Functional analysis of RNA-seq data

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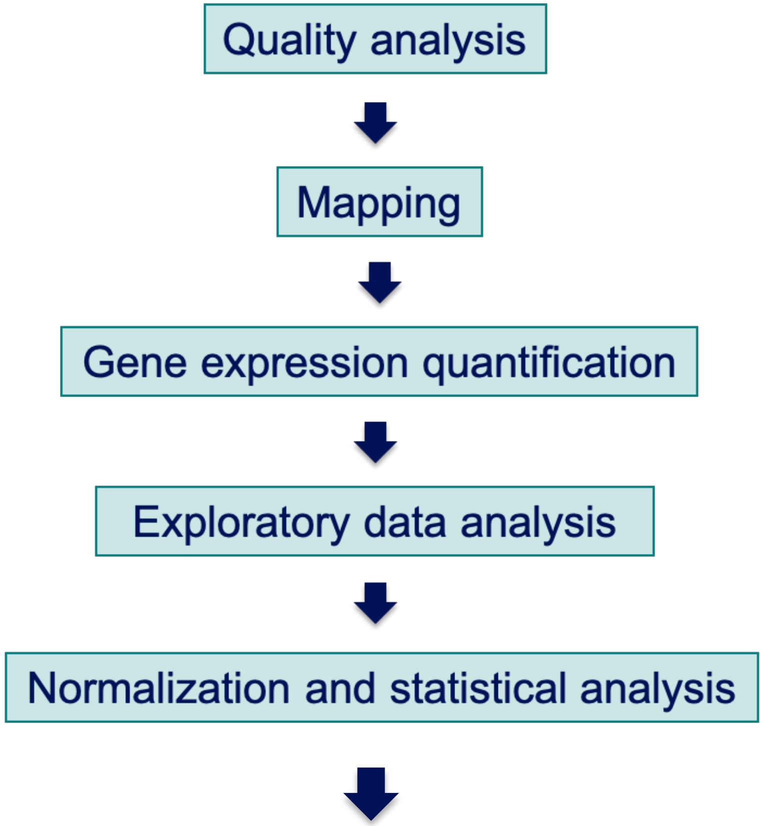
Analysis of RNA-seq data



Quality analysis

Mapping

Gene expression quantification

Exploratory data analysis

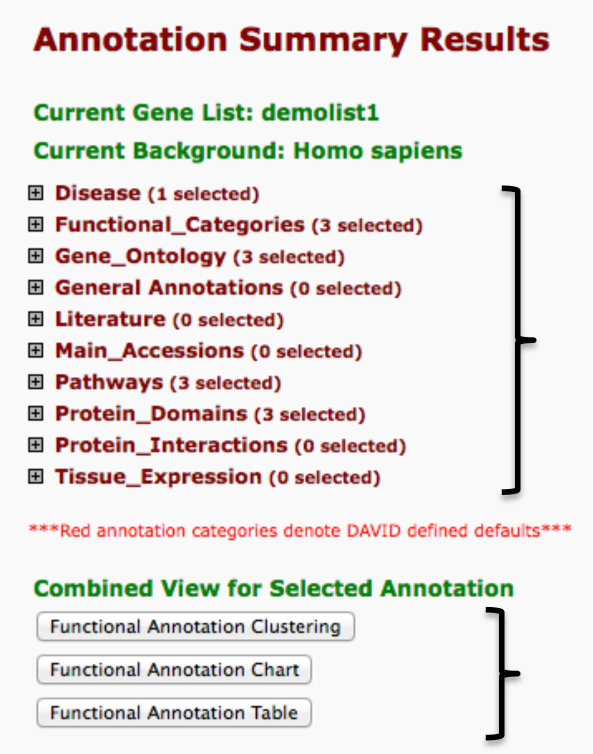
Normalization and statistical analysis

Functional enrichment analysis, pathway analysis, integration with other data, …

Functional analysis

- A lot of functional analysis tools available
  - Initially developed for microarray data
  - e.g. GO tools listed in
    http://geneontology.org/docs/go-enrichment-analysis/
  - Methods specific to RNA-seq data
    - Bioconductor packages
      - Goseq (Young et al., Genome Biology 2010;11:R14)
      - SeqGSEA (Wang et al. BMC Bioinformatics 2013, 14(Sup5):S16)
    - GSAASeqSP (Xiong et al Scientific Reports 2014; 4:6347)
- DAVID will be used for this practical session because
  - graphical interface & free software
- DAVID
  - **D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery
  - https://david.ncifcrf.gov/
  - A very interested article describing how to use DAVID :
    Huang et al. Nature Protocols 2009;4(1):44-57.

DAVID



Annotation Summary Results

Current Gene List: demolist1

Current Background: Homo sapiens

- ⊞ **Disease** (1 selected)
- ⊞ **Functional_Categories** (3 selected)
- ⊞ **Gene_Ontology** (3 selected)
- ⊞ **General Annotations** (0 selected)
- ⊞ **Literature** (0 selected)
- ⊞ **Main_Accessions** (0 selected)
- ⊞ **Pathways** (3 selected)
- ⊞ **Protein_Domains** (3 selected)
- ⊞ **Protein_Interactions** (0 selected)
- ⊞ **Tissue_Expression** (0 selected)

Red annotation categories denote DAVID defined defaults

Combined View for Selected Annotation

Functional Annotation Clustering
Functional Annotation Chart
Functional Annotation Table

Different sources of annotation

- Disease (OMIM)
- Gene Ontology
- Pathways (KEGG, Biocarta)
- Protein Domains (InterPro, SMART)
- Protein Interaction (BIND)
- …

Different tools

- Functional Annotation Clustering
  - Cluster functionally similar terms associated with a gene list into groups
- Functional Annotation Chart
  - Identify enriched annotation terms associated with a gene list
- Functional Annotation Table
  - Query associated annotations for all genes from a list

Exercise : functional analysis

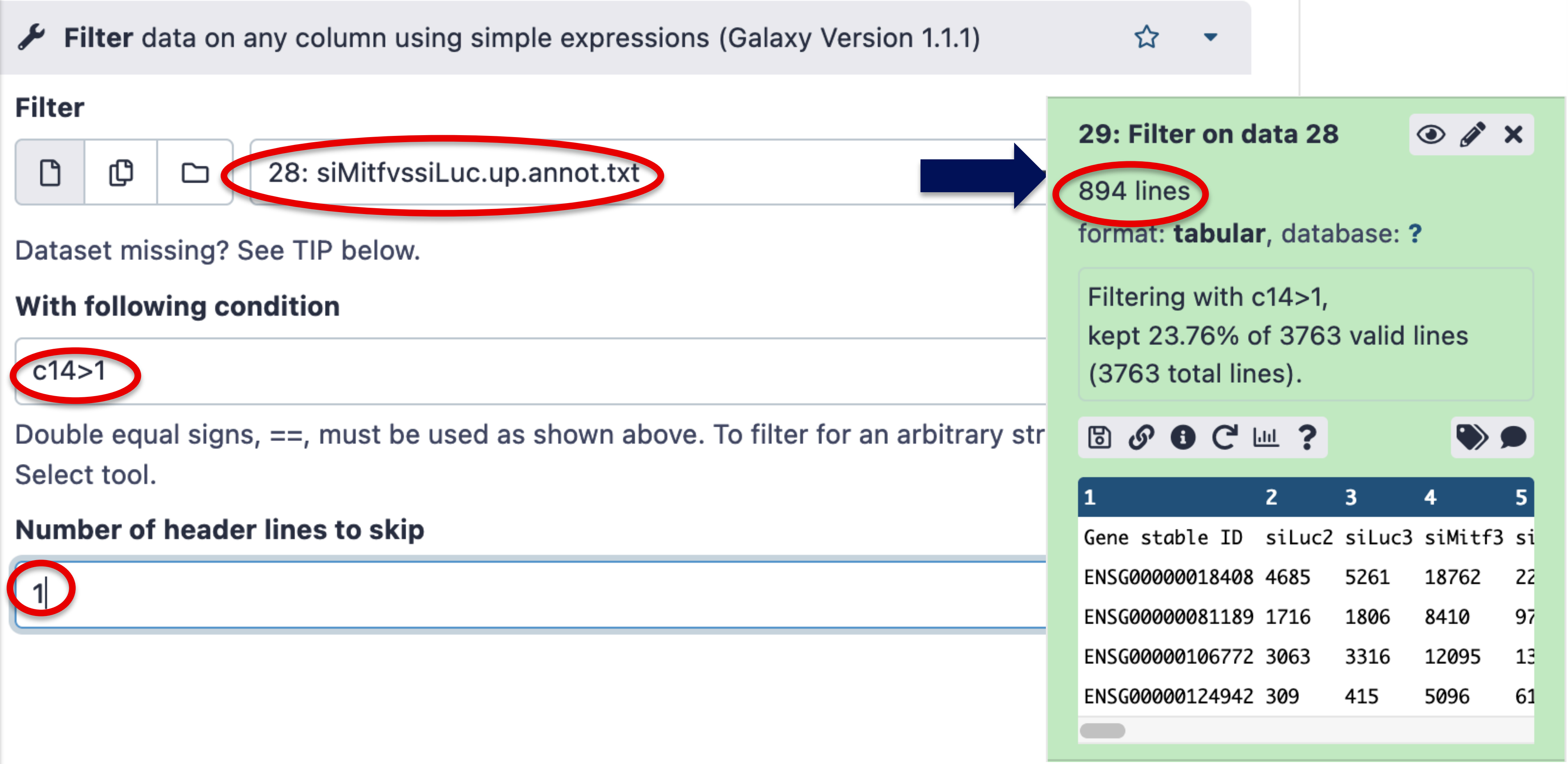
- Use DAVID to perform functional analysis of genes significantly over-expressed in siMitf vs siLuc samples
  - Using the thresholds : adjusted p-value < 0.05 and $\log_2$(Fold-Change) > 1
- For this purpose :
  1. Select over-expressed genes using the **Filter** tool on Galaxy
     - Input dataset : siMitfvssiLuc.up.annot.txt

       In your history or dataset 21 in “NGS data analysis training Strasbourg” history
     - Threshold : $\log_2$(Fold-Change) > 1

       Indeed, genes in siMitfvssiLuc.up.annot.txt file have already been selected with adjusted p-value < 0.05
       (cf “Threshold of statistical significance” in SARTools advanced parameters)
  2. Create a file with gene name for all these genes using the **Cut** tool on Galaxy
  3. Analyse this gene list using DAVID

1. Select over-expressed genes

- Among significantly differentially expressed genes, select genes with $\log_2$(Fold-Change) > 1



Filter data on any column using simple expressions (Galaxy Version 1.1.1)

Filter

28: siMitfvssiLuc.up.annot.txt

Dataset missing? See TIP below.

With following condition

c14>1

Double equal signs, ==, must be used as shown above. To filter for an arbitrary str
Select tool.

Number of header lines to skip

1

29: Filter on data 28

894 lines

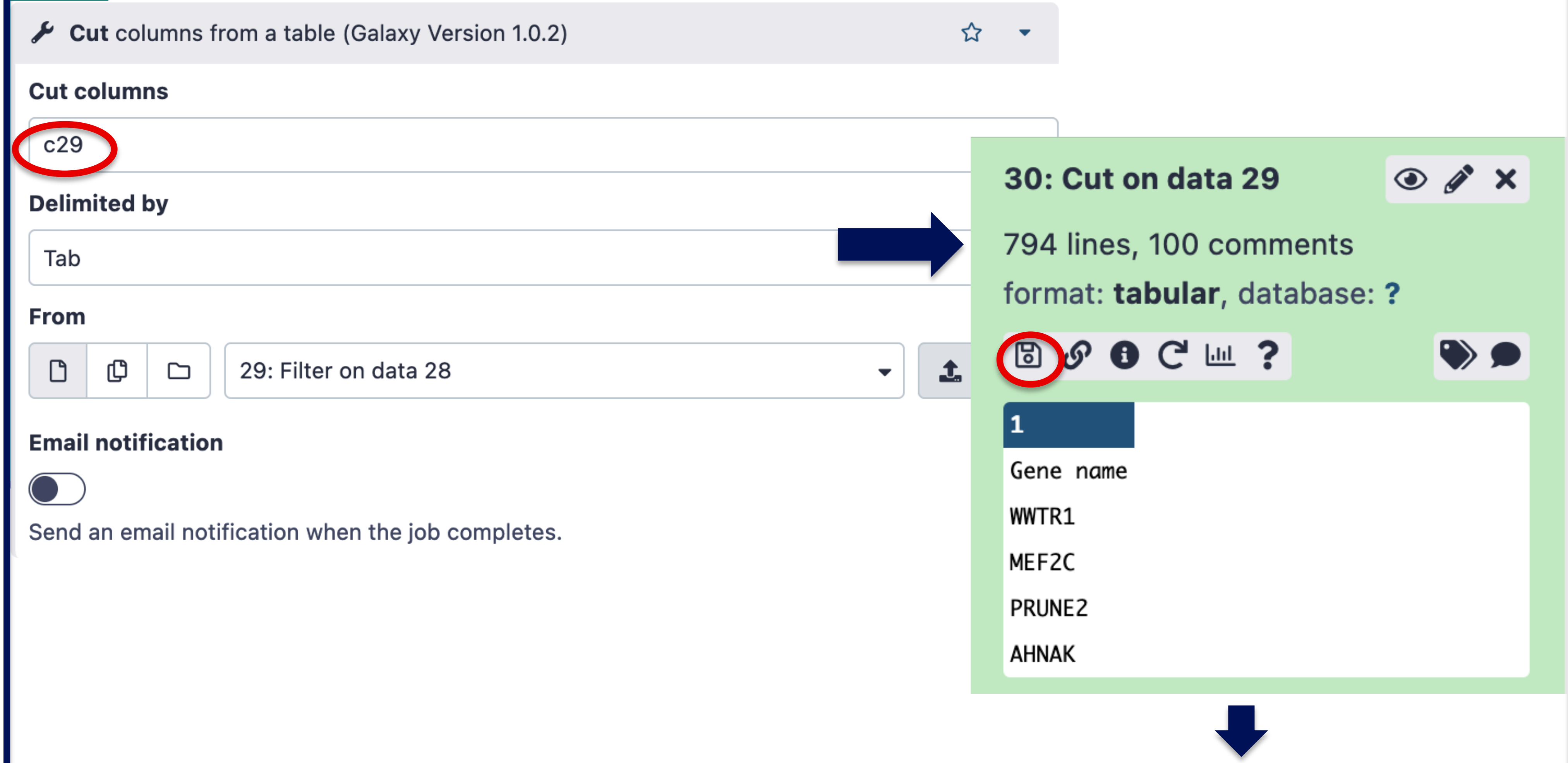
format: **tabular**, database: **?**

Filtering with c14>1,
kept 23.76% of 3763 valid lines
(3763 total lines).

1	2	3	4	5
Gene stable ID	siLuc2	siLuc3	siMitf3	si
ENSG00000018408	4685	5261	18762	22
ENSG00000081189	1716	1806	8410	97
ENSG00000106772	3063	3316	12095	13
ENSG00000124942	309	415	5096	61

2. Create a list of gene names

- Select associated gene names in the previous table



Cut columns from a table (Galaxy Version 1.0.2)

Cut columns

c29

Delimited by

Tab

From

29: Filter on data 28

Email notification

Send an email notification when the job completes.

30: Cut on data 29

794 lines, 100 comments

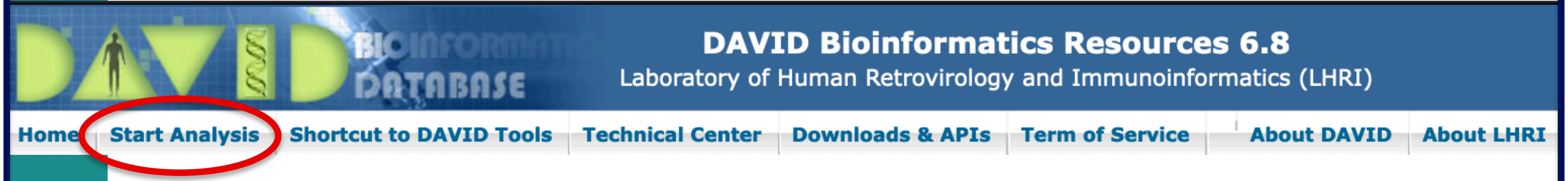
format: **tabular**, database: **?**

1
Gene name
WWTR1
MEF2C
PRUNE2
AHNAK

siMitfvssiLuc_upgenes_lfc1_padj005**.txt** file

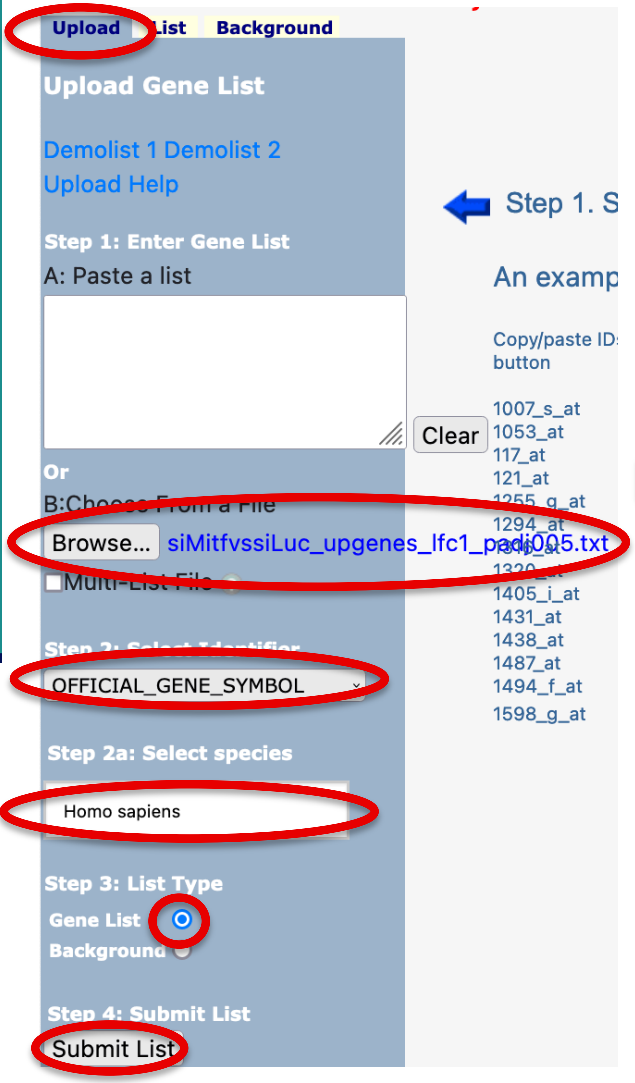
3. Analyse your gene list using DAVID

- Go to https://david.ncifcrf.gov
- Click on Start Analysis



3. Analyze your gene list using DAVID

- Enter your gene list



Upload List Background

Upload Gene List

Demolist 1 Demolist 2

Upload Help

Step 1: Enter Gene List

A: Paste a list

Clear

Or

B:Choose From a File

Browse... siMitfvssiLuc_upgenes_lfc1_padj005.txt

☐ Multi-List File

Step 2: Select Identifier

OFFICIAL_GENE_SYMBOL

Step 2a: Select species

Homo sapiens

Step 3: List Type

Gene List ●

Background ○

Step 4: Submit List

Submit List

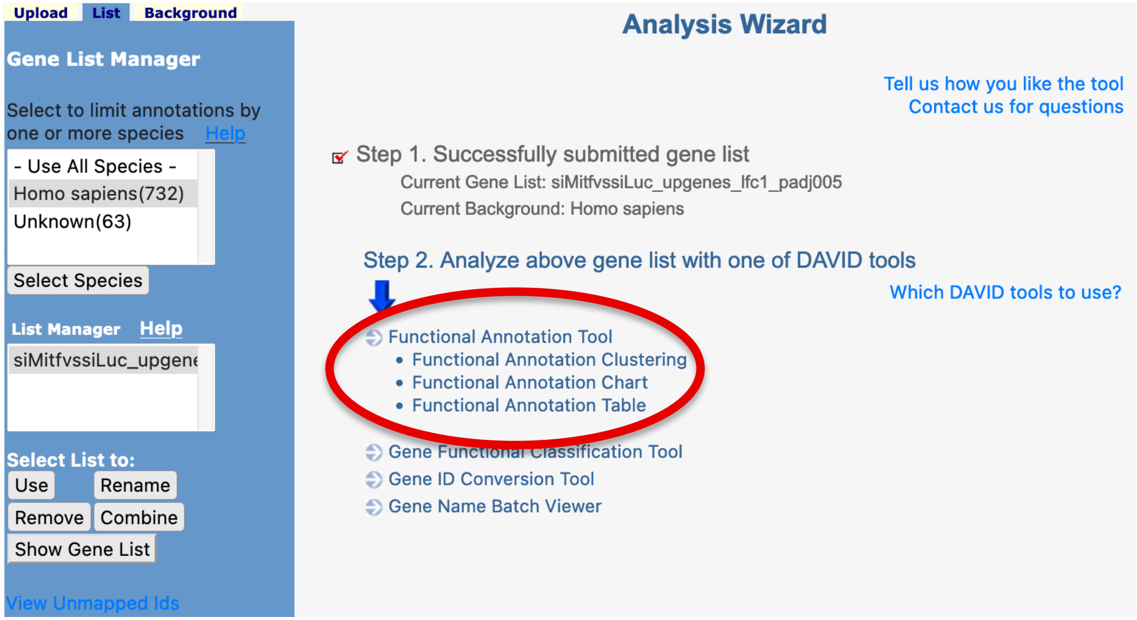
Step 1. S

An examp

Copy/paste ID
button

1007_s_at
1053_at
117_at
121_at
1255_g_at
1294_at
1316_at
1320_at
1405_i_at
1431_at
1438_at
1487_at
1494_f_at
1598_g_at

- Analyze your list



Upload List Background

Gene List Manager

Select to limit annotations by one or more species Help

- Use All Species -
Homo sapiens(732)
Unknown(63)

Select Species

List Manager Help

siMitfvssiLuc_upgene

Select List to:

Use Rename
Remove Combine
Show Gene List

View Unmapped Ids

Analysis Wizard

Tell us how you like the tool
Contact us for questions

☑ Step 1. Successfully submitted gene list

Current Gene List: siMitfvssiLuc_upgenes_lfc1_padj005

Current Background: Homo sapiens

Step 2. Analyze above gene list with one of DAVID tools

Which DAVID tools to use?

Functional Annotation Tool
- Functional Annotation Clustering
- Functional Annotation Chart
- Functional Annotation Table

Gene Functional Classification Tool

Gene ID Conversion Tool

Gene Name Batch Viewer

Exercise : functional analysis

1. What are the 10 most enriched functional annotation terms among annotations of the genes from your list ?

   How many genes are annotated with each of these terms ?

   Which genes are annotated with the most enriched GO term ?

2. *KIT ligand* (*KITLG*) gene is annotated with this GO term.

   What are all associated annotations for this gene ?

   Among these annotations you will find the KEGG pathway "PI3K-Akt signalling pathway".
   Are other genes from your list member of this pathway ?

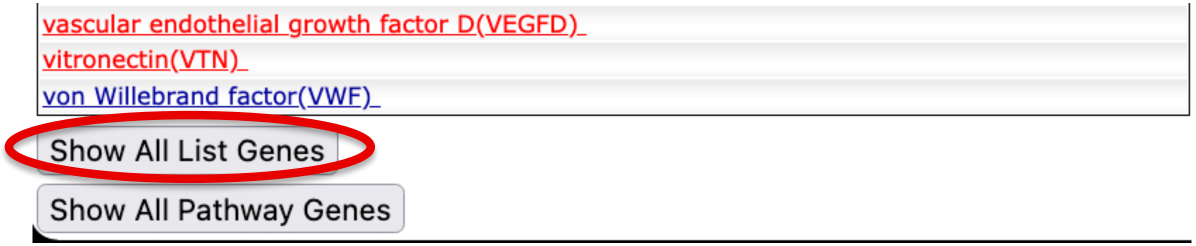
Exercise : functional analysis

3. We would like to represent on an heatmap the variation of expression of all these genes (list genes in PI3K-Akt signalling pathway) in the four samples

   → Prepare a file with the normalized read counts for these genes in all samples using Galaxy, and use Heatmapper (http://www.heatmapper.ca/expression/) to perform the heatmap

   1. Download list genes in PI3K-Akt signalling pathway from DAVID :

      Click on "Show all list genes" on the bottom of the page representing PI3K-AKT signalling pathway*

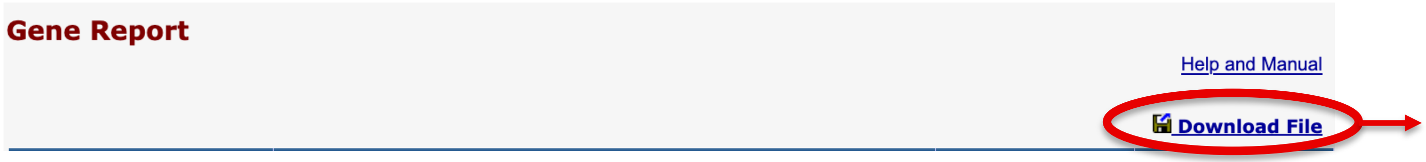


vascular endothelial growth factor D(VEGFD)

vitronectin(VTN)

von Willebrand factor(VWF)

then right click on Download File (top right) and save link target on disk



→ pi3k_akt_signalling_genes.txt

* You should be on this page at the end of question 2. Otherwise you will find this page in DAVID Functional Annotation Table by searching « PI3K » and clicking on the corresponding link (PI3K-Akt signalling pathway)

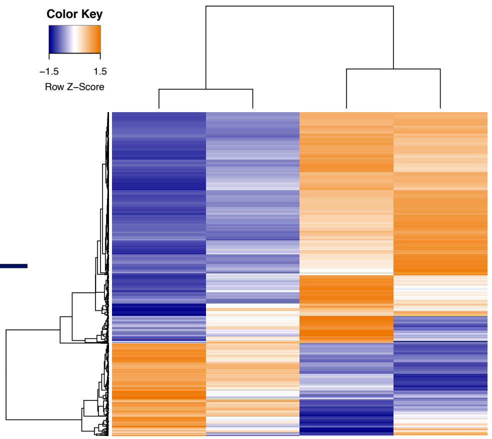
Exercise : functional analysis

We will join the file obtained at step 1 with siMitfvssiLuc.up.annot.txt using the common column (containing gene symbol) → We will thus retain only PI3K-Akt signalling genes from siMitfvssiLuc.up.annot.txt file.

2. Import pi3k_akt_signalling_genes.txt file on Galaxy
3. On Galaxy, join siMitfvssiLuc.up.annot.txt with pi3k_akt_signalling_genes.txt on their common column (Gene name)
4. On Galaxy, prepare a file with 5 columns : Gene name and four columns containing normalized read counts in the four samples (use the **Cut** tool and the results obtained at step 4).
5. Download this file and change file extension to txt
6. Use this file to perform an heatmap representing the variation of expression of these genes in the four RNAseq samples using Heatmapper (http://www.heatmapper.ca/expression/)

   after changing the name of the first column to NAME

Heatmap and clustering



- Heatmap

  Colour-scaled representation of the data

  Data represented :

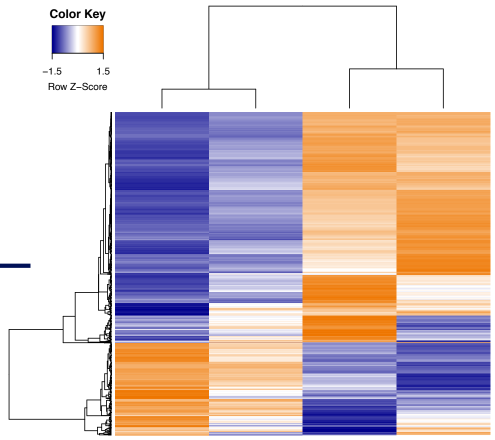
  - Expression
    - Normalized and divided by gene length

    → to compare the expression level of several genes
  - Expression variation
    - $\log_2$(Fold-Change)

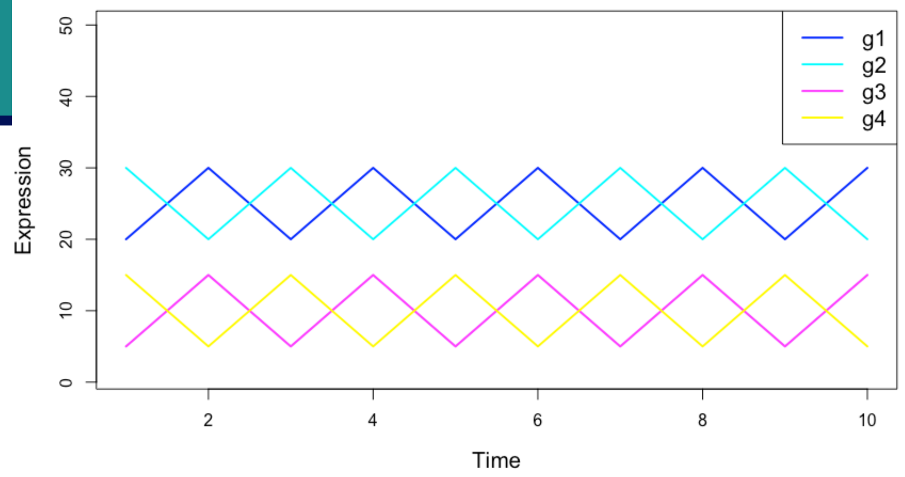
    log2 → over- and under-expression are on symmetric scales
    - Z-score

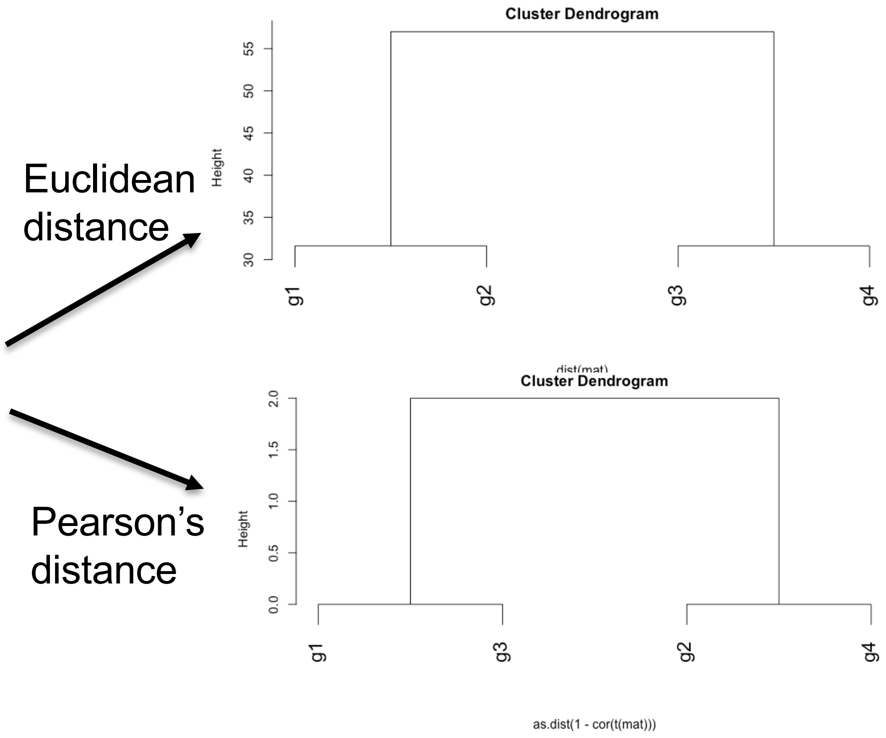
    → row z-score = [Value – mean(row)] / standard deviation(row)

Heatmap and clustering



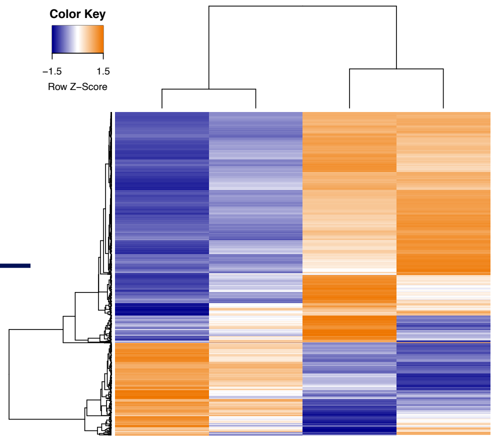
- Hierarchical clustering
  - Distance measure
    - Pairwise distance of all data points
    - Default in a lot of clustering software : Euclidean
    - If you want to group genes with similar expression patterns (i.e. on the shape of the expression profiles) : 1-correlation




Euclidean distance

Pearson’s distance

Heatmap and clustering



- Hierarchical clustering
  - Distance measure
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    - If you want to group genes with similar expression patterns (i.e. on the shape of the expression profile) : 1-correlation
    - To group points
  - Clustering method
    - To join groups of points
    - Average : distance between two groups = average distance between all pairs of points from the two different groups