ABRF 2017 Satellite workshop - Hands-on 4 : Statistical methods for high-throughput biology

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Summary

- Significance analysis for MS proteomics based peak intenesities data
- Significance analysis for MS proteomics based peak count data
- Compare the results with different statistical methods.

Data

- the quantified peak intensities data from ABRF 2015, processed by Skyline.
- the spectral count data from ABRF 2015 is used.

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1. Load example datasets

```
load(file='Section4.RData')
```

---

2. Read Skyline output

The required input data is generated automatically when using `MSstats` report format in Skyline. We first load and access the dataset processed by Skyline. The name of saved file from Skyline using `MSstats report format` is 'iPRG_10ppm_2rt_15cut_nosingle.csv'. or you can use the published data from **todo : add link, This file is available in panorama.**

```
# Read output from skyline : Cox.skyline.csv
raw <- read.csv(file="iPRG_10ppm_2rt_15cut_nosingle.csv")
```

We can read csv file. Here we will load R data file which is the exactly same data in iPRG_10ppm_2rt_15cut_nosingle.csv file.

---

3. Load MSstats

Load MSstats first. Then you are ready to start MSstats.

```
library(MSstats)
?MSstats
```

4. Preprocessing for skyline output

4.1. Set annotation file

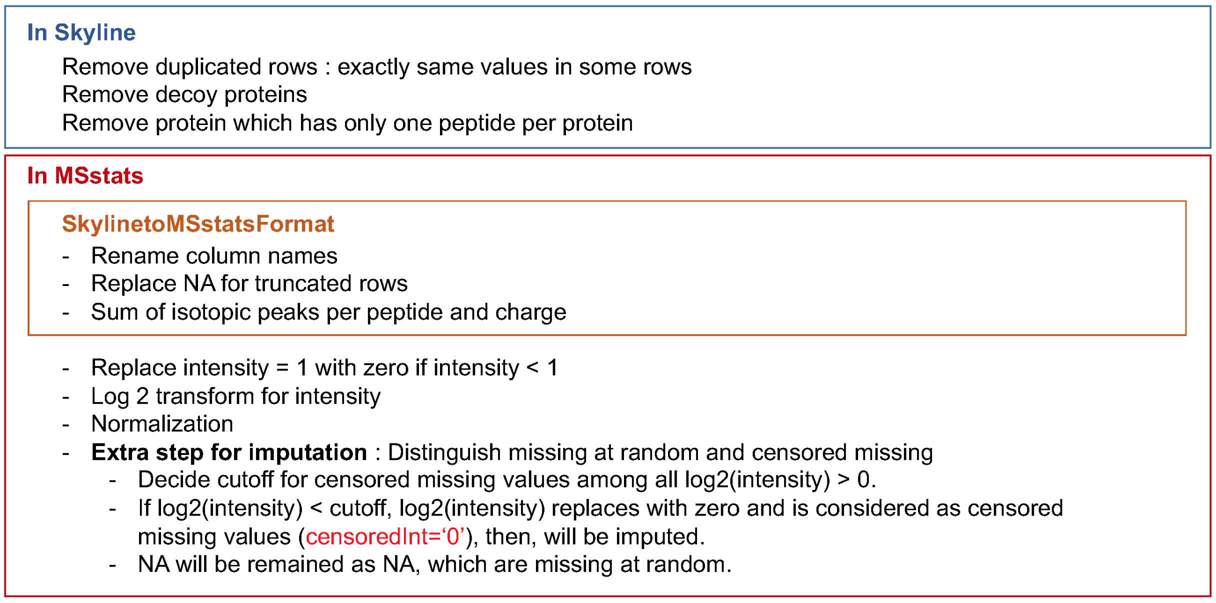
Annotation information is required to fill in `Condition` and `BioReplicate` for corresponding `Run` information. Users have to prepare as csv or txt file like ‘iPRG_skyline_annotation.csv’, which includes `Run`, `Condition`, and `BioReplicate` information, and load it in R.

```r
annot <- read.csv("iPRG_skyline_annotation.csv", header=TRUE)
annot
```

4.2. Preprocessing with `SkylinetoMSstatsFormat`

The input data for `MSstats` is required to contain variables of `ProteinName`, `PeptideSequence`, `PrecursorCharge`, `FragmentIon`, `ProductCharge`, `IsotopeLabelType`, `Condition`, `BioReplicate`, `Run`, `Intensity`. These variable names should be fixed. `MSstats` input from Skyline adapts the column scheme of the dataset so that it fits `MSstats` input format. However there are several extra column names and also some of them need to be changed. `SkylinetoMSstatsFormat` function helps pre-processing for making right format of MSstats input from Skyline output. For example, it renames some column name, and replace truncated peak intensities with NA. Another important step is to handle isotopic peaks before using `dataProcess`. The output from Skyline for DDA experiment has several measurements of peak area from the monoisotopic, M+1 and M+2 peaks. To get a robust measure of peptide intensity, we can sum over isotopic peaks per peptide or use the highest peak. Here we take a summation per peptide ion.

Here is the summary of pre-processing steps in `SkylinetoMSstatsFormat` function (in orange box below).



In Skyline

Remove duplicated rows : exactly same values in some rows
Remove decoy proteins
Remove protein which has only one peptide per protein

In MSstats

SkylinetoMSstatsFormat

- Rename column names
- Replace NA for truncated rows
- Sum of isotopic peaks per peptide and charge

- Replace intensity = 1 with zero if intensity < 1
- Log 2 transform for intensity
- Normalization
- **Extra step for imputation** : Distinguish missing at random and censored missing
  - Decide cutoff for censored missing values among all log2(intensity) > 0.
  - If log2(intensity) < cutoff, log2(intensity) replaces with zero and is considered as censored missing values (censoredInt=‘0’), then, will be imputed.
  - NA will be remained as NA, which are missing at random.

‘

```r
# reformating and pre-processing for Skyline output.
quant <- SkylinetoMSstatsFormat(raw, annotation=annot)
```

```
## ** Proteins, which names include DECOY, are removed.

## Peptides, that are used in more than one proteins, are removed.

## Warning in SkylinetoMSstatsFormat(raw, annotation = annot): NAs introduced
## by coercion

## ** Truncated peaks are replaced with NA.

## ** For DDA datasets, three isotopic peaks per feature and run are summed.
```

```r
head(quant)
```

```
##            ProteinName        PeptideSequence PrecursorCharge FragmentIon
## 1 sp|P38915|SPT8_YEAST      AAAAGAGGAGDSGDAVTK               2         sum
## 2 sp|P40457|MLP2_YEAST               QLLDESSEQK               2         sum
## 3 sp|P42943|TCPH_YEAST             GGAEQVIAEVER               2         sum
## 4 sp|P40202|CCS1_YEAST               DYSFLGVIAR               2         sum
## 5 sp|Q12049|THP3_YEAST LSWNNVNQVSNPLMVTPLPGLQK               3         sum
## 6 sp|P47122|NPA3_YEAST           TPPYVINLDPAVLR               3         sum
##   ProductCharge IsotopeLabelType  Condition BioReplicate
## 1            NA                L Condition1            1
## 2            NA                L Condition1            1
## 3            NA                L Condition1            1
## 4            NA                L Condition1            1
## 5            NA                L Condition1            1
## 6            NA                L Condition1            1
##                         Run Intensity
## 1 JD_06232014_sample1_B.raw         0
## 2 JD_06232014_sample1_B.raw   6686797
## 3 JD_06232014_sample1_B.raw 284344776
## 4 JD_06232014_sample1_B.raw 200172818
## 5 JD_06232014_sample1_B.raw   2841395
## 6 JD_06232014_sample1_B.raw   5602300
```

For further details, visit the help file using the following code.

```r
?SkylinetoMSstatsFormat
```

---

5. Processing the data for analysis : Normalizing and summarizing data with dataProcess

! Always pay attention to the default options

After reading the datasets, `MSstats` performs 1) logarithm transformation of `Intensity` column, 2) normalization, 3) feature selection, (all features vs subset of features), 4) imputation for censored missing value, which are below the cutoff and undetectable, 5) run-level summarization.

To get started with this function, visit the help section of `dataProcess` first:

Default normalization and summarization options

Then (1) normalization will be performed first. The default option for normalization is `equalizeMedians`. 'equalizeMedians' fits for label-based SRM experiments, which we can use reference signals. There are three more options for normalization. Depending on the suitable assumption for your experiment, you can choose one of them.

Then, (2) run level summarization will be performed including missing value imputation by accerelated failure model and robust parameter estimation by TMP (Tukey's median polish).

Below show the default for all options in dataProcess except `skylineReport` and `censoredInt`. For input from Skyline, `skylineReport=TRUE` snd `censoredInt='NA'`

```
# `TMP` is the default model-based summarization option
quant.processed <- dataProcess(raw = quant,
                               logTrans=2,
                               normalization = 'equalizeMedians',
                               summaryMethod = 'TMP',
                               MBimpute=TRUE,
                               censoredInt='0',
                               cutoffCensored='minFeature',
                               maxQuantileforCensored = 0.999)
```

Let's check output from `dataProcess`

```
# show the name of outputs
names(quant.processed)
```

```
## [1] "ProcessedData"     "RunlevelData"      "SummaryMethod"
## [4] "ModelQC"           "PredictBySurvival"
```

```
# show reformated and normalized data.
# 'ABUNDANCE' column has normalized log2 transformed intensities.
head(quant.processed$ProcessedData)
```

```
##                     PROTEIN                         PEPTIDE TRANSITION
## 27092 sp|D6VTK4|STE2_YEAST EGEVEPVDM[+16]YTPDTAADEEARK_3     sum_NA
## 10700 sp|D6VTK4|STE2_YEAST      EGEVEPVDMYTPDTAADEEARK_3     sum_NA
## 20407 sp|D6VTK4|STE2_YEAST         FYPGTLSSFQTDSINNDAK_2     sum_NA
## 2213  sp|D6VTK4|STE2_YEAST                  IGPFADASYK_2     sum_NA
## 938   sp|D6VTK4|STE2_YEAST                     KETTSDK_2     sum_NA
## 26116 sp|D6VTK4|STE2_YEAST               NQFYQLPTPTSSK_2     sum_NA
##                                FEATURE LABEL GROUP_ORIGINAL
## 27092 EGEVEPVDM[+16]YTPDTAADEEARK_3_sum_NA     L     Condition1
## 10700      EGEVEPVDMYTPDTAADEEARK_3_sum_NA     L     Condition1
## 20407         FYPGTLSSFQTDSINNDAK_2_sum_NA     L     Condition1
## 2213                   IGPFADASYK_2_sum_NA     L     Condition1
## 938                       KETTSDK_2_sum_NA     L     Condition1
## 26116            NQFYQLPTPTSSK_2_sum_NA     L     Condition1
##       SUBJECT_ORIGINAL RUN GROUP SUBJECT SUBJECT_NESTED INTENSITY
## 27092                1   1     1       1            1.1   5222795
## 10700                1   1     1       1            1.1 182195648
## 20407                1   1     1       1            1.1  86229170
## 2213                 1   1     1       1            1.1 157996653
## 938                  1   1     1       1            1.1 177684007
## 26116                1   1     1       1            1.1 140368798
##       ABUNDANCE METHOD               originalRUN censored
## 27092  22.07353      1 JD_06232014_sample1_B.raw    FALSE
## 10700  27.19805      1 JD_06232014_sample1_B.raw    FALSE
## 20407  26.11881      1 JD_06232014_sample1_B.raw    FALSE
## 2213   26.99246      1 JD_06232014_sample1_B.raw    FALSE
## 938    27.16188      1 JD_06232014_sample1_B.raw    FALSE
## 26116  26.82179      1 JD_06232014_sample1_B.raw    FALSE
```

```
# This table includes run-level summarized log2 intensities. (column : LogIntensities)
# Now one summarized log2 intensities per Protein and Run.
# NumMeasuredFeature : show how many features are used for run-level summarization.
#         If there is no missing value, it should be the number of features in certain protein.
# MissingPercentage : the number of missing features / the number of features in certain protein.
head(quant.processed$RunlevelData)
```

```
##   RUN              Protein LogIntensities NumMeasuredFeature
## 1   1 sp|D6VTK4|STE2_YEAST       26.81232                  8
## 2   2 sp|D6VTK4|STE2_YEAST       26.60786                  8
## 3   3 sp|D6VTK4|STE2_YEAST       26.58301                  8
## 4   4 sp|D6VTK4|STE2_YEAST       26.83563                  8
## 5   5 sp|D6VTK4|STE2_YEAST       26.79430                  8
## 6   6 sp|D6VTK4|STE2_YEAST       26.60863                  8
##   MissingPercentage more50missing NumImputedFeature
## 1                 0         FALSE                 0
## 2                 0         FALSE                 0
## 3                 0         FALSE                 0
## 4                 0         FALSE                 0
## 5                 0         FALSE                 0
## 6                 0         FALSE                 0
##                 originalRUN GROUP GROUP_ORIGINAL SUBJECT_ORIGINAL
## 1 JD_06232014_sample1_B.raw     1     Condition1                1
## 2 JD_06232014_sample1_C.raw     1     Condition1                1
## 3 JD_06232014_sample1-A.raw     1     Condition1                1
## 4 JD_06232014_sample2_A.raw     2     Condition2                2
## 5 JD_06232014_sample2_B.raw     2     Condition2                2
## 6 JD_06232014_sample2_C.raw     2     Condition2                2
##   SUBJECT_NESTED SUBJECT
## 1            1.1       1
## 2            1.1       1
## 3            1.1       1
## 4            2.2       2
## 5            2.2       2
## 6            2.2       2
```

```
# show which summarization method is used.
head(quant.processed$SummaryMethod)
```

```
## [1] "TMP"
```

---

6. Visualization of processed data

6.1. Quality control plots

Now let's look at what the equalize medians procedure did to our data. QC plot is good to see the distribution of intensities per MS run and outliers. So, it is good visualization to check normalization. However, not good to see individual intensities.

```
# QC plot for normalized data with equalize median method
dataProcessPlots(data = quant.processed,
                 type="QCplot",
                 width=7, height=7,
                 which.Protein = 1,
                 address='iPRG_skyline_equalizeNorm_')
```

Then, `iPRG_skyline_equalizeNorm_QCPlot.pdf` are generated in the currect directory.

Now the median log2 intensities per run across MS runs are the same.

6.2. Profile plots

Profile plot is good visualization to check individual measurements. Each dot means one intensity. The dots are linked with line per feature. If line is disconnected, that means there is no value (missing value). Color means different peptides and charge stages. different line type means different transition.

```
# if you have many MS runs, adjust width of plot (makd wider)
# Profile plot for the data with equalized median method
dataProcessPlots(data = quant.processed,
                 type="Profileplot",
                 width=7, height=7,
                 address="iPRG_skyline_equalizeNorm_")
```

`iPRG_skyline_equalizeNorm_ProfilePlot.pdf` and `iPRG_skyline_equalizeNorm_ProfilePlot_wSummarization.pdf` are generated in the current directory.

Then, Let's go though profile plots to see overall quality of data.

There are two pdfs for each protein, first is profile plot with normalized data and second plot is profile plot with normalilzed data and summarized data. This profile plot shows each peptide transition across runs, grouped per condition. Ech peptide has a different colour/type layout.

This plot shows The panel on the right shows the same transitions in grey, with the values as summarized by the model overlayed in red.

Instead of making all profiles plots for all proteins, we can make plot for individual protein. Here is the example of spike-in protein, `sp|P44015|VAC2_YEAST`

```
dataProcessPlots(data = quant.processed,
                 type="Profileplot",
                 featureName="NA",
                 width=7, height=7,
                 which.Protein = 'sp|P44015|VAC2_YEAST',
                 address="iPRG_skyline_equalizeNorm_P44015_")
```

```
## Warning: Ignoring unknown aesthetics: linetype
```

```
## Drew the Profile plot for  sp|P44015|VAC2_YEAST ( 1  of  1 )
```

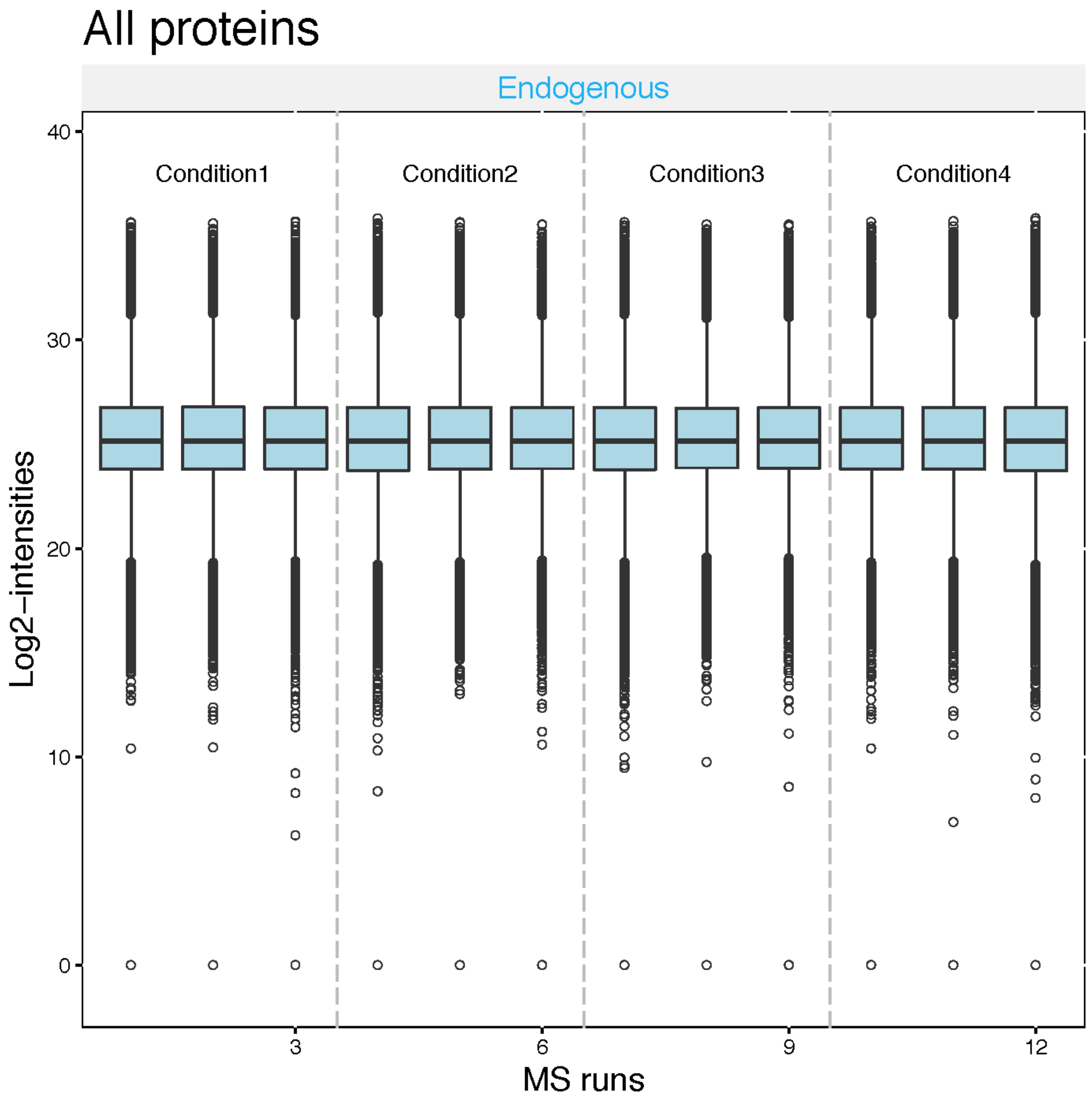


Figure 1: QC plot

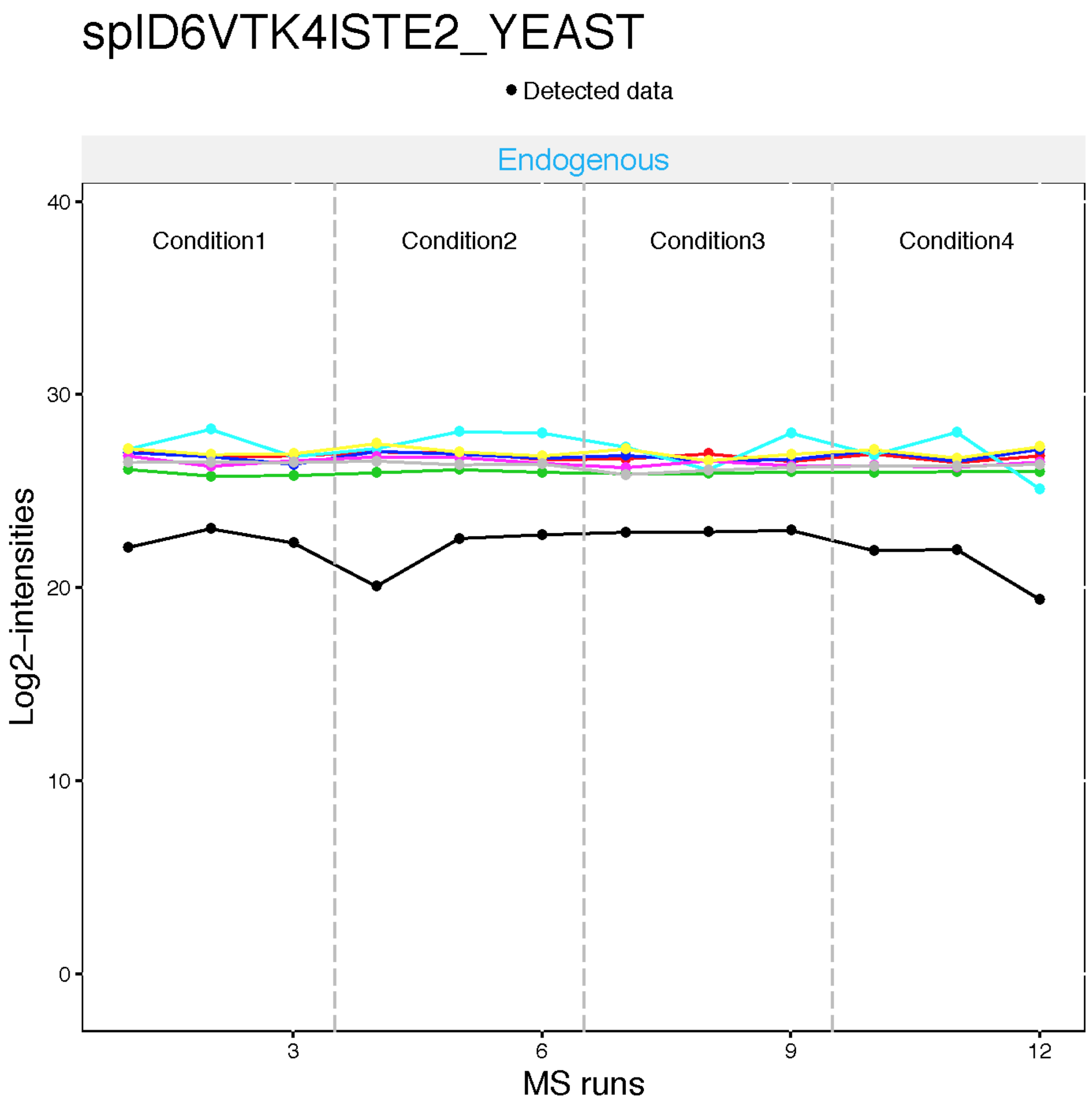


Figure 2: Profile plot for protein D6VTK4

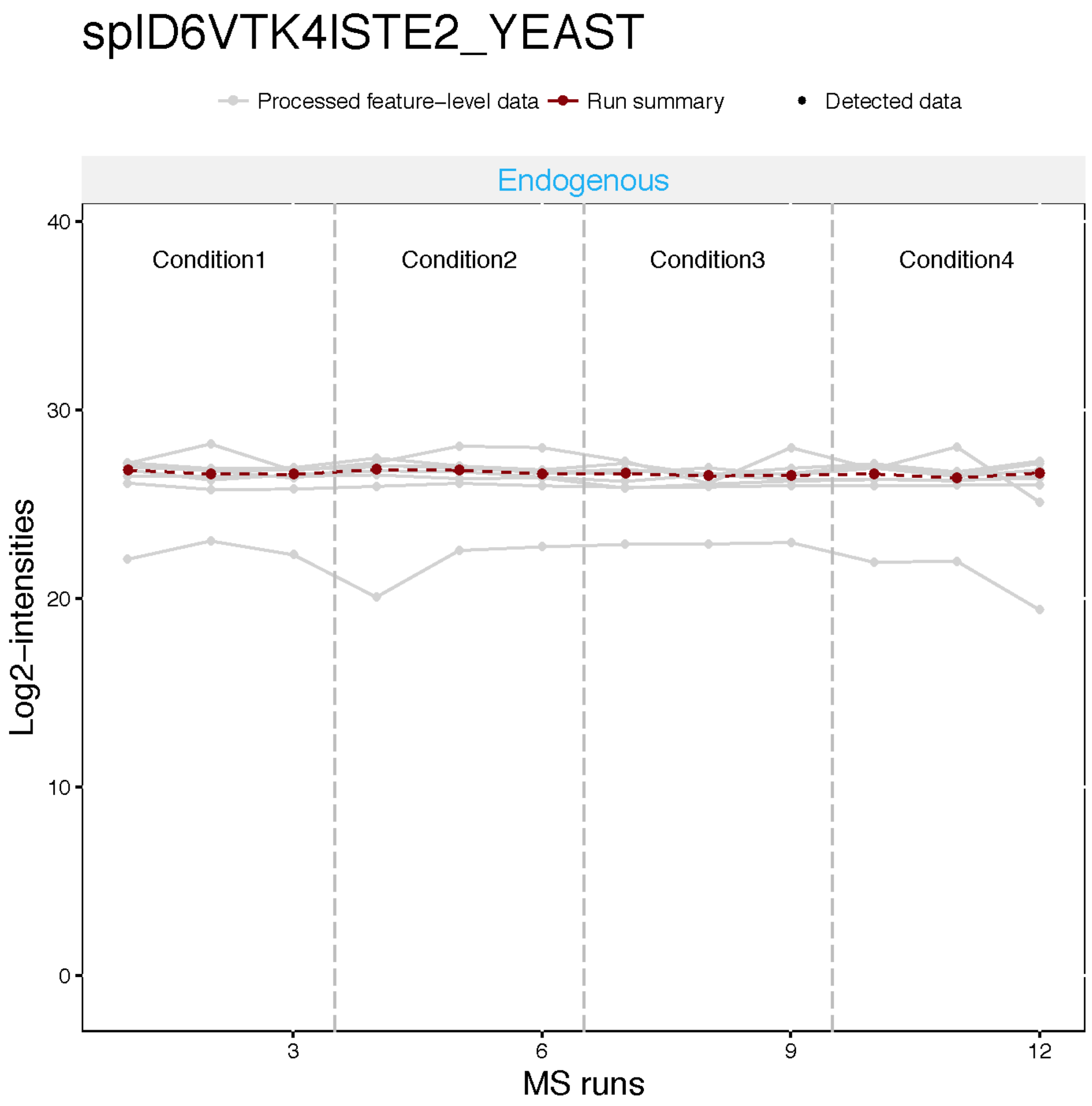


Figure 3: Profile plot with summarized value

```
## Warning: Ignoring unknown aesthetics: linetype
## Drew the Profile plot with summarization for  sp|P44015|VAC2_YEAST ( 1  of  1 )
```

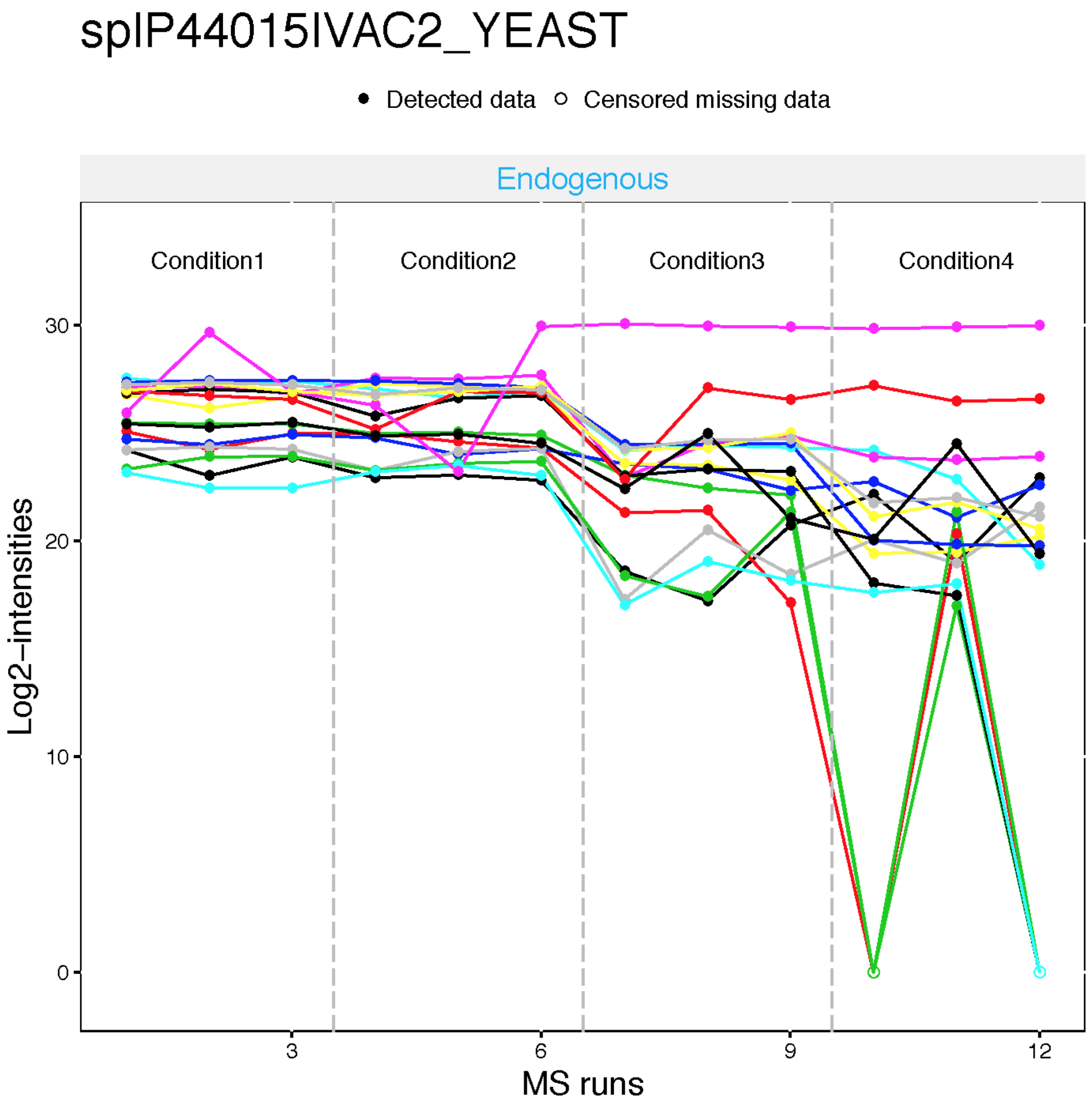
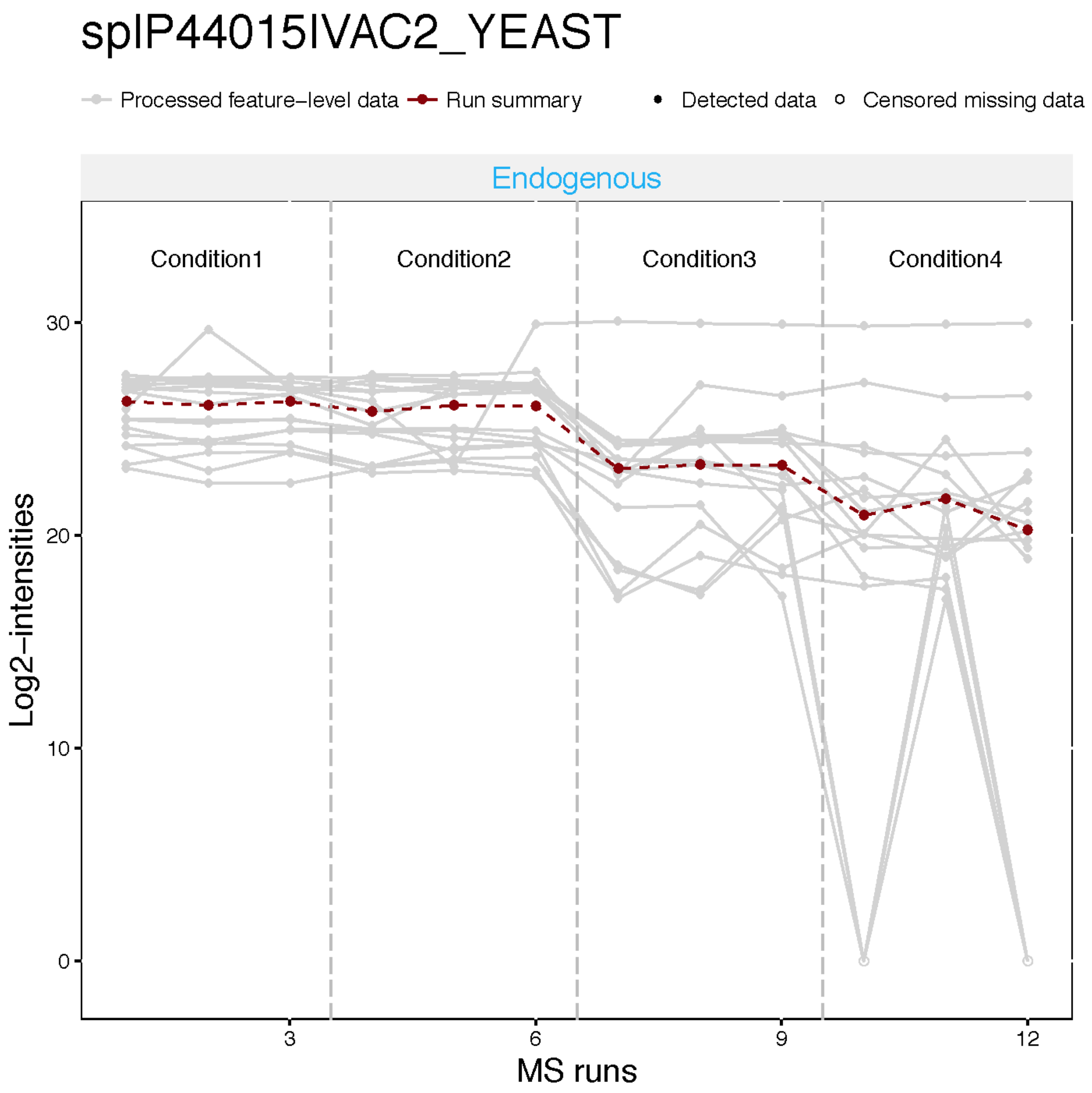


Figure 4:

This is the study design. Let's check visualization for those proteins.

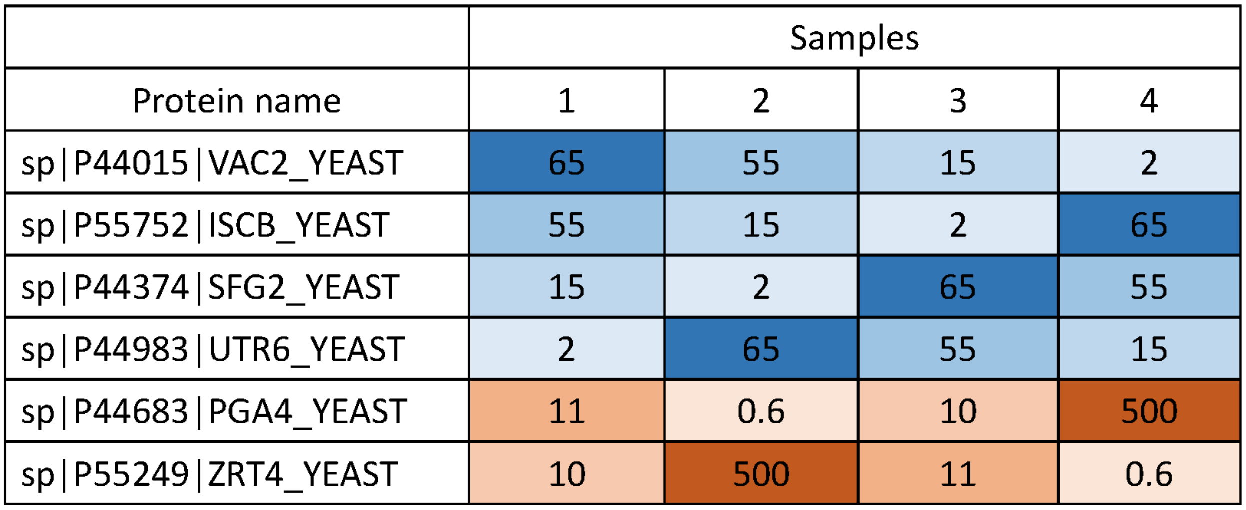
** ! Extreme case : no measurement in certain run.**

MSstats needs at least one measurement per run. If not, can not get run-summarized value.



sp|P44015|VAC2_YEAST
Processed feature–level data
Run summary
Detected data
Censored missing data
Endogenous
Condition1
Condition2
Condition3
Condition4
Log2–intensities
0
10
20
30
3
6
9
12
MS runs

Figure 5:



	Samples			
Protein name	1	2	3	4
sp|P44015|VAC2_YEAST	65	55	15	2
sp|P55752|ISCB_YEAST	55	15	2	65
sp|P44374|SFG2_YEAST	15	2	65	55
sp|P44983|UTR6_YEAST	2	65	55	15
sp|P44683|PGA4_YEAST	11	0.6	10	500
sp|P55249|ZRT4_YEAST	10	500	11	0.6

Figure 6: Experimental design for spike-in proteins

6.3. Condition plots

Condition plots illustrate the systematic difference between conditions. The dots indicates the mean of all measurments (Peptide ion and multiple MS runs) in each condition and default error bar is CI with 0.95 significant level. However, it is not related with model-based analysis.

```
dataProcessPlots(data = quant.processed,
                 type="conditionplot",
                 width=7, height=7,
                 address="iPRG_skyline_equalizeNorm_")
```

This is the condition plot for protein, `sp|P44015|VAC2_YEAST` .

---

7. Finding differentially abundant proteins across conditions

7.1 Assign contrast matrix

After we normalized the data and summarized each protein's behaviour across conditions in `dataProcess` step, we are all set to compare protein changes between groups of conditions. Within MSstats we can do this with the `groupComparison` function, which takes as input the output of the `dataProcess` function.

```
?groupComparison
```

We have to tell `groupComparison` which are the conditions we would like to compare. You can make your `contrast.matrix` in R in a text editor. We define our contrast matrix by adding a column for every condition, **in alphabetical order**. We add a row for every comparison we would like to make between groups of conditions.

0 is for conditions we would like to ignore. **1** is for conditions we would like to put in the numerator of the ratio or fold-change. **-1** is for conditions we would like to put in the denumerator of the ratio or fold-change.

If you have multiple groups, you can assign any gruop comparisons you are interested in. For example, if you have 4 different conditions, Condition1, Condition2, Condition3, Condition4, there are many possible

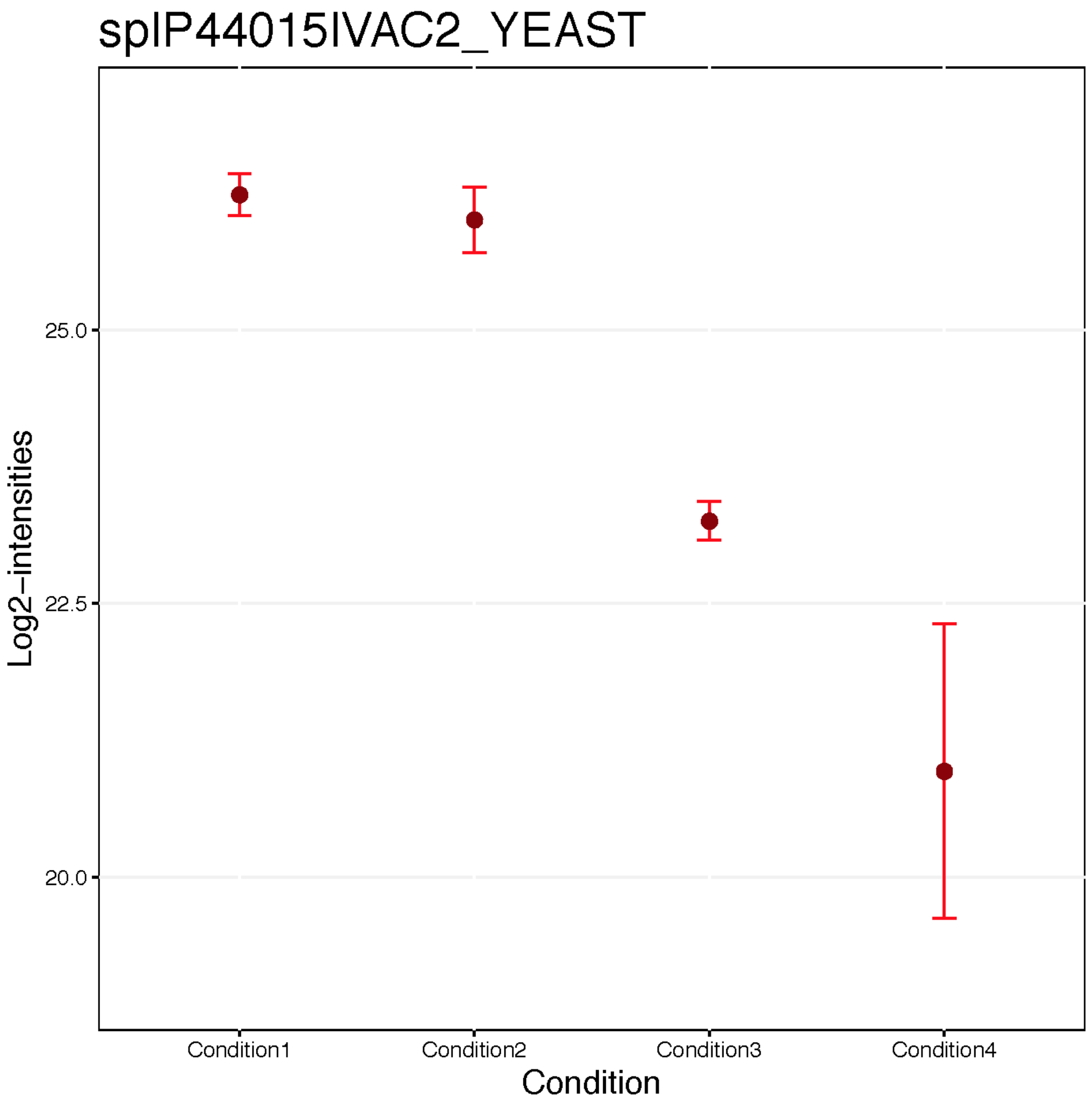


Figure 7: Condition plot for descriptive purpose

comparisons.

```
# check unique conditions and check order of condition information
# In this case, Disease and Healthy
unique(quant.processed$ProcessedData$GROUP_ORIGINAL)
```

```
## [1] Condition1 Condition2 Condition3 Condition4
## Levels: Condition1 Condition2 Condition3 Condition4
```

```
comparison1<-matrix(c(-1,1,0,0),nrow=1)
comparison2<-matrix(c(-1,0,1,0),nrow=1)
comparison3<-matrix(c(-1,0,0,1),nrow=1)
comparison4<-matrix(c(0,-1,1,0),nrow=1)
comparison5<-matrix(c(0,-1,0,1),nrow=1)
comparison6<-matrix(c(0,0,-1,1),nrow=1)
comparison<-rbind(comparison1, comparison2, comparison3, comparison4, comparison5, comparison6)
row.names(comparison)<-c("C2-C1","C3-C1","C4-C1","C3-C2","C4-C2","C4-C3")

comparison
```

```
##       [,1] [,2] [,3] [,4]
## C2-C1   -1    1    0    0
## C3-C1   -1    0    1    0
## C4-C1   -1    0    0    1
## C3-C2    0   -1    1    0
## C4-C2    0   -1    0    1
## C4-C3    0    0   -1    1
```

7.2. Comparing conditions with groupComparison

`groupComparison` uses the run-level summarized data (`$RunlevelData` from `dataProcess` function) for hypothesis testing.

```
test <- groupComparison(contrast.matrix=comparison, data=quant.processed)
```

Let's check the output.

```
names(test)
```

```
## [1] "ComparisonResult" "ModelQC"          "fittedmodel"
```

```
# Show test result
# Label : which comparison is used
# log2FC : estimated log2 fold change between Diseased and Healthy
# adj.pvalue : adjusted p value
# issue : detect whether this protein has any issue for comparison
#    such as, there is measurement in certain group, or no measurement at all.
# MissingPercentage : the number of missing intensities/total number of intensities
#     in conditions your are interested in for comparison
# ImputationPercentage : the number of imputed intensities/total number of intensities
#     in conditions your are interested in for comparison
head(test$ComparisonResult)
```

```
##                 Protein Label      log2FC         SE     Tvalue DF
## 1   sp|D6VTK4|STE2_YEAST C2-C1  0.07846162 0.09648008  0.8132416  8
## 7   sp|O13297|CET1_YEAST C2-C1 -0.11119732 0.07749333 -1.4349278  8
## 13  sp|O13329|FOB1_YEAST C2-C1 -0.16209422 0.29090086 -0.5572146  8
## 19  sp|O13539|THP2_YEAST C2-C1 -0.43742107 0.82871351 -0.5278315  8
## 25 sp|O13547|CCW14_YEAST C2-C1 -0.05774773 0.14672405 -0.3935805  8
## 31 sp|O13563|RPN13_YEAST C2-C1 -0.16945187 0.09518591 -1.7802201  8
##       pvalue adj.pvalue issue MissingPercentage ImputationPercentage
## 1  0.4396100  0.9991155    NA                 0                    0
## 7  0.1892233  0.9991155    NA                 0                    0
## 13 0.5926243  0.9991155    NA                 0                    0
## 19 0.6119387  0.9991155    NA                 0                    0
## 25 0.7041724  0.9991155    NA                 0                    0
## 31 0.1129126  0.9991155    NA                 0                    0
```

```
# After fitting linear model, residuals and fitted values can be shown.
head(test$ModelQC)
```

```
##   RUN              PROTEIN ABUNDANCE NumMeasuredFeature MissingPercentage
## 1   1 sp|D6VTK4|STE2_YEAST  26.81232                  8                 0
## 2   2 sp|D6VTK4|STE2_YEAST  26.60786                  8                 0
## 3   3 sp|D6VTK4|STE2_YEAST  26.58301                  8                 0
## 4   4 sp|D6VTK4|STE2_YEAST  26.83563                  8                 0
## 5   5 sp|D6VTK4|STE2_YEAST  26.79430                  8                 0
## 6   6 sp|D6VTK4|STE2_YEAST  26.60863                  8                 0
##   more50missing NumImputedFeature               originalRUN GROUP
## 1         FALSE                 0 JD_06232014_sample1_B.raw     1
## 2         FALSE                 0 JD_06232014_sample1_C.raw     1
## 3         FALSE                 0 JD_06232014_sample1-A.raw     1
## 4         FALSE                 0 JD_06232014_sample2_A.raw     2
## 5         FALSE                 0 JD_06232014_sample2_B.raw     2
## 6         FALSE                 0 JD_06232014_sample2_C.raw     2
##   GROUP_ORIGINAL SUBJECT_ORIGINAL SUBJECT_NESTED SUBJECT   residuals
## 1     Condition1                1            1.1       1  0.14458899
## 2     Condition1                1            1.1       1 -0.05986794
## 3     Condition1                1            1.1       1 -0.08472105
## 4     Condition2                2            2.2       2  0.08944428
## 5     Condition2                2            2.2       2  0.04811446
## 6     Condition2                2            2.2       2 -0.13755874
##     fitted
## 1 26.66773
## 2 26.66773
## 3 26.66773
## 4 26.74619
## 5 26.74619
## 6 26.74619
```

```
# Fitted model per protein
head(test$fittedmodel)
```

```
## [[1]]
##
## Call:
## lm(formula = ABUNDANCE ~ GROUP, data = data2)
##
## Coefficients:
## (Intercept)       GROUP2       GROUP3       GROUP4
##    26.66773      0.07846     -0.11566     -0.11841
##
##
```

```
## [[2]]
##
## Call:
## lm(formula = ABUNDANCE ~ GROUP, data = data2)
##
## Coefficients:
## (Intercept)       GROUP2       GROUP3       GROUP4
##    24.70386     -0.11120     -0.14451     -0.09633
##
##
## [[3]]
##
## Call:
## lm(formula = ABUNDANCE ~ GROUP, data = data2)
##
## Coefficients:
## (Intercept)       GROUP2       GROUP3       GROUP4
##     23.6207      -0.1621       0.1209      -0.3511
##
##
## [[4]]
##
## Call:
## lm(formula = ABUNDANCE ~ GROUP, data = data2)
##
## Coefficients:
## (Intercept)       GROUP2       GROUP3       GROUP4
##     26.4006      -0.4374      -0.4318      -0.5024
##
##
## [[5]]
##
## Call:
## lm(formula = ABUNDANCE ~ GROUP, data = data2)
##
## Coefficients:
## (Intercept)       GROUP2       GROUP3       GROUP4
##    27.04775     -0.05775     -0.29114     -0.09482
##
##
## [[6]]
##
## Call:
## lm(formula = ABUNDANCE ~ GROUP, data = data2)
##
## Coefficients:
## (Intercept)       GROUP2       GROUP3       GROUP4
##    26.18595     -0.16945     -0.07609     -0.21625
```

Let's save the testing result as .csv file.

```
Skyline.intensity.comparison.result <- test$ComparisonResult

write.csv(Skyline.intensity.comparison.result, file='testResult_iprg_skyline.csv')
```

Let's inspect the results to see what proteins are changing significantly between Diseased and Healthy.

```
head(Skyline.intensity.comparison.result)
```

```
##                    Protein Label      log2FC         SE     Tvalue DF
## 1   sp|D6VTK4|STE2_YEAST C2-C1  0.07846162 0.09648008  0.8132416  8
## 7   sp|O13297|CET1_YEAST C2-C1 -0.11119732 0.07749333 -1.4349278  8
## 13  sp|O13329|FOB1_YEAST C2-C1 -0.16209422 0.29090086 -0.5572146  8
## 19  sp|O13539|THP2_YEAST C2-C1 -0.43742107 0.82871351 -0.5278315  8
## 25 sp|O13547|CCW14_YEAST C2-C1 -0.05774773 0.14672405 -0.3935805  8
## 31 sp|O13563|RPN13_YEAST C2-C1 -0.16945187 0.09518591 -1.7802201  8
##       pvalue adj.pvalue issue MissingPercentage ImputationPercentage
## 1  0.4396100  0.9991155    NA                 0                    0
## 7  0.1892233  0.9991155    NA                 0                    0
## 13 0.5926243  0.9991155    NA                 0                    0
## 19 0.6119387  0.9991155    NA                 0                    0
## 25 0.7041724  0.9991155    NA                 0                    0
## 31 0.1129126  0.9991155    NA                 0                    0
```

```
SignificantProteins <-
  Skyline.intensity.comparison.result[Skyline.intensity.comparison.result$adj.pvalue < 0.05 ,]
nrow(SignificantProteins)
```

```
## [1] 29
```

```
SignificantProteinsUpInDiseased <- SignificantProteins[SignificantProteins$log2FC > 2 ,]
nrow(SignificantProteinsUpInDiseased)
```

```
## [1] 14
```

---

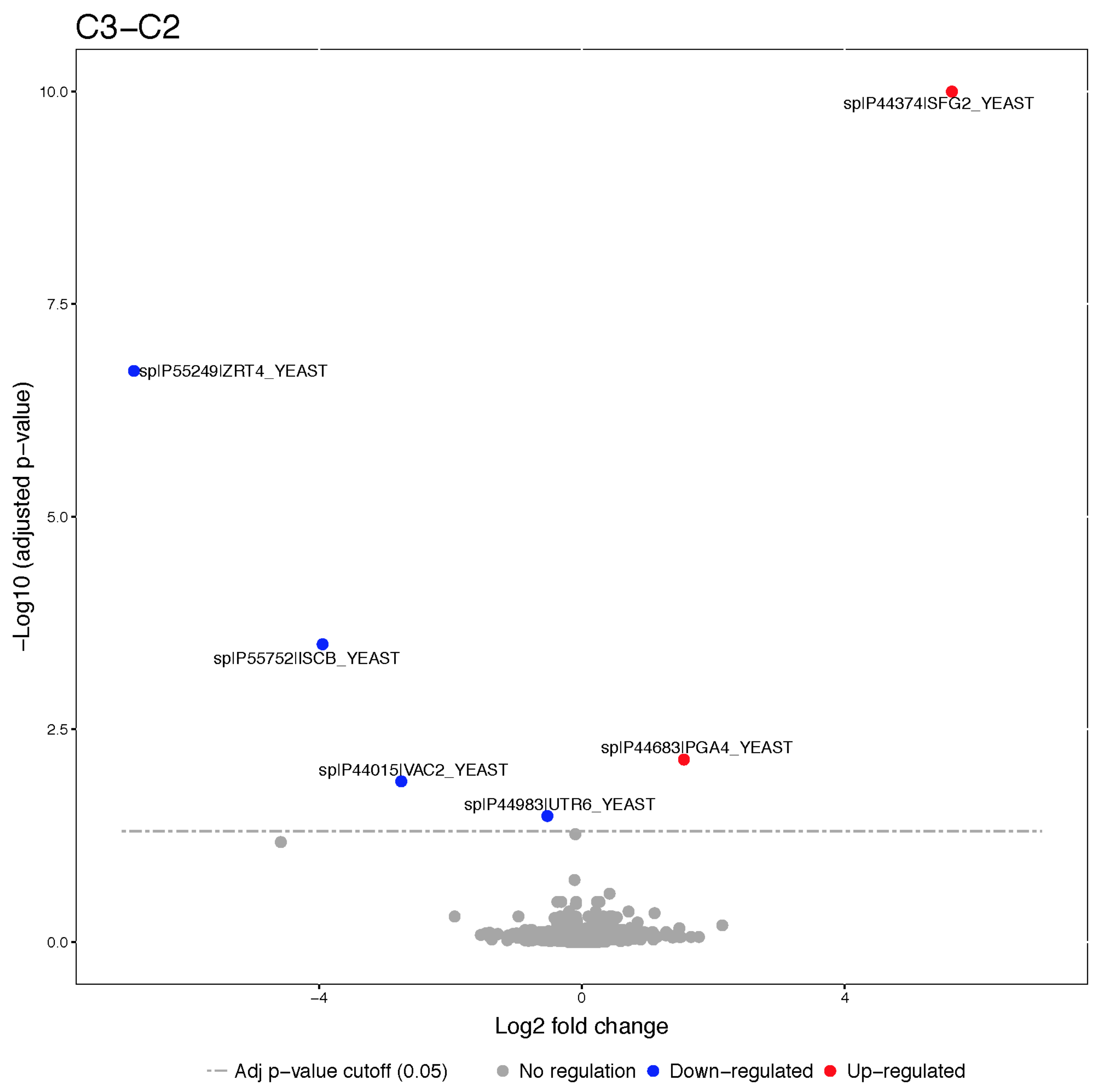
8. Visualization of differentially abundant proteins

```
?groupComparisonPlots
```

8.1. Volcano plot

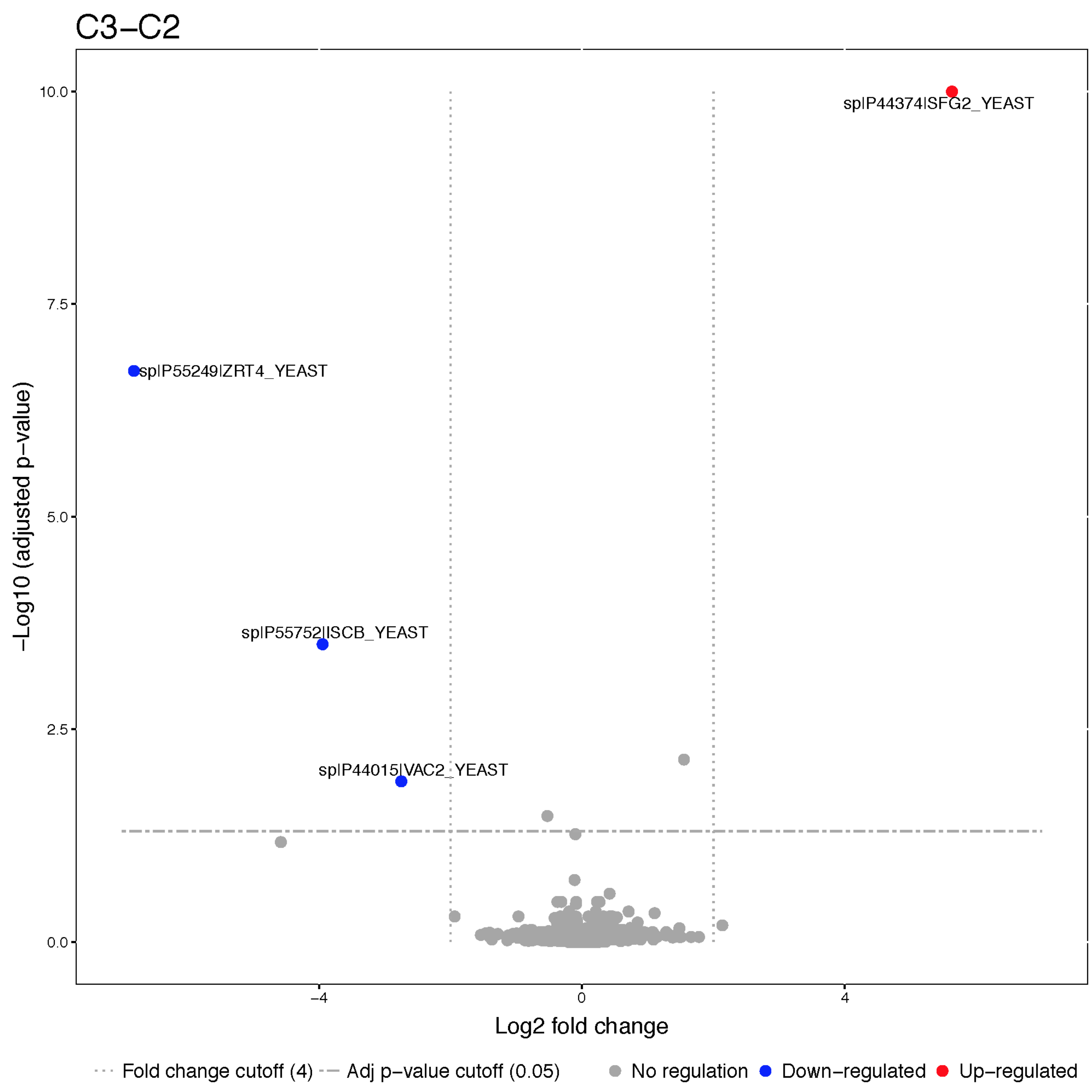
Volcano plots allow us to visually separate strong changes, which are not significant, from strong and significant changes. Look for these subjects in the upper right and upper left quadrants of the plot. Protein name will be shown only for siginificant proteins.

```
groupComparisonPlots(data = Skyline.intensity.comparison.result,
                     type = 'VolcanoPlot',
                     address = 'iprg_skyline_')
```



We can set up estimated fold change cutoff.

```r
groupComparisonPlots(data = Skyline.intensity.comparison.result,
                     type = 'VolcanoPlot',
                     sig = 0.05,
                     FCcutoff = 2^2,
                     address = 'iprg_skyline_FCcutoff4_')
```



## 8.2. Comparison plot

Comparison plots illustrate model-based estimates of log-fold changes, and the associated uncertainty, in several comparisons of conditions for one protein. X-axis is the comparison of interest. Y-axis is the log fold change. The dots are the model-based estimates of log-fold change, and the error bars are the model-based 95% confidence intervals (the option sig can be used to change the significance level of significance). For simplicity, the confidence intervals are adjusted for multiple comparisons within protein only, using the Bonferroni approach. For proteins with N comparisons, the individual confidence intervals are at the level of 1-sig/N.

```
groupComparisonPlots(Skyline.intensity.comparison.result,
                     type="ComparisonPlot",
                     address="testResult_iprg_skyline_")
```

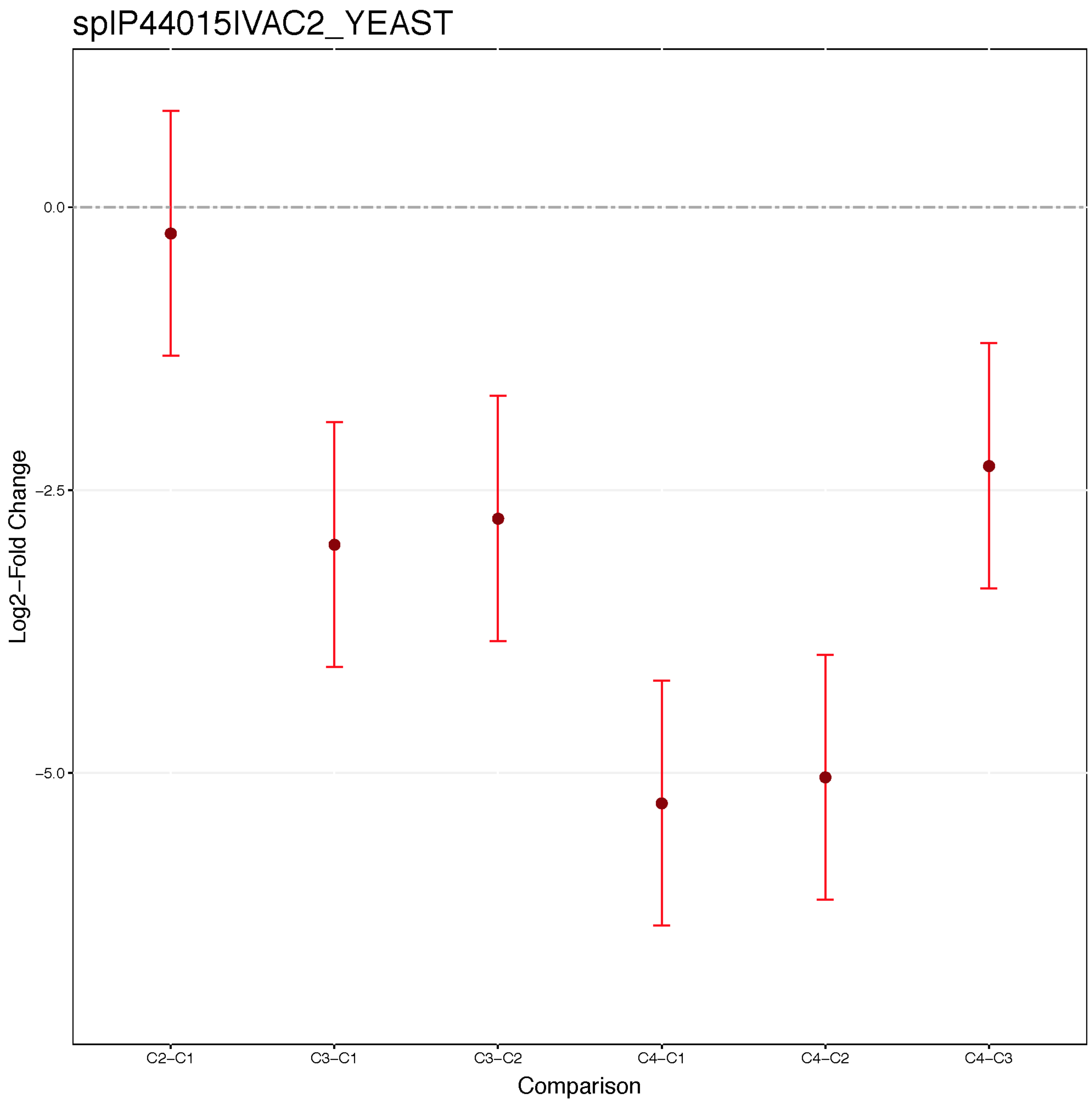


Figure 8: Comparison plot with model-based result

9. Planning future experimental designs

This last analysis step views the dataset as a pilot study of a future experiment, utilizes its variance components, and calculates the minimal number of replicates required in a future experiment to achieve the desired statistical power. The calculation is performed by the function `designSampleSize`, which takes as input the fitted model in `groupComparison`. Sample size calculation assumes same experimental design (i.e. group comparison, time course or paired design) as in the current dataset, and uses the model fit to estimate the median variance components across all the proteins. Finally, sample size calculation assumes that a large proportion of proteins (specifically, 99%) will not change in abundance in the future experiment. This assumption also provides conservative results. Using the estimated variance components, the function relates the number of biological replicates per condition (`numSample`, rounded to 0 decimal), average statistical power across all the proteins (`power`), minimal fold change that we would like to detect (can be specified as a range, e.g. `desiredFC=c(1.1, 2)`), and the False Discovery Rate (`FDR`). The user should specify all these quantities but one, and the function will solve for the remainder. The quantity to solve for should be set to `= TRUE`.

```
?designSampleSize
```

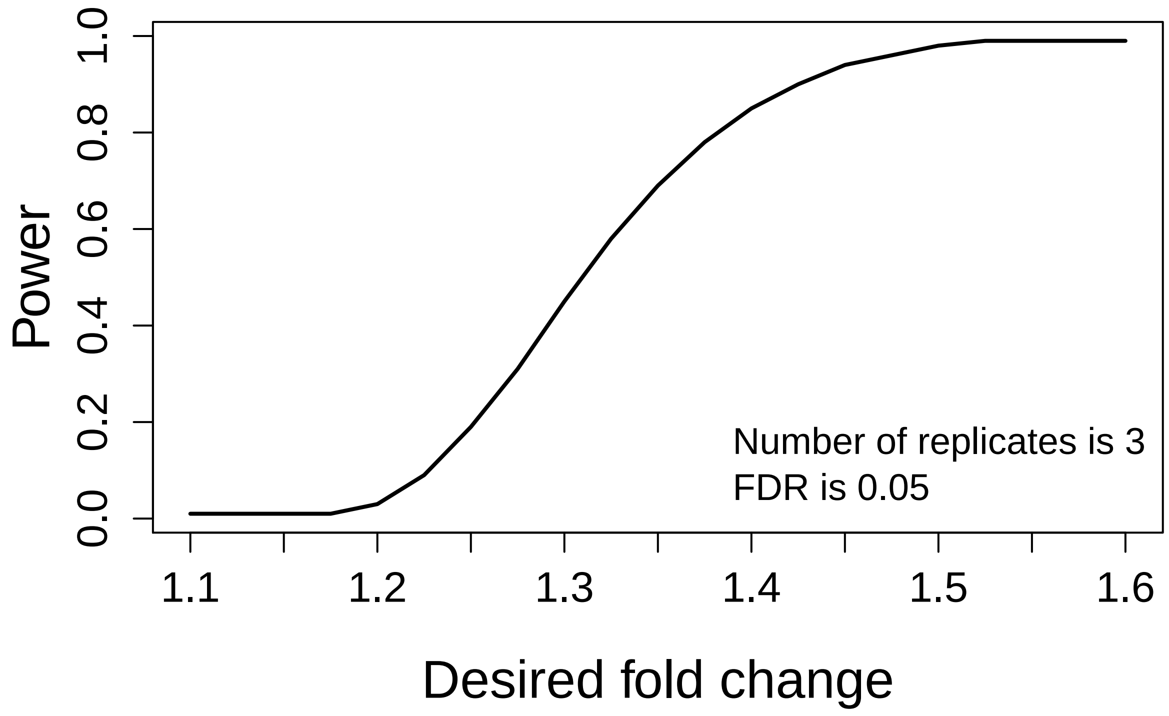
9.1. Calculating statistical power

```
# calculate the power
test.power <- designSampleSize(data = test$fittedmodel,
                               desiredFC = c(1.1, 1.6),
                               FDR = 0.05,
                               power = TRUE,
                               numSample = 3)
test.power
```

```
##    desiredFC numSample  FDR power    CV
## 1      1.100         3 0.05  0.01 0.010
## 2      1.125         3 0.05  0.01 0.010
## 3      1.150         3 0.05  0.01 0.010
## 4      1.175         3 0.05  0.01 0.010
## 5      1.200         3 0.05  0.03 0.009
## 6      1.225         3 0.05  0.09 0.009
## 7      1.250         3 0.05  0.19 0.009
## 8      1.275         3 0.05  0.31 0.009
## 9      1.300         3 0.05  0.45 0.009
## 10     1.325         3 0.05  0.58 0.009
## 11     1.350         3 0.05  0.69 0.008
## 12     1.375         3 0.05  0.78 0.008
## 13     1.400         3 0.05  0.85 0.008
## 14     1.425         3 0.05  0.90 0.008
## 15     1.450         3 0.05  0.94 0.008
## 16     1.475         3 0.05  0.96 0.008
## 17     1.500         3 0.05  0.98 0.008
## 18     1.525         3 0.05  0.99 0.007
## 19     1.550         3 0.05  0.99 0.007
## 20     1.575         3 0.05  0.99 0.007
## 21     1.600         3 0.05  0.99 0.007
```

9.2. Visualizing the relationship between desired fold-change and power

```r
designSampleSizePlots(data = test.power)
```



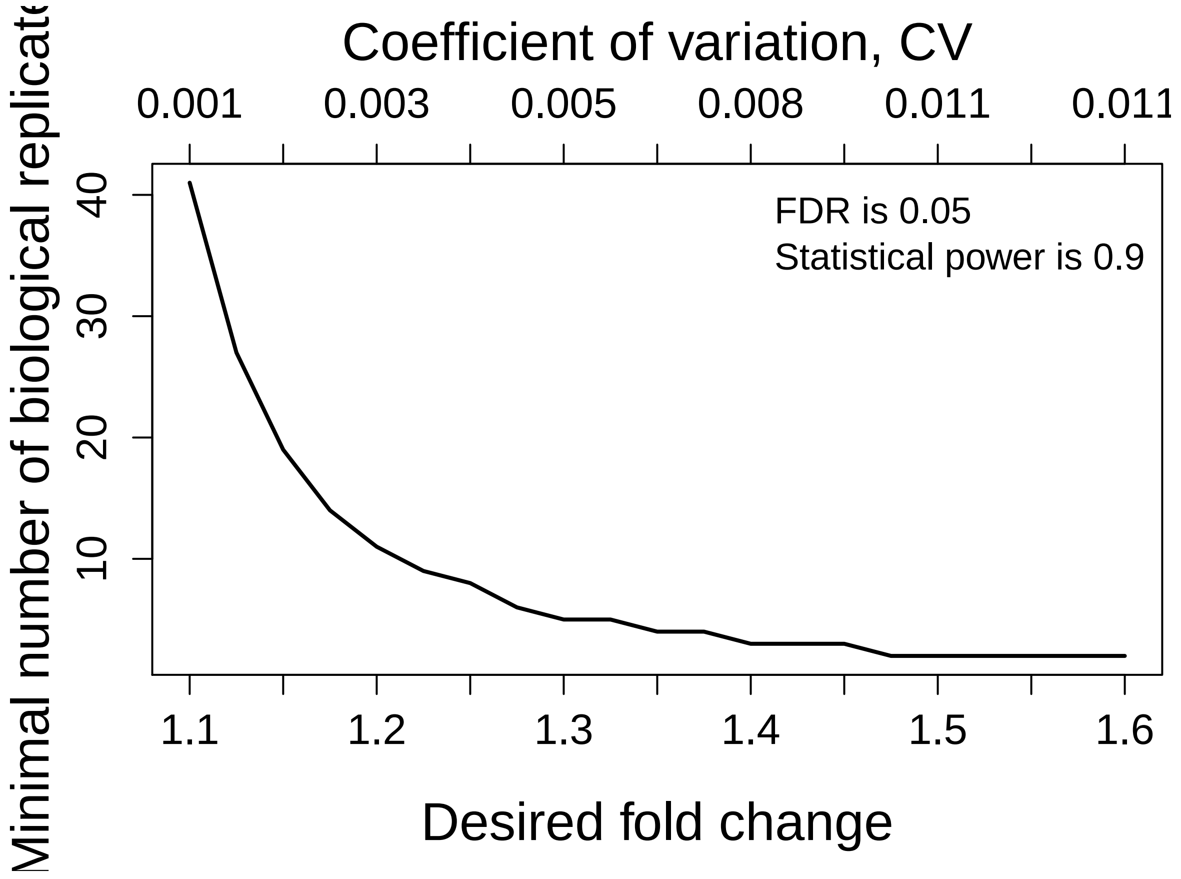
9.3. Designing sample size for desired fold-change

```r
# Minimal number of biological replicates per condition
samplesize <- designSampleSize(data = test$fittedmodel,
                               desiredFC = c(1.1, 1.6),
                               FDR = 0.05,
                               power = 0.9,
                               numSample = TRUE)
samplesize
```

```
##    desiredFC numSample  FDR power    CV
## 1      1.100        41 0.05   0.9 0.001
## 2      1.125        27 0.05   0.9 0.001
## 3      1.150        19 0.05   0.9 0.002
## 4      1.175        14 0.05   0.9 0.002
## 5      1.200        11 0.05   0.9 0.003
## 6      1.225         9 0.05   0.9 0.003
## 7      1.250         8 0.05   0.9 0.003
## 8      1.275         6 0.05   0.9 0.004
## 9      1.300         5 0.05   0.9 0.005
## 10     1.325         5 0.05   0.9 0.005
## 11     1.350         4 0.05   0.9 0.006
## 12     1.375         4 0.05   0.9 0.006
## 13     1.400         3 0.05   0.9 0.008
## 14     1.425         3 0.05   0.9 0.008
## 15     1.450          3 0.05   0.9 0.008
## 16     1.475          2 0.05   0.9 0.012
## 17     1.500          2 0.05   0.9 0.011
## 18     1.525          2 0.05   0.9 0.011
## 19     1.550          2 0.05   0.9 0.011
## 20     1.575          2 0.05   0.9 0.011
## 21     1.600          2 0.05   0.9 0.011
```

9.4. Visualizing the relationship between desired fold-change and mininum sample size number

```
designSampleSizePlots(data = samplesize)
```



10. Protein subject quantification

If there is no technical replicate, subject (or sample) quantification should be the same as run-level summarization. If there are technical replicates, subjet-level summarization(quantification) with run-level summarization will be useful for downstream analysis, such as classification.

```
?quantification
```

```r
sampleQuant <- quantification(quant.processed)
head(sampleQuant)
```

```
##                  Protein Condition1_1 Condition2_2 Condition3_3
## 1  sp|D6VTK4|STE2_YEAST     26.60786     26.79430     26.53029
## 2  sp|O13297|CET1_YEAST     24.71809     24.57865     24.62652
## 3  sp|O13329|FOB1_YEAST     23.47075     23.43427     23.73741
## 4  sp|O13539|THP2_YEAST     27.38510     25.90646     25.91799
## 5 sp|O13547|CCW14_YEAST     27.11638     26.91302     26.75541
## 6 sp|O13563|RPN13_YEAST     26.17056     26.01078     26.11412
##   Condition4_4
## 1     26.60612
## 2     24.64886
## 3     23.16646
## 4     25.91781
## 5     26.98082
## 6     26.05415
```

---

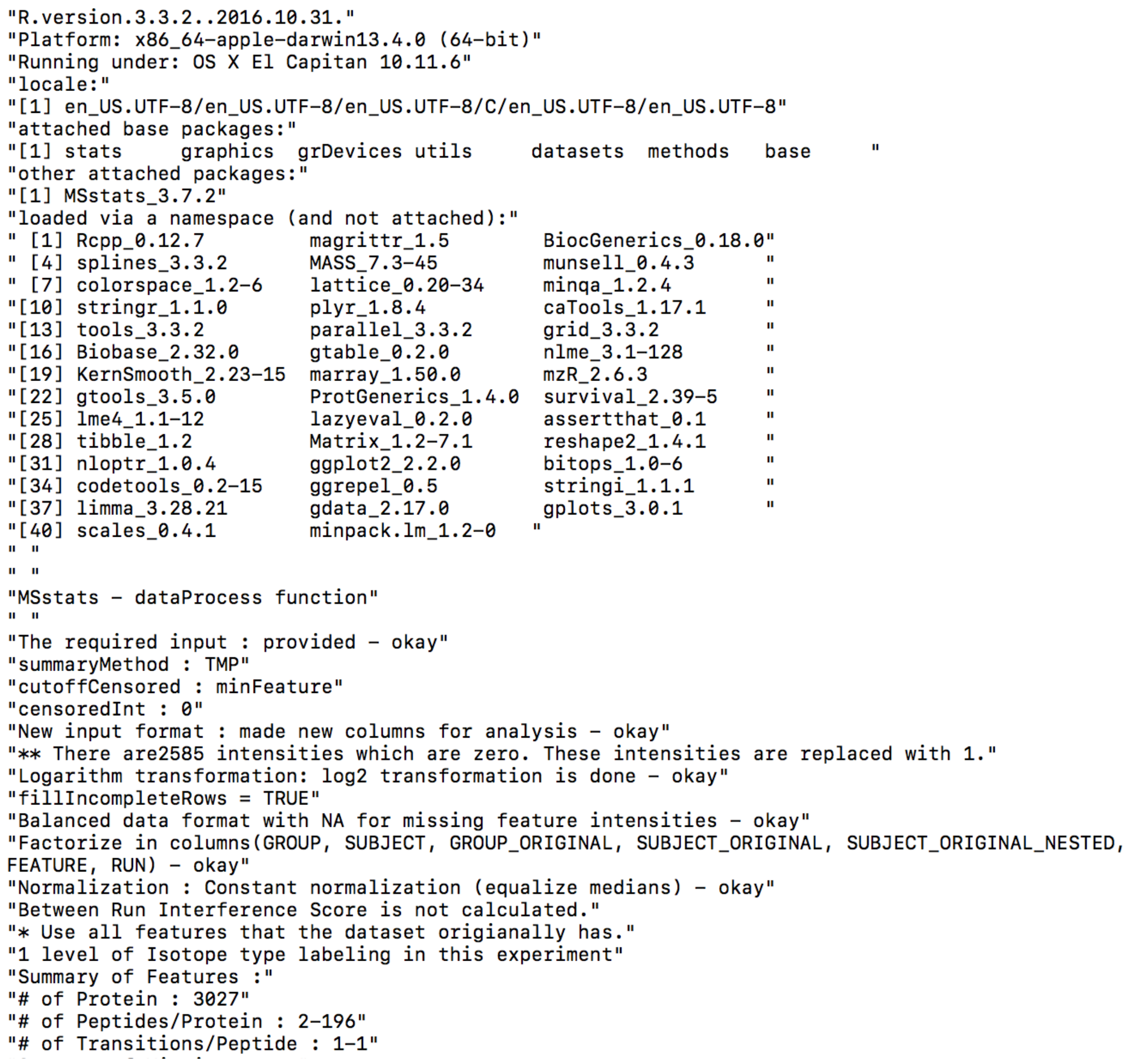
11. msstats.log and sessionInfo.txt

These two files are important to keep the records of package versions and options in functions.

---

12. Example codes for using Limma

```r
library(limma)

## use run-level summarized value from MSstats
input <- quant.processed$RunlevelData

## reformat
input2 <- dcast(Protein ~ originalRUN, data=input, value.var = 'LogIntensities')
head(input2)

## annotate protein id in rowname
rownames(input2) <- input2$Protein
input2 <- input2[, -1]

design <- model.matrix(~0+factor(c(1,1,1,2,2,2,3,3,3,4,4,4)))
colnames(design) <- c('Condition1', 'Condition2', 'Condition3', 'Condition4')
contrast.matrix <- makeContrasts(Condition2-Condition1, levels=design)

fit <- lmFit(input2, design)
fit2 <- contrasts.fit(fit, contrast.matrix)
fit2 <- eBayes(fit2)

test.limma <- data.frame(Label='C2-C1',
                         log2FC=fit2$coefficients,
```



```
"R.version.3.3.2..2016.10.31."
"Platform: x86_64-apple-darwin13.4.0 (64-bit)"
"Running under: OS X El Capitan 10.11.6"
"locale:"
"[1] en_US.UTF-8/en_US.UTF-8/en_US.UTF-8/C/en_US.UTF-8/en_US.UTF-8"
"attached base packages:"
"[1] stats     graphics  grDevices utils     datasets  methods   base     "
"other attached packages:"
"[1] MSstats_3.7.2"
"loaded via a namespace (and not attached):"
" [1] Rcpp_0.12.7         magrittr_1.5        BiocGenerics_0.18.0"
" [4] splines_3.3.2       MASS_7.3-45         munsell_0.4.3      "
" [7] colorspace_1.2-6    lattice_0.20-34     minqa_1.2.4        "
"[10] stringr_1.1.0       plyr_1.8.4          caTools_1.17.1     "
"[13] tools_3.3.2         parallel_3.3.2      grid_3.3.2         "
"[16] Biobase_2.32.0      gtable_0.2.0        nlme_3.1-128       "
"[19] KernSmooth_2.23-15  marray_1.50.0       mzR_2.6.3          "
"[22] gtools_3.5.0        ProtGenerics_1.4.0  survival_2.39-5    "
"[25] lme4_1.1-12         lazyeval_0.2.0      assertthat_0.1     "
"[28] tibble_1.2          Matrix_1.2-7.1      reshape2_1.4.1     "
"[31] nloptr_1.0.4        ggplot2_2.2.0       bitops_1.0-6       "
"[34] codetools_0.2-15    ggrepel_0.5         stringi_1.1.1      "
"[37] limma_3.28.21       gdata_2.17.0        gplots_3.0.1       "
"[40] scales_0.4.1        minpack.lm_1.2-0   "
" "
" "
"MSstats - dataProcess function"
" "
"The required input : provided - okay"
"summaryMethod : TMP"
"cutoffCensored : minFeature"
"censoredInt : 0"
"New input format : made new columns for analysis - okay"
"** There are2585 intensities which are zero. These intensities are replaced with 1."
"Logarithm transformation: log2 transformation is done - okay"
"fillIncompleteRows = TRUE"
"Balanced data format with NA for missing feature intensities - okay"
"Factorize in columns(GROUP, SUBJECT, GROUP_ORIGINAL, SUBJECT_ORIGINAL, SUBJECT_ORIGINAL_NESTED,
FEATURE, RUN) - okay"
"Normalization : Constant normalization (equalize medians) - okay"
"Between Run Interference Score is not calculated."
"* Use all features that the dataset origianally has."
"1 level of Isotope type labeling in this experiment"
"Summary of Features :"
"# of Protein : 3027"
"# of Peptides/Protein : 2-196"
"# of Transitions/Peptide : 1-1"
```

Figure 9: log file

```
                                pvalue=fit2$p.value)
head(test.limma)
colnames(test.limma)[2] <- 'log2FC'
colnames(test.limma)[3] <- 'pvalue'
test.limma$adj.pvalue <- p.adjust(test.limma$pvalue, method="BH")
test.limma$Protein <- rownames(test.limma)

save(test.limma, file='test.limma.RData')
```

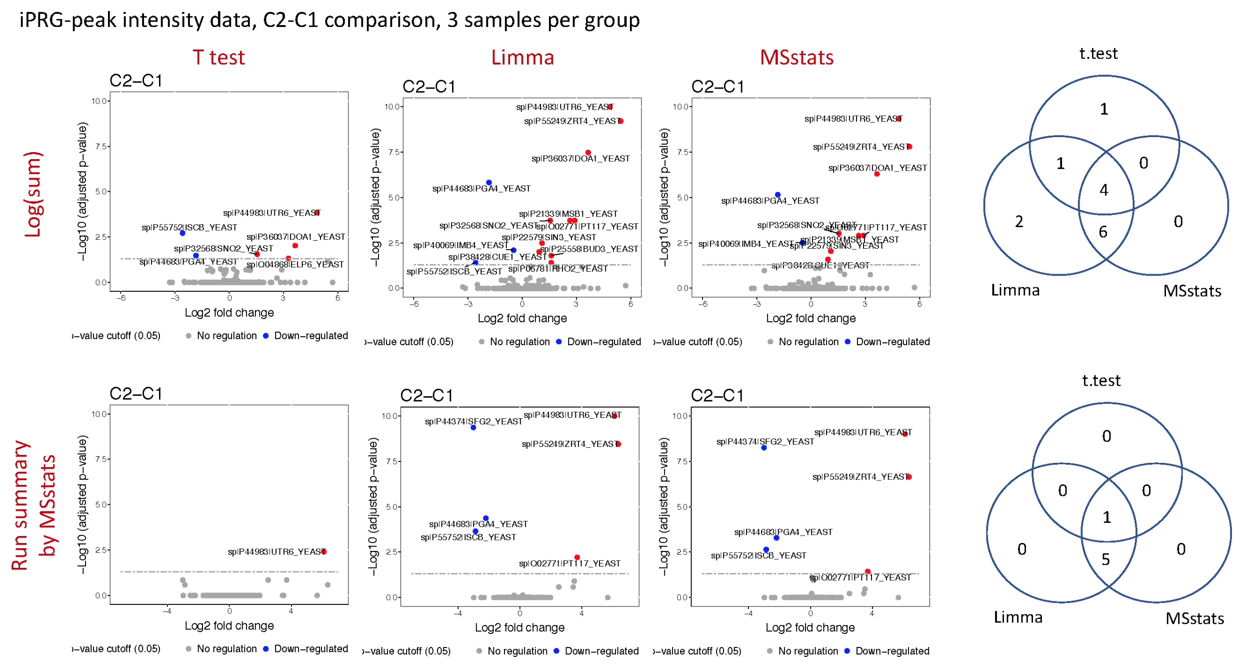


Figure 10: Comparison of results between different statistical methods

13. Example codes for using DESeq2 for MS Proteomics, iPRG spectral count data

```
## load count data
Load(iprg.count)

## Reformat
library(reshape2)

Y <- dcast(Protein ~ Run, data=iprg.count)
head(Y)

countData <- as.matrix(Y[,-c(1)])
protName <- as.character(Y[,1])
```

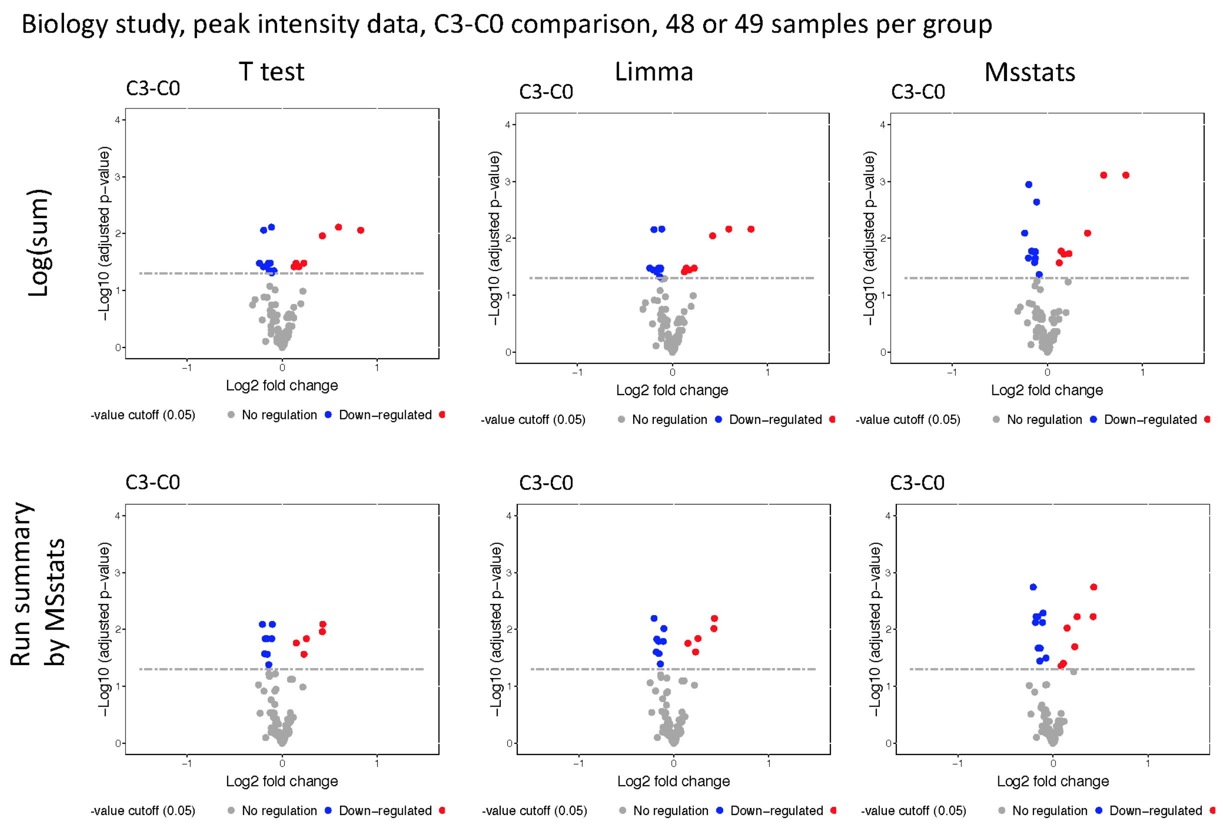


Figure 11: Comparison of results between different statistical methods with biology study with around 50 samples per group

```r
##############################
## DESeq2: NB GLM + Wald test
##############################

library(DESeq2)

## Format the data for DESeq
## use M1 as reference in the design matrix
colData <- data.frame(M1=factor(c(rep(1, 3),rep(0, 9))),
                      M2=factor(c(rep(0, 3), rep(1,3), rep(0, 6))),
                      M3=factor(c(rep(0, 6), rep(1,3),rep(0, 3))),
                      M4=factor(c(rep(0, 9), rep(1,3))))

## Comparisons with 1st one

## Design model ~ M2+M3+M4
dds <- DESeqDataSetFromMatrix(countData = countData, colData = colData, design = ~ M2+M3+M4)

## Test for differential expression with NB distribution and exact test
dds <- DESeq(dds)
dds # object of DESeqDataSet class
resultsNames(dds)

## block automatic independent filtering after testing
c1vs2 <- results(dds, contrast=c('M2', '0', '1'), independentFiltering = FALSE)
c1vs3 <- results(dds, contrast=c('M3', '0', '1'), independentFiltering = FALSE)
c1vs4 <- results(dds, contrast=c('M4', '0', '1'), independentFiltering = FALSE)

## Combine everything
results_count <- data.frame(rbind(c1vs2, c1vs3, c1vs4))
results_count$Label <- factor(c(rep(c("C2-C1"),dim(Y)[1]),rep(c("C3-C1"),
                                                                dim(Y)[1]),rep(c("C4-C1"),dim(Y)[1])))
results_count$Protein <- Y$Protein

results_count <- results_count[, c(8,7,1,2,3,4,5,6)]
head(results_count)
colnames(results_count)[4] <- 'log2FC'
colnames(results_count)[8] <- 'adj.pvalue'

## Save the result
test.count.deseq2 <- results_count
save(test.count.deseq2, file='test.count.deseq2.RData')
```

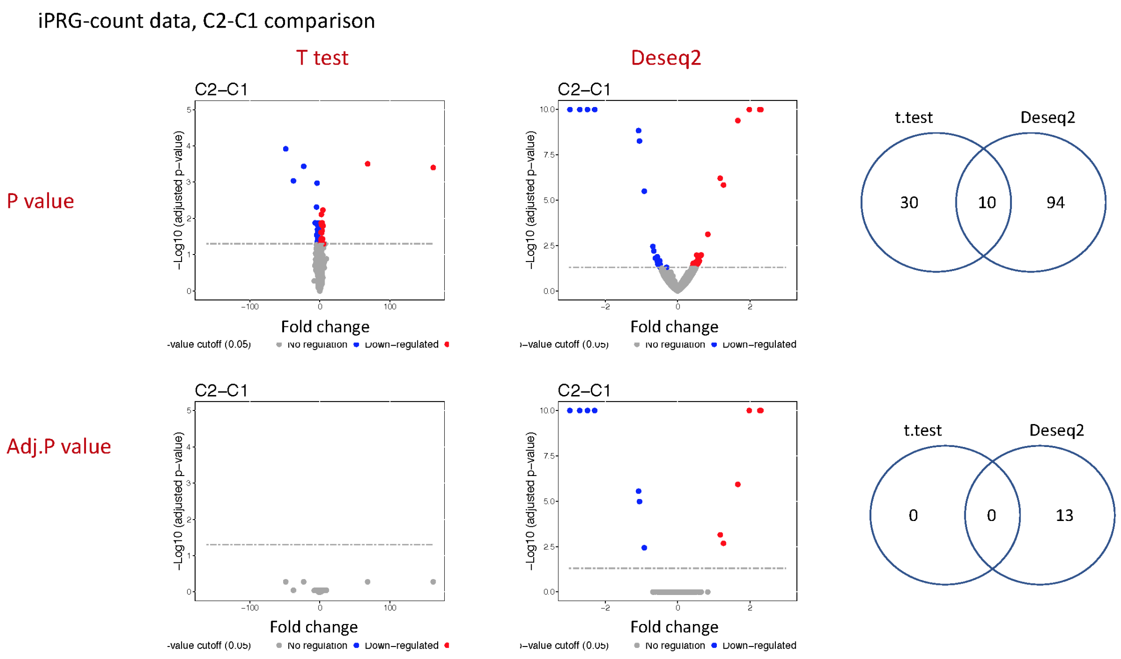


Figure 12: Comparison of results between different statistical methods for spectral count data