

msmsTests package

LC-MS/MS post test filters to improve reproducibility

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1 Introduction

The *omics* technologies are in general challenged by a high dimensionality problem, whereby a high number of statistical tests are carried out at the same time on very few samples. The high number of tests that are carried out simultaneously requires a p-value adjustment to control the false discovery rate (FDR). The low number of replicates, combined with a high number of statistical tests easily leads to low reproducibility in the results. Shi et al. addressed this issue in a systematic manner using a large dataset elaborated within the studies of the MicroArray Quality Control Consortium (MAQC) [1]. The MAQC studied the possible sources of the limitations of microarray (MA) studies that precluded obtaining reproducible results across laboratories and platforms. Their conclusions underscored the influence of batch effects and the limitation introduced by

using only the p-value criteria to get ordered lists of differentially expressed features as the main weaknesses in MA studies [2] [3]. This recommendation makes an intuitive appeal to the use of a post-test filter, excluding features that show low p-values but poor effect size or low signal strength, as a mean to guaranty a minimal level of reproducibility.

Label-free differential proteomics is based on comparing the expression of proteins between different biological conditions [4] [5]. The expression of a protein by LC-MS/MS may be obtained by MS peak intensity measures or by the number of spectral counts assigned to that protein in a LC-MS/MS run. Here we concentrate on spectral counts.

In proteomics reproducibility is also of great concern, and the use of such post-test filters may help notably [6].

2 An example LC-MS/MS dataset

The example dataset [6] is the result of multiple spiking experiments, showing real LC-MS/MS data. Samples of 500 micrograms of a standard yeast lysate are spiked with 100, 200, 400 and 600fm of a complex mix of 48 equimolar human proteins (UPS1, Sigma-Aldrich). The number of technical replicates vary between 3 to 6.

The dataset consists in an instance of the *MSnSet* class, defined in the MSnbase package [7], a S4 class [8] [9]. This *MSnSet* object contains a spectral counts (SpC) matrix in the *assayData* slot, and a factor treatment in the *phenoData* slot. (See also the expressionSet vignette by vignette("ExpressionSetIntroduction",package="Biobase") [10])

```
> library(msmsTests)
> data(msms.spk)
> msms.spk

MSnSet (storageMode: lockedEnvironment)
assayData: 685 features, 19 samples
  element names: exprs
protocolData: none
phenoData
  sampleNames: Y500U100_001 Y500U100_002 ... Y500U600_006 (19 total)
  varLabels: treat
  varMetadata: labelDescription
featureData: none
experimentData: use 'experimentData(object)'
  pubMedIds: http://www.ncbi.nlm.nih.gov/pubmed/23770383
Annotation:
- - - Processing information - - -
  MSnbase version: 1.8.0

> dim(msms.spk)

[1] 685  19

> head(pData(msms.spk))
```

```

          treat
Y500U100_001 U100
Y500U100_002 U100
Y500U100_003 U100
Y500U100_004 U100
Y500U200_001 U200
Y500U200_002 U200

> table(pData(msms.spk)$treat)

U100 U200 U400 U600
   4    6    3    6

```

Although the mix is equimolar the signal strength of each protein is markedly different, allowing to cover the full range of SpC values, what makes it specially worth in this sort of experiments:

```

> msms.spc <- exprs(msms.spk)
> treat <- pData(msms.spk)
> idx <- grep("HUMAN",rownames(msms.spc))
> mSpC <- t( apply(msms.spc[idx,],1,function(x) tapply(x,treat,mean)) )
> apply(mSpC,2,summary)

```

	U100	U200	U400	U600
Min.	0.000000	0.000000	0.000000	0.1666667
1st Qu.	0.000000	0.250000	1.500000	2.1666667
Median	0.500000	1.000000	2.666667	4.6666667
Mean	1.384615	2.752137	6.837607	9.7521368
3rd Qu.	1.500000	3.333333	7.833333	11.4166667
Max.	9.750000	20.500000	43.333333	60.3333333

3 Differential expression tests on spectral counts

Spectral counts (SpC) is an integer measure which requires of tests suited to compare counts, as the GML methods based in the Poisson distribution, the negative-binomial, or the quasiliikelihood [11]

Generally speaking no test is superior to the other. They are just more or less indicated in some cases. The Poisson regression requires the estimation of just one parameter, and is indicated when the number of replicates is low, two or three. A drawback of the Poisson distribution is that its variance equals its mean, and it is not able to explain extra sources of variability a part of the sampling. Quasi-likelihood is a distribution independent GLM, but requires of the estimation of two parameters and hence needs a higher number of replicates, i.e. not less than four. The negative-binomial requires two parameters too, but we may use the implementation of this GLM in the edgeR package [12] which uses an empirical Bayes method to share information across features and may be employed with a restricted number of replicates; in the worst case it limits with the Poisson solution.

4 Poisson GLM regression example

When using the Poisson distribution we implicitly accept a model not sensitive to biological variability [11]. So it is just recommended in cases where we have very few replicates, if any, and we do not expect a significant biological variability between samples.

```
> ### Subset to the 200 and 600fm spikings
> fl <- treat=="U200" | treat=="U600"
> e <- msms.spk[,fl]
> table(pData(e))

U100 U200 U400 U600
    0     6     0     6

> ### Remove all zero rows
> e <- pp.msms.data(e)
> dim(e)

[1] 664  12

> ### Null and alternative model
> null.f <- "y~1"
> alt.f <- "y~treat"
> ### Normalizing condition
> div <- apply(exprs(e),2,sum)
> ### Poisson GLM
> pois.res <- msms.glm.pois(e,alt.f,null.f,div=div)
> str(pois.res)

'data.frame':      664 obs. of  3 variables:
 $ LogFC  : num  0.12061 -0.18016 0.40229 0.00168 -0.37157 ...
 $ D      : num  5.22 8.93 4.11e+01 7.72e-04 3.10e+01 ...
 $ p.value: num  2.23e-02 2.80e-03 1.41e-10 9.78e-01 2.59e-08 ...

> ### DEPs on unadjusted p-values
> sum(pois.res$p.value<=0.01)

[1] 44

> ### DEPs on multitest adjusted p-values
> adjp <- p.adjust(pois.res$p.value,method="BH")
> sum(adjp<=0.01)

[1] 26

> ### The top features
> o <- order(pois.res$p.value)
> head(pois.res[o,],20)
```

	LogFC	D	p.value
TRFL_HUMAN	1.8306054	150.41594	1.406200e-34
ALBU_HUMAN	1.9625212	104.51026	1.563819e-24
TRFE_HUMAN	1.3100859	81.53741	1.719756e-19
YPL037C	0.7416385	79.62566	4.525092e-19
CAH2_HUMAN	2.3041307	63.81053	1.369793e-15
CATA_HUMAN	1.5343714	50.12093	1.445571e-12
CAH1_HUMAN	4.4542343	47.82775	4.653585e-12
MYG_HUMAN	2.1323062	46.76436	8.005636e-12
YOL086C	0.4022934	41.14620	1.412577e-10
KCRM_HUMAN	1.4294782	37.83644	7.693109e-10
YGR192C	-0.3715658	30.99581	2.585863e-08
ANT3_HUMAN	1.4013022	27.39627	1.657502e-07
GSTA1_HUMAN	2.5495822	27.11728	1.914789e-07
PRDX1_HUMAN	1.6359291	26.61692	2.480616e-07
UBE2C_HUMAN	3.0181352	25.68582	4.017692e-07
NQO1_HUMAN	3.7380273	25.66094	4.069826e-07
CATD_HUMAN	1.6748334	24.58937	7.094204e-07
CYC_HUMAN	32.2452233	24.09871	9.152148e-07
ANXA5_HUMAN	2.5156349	22.35721	2.263617e-06
YJL136C	0.7517022	21.60136	3.356135e-06

```
> ### How the UPS1 proteins get ordered in the list
> grep("HUMAN",rownames(pois.res[o,]))
```

```
[1] 1 2 3 5 6 7 8 10 12 13 14 15 16 17 18 19 21 22 23
[20] 29 30 31 32 33 36 37 39 44 45 46 57 62 64 87 88 115 126 236
[39] 240
```

```
> ### Truth table
> nh <- length(grep("HUMAN",featureNames(e)))
> ny <- length(grep("HUMAN",featureNames(e),invert=TRUE))
> tp <- length(grep("HUMAN",rownames(pois.res)[adjp<=0.01]))
> fp <- sum(adjp<=0.01)-tp
> (tt.pois1 <- data.frame(TP=tp,FP=fp,TN=ny-fp,FN=nh-tp))
```

```
TP FP TN FN
1 19 7 618 20
```

5 Quasi-likelihood GLM regression example

The quasi-likelihood is a distribution free model that allows for overdispersion, and could be indicated where an appreciable source of biological variability is expected. In this model, instead of specifying a probability distribution for the data, we just provide a relationship between mean and variance. This relationship takes the form of a function, with a multiplicative factor known as the overdispersion, which has to be estimated from the data [11]. Its use in proteomics has been documented by Li et al. (2010)[13].

```

> ### Quasi-likelihood GLM
> ql.res <- msms.glm.qlll(e,alt.f,null.f,div=div)
> str(ql.res)

'data.frame':      664 obs. of  3 variables:
 $ LogFC   : num  0.12061 -0.18016 0.40229 0.00168 -0.37157 ...
 $ D       : num  5.22 8.93 4.11e+01 7.72e-04 3.10e+01 ...
 $ p.value: num  0.314295 0.001513 0.048548 0.991762 0.000234 ...

> ### DEPs on unadjusted p-values
> sum(ql.res$p.value<=0.01)

[1] 75

> ### DEPs on multitest adjusted p-values
> adjp <- p.adjust(ql.res$p.value,method="BH")
> sum(adjp<=0.01)

[1] 22

> ### The top features
> o <- order(ql.res$p.value)
> head(ql.res[o,],20)

```

	LogFC	D	p.value
PRDX1_HUMAN	1.6359291	26.61692	4.478445e-09
TRFE_HUMAN	1.3100859	81.53741	7.691259e-09
TRFL_HUMAN	1.8306054	150.41594	2.767215e-08
ALBU_HUMAN	1.9625212	104.51026	4.782251e-08
CYC_HUMAN	32.2452234	24.09871	6.946465e-08
CATA_HUMAN	1.5343714	50.12093	1.533212e-07
CAH2_HUMAN	2.3041307	63.81053	2.984566e-07
GSTA1_HUMAN	2.5495822	27.11728	5.778069e-07
CAH1_HUMAN	4.4542343	47.82775	3.020100e-06
UBE2C_HUMAN	3.0181352	25.68582	7.086311e-06
CATG_HUMAN	1.4749929	21.35193	7.428511e-06
CATD_HUMAN	1.6748334	24.58937	1.287976e-05
ANT3_HUMAN	1.4013022	27.39627	2.087493e-05
SODC_HUMAN	1.3712449	10.38483	2.928947e-05
MYG_HUMAN	2.1323062	46.76436	5.971058e-05
NQO1_HUMAN	3.7380273	25.66094	9.621658e-05
KCRM_HUMAN	1.4294782	37.83644	1.056421e-04
ANXA5_HUMAN	2.5156349	22.35721	2.173514e-04
YGR192C	-0.3715658	30.99581	2.338676e-04
TAU_HUMAN	1.5156349	11.43236	2.481070e-04

```

> ### How the UPS1 proteins get ordered in the list
> grep("HUMAN",rownames(ql.res[o,]))

```

```
[1] 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 20
[20] 23 24 27 31 32 34 35 42 44 47 54 58 60 61 122 153 158 248 249
[39] 281
```

```
> ### Truth table
> tp <- length(grep("HUMAN",rownames(q1.res)[adjp<=0.01]))
> fp <- sum(adjp<=0.01)-tp
> (tt.q11 <- data.frame(TP=tp,FP=fp,TN=ny-fp,FN=nh-tp))
```

```
TP FP TN FN
1 19 3 622 20
```

6 edgeR: negative binomial GLM regression example

The negative-binomial provides another model that allows for overdispersion. The implementation adopted in this package is entirely based in the solution provided by the package edgeR [12] which includes empirical Bayes methods to share information among features, and thus may be employed even when the number of replicates is as low as two. The negative-binomial is downward limited, when no overdispersion is observed, by the Poisson distribution.

```
> ### Negative-binomial
> nb.res <- msms.edgeR(e,alt.f,null.f,div=div,fnm="treat")
> str(nb.res)

'data.frame':      664 obs. of  3 variables:
 $ LogFC   : num  0.1279 -0.1801 0.4264 0.0122 -0.3724 ...
 $ LR      : num  1.3141 8.79604 6.94406 0.00671 27.21512 ...
 $ p.value: num  2.52e-01 3.02e-03 8.41e-03 9.35e-01 1.82e-07 ...
```

```
> ### DEPs on unadjusted p-values
> sum(nb.res$p.value<=0.01)
```

```
[1] 40
```

```
> ### DEPs on multitest adjusted p-values
> adjp <- p.adjust(nb.res$p.value,method="BH")
> sum(adjp<=0.01)
```

```
[1] 22
```

```
> ### The top features
> o <- order(nb.res$p.value)
> head(nb.res[o,],20)
```

	LogFC	LR	p.value
TRFL_HUMAN	1.8228068	149.96447	1.764927e-34
ALBU_HUMAN	1.9486266	104.31877	1.722494e-24
TRFE_HUMAN	1.3049775	81.29440	1.944770e-19
CAH2_HUMAN	2.2665908	63.75510	1.408883e-15
CATA_HUMAN	1.5209383	50.05208	1.497186e-12
CAH1_HUMAN	4.0281168	47.49324	5.519398e-12
MYG_HUMAN	2.0923677	45.65420	1.410831e-11
KCRM_HUMAN	1.4146191	36.29746	1.693845e-09
ANT3_HUMAN	1.3827898	27.30592	1.736790e-07
YGR192C	-0.3723619	27.21512	1.820296e-07
GSTA1_HUMAN	2.4316185	26.92058	2.119899e-07
PRDX1_HUMAN	1.6054234	26.57308	2.537549e-07
UBE2C_HUMAN	2.8079615	25.49225	4.441628e-07
NQO1_HUMAN	3.3269851	25.47239	4.487582e-07
CATD_HUMAN	1.6393037	24.37058	7.947301e-07
CYC_HUMAN	4.6109504	24.00066	9.630245e-07
ANXA5_HUMAN	2.3800320	22.17535	2.488488e-06
CATG_HUMAN	1.4473996	21.18447	4.171299e-06
SYH_HUMAN	3.1111352	20.73575	5.272237e-06
IL8_HUMAN	3.2058569	15.17681	9.789825e-05

```
> ### How the UPS1 proteins get ordered in the list
> grep("HUMAN",rownames(nb.res[o,]))
```

```
[1] 1 2 3 4 5 6 7 8 9 11 12 13 14 15 16 17 18 19 20
[20] 23 25 26 27 28 31 32 34 40 41 42 53 58 60 81 82 111 123 229
[39] 230
```

```
> ### Truth table
> tp <- length(grep("HUMAN",rownames(nb.res)[adjp<=0.01]))
> fp <- sum(adjp<=0.01)-tp
> (tt.nb1 <- data.frame(TP=tp,FP=fp,TN=ny-fp,FN=nh-tp))
```

```
TP FP TN FN
1 19 3 622 20
```

7 Reproducibility

In the *omics* field, reproducibility is of biggest concern. Very low p-values for a protein in an experiment are not enough to declare that protein as of interest as a biomarker. A good biomarker should give as well a reproducible signal, and posses a biologically significant effect size. According to our experience, a protein giving less than three counts in the most abundant condition results of poor reproducibility, to be declared as statistically significant, experiment after experiment. On the other hand most of the false positives in an spiking experiment show log fold changes below 1. These two observations [6] allow

to improve the results obtained in the previous sections. The trick is to flag as relevant those proteins which have low p-values, high enough signal, and good effect size. In performing this relevance filter we may even accept higher adjusted p-values than usual. This flagging is provided by the function `test.results`.

```
> ### Cut-off values for a relevant protein as biomarker
> alpha.cut <- 0.05
> SpC.cut <- 2
> lFC.cut <- 1
> ### Relevant proteins according to the Poisson GLM
> pois.tbl <- test.results(pois.res,e,pData(e)$treat,"U600","U200",div,
                           alpha=alpha.cut,minSpC=SpC.cut,minLFC=lFC.cut,
                           method="BH")$tres
> (pois.nms <- rownames(pois.tbl)[pois.tbl$DEP])

[1] "TRFL_HUMAN" "ALBU_HUMAN" "TRFE_HUMAN" "CAH2_HUMAN" "CATA_HUMAN"
[6] "CAH1_HUMAN" "MYG_HUMAN" "KCRM_HUMAN" "ANT3_HUMAN" "GSTA1_HUMAN"
[11] "PRDX1_HUMAN" "UBE2C_HUMAN" "NQO1_HUMAN" "CATD_HUMAN" "CYC_HUMAN"
[16] "ANXA5_HUMAN" "CATG_HUMAN" "SYH_HUMAN" "IL8_HUMAN" "LYSC_HUMAN"
[21] "PDGFB_HUMAN" "TAU_HUMAN" "SODC_HUMAN" "YLR388W" "LALBA_HUMAN"

> ### Truth table
> ridx <- grep("HUMAN",pois.nms)
> tp <- length(ridx)
> fp <- length(pois.nms)-length(ridx)
> (tt.pois2 <- data.frame(TP=tp,FP=fp,TN=ny-fp,FN=nh-tp))

  TP FP  TN FN
1 24  1 624 15

> ### Relevant proteins according to the quasi-likelihood GLM
> ql.tbl <- test.results(ql.res,e,pData(e)$treat,"U600","U200",div,
                           alpha=0.05,minSpC=2,minLFC=1,method="BH")$tres
> (ql.nms <- rownames(ql.tbl)[ql.tbl$DEP])

[1] "PRDX1_HUMAN" "TRFE_HUMAN" "TRFL_HUMAN" "ALBU_HUMAN" "CYC_HUMAN"
[6] "CATA_HUMAN" "CAH2_HUMAN" "GSTA1_HUMAN" "CAH1_HUMAN" "UBE2C_HUMAN"
[11] "CATG_HUMAN" "CATD_HUMAN" "ANT3_HUMAN" "SODC_HUMAN" "MYG_HUMAN"
[16] "NQO1_HUMAN" "KCRM_HUMAN" "ANXA5_HUMAN" "TAU_HUMAN" "SYH_HUMAN"
[21] "UB2E1_HUMAN" "IL8_HUMAN" "LALBA_HUMAN" "UBC9_HUMAN" "YOR098C"
[26] "LYSC_HUMAN" "EGF_HUMAN" "PDGFB_HUMAN" "YDR012W" "RASH_HUMAN"

> ### Truth table
> ridx <- grep("HUMAN",ql.nms)
> tp <- length(ridx)
> fp <- length(ql.nms)-length(ridx)
> (tt.ql2 <- data.frame(TP=tp,FP=fp,TN=ny-fp,FN=nh-tp))
```

```

TP FP  TN FN
1 28  2 623 11

```

```

> ### Relevant proteins according to the negative-binomial GLM
> nb.tbl <- test.results(nb.res,e,pData(e)$treat,"U600","U200",div,
                        alpha=0.05,minSpC=2,minLFC=1,method="BH")$tres
> (nb.nms <- rownames(nb.tbl)[nb.tbl$DEP])

[1] "TRFL_HUMAN"  "ALBU_HUMAN"  "TRFE_HUMAN"  "CAH2_HUMAN"  "CATA_HUMAN"
[6] "CAH1_HUMAN"  "MYG_HUMAN"   "KCRM_HUMAN"  "ANT3_HUMAN"  "GSTA1_HUMAN"
[11] "PRDX1_HUMAN" "UBE2C_HUMAN" "NQO1_HUMAN"  "CATD_HUMAN"  "CYC_HUMAN"
[16] "ANXA5_HUMAN" "CATG_HUMAN"  "SYH_HUMAN"   "IL8_HUMAN"   "LYSC_HUMAN"
[21] "PDGFB_HUMAN" "TAU_HUMAN"   "SODC_HUMAN"  "YLR388W"

> ### Truth table
> ridx <- grep("HUMAN",nb.nms)
> tp <- length(ridx)
> fp <- length(nb.nms)-length(ridx)
> (tt.nb2 <- data.frame(TP=tp,FP=fp,TN=ny-fp,FN=nh-tp))

```

```

TP FP  TN FN
1 23  1 624 16

```

As you may see, without the post-test filter a relatively low adjusted p-value cut-off is required to keep an acceptable number of false positives. The post-test filter allows to relax the p-value cut-off improving at the same time both the number of true positives and false positives.

	Test	Significance	Filtered	TP	FP	TN	FN
	Poisson	0.05	No	26	12	613	13
	Quasi-likelihood	0.05	No	30	24	601	9
	Negative-binomial	0.05	No	24	6	619	15
	Poisson	0.01	No	19	7	618	20
	Quasi-likelihood	0.01	No	19	3	622	20
	Negative-binomial	0.01	No	19	3	622	20
	Poisson	0.05	Yes	24	1	624	15
	Quasi-likelihood	0.05	Yes	28	2	623	11
	Negative-binomial	0.05	Yes	23	1	624	16

Table 1: Truth tables

8 Other functions in the package

A useful tool to visualize the global results of differential expression tests is a table of accumulated frequencies of features by p-values in bins of log fold changes. It may help in finding the most appropriate post-test filter cut-off values in a given experiment.

```
> ### All features
> pval.by.fc(ql.tbl$adjp,ql.tbl$LogFC)
```

LogFC	p.vals						
	<=0.001	<=0.005	<=0.01	<=0.05	<=0.1	<=0.2	<=1
(-Inf,-10]	0	0	2	6	13	21	40
(-10,-2]	0	0	0	0	2	5	14
(-2,-1]	0	0	0	2	4	8	41
(-1,-0.848]	0	0	0	1	1	2	7
(-0.848,-0.585]	0	0	0	4	6	15	52
(-0.585,0]	0	0	1	10	16	36	282
(0,0.585]	0	0	0	0	0	0	123
(0.585,0.848]	0	0	0	0	2	3	16
(0.848,1]	0	0	0	0	0	1	15
(1,2]	7	10	11	16	17	22	30
(2,10]	4	6	7	11	12	13	17
(10, Inf]	1	1	1	4	8	17	27
Tot	12	17	22	54	81	143	664

```
> ### Filtering by minimal signal
> f1 <- ql.tbl$U600 > 2
> pval.by.fc(ql.tbl$adjp[f1],ql.tbl$LogFC[f1])
```

LogFC	p.vals						
	<=0.001	<=0.005	<=0.01	<=0.05	<=0.1	<=0.2	<=1
(-Inf,-10]	0	0	0	0	0	0	0
(-10,-2]	0	0	0	0	0	0	0
(-2,-1]	0	0	0	0	0	0	0
(-1,-0.848]	0	0	0	1	1	2	3
(-0.848,-0.585]	0	0	0	3	4	10	13
(-0.585,0]	0	0	1	10	16	36	195
(0,0.585]	0	0	0	0	0	0	85
(0.585,0.848]	0	0	0	0	2	3	7
(0.848,1]	0	0	0	0	0	1	2
(1,2]	7	10	11	16	17	20	21
(2,10]	4	6	7	11	12	12	12
(10, Inf]	1	1	1	1	1	1	1
Tot	12	17	20	42	53	85	339

Another usual tool is a volcano plot with the ability to visualize the effect of different post-test filter cut-off values.

```
> par(mar=c(5,4,0.5,2)+0.1)
> res.volcanoplot(ql.tbl,max.pval=0.05,min.LFC=1,maxx=3,maxy=NULL,
  ylbls=3)
```



Figure 1: Volcanoplot

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