RNA-Seq Analysis Training

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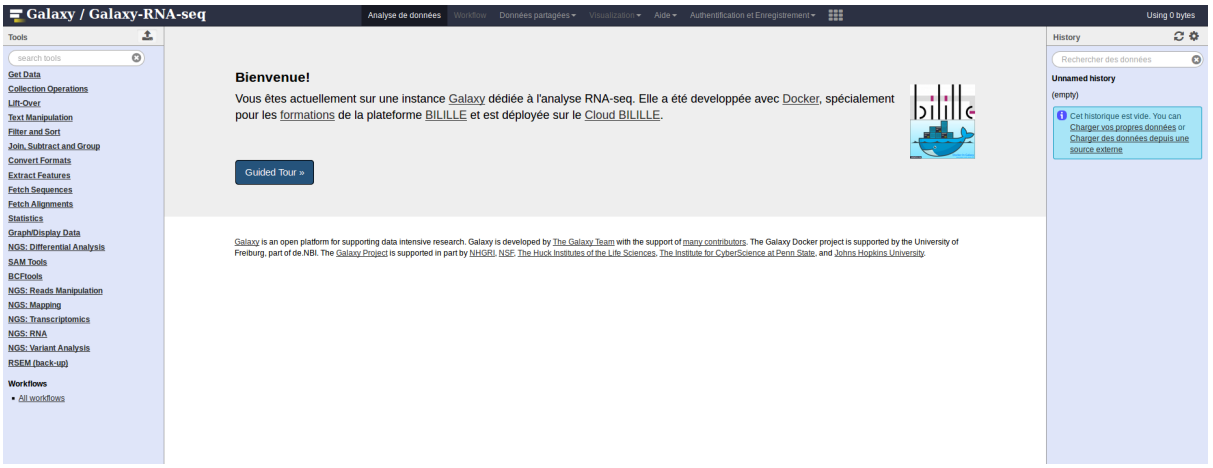
Introduction

Connecting to your Galaxy instance

- Open an internet browser and type in the IP address given by the trainers in the address bar.
- the following home page is displayed:

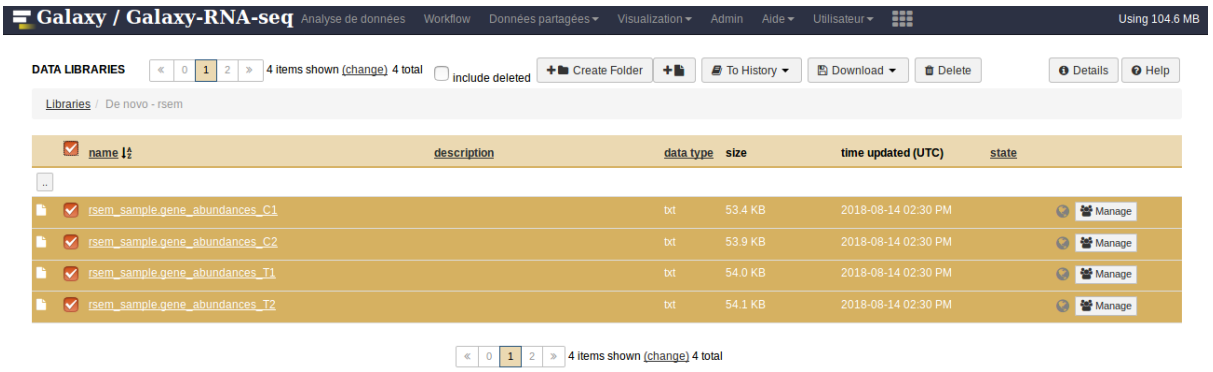


- Click on User -> login and enter the ID userX@bilille.fr and the password "bililleuser" (with X a number between 1 and 6 given by the trainer)
- The home page is displayed again. Click on User and "logged in as userX@bilille.fr" should appear. (with X a figure between 1 and 6 given by the trainer)

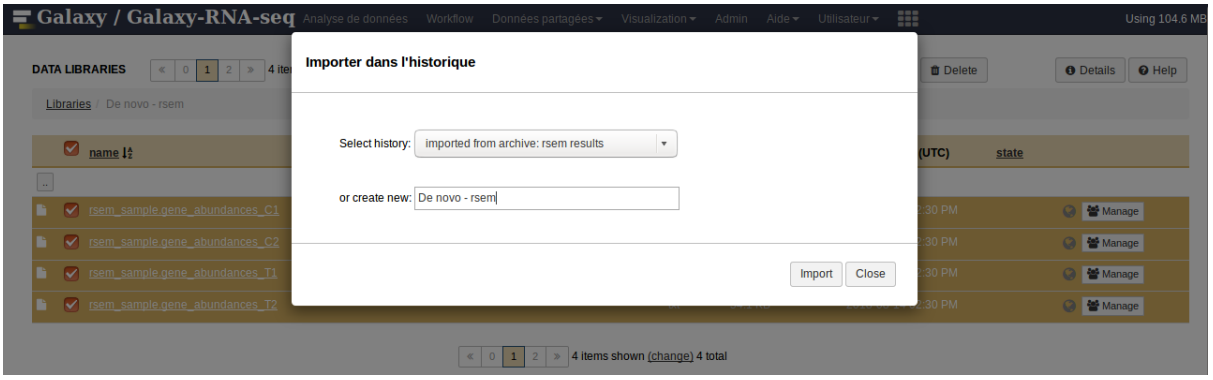
Data analysis De novo - rsem

Import the data into a new history

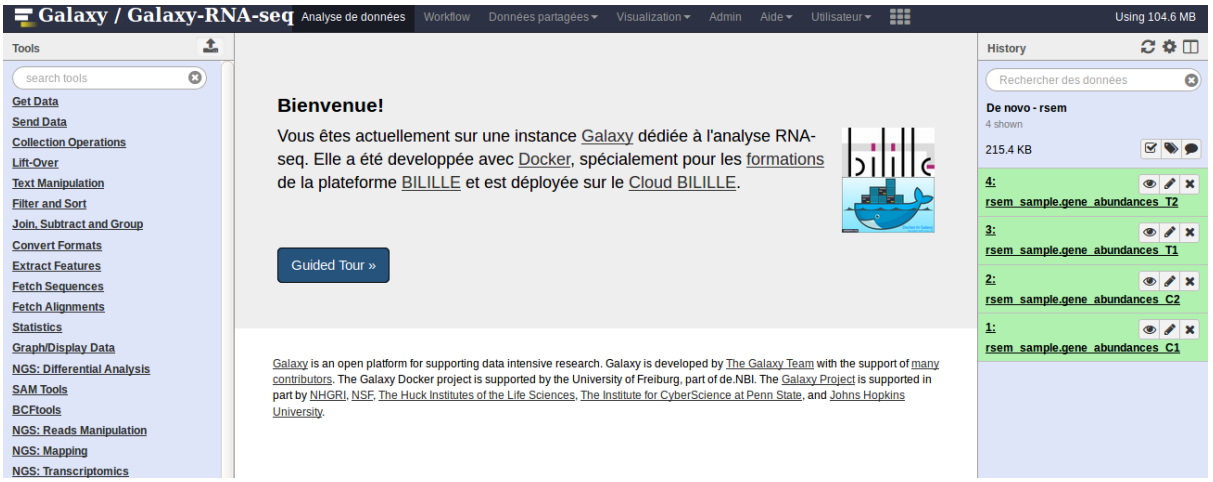
- Within Galaxy, click on Shared data -> Data library
- Click on De novo - rsem
- Select the 4 .txt files



- Click on "To History" then "as Datasets"
- Give a name to the new history in the "or create new" field (eg De novo rsem ").



- Your new history now appears in the "Analyze Data" section.



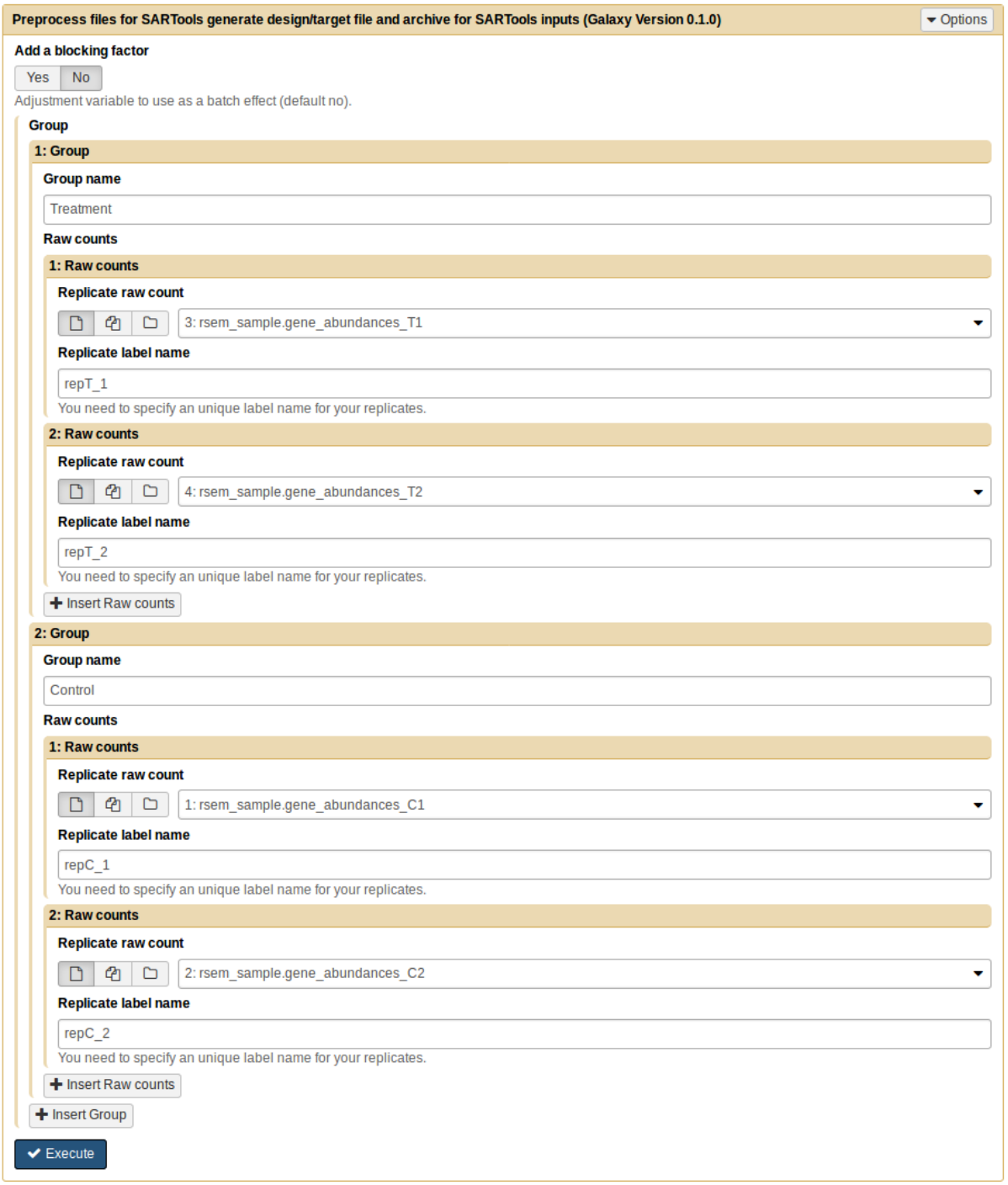
The data correspond to RNA-Seq count data from rsem for 4 replicates under 2 conditions: 2 replicates per condition, C (Control) and T (Treatment).

Preprocessing phase with SARTools.

Goal: This step creates datasets adapted to SARTools.

In the Tools panel, in the NGS: Differential Analysis section, click on the "preprocess files for SARTools" tool.

- Create 2 groups: Control and Treatment and add the 2 corresponding replicates to each of the 2 conditions
- Choose different replicate names for each replicate (for example repC_1, rep C_2 for the Control group and repT_1, rep T_2 for the Treatment group)



The tool returns 2 outputs

- a design file containing the conditions of the experiment in the .txt format.

1	2	3
label	files	group
repT_1	dataset_216.dat	Treatment
repT_2	dataset_217.dat	Treatment
repC_1	dataset_214.dat	Control
repC_2	dataset_215.dat	Control

- a .zip file containing all the count files.

Analysis with Sartools

Goal : Perform differential analysis of loaded data.

In the panel tool, click on the "SARTools DESeq2" tool

- Fill in the design / target file and the Zip file containing the raw counts.
- In the field "Reference biological condition" enter the value "Control" corresponding to the reference condition of the data.
- Leave the other fields unchanged.

SARTools DESeq2 Compare two or more biological conditions in a RNA-Seq framework with DESeq2 (Galaxy Version 1.3.2.0) Options

Name of the project used for the report

Project

(-P, --projectName) No space allowed.

Name of the report author

Galaxy

(-A, --author) No space allowed.

Design / target file

9: design file for SARTools (on data 2, data 1, and others)

(-t, --targetFile) See the help section below for details on the required format.

Zip file containing raw counts files

10: counts files for SARTools (on data 2, data 1, and others)

(-r, --rawDir) See the help section below for details on the required format.

Names of the features to be removed

alignment_not_unique,ambiguous,no_feature,not_aligned,too_low_aQual

(-F, --featuresToRemove) Separate the features with a comma, no space allowed. More than once can be specified. Specific HTSeq-count information and rRNA for example. Default are 'alignment_not_unique,ambiguous,no_feature,not_aligned,too_low_aQual'.

Factor of interest

group

(-v, --varInt) Biological condition in the target file. Default is 'group'.

Reference biological condition

Control

(-c, --condRef) Reference biological condition used to compute fold-changes, must be one of the levels of 'Factor of interest'.

Advanced Parameters

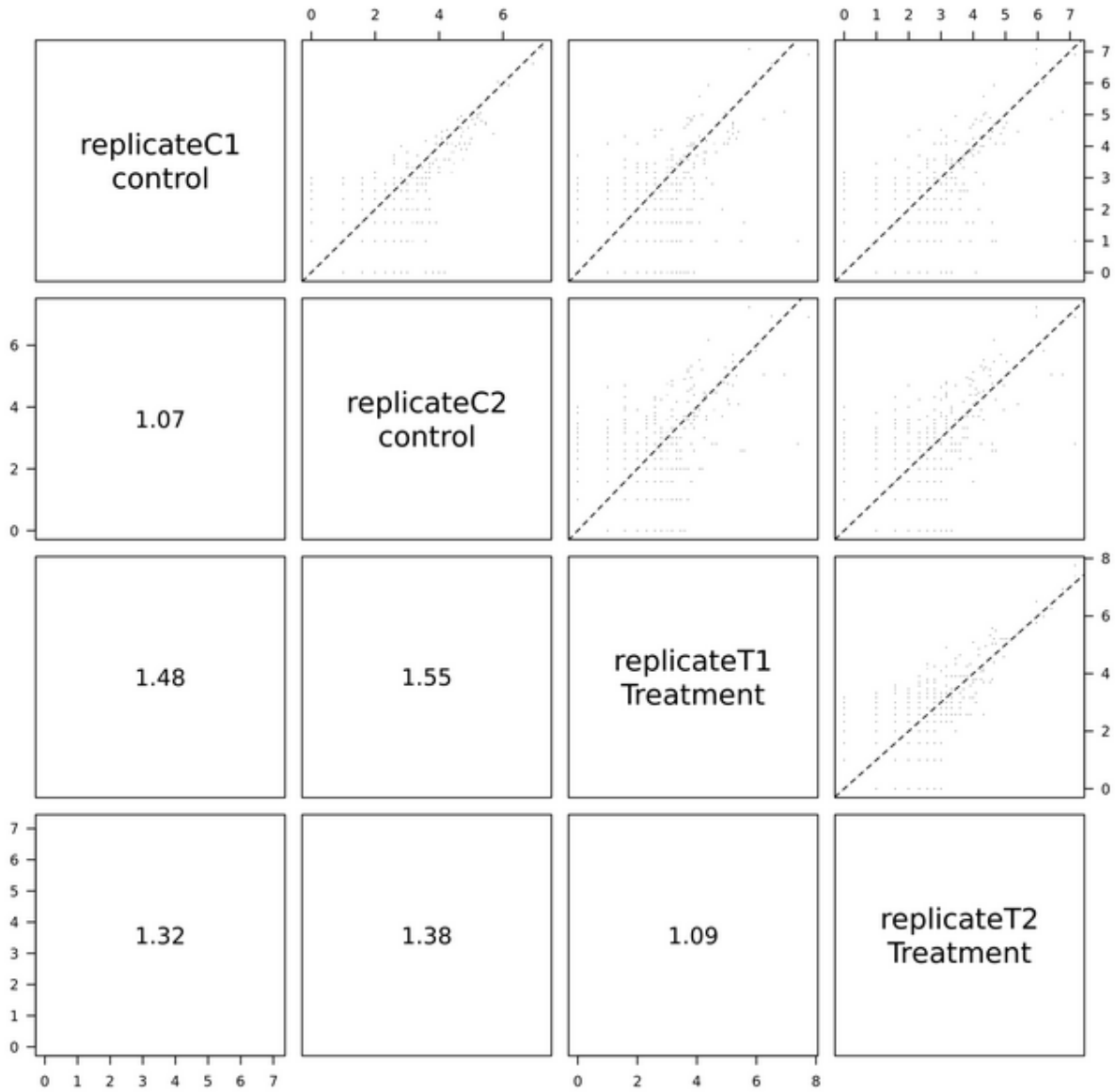
Hide

Execute

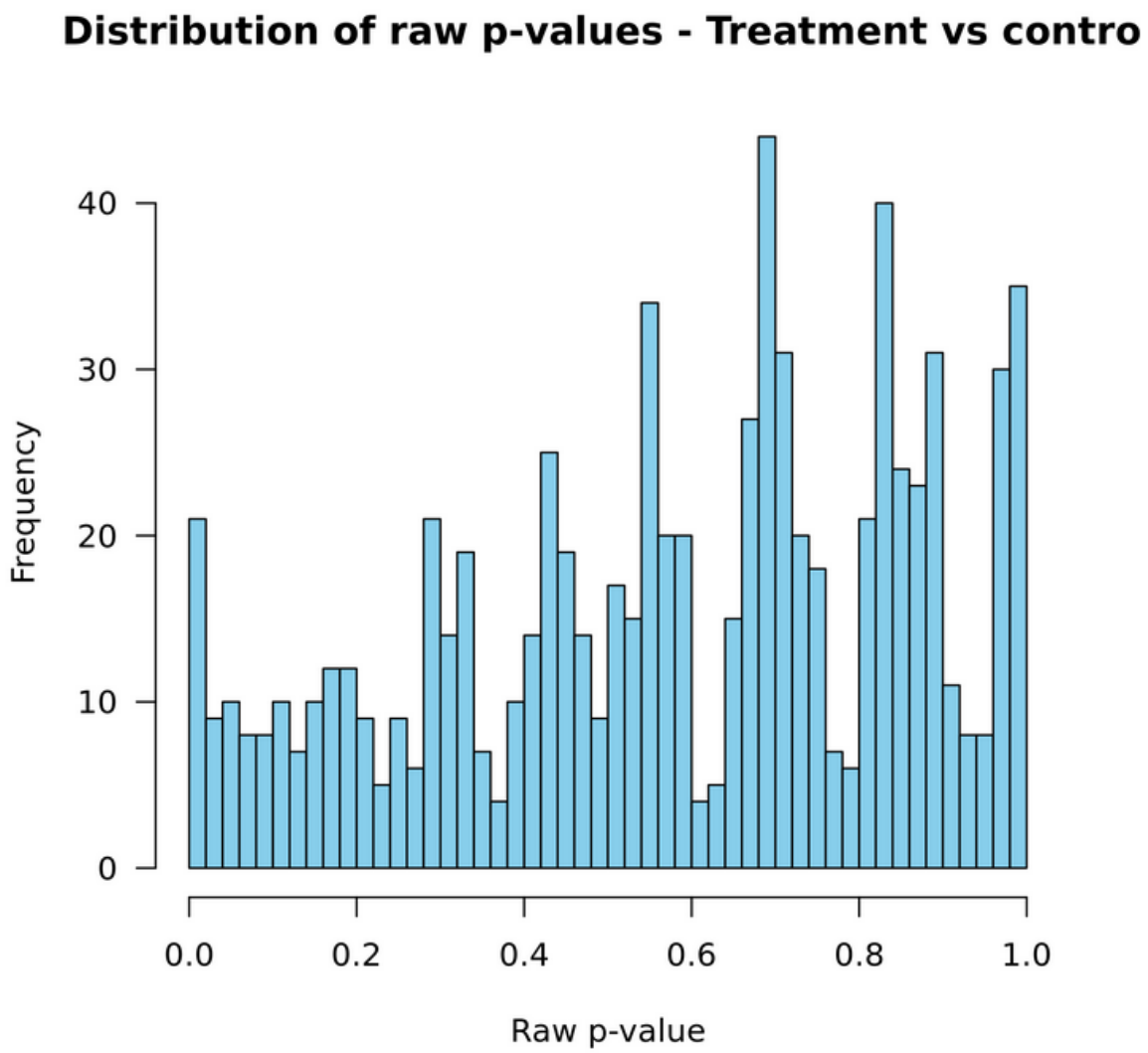
SARTools returns 5 datasets:

- A complete report in HTML to evaluate the quality of the data and the quality of differential analysis
- The complete list of the analyzed genes, as well as the under-expressed and over-expressed genes.
- The list of figures used in the HTML report
- A log check that all the stages of the analysis went well.
- An RData object that can be exported and used under R.

In the report, we can check that the SERE indicators are very close to 1, suggesting that the replicates of this dataset are technical and non-biological replicates.



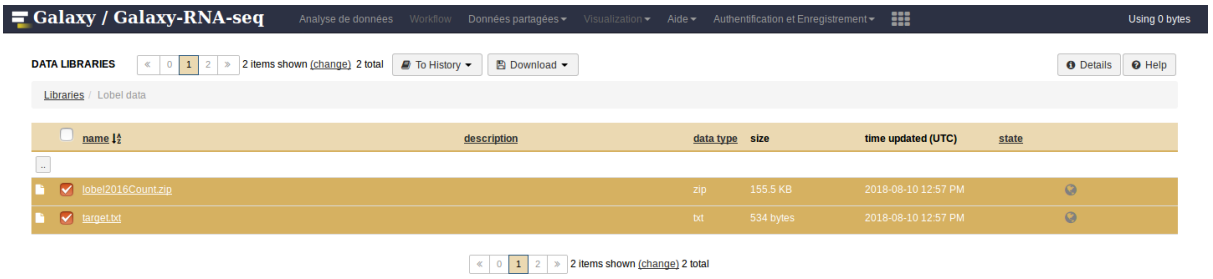
A little later in the report, the histogram of raw p-values suggests that the statistical model used is not appropriate for this dataset.



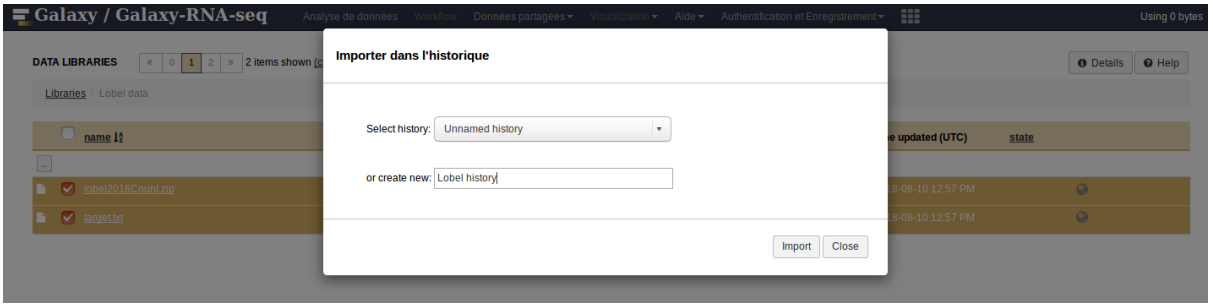
Analyze "Lobel" data with SARTools

Import data into new history

- Under Galaxy click Shared Data -> Data Library
- Click Lobel Data.
- Select the 2 files "lobel2016Count.zip" and "target.txt".



- Click on "To History" then "as Datasets"
- Give a name to the new history in the "or create new" field (eg "Lobel history")



- Your new history now appears in the "Analyze Data" section.

The lobel.zip file contains counts from Lobel L, Herskovits AA (2016) Systems Level Analyzes Reveal Multiple Regulatory Activities of CodY Controlling Metabolism, Motility and Virulence in Listeria monocytogenes. PLoS Genet 12 (2): e1005870. doi: 10.1371 / journal.pgen.1005870.

The target.txt file contains the description of the conditions of the experiment for its analysis by SARTools: 11 replicates for 2 conditions (6 WT for 5 codY)

Analysis with SARTools

- Fill in the design / target file and the Zip file containing the raw counts.
- In the field "Factor of interest" enter the value "strain" corresponding to the 3rd column of the file target and containing the 2 conditions to be compared.
- In the "Reference biological condition" field enter the value “WT”.
- Leave the other fields unchanged.

SARTools DESeq2 Compare two or more biological conditions in a RNA-Seq framework with DESeq2 (Galaxy Version 1.3.2.0) Options

Name of the project used for the report

Project

(-P, --projectName) No space allowed.

Name of the report author

Galaxy

(-A, --author) No space allowed.

Design / target file

1: target.txt

(-t, --targetFile) See the help section below for details on the required format.

Zip file containing raw counts files

2: lobel2016Count.zip

(-r, --rawDir) See the help section below for details on the required format.

Names of the features to be removed

alignment_not_unique,ambiguous,no_feature,not_aligned,too_low_aQual

(-F, --featuresToRemove) Separate the features with a comma, no space allowed. More than once can be specified. Specific HTSeq-count information and rRNA for example. Default are 'alignment_not_unique,ambiguous,no_feature,not_aligned,too_low_aQual'.

Factor of interest

strain

(-v, --varInt) Biological condition in the target file. Default is 'group'.

Reference biological condition

WT

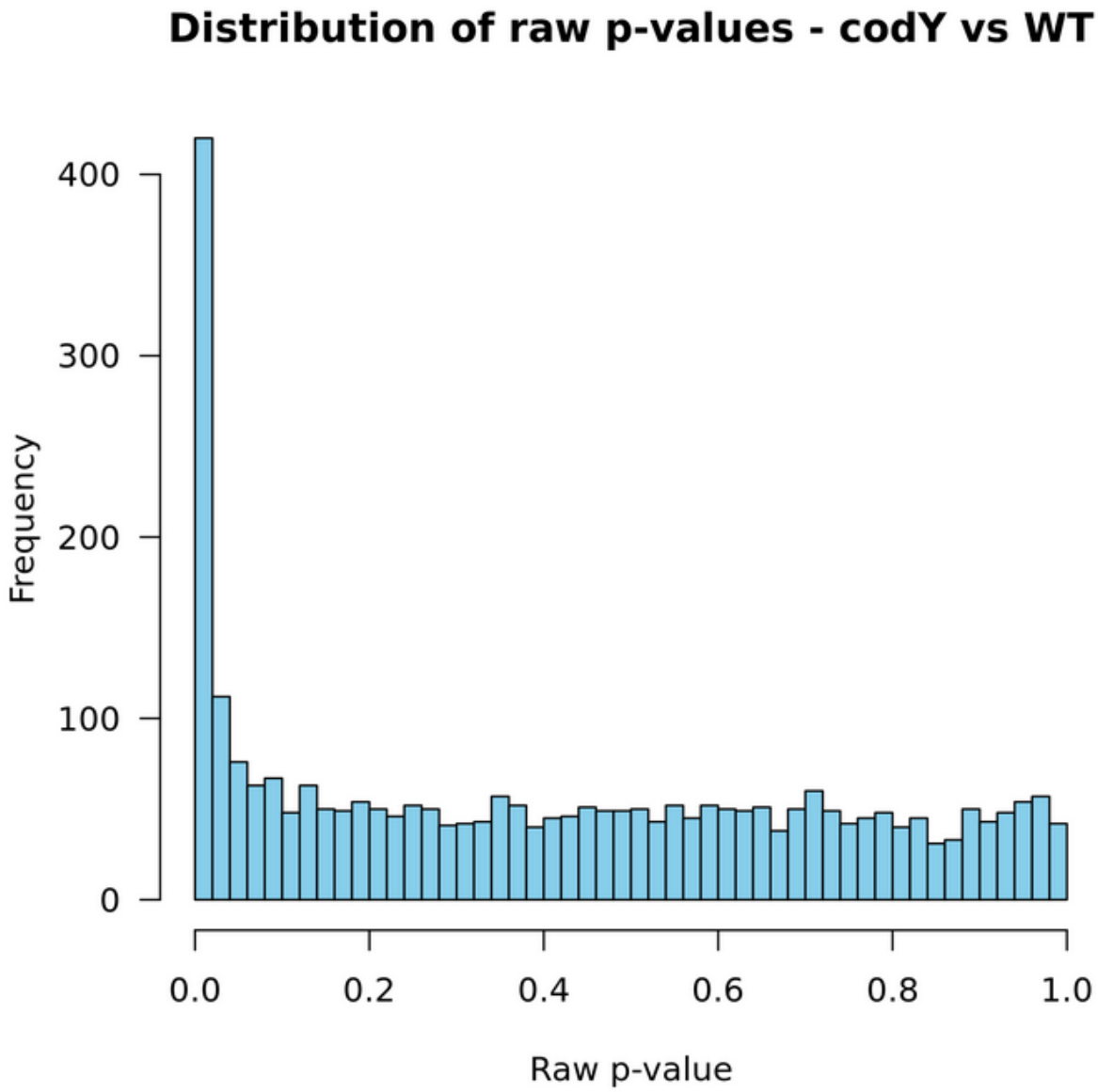
(-c, --condRef) Reference biological condition used to compute fold-changes, must be one of the levels of 'Factor of interest'.

Advanced Parameters

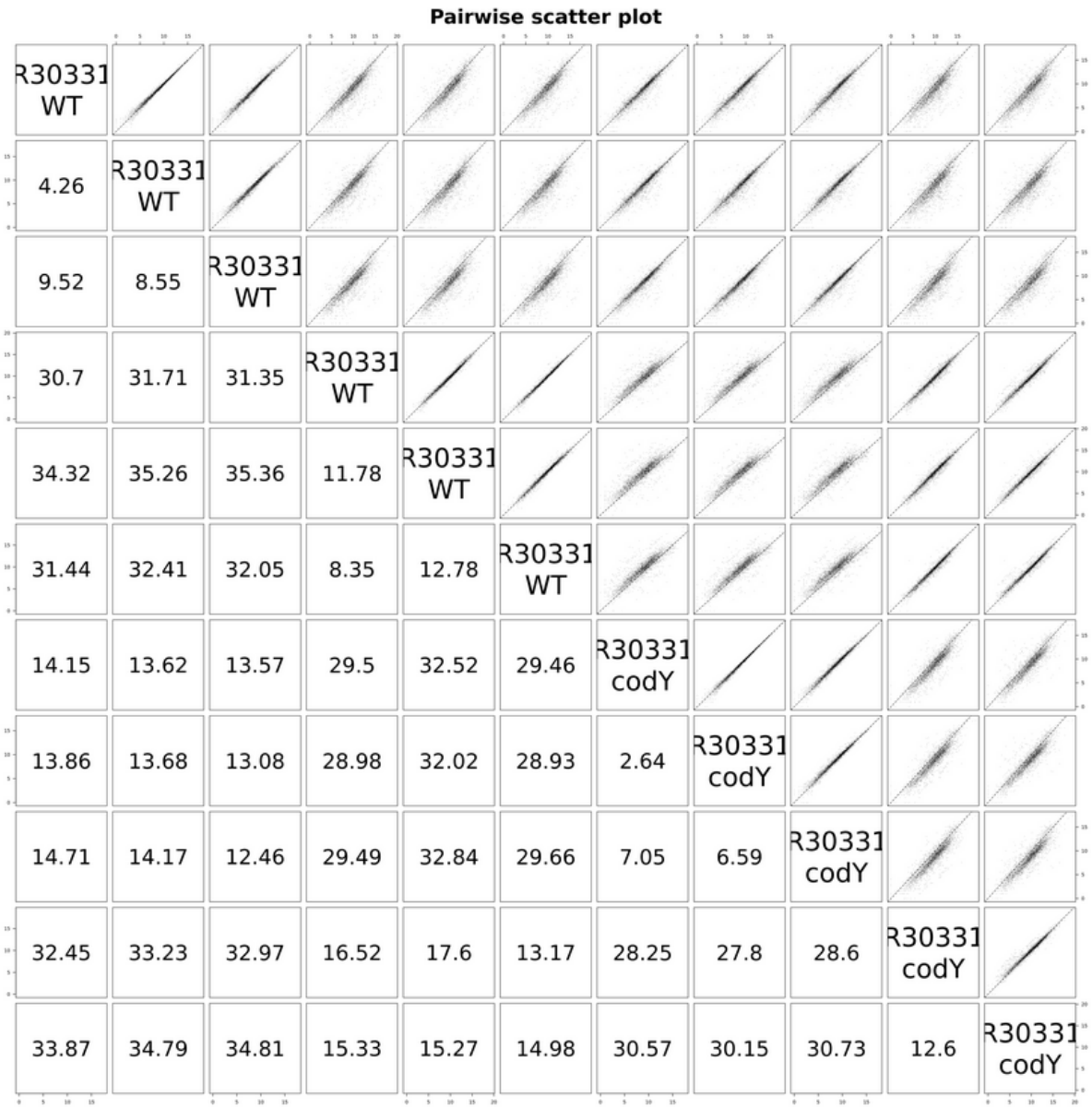
Hide

Execute

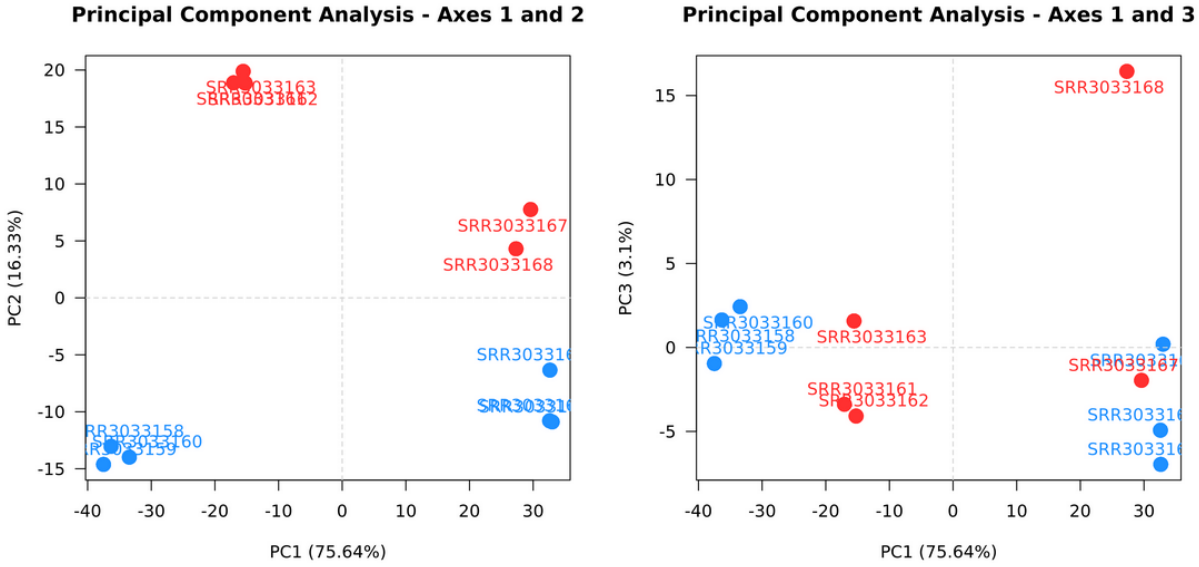
In the generated report, we notice that the histogram of the raw p-values has an expected shape: a left peak corresponding to the differentially expressed genes and a uniform distribution elsewhere.



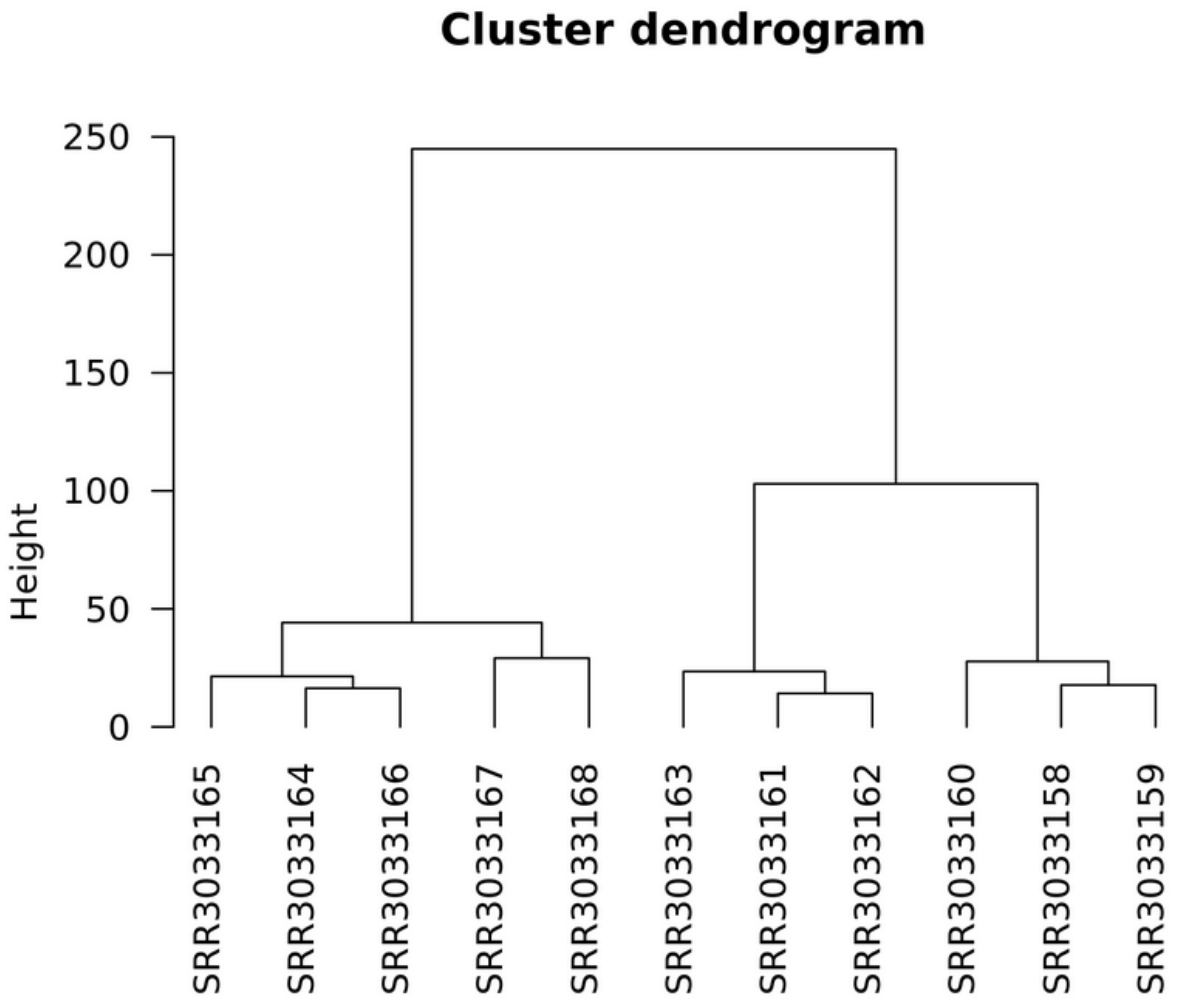
Let see the exploratory analysis:



All SERE coefficients are much higher than 1 suggesting that there are only biological replicates here. Note that the coefficient between the 3rd WT and the 4th WT (31,35) is greater than the coefficient between the 3rd WT and the first CodY (13,57). This is explained very well a little further thanks to the ACP



The first axis that explains more than 75% of the variability separates the samples according to their culture environment ("medium" column in the target file).



The dendrogram shows that the BHI medium separates the WT from the CodY better than the LBMM medium.

In order to take into account the effect of the culture medium, the analysis is restarted by including this effect as a blocking factor.

To do this:

- Click on "show" at the end of the parameters
- Click on "YES" in the blocking factor field and indicate the value "medium".
- Relaunch the analysis

Advanced Parameters

Show

Add a blocking factor

Yes No

(-b, --batch) Adjustment variable to use as a batch effect. Default: unchecked if no batch effect needs to be taken into account.

Blocking factor value

medium

Must be a column of the target file

Mean-variance relationship

parametric

(-f, --fitType) Type of model for the mean-dispersion relationship. Parametric by default.

Perform the outliers detection

Yes No

(-o, --cooksCutoff) Checked by default.

Perform independent filtering

Yes No

(-i, --independentFiltering) Checked by default.

Threshold of statistical significance

0.05

(-a, --alpha) Significance threshold applied to the adjusted p-values to select the differentially expressed features. Default is 0.05. The comma is not allowed as decimal separator, use a point instead.

p-value adjustment method

BH

(-p, --pAdjustMethod) p-value adjustment method for multiple testing. 'BH' by default, 'BY' or any value of p.adjust.methods.

Transformation for PCA/clustering

VST

(-T --typeTrans) Method of transformation of the counts for the clustering and the PCA: 'VST' (default) for Variance Stabilizing Transformation, or 'rlog' for Regularized Log Transformation.

Estimation of the size factors

median

(-l --locfunc) 'median' (default) or 'shorth' from the genefilter package.

Colors of each biological condition on the plots: 'col1,col2,col3,col4'

dodgerblue,firebrick1,MediumVioletRed,SpringGreen,chartreuse,cyan,darkorchid,darkorange

(-C, --colors) Separate the colors with a comma, no space allowed. Default are 'dodgerblue,firebrick1,MediumVioletRed,SpringGreen,chartreuse,cyan,darkorchid,darkorange'.

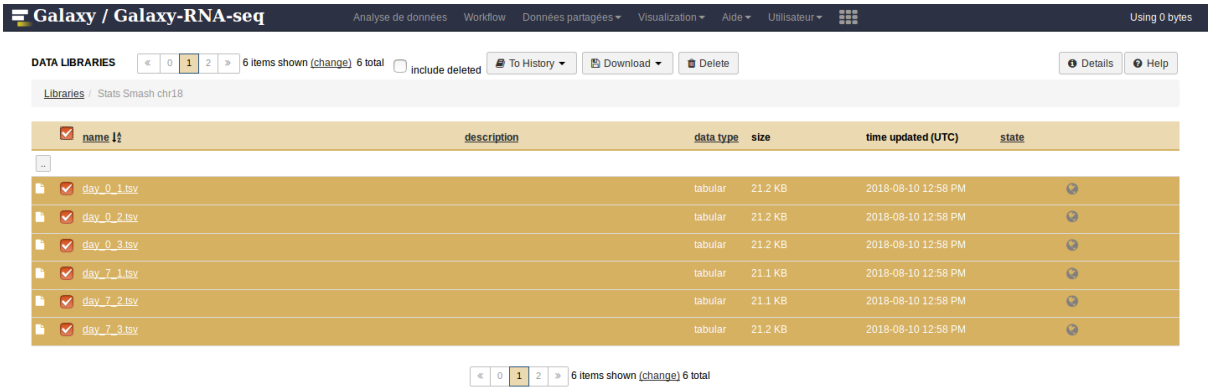
Execute

This second analysis returns more genes differentially expressed than the previous one.

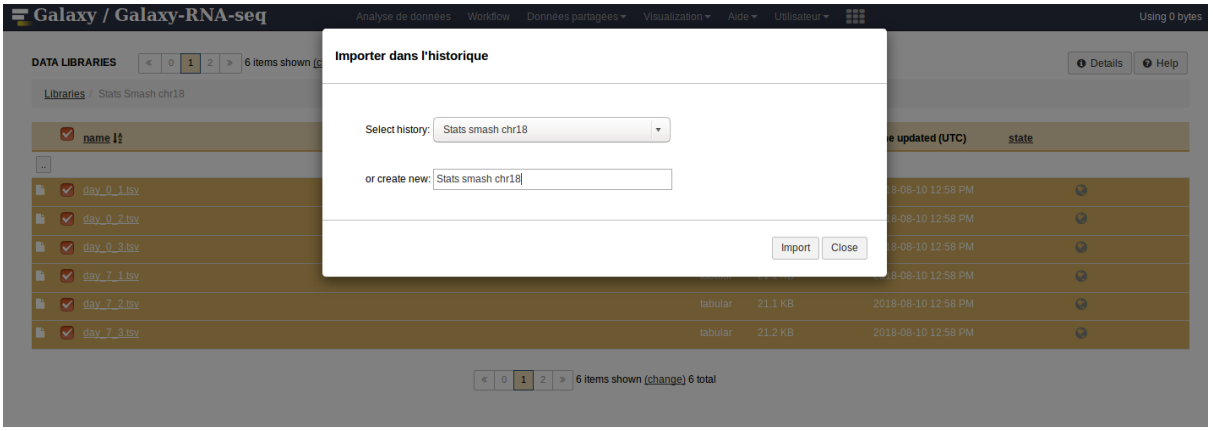
Data analysis "Stats Smash chr18" with SARTools

Import the data in a new history

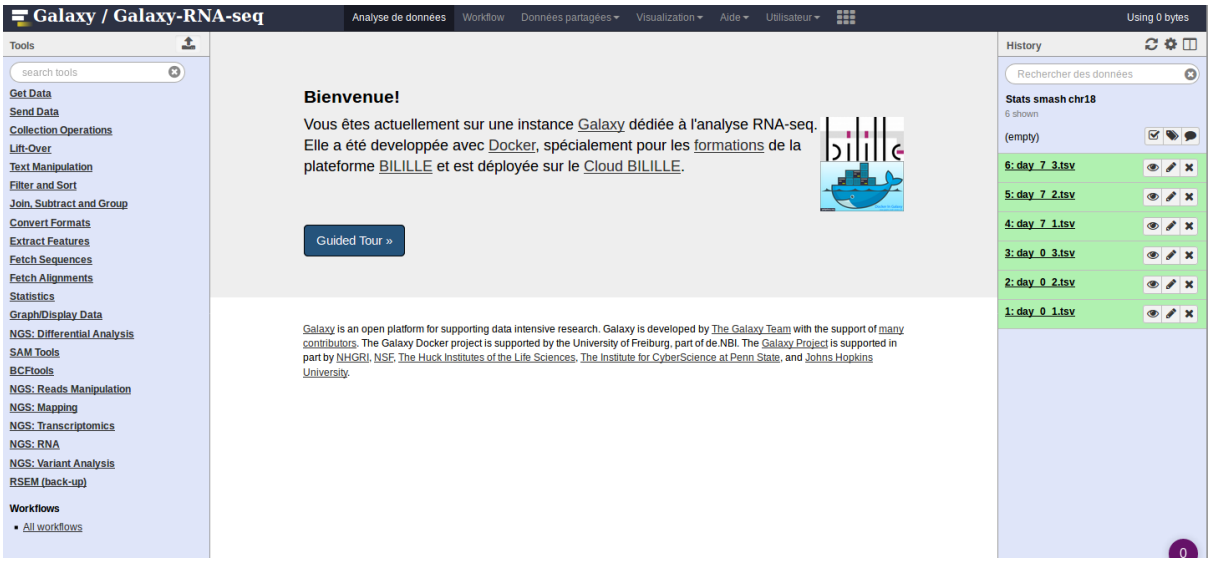
- Click on Shared Data -> Data Library
- Click on Stats Smash chr18.
- Select the 6 .tsv files



- Click on "To History" then "as Datasets"
- Give a name to the new history in the "or create new" field (eg Stats smash chr18 ").



- Your new history now appears in the "Data Analysis" section.



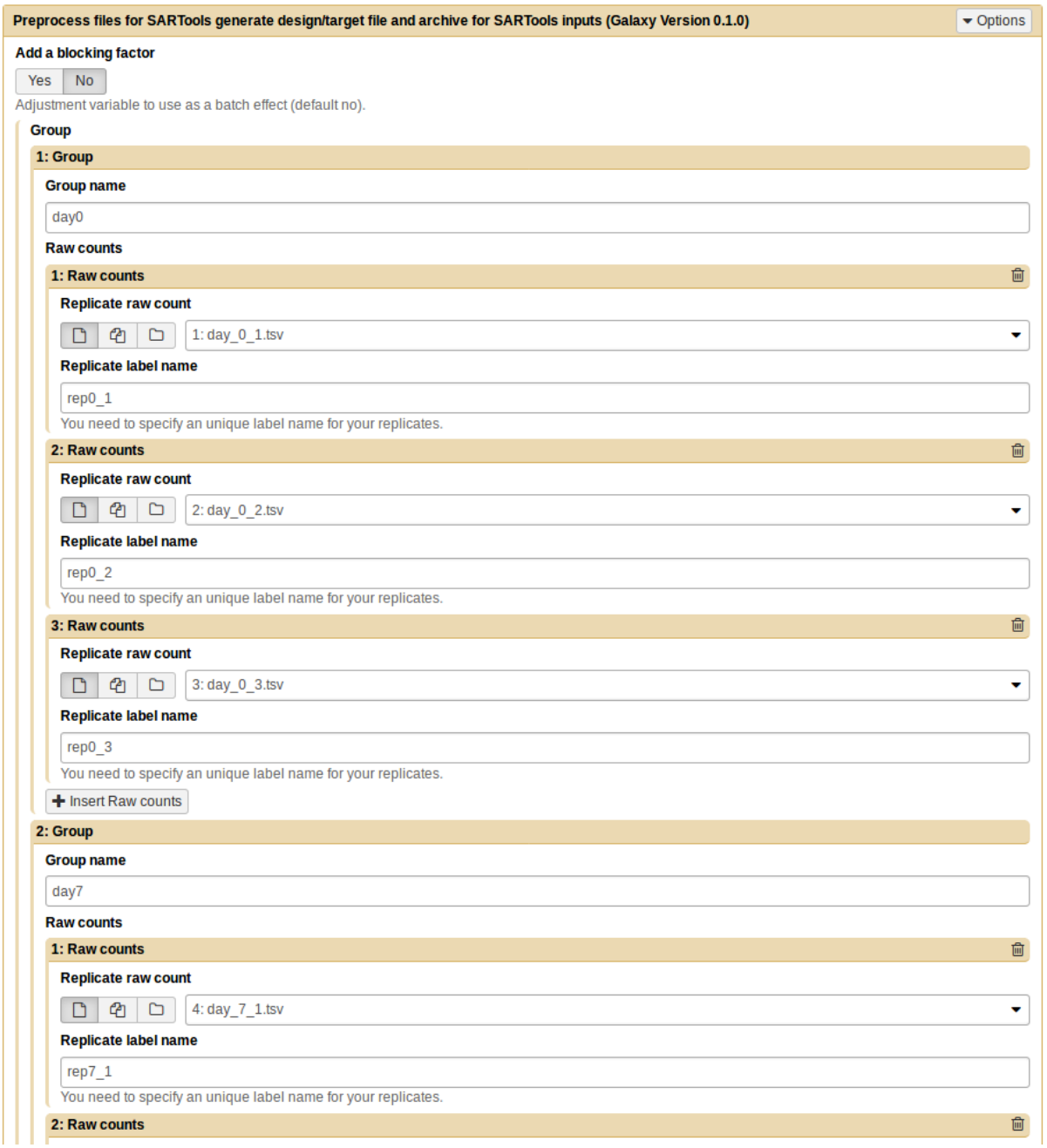
The data correspond to RNA-Seq count data for 6 replicates under 2 conditions (3 replicates per condition, day0 and day7).

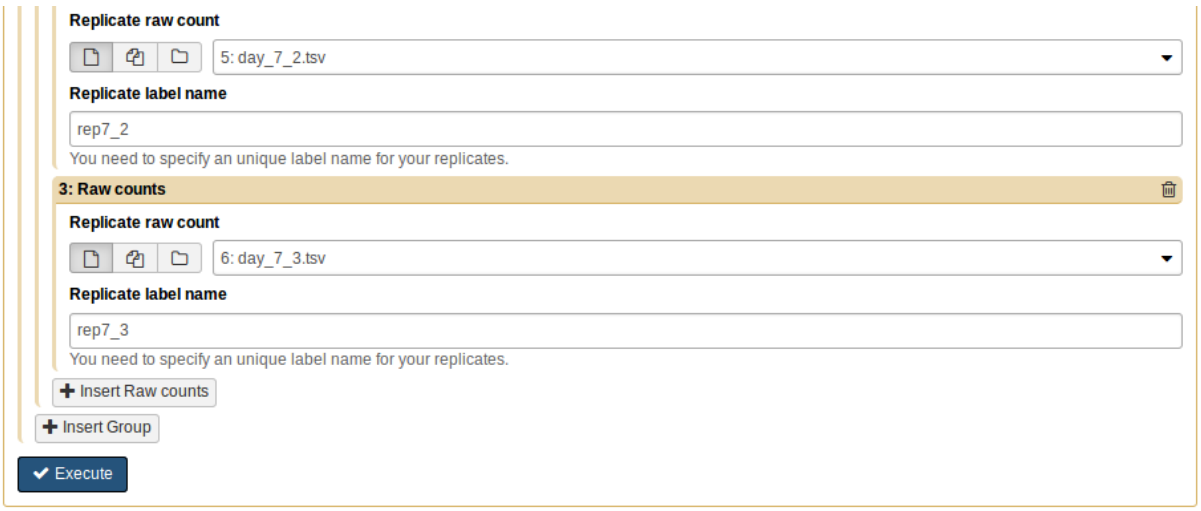
Preprocessing phase with SARTools.

Goal: This step creates datasets adapted to SARTools.

In the Tools panel, click the "preprocess files for SARTools" tool.

- Create 2 groups: day0 and day7 and add the 3 corresponding replicates to each of the 2 conditions (click "Insert Raw counts" to add a replicate to the groups)
- Choose different replicate names for each replicate (for example rep0_1, rep 0_2 and rep0_3 for the group day0 and rep7_1, rep 7_2 and rep7_3 for the group day7)





The tool returns 2 outputs

- a design file containing the conditions of the experiment in format txt

1	2	3
label	files	group
rep0_1	dataset_87.dat	day0
rep0_2	dataset_86.dat	day0
rep0_3	dataset_85.dat	day0
rep7_1	dataset_84.dat	day7
rep7_2	dataset_83.dat	day7
rep7_3	dataset_82.dat	day7

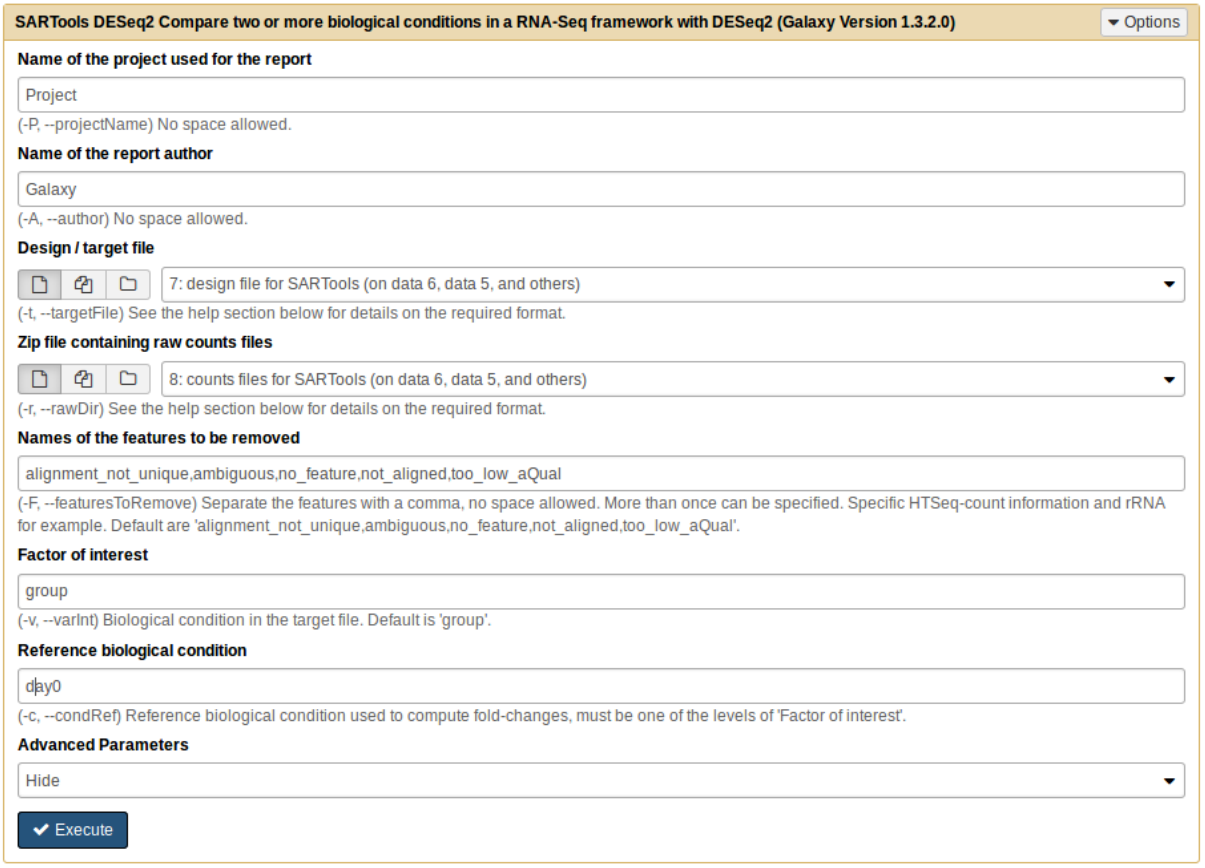
- a zip file containing all the count files.

Analysis with Sartools

Goal: Carry out the differential analysis.

In the panel tool, click on the "SARTools DESeq2" tool

- Fill in the design / target file and the Zip file containing the raw counts.
- In the field "Reference biological condition" enter the value "Day0" corresponding to the reference condition of the data.
- Leave the other fields unchanged.



The histogram of raw p-values has peaks in unexpected places. We restart the analysis with edgeR, keeping the same parameters as for DESeq2:

In the Tools panel, in the NGS: Differential Analysis part, click on the "SARTools edgeR" tool

- Fill in the design / target file and the Zip file containing the gross counts.
- In the field "Reference biological condition" enter the value "Day0" corresponding to the reference condition of the data.
- Leave the other fields unchanged.

SARTools edgeR Compare two or more biological conditions in a RNA-Seq framework with edgeR (Galaxy Version 1.3.2.0) Options

Name of the project used for the report

Project

(-P, --projectName) No space allowed.

Name of the report author

Galaxy

(-A, --author) No space allowed.

Design / target file

7: design file for SARTools (on data 6, data 5, and others)

(-t, --targetFile) See the help section below for details on the required format.

Zip file containing raw counts files

8: counts files for SARTools (on data 6, data 5, and others)

(-r, --rawDir) See the help section below for details on the required format.

Names of the features to be removed

alignment_not_unique,ambiguous,no_feature,not_aligned,too_low_aQual

(-F, --featuresToRemove) Separate the features with a comma, no space allowed. More than once can be specified. Specific HTSeq-count information and rRNA for example. Default are 'alignment_not_unique,ambiguous,no_feature,not_aligned,too_low_aQual'.

Factor of interest

group

(-v, --varInt) Biological condition in the target file. Default is 'group'.

Reference biological condition

day0

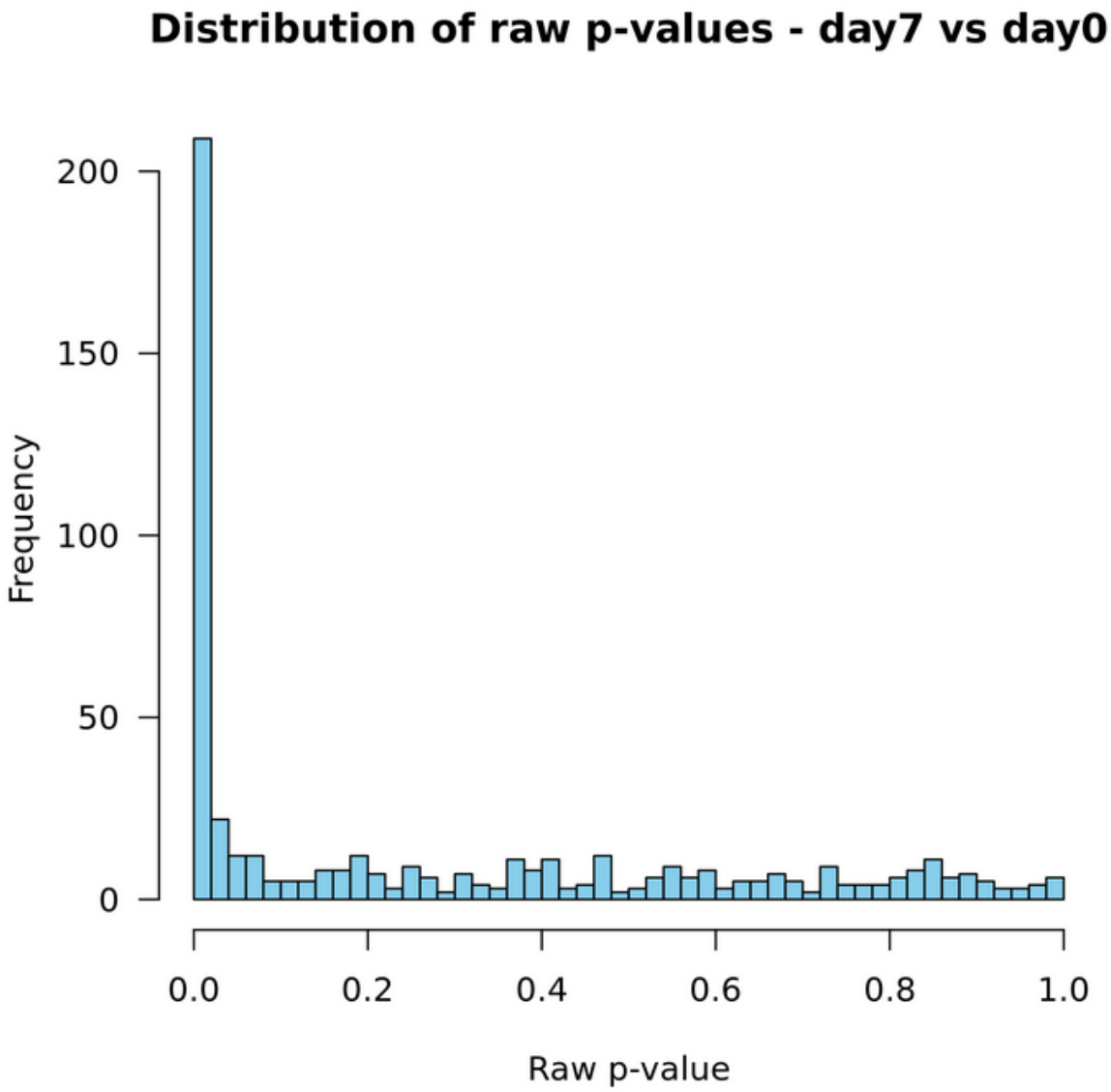
(-c, --condRef) Reference biological condition used to compute fold-changes, must be one of the levels of 'Factor of interest'.

Advanced Parameters

Hide

Execute

It now becomes clear that EdgeR is more suited to this dataset than DESeq2.



GSEA Analysis

Data Preparation

In order to perform the GSEA analysis of these data, we need to retrieve the identifiers of the differentially expressed genes. For this example we will focus on the overexpressed genes.

Reports generated by SARTools produce files that are not directly usable in Galaxy. So we will have to recover the file of interest and re-import it into Galaxy.

- Click on the "eye" icon of the "SARtools EdgeR tables" dataset, the following page appears.

Galaxy Tool SARTools_edgeR

Run at 17/08/2018 09:07:20

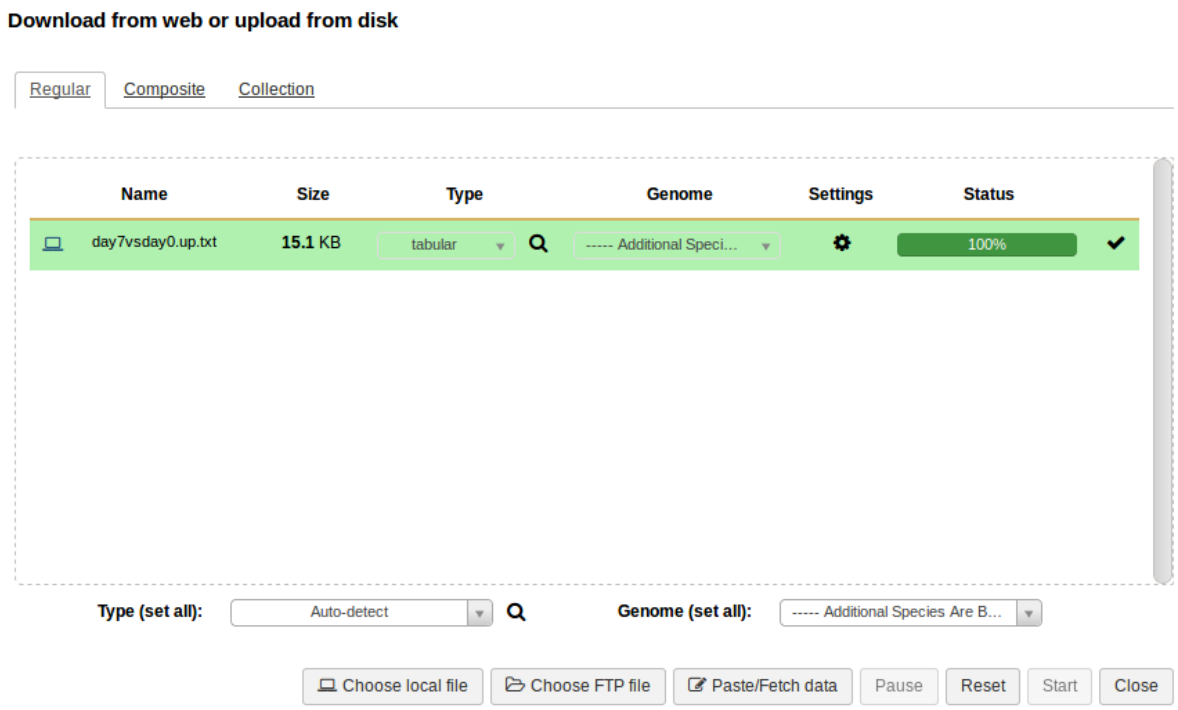
Tables available for downloading

Output File Name (click to view)	Size
day7vsday0.complete.txt	111.7 KB
day7vsday0.down.txt	14.2 KB
day7vsday0.up.txt	15.1 KB

With a right click of the mouse, we get the day7vsday0.up.txt file by clicking on "save link as".

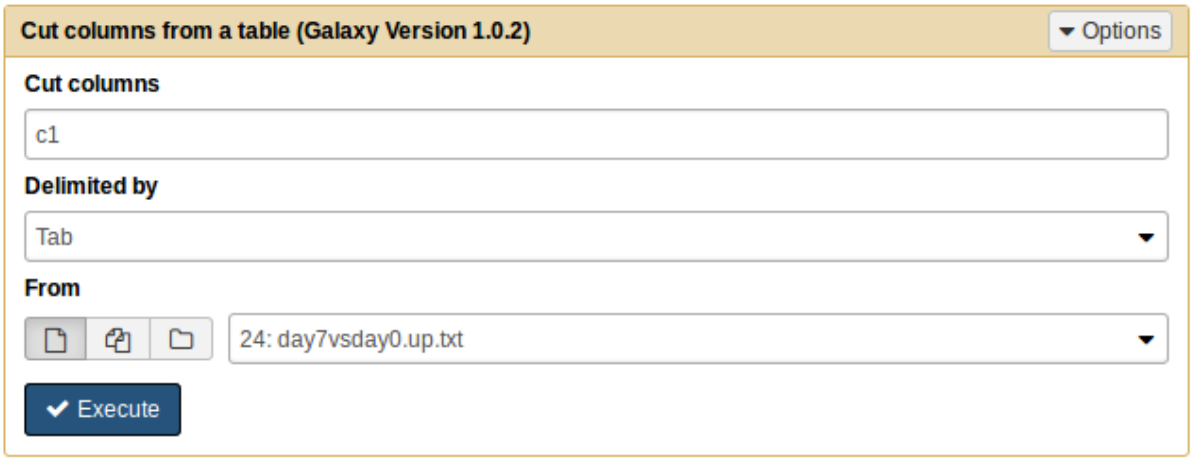
Then upload this file on the current Galaxy history :

- Click Get Data -> Upload in the panel tools
- Choose type "tabular" to make sure of its good display under Galaxy.



Once the file has been uploaded, the first column containing the identifiers of the differentially expressed genes is retrieved:

- Click on Text Manipulation -> Cut in the panel tools
- In the Cut columns field, enter "c1" to keep only the first column.



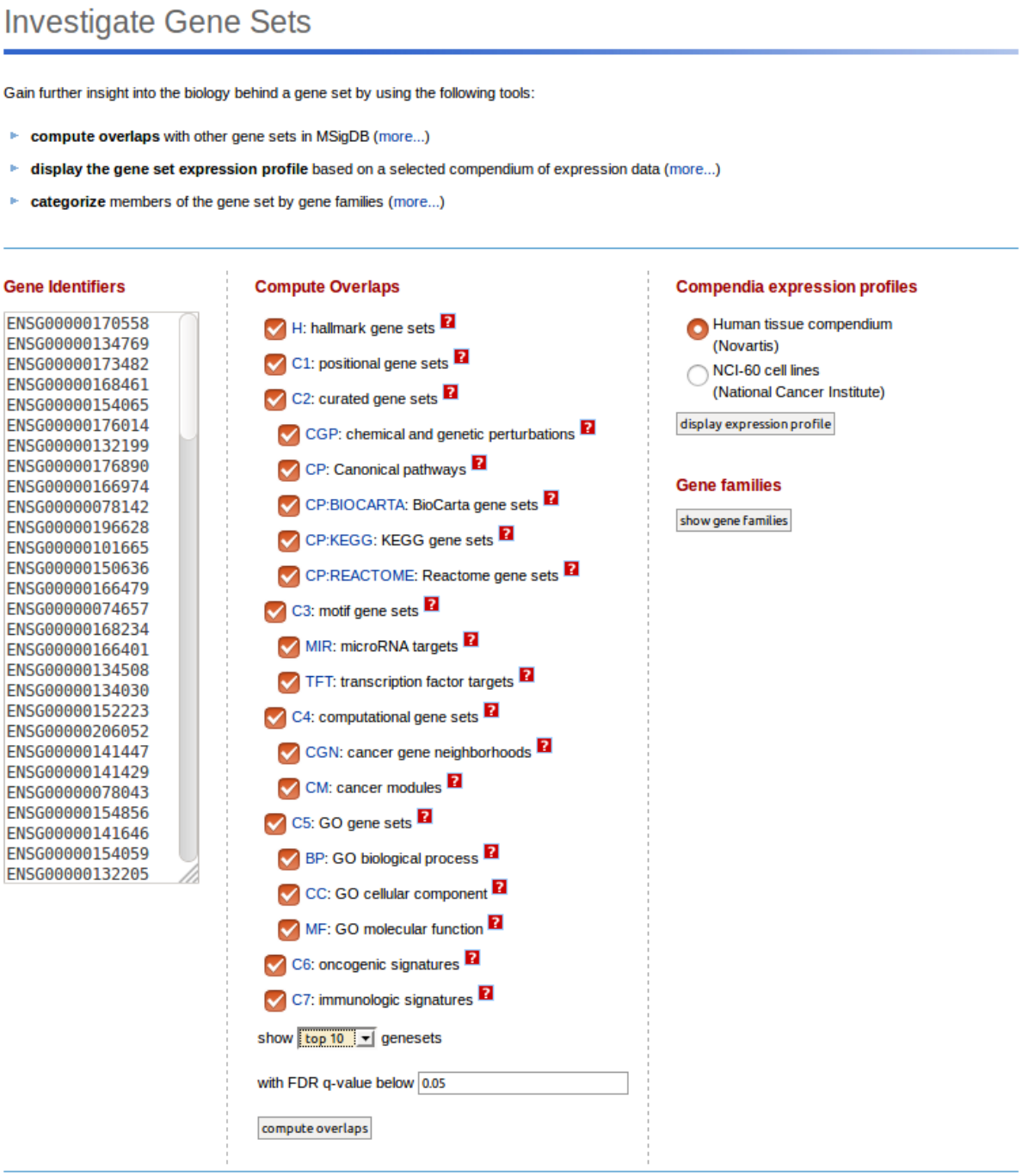
The list of ENSEMBL identifiers of the overexpressed genes is then obtained.

To carry out the GSEA analysis, go to the following link:
http://software.broadinstitute.org/gsea/msigdb/annotate.jsp

This analysis will make it possible to leave gene clusters whose genes are overrepresented among the list overexpressed genes.

Once identified on the site:

- Copy / paste the list of identifiers of our genes in the field on the left
- Select the gene sets of interest (in this example we select all the groups).
- Choose to display only the top 10 gene groups.



Clicking on "compute overlaps" gives the following results:

- The list of gene sets that are overrepresented in the list of differentially expressed genes

Compute Overlaps for Selected Genes

Converted 110 submitted identifiers into 77 entrez genes. click here for details.

Collections	# Overlaps Shown	# Gene Sets in Collections	# Genes in Comparison (n)	# Genes in Universe (N)
C1, C2, C3, C4, C5, C6, C7, H	10	17810	77	45956

Click the gene set name to see the gene set page. Click the number of genes [in brackets] to download the list of genes.

Color bar shading from light green to black, where lighter colors indicate more significant FDR q-values (< 0.05) and black indicates less significant FDR q-values (>= 0.05).

Save to: Excel | GenomeSpace

Gene Set Name [# Genes (K)]	Description	# Genes in Overlap (k)	k/K	p-value	FDR q-value
chr18p11 [149]	Genes in cytogenetic band chr18p11	16		$7.51\ e^{-25}$	$1.34\ e^{-20}$
chr18q21 [128]	Genes in cytogenetic band chr18q21	15		$6.15\ e^{-24}$	$5.48\ e^{-20}$
chr18q12 [70]	Genes in cytogenetic band chr18q12	13		$1.24\ e^{-23}$	$7.36\ e^{-20}$
chr18q22 [38]	Genes in cytogenetic band chr18q22	10		$4.32\ e^{-20}$	$1.92\ e^{-16}$
chr18q11 [55]	Genes in cytogenetic band chr18q11	9		$3.83\ e^{-16}$	$1.36\ e^{-12}$
chr18q23 [36]	Genes in cytogenetic band chr18q23	8		$1.24\ e^{-15}$	$3.69\ e^{-12}$
GO_SEQUENCE_SPECIFIC_DNA_BINDING [1037]	Interacting selectively and non-covalently with DNA of a specific nucleotide composition, e.g. GC-rich DNA binding, or with a specific sequence motif or type of DNA e.g. promotor binding or rDNA binding.	13		$1.77\ e^{-8}$	$4.5\ e^{-5}$
PILON_KLF1_TARGETS_DN [1972]	Genes down-regulated in erythroid progenitor cells from fetal livers of E13.5 embryos with KLF1 [GeneID=10661] knockout compared to those from the wild type embryos.	17		$2.24\ e^{-8}$	$4.99\ e^{-5}$
GO_TRANSCRIPTION_FROM_RNA_POLYMERASE_IE_II_PROMOTER [724]	The synthesis of RNA from a DNA template by RNA polymerase II, originating at an RNA polymerase II promoter. Includes transcription of messenger RNA (mRNA) and certain small nuclear RNAs (snRNAs).	11		$3.58\ e^{-8}$	$7.09\ e^{-5}$
GSE11924_TH2_VS_TH17_CD4_TCELL_DN [200]	Genes down-regulated in comparison of Th2 cells versus Th17 cells.	7		$4.95\ e^{-8}$	$8.81\ e^{-5}$

- The overlay matrix between over-expressed genes and gene clusters.

Entrez Gene Id	Gene Symbol	chr18p11	chr18q21	chr18q12	chr18q22	chr18q11	chr18q23	GO_SEQUENCE_SPECIFIC_DNA_BINDING	PILON_KLF1_TARGETS_DN	GO_TRANSCRIPTION_FROM_RNA_POLYMERASE_II_PROMOTER	GSE11924_TH2_VS_TH17_CD4_TCELL_DN	Entrez	Source	Gene Description
8731	RNMT	■							■	■	■		S	RNA (guanine-7-) methyltransferase
11031	RAB31	■							■				S	RAB31, member RAS oncogene family
9989	PPP4R1	■							■				S	protein phosphatase 4, regulatory subunit 1
65258	MPPE1	■							■				S	metallophosphoesterase 1
9984	THOC1	■								■			S	THO complex 1
8774	NAPG	■											S	N-ethylmaleimide-sensitive factor attachment protein, gamma
84617	TUBB6	■											S	tubulin, beta 6 class V
84034	EMILIN2	■											S	elastin microfibril interfacer 2
23253	ANKRD12	■											S	ankyrin repeat domain 12
9229	DLGAP1	■											S	discs, large (Drosophila) homolog-associated protein 1
5797	PTPRM	■											S	protein tyrosine phosphatase, receptor type, M
55556	ENOSF1	■											S	enolase superfamily member 1
7298	TYMS	■											S	thymidylate synthetase
10939	AFG3L2	■											S	AFG3 ATPase family gene 3-like 2 (S. cerevisiae)
147495	APCDD1	■											S	adenomatosis polyposis coli down-regulated 1
339290	LOC339290	■											S	uncharacterized LOC339290
4089	SMAD4		■					■	■	■			S	SMAD family member 4
4087	SMAD2		■					■	■	■			S	SMAD family member 2
4152	MBD1		■					■	■	■			S	methyl-CpG binding domain protein 1
6925	TCF4		■					■		■	■		S	transcription factor 4
55205	ZNF532		■					■					S	zinc finger protein 532
115701	ALPK2		■								■		S	alpha-kinase 2
4092	SMAD7		■								■		S	SMAD family member 7
2235	FECH		■								■		S	ferrochelatase
83473	KATNAL2		■										S	katanin p60 subunit A-like 2
57614	KIAA1468		■										S	KIAA1468
23335	WDR7		■										S	WD repeat domain 7
497661	C18orf32		■										S	chromosome 18 open reading frame 32
9811	CTIF		■										S	CBP80/20-dependent translation initiation factor
11201	POLI		■										S	polymerase (DNA directed) iota
5271	SERPINB8		■										S	serpin peptidase inhibitor, clade B (ovalbumin), member 8
30827	CXXC1			■				■					S	CXXC finger protein 1
7572	ZNF24			■				■					S	zinc finger protein 24
10982	MAPRE2			■					■				S	microtubule-associated protein, RP/EB family, member 2

Analysis of data from the recount project

Retrieving data via the recount tool

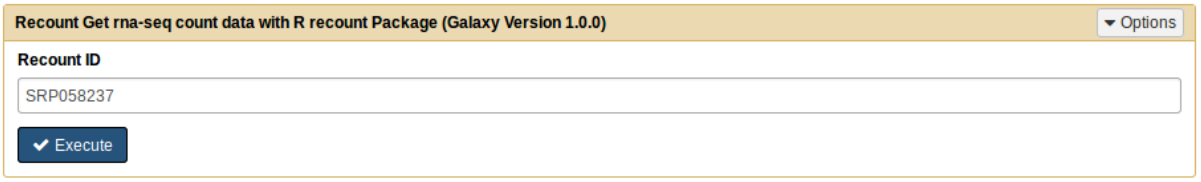
Goal: To retrieve count data via the recount2 tool (https://jhubiostatistics.shinyapps.io/recount/).

In this example we will treat the dataset SRP058237: This dataset contains 17 samples related to lung cancer.

- 2 conditions: Tumor (tumor cells) and adjacent (healthy cells taken next to the tumor)
- 3 types of cells (IMMCs, Neutrophil, Epithelial)

In the Tools panel, in the NGS: Differential Analysis part, click on the Recount tool .

- Fill in the "Recount ID" field using ID SRP058237.



The Recount tool returns 1 count file per sample, here 17 files, and 1 file summarizing the conditions of the samples.

1	2
"ENSG00000000003.14"	0
"ENSG00000000005.5"	0
"ENSG00000000419.12"	515
"ENSG00000000457.13"	91
"ENSG00000000460.16"	182
"ENSG00000000938.12"	13683
"ENSG00000000971.15"	136
"ENSG00000001036.13"	2538

Header of a count file generated by Recount.

Exercise: Run the differential analysis between tum-IMMC and adj-IMMC conditions.

GSEA analysis on the msigdb site

Preparation of the data:

In order to carry out the GSEA analysis, it is necessary to carry out some pre-treatments.

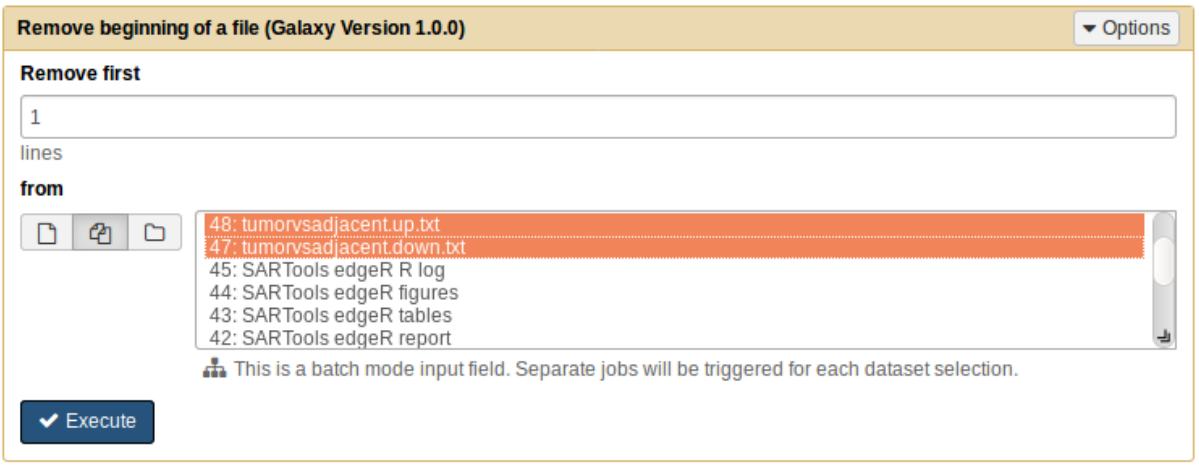
For this analysis we will retrieve the set of differentially expressed genes. First, it is necessary to retrieve the list of the differentially expressed genes generated by SARTools. We will proceed in the same way as in the previous chapter :

- Save the 2 files locally
- Reimport them under Galaxy using the upload tool.

Once reimported in Galaxy, it is necessary to concatenate and to modify the ENSEMBL identifiers because the broad institute website does not accept the suffixes of these identifiers.

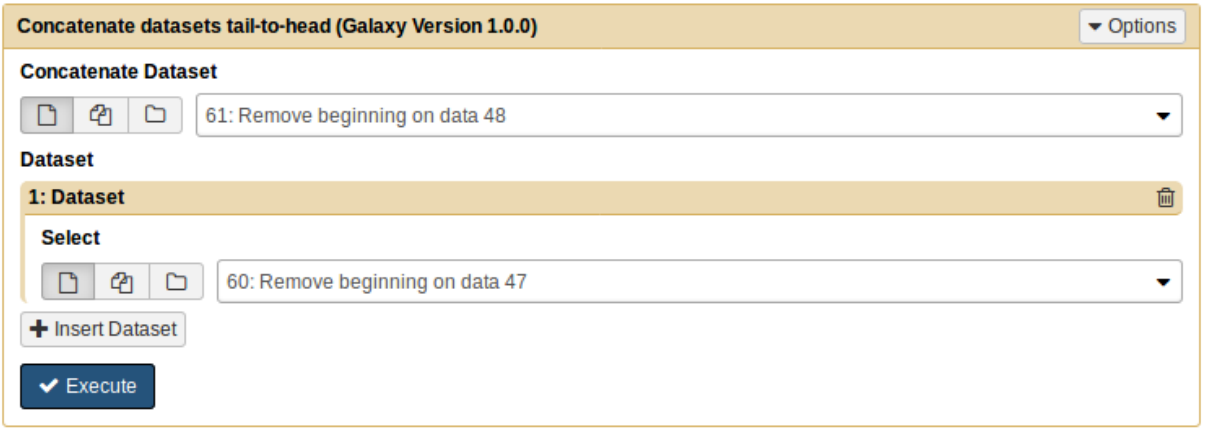
First we will delete the first header line of the file.
In the "Text Manipulation" section click on the "Remove beginning of a file" tool

- Enter "1" in the "Remove first" field
- Click on the icon  to select the 2 files to be processed

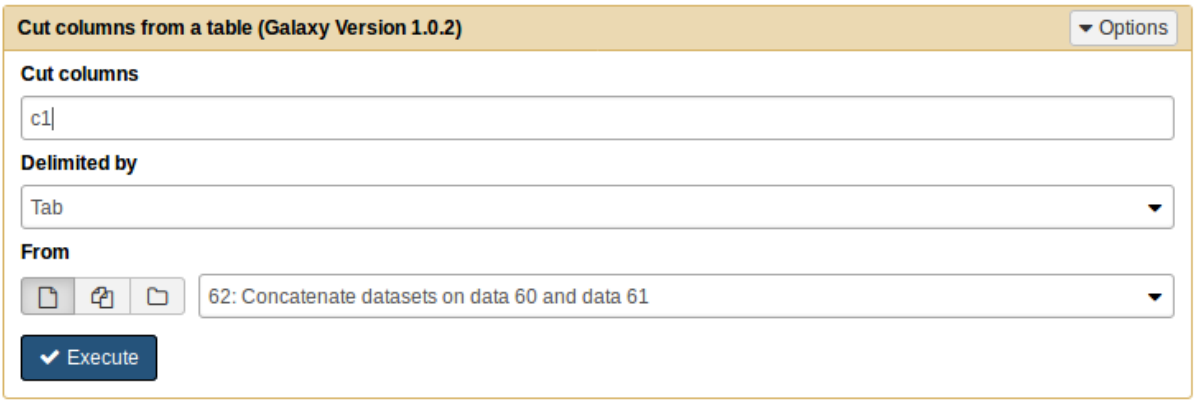


To concatenate the files, in the section "Text Manipulation" click on the tool "Concatenate datasets tail-to-head"

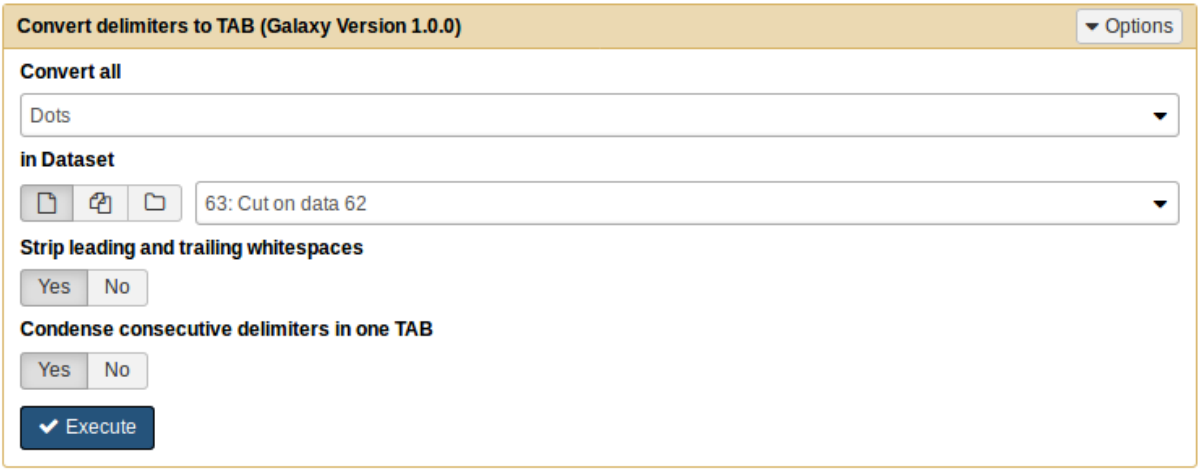
- Choose the 2 files corresponding to the 2 resulting files of the previous step



Then retrieve the first column of the resulting file with the tool "Cut" of the "Text Manipulation" section:



We get the list of differentially expressed genes, but the identifiers still contain the suffixes. To delete them, use the "convert" tool in the "Text Manipulation" section and replace the points with tabs:



Finally, use the “cut” tool again to get the first column of the last resulting file and you should get the list of ENSEMBL identifiers of differentially expressed genes.

1
ENSG00000116774
ENSG00000091409
ENSG00000131747
ENSG00000133063
ENSG00000134061
ENSG00000114251
ENSG00000262406
ENSG00000088325
ENSG00000166165
ENSG00000143195
ENSG00000143891
ENSG00000117394

Exercise: Run the GSEA analysis