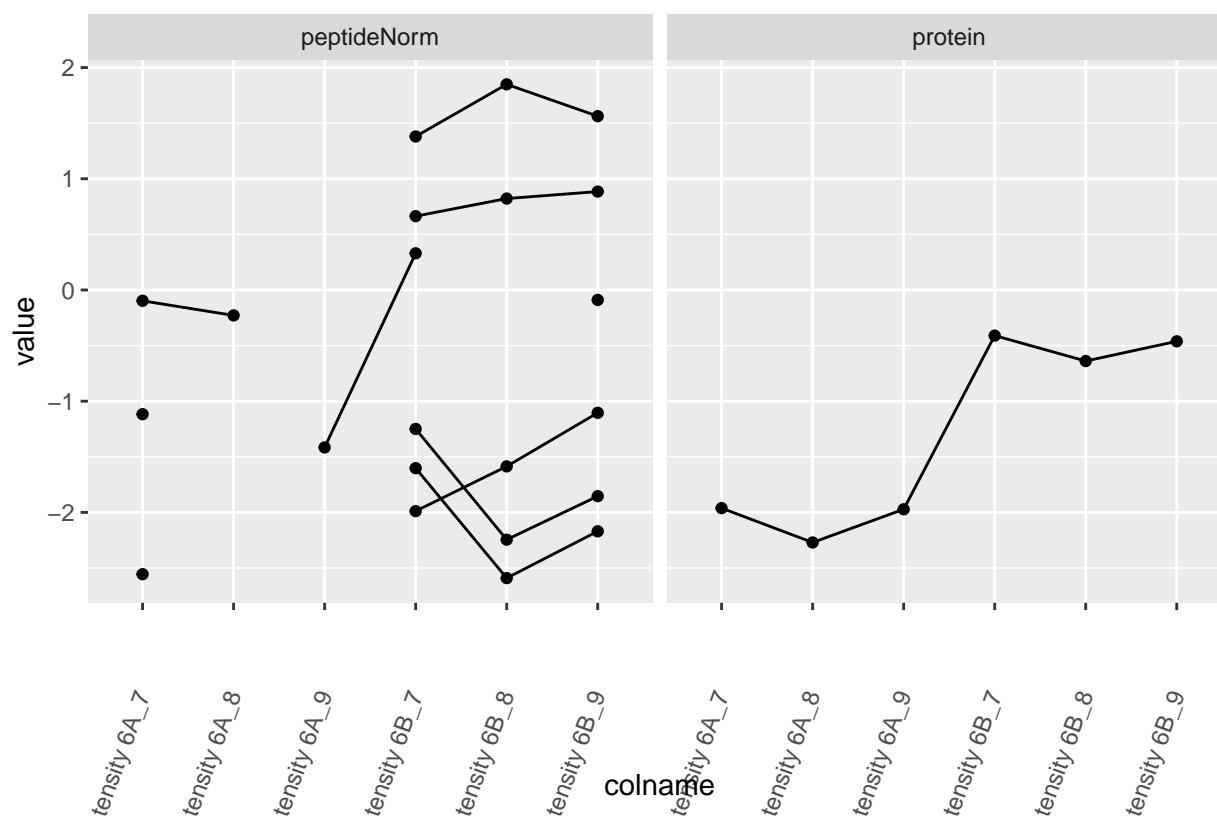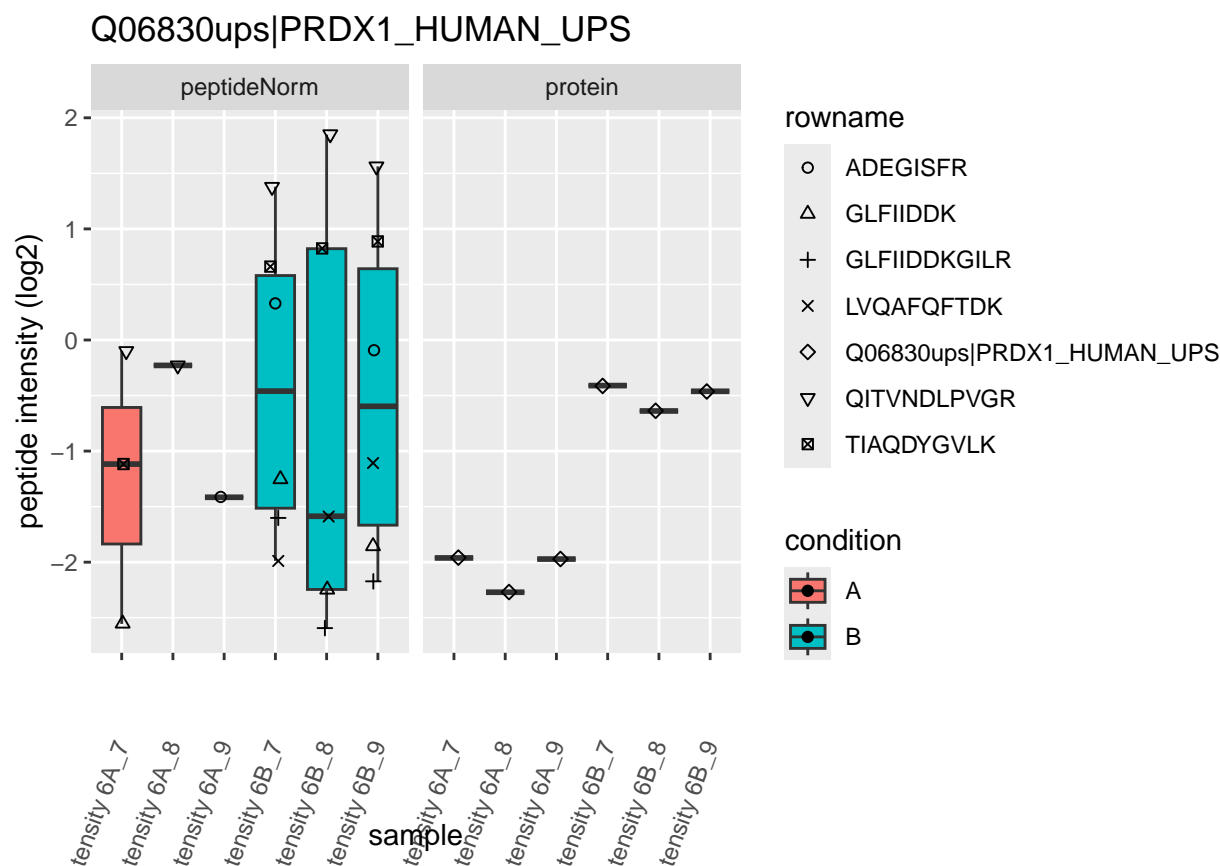# P99999ups|CYC_HUMAN_UPS

P99999ups|CYC_HUMAN_UPS
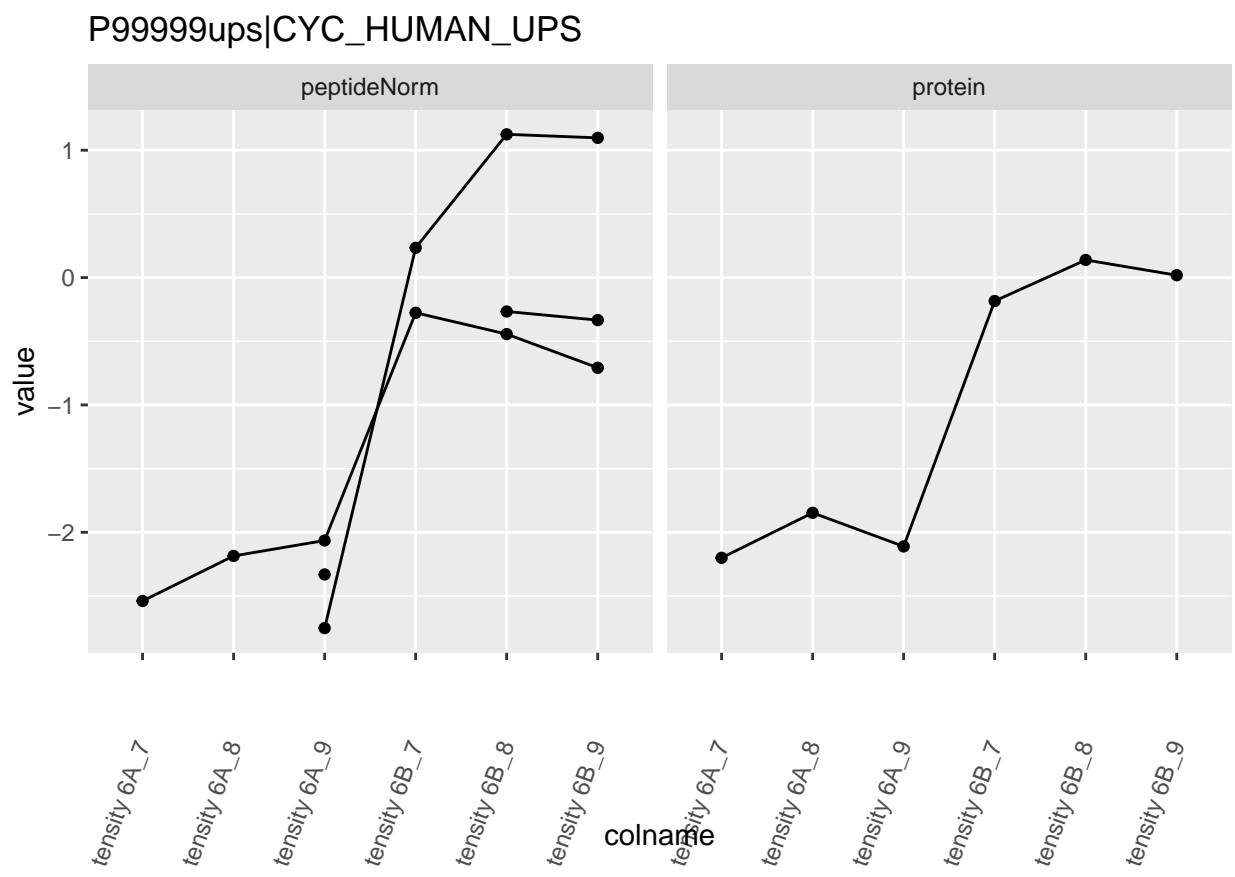
Q06830ups|PRDX1_HUMAN_UPS

Note, that the yeast protein is only covered by 3 peptides. Only one peptide is picked up in condition A. This peptide is also only once observed in spike-in condition B. This puts a considerable burden upon the inference and could be avoided by more stringent filtering.

5 Session Info

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

```r
sessionInfo()
```

```
## R version 4.4.0 RC (2024-04-16 r86468)
## Platform: aarch64-apple-darwin20
## Running under: macOS 15.5
##
## Matrix products: default
## BLAS:   /Library/Frameworks/R.framework/Versions/4.4-arm64/Resources/lib/libRblas.0.dylib
## LAPACK: /Library/Frameworks/R.framework/Versions/4.4-arm64/Resources/lib/libRlapack.dylib;  LAPACK v
##
## locale:
## [1] en_US.UTF-8/en_US.UTF-8/en_US.UTF-8/C/en_US.UTF-8/en_US.UTF-8
##
## time zone: Europe/Brussels
## tzcode source: internal
##
## attached base packages:
```

```
## [1] stats4    stats    graphics  grDevices utils    datasets  methods
## [8] base
##
## other attached packages:
##  [1] ExploreModelMatrix_1.16.0    pcaMethods_1.96.0
##  [3] data.table_1.17.6            plotly_4.10.4
##  [5] msqrob2_1.15.1               QFeatures_1.19.3
##  [7] MultiAssayExperiment_1.34.0  SummarizedExperiment_1.34.0
##  [9] Biobase_2.64.0               GenomicRanges_1.56.1
## [11] GenomeInfoDb_1.40.1          IRanges_2.38.1
## [13] S4Vectors_0.42.1             BiocGenerics_0.54.0
## [15] generics_0.1.3               MatrixGenerics_1.16.0
## [17] matrixStats_1.4.1            limma_3.60.6
## [19] lubridate_1.9.3              forcats_1.0.0
## [21] stringr_1.5.1                dplyr_1.1.4
## [23] purrr_1.0.2                  readr_2.1.5
## [25] tidyr_1.3.1                  tibble_3.2.1
## [27] ggplot2_3.5.1                tidyverse_2.0.0
##
## loaded via a namespace (and not attached):
##  [1] rlang_1.1.4            magrittr_2.0.3        shinydashboard_0.7.2
##  [4] clue_0.3-65            compiler_4.4.0        vctrs_0.6.5
##  [7] reshape2_1.4.4         ProtGenerics_1.36.0   pkgconfig_2.0.3
## [10] crayon_1.5.3           fastmap_1.2.0         XVector_0.44.0
## [13] fontawesome_0.5.2      labeling_0.4.3        utf8_1.2.4
## [16] promises_1.3.0         rmarkdown_2.28        tzdb_0.4.0
## [19] UCSC.utils_1.0.0       nloptr_2.1.1          bit_4.5.0
## [22] xfun_0.47              zlibbioc_1.50.0       cachem_1.1.0
## [25] jsonlite_1.8.9         later_1.3.2           highr_0.11
## [28] DelayedArray_0.30.1    BiocParallel_1.38.0   parallel_4.4.0
## [31] cluster_2.1.6          R6_2.5.1              bslib_0.8.0
## [34] stringi_1.8.4          boot_1.3-31           jquerylib_0.1.4
## [37] Rcpp_1.0.13-1          bookdown_0.40         knitr_1.48
## [40] BiocBaseUtils_1.10.0   httpuv_1.6.15         Matrix_1.7-0
## [43] splines_4.4.0          igraph_2.0.3          timechange_0.3.0
## [46] tidyselect_1.2.1       rstudioapi_0.16.0     abind_1.4-8
## [49] yaml_2.3.10            codetools_0.2-20      lattice_0.22-6
## [52] plyr_1.8.9             shiny_1.9.1           withr_3.0.1
## [55] evaluate_1.0.0         pillar_1.9.0          DT_0.33
## [58] shinyjs_2.1.0          hms_1.1.3             munsell_0.5.1
## [61] scales_1.3.0           minqa_1.2.8           xtable_1.8-4
## [64] glue_1.8.0             lazyeval_0.2.2        tools_4.4.0
## [67] lme4_1.1-35.5          cowplot_1.1.3         grid_4.4.0
## [70] MsCoreUtils_1.16.1     colorspace_2.1-1      nlme_3.1-166
## [73] GenomeInfoDbData_1.2.12 cli_3.6.3            fansi_1.0.6
## [76] S4Arrays_1.4.1         viridisLite_0.4.2     AnnotationFilter_1.28.0
## [79] gtable_0.3.5           rintrojs_0.3.4        sass_0.4.9
## [82] digest_0.6.37          SparseArray_1.4.8     farver_2.1.2
## [85] htmlwidgets_1.6.4      htmltools_0.5.8.1     lifecycle_1.0.4
## [88] httr_1.4.7             mime_0.12             statmod_1.5.0
## [91] bit64_4.5.2            MASS_7.3-61
```