IRIS-EDA Supplementary Material

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Abstract

Next-Generation Sequencing has made available much more large-scale genomic data. Studies with RNA-sequencing (RNA-seq) data typically involve generation of genetic expression profiles that can be further analyzed, many times involving differential gene expression (DGE). This process enables a comparison across samples of two or more factor levels. A recurring issue with DGE analyses is the complicated nature of the comparisons to be made, in which a variety of factor combinations, pairwise comparisons, and main or blocked main effects need to be tested. Here we present a tool called IRIS-EDA, which is a server-based DGE tool developed using Shiny providing a straightforward, user-friendly platform for performing comprehensive DGE analysis. This tool provides crucial analyses that help design hypotheses and to determine key genomic features. IRIS-EDA integrates the three most commonly used R-based DGE tools to determine differentially expressed genes (DEGs) and includes numerous methods for performing preliminary analysis on user-provided genetic expression information. Additionally, this tool integrates a variety of visualizations, in a highly interactive manner, for improved interpretation of preliminary and DGE analyses.

Contents

S1: Citation information

Table 1: A comparative overview of citation counts for DGE tools and DGE servers. DGE analytical tools (*Tool*) are compared based on the following criteria: Current number of citations (*Citations*), percentage of total citations from the analytical tools presented (*Citation %*), year the analytical tool was published (*Year*), approximate citations per year based on data accrued through 2017 (*Citations/Year*), and if the analytical tool has an R-based application (*R-based*).

Tool	Citations	Citation %	Year	Citations/Year (through 2017)	R-based?
edgeR (Robinson et al. 2010)	7093	32.39700375	2010	1013.285714	Yes
Cuffdiff (Trapnell et al. 2012)	4533	20.70430255	2012	906.6	No
Cuffdiff2 (Trapnell et al. 2013)	1508	6.887731799	2013	377	No
DESeq2 (Love et al. 2014)	4249	19.40714351	2014	1416.333333	Yes
limma (Ritchie et al. 2015)	2399	10.95733991	2015	1199.5	Yes
DEGseq (Wang et al. 2009)	1237	5.649949758	2009	154.625	Yes
baySeq (Hardcastle et al. 2010)	562	2.56691331	2010	80.28571429	Yes
SAMseq (Li et al. 2013)	275	1.256051886	2013	68.75	Yes
NOIseq (Tarazona et al. 2012)	38	0.173563533	2012	7.6	Yes

S2: Accessibility

IRIS-EDA can be freely accessed directly through this link or through R using the following code in the proceeding sections.

S2.1: Install CRAN packages

IRIS-EDA requires several packages to operate. To get these pacakages, the following commands can be entereed into an R terminal to check if you already have the necessary packages. If not, the following code will install any missing packages:

```
# CRAN
packages <- c(
    "crosstalk", "dplyr", "DT", "gtools", "plotly", "shiny", "plyr",
    "shinyBS", "shinycssloaders", "shinythemes", "tibble", "tidyr",
    "Rcpp", "Hmisc", "ggplot2", "locfit", "GGally", "pheatmap",
    "reshape2", "backports", "digest", "fields", "psych", "stringr",
    "tools", "openxlsx", "Rtsne", "WGCNA", "flashClust", "paraellel",
    "MCL", "kmed", "ape"
)
np <- packages[!(packages %in% installed.packages()[, "Package"])]
if(length(np)) install.packages(np)
```

S2.2: Install Bioconductor packages

You will also need several Bioconductor packages. Similar to the prior section, the following code will check and install any missing Bioconductor packages into your R library:

```
# Bioconductor
bioc.packages <- c("DESeq2", "edgeR", "limma", "QUBIC")
np <- bioc.packages[!(bioc.packages %in% installed.packages()[,"Package"])]
source("https://bioconductor.org/biocLite.R")
if (length(np)) biocLite(np)
```

S2.3: Run the Shiny application

Once you have installed all of the necessary packages, you can now run the application locally by entering the following code into an R terminal:

```
shiny::runGitHub("iris", "btmonier")
```

Typically, the link will provide an easier route to using IRIS-EDA. In circumstances where internet connections will be limited (such as during travel), loading IRIS-EDA through R while internet is still available will allow users to utilize IRIS-EDA without an internet connection later on.

Installing the local application via GitHub will also let the user have access to more "developmental" versions of IRIS-EDA. Be warned though; using developmental versions of this application may be "cutting edge", however, this could potentially break various features in the application. Therefore, using the server (http://bmbl.sdstate.edu/IRIS/) will provide a more "stable", peer-reviewed release of the application.

S3: Single-cell RNA-seq

S3.1: Overview

Upon sample submission to IRIS-EDA, you have two options: (1) Perform analyses on a normal RNA-seq experiment or (2) run single-cell RNA-seq analysis.

If you choose the latter option, you will need to provide a file containing ID lengths to help reduce variability. This is crucial since filtration of low TPM (transcripts per million) reads can lead to better results. In order to calculate TPM, ID length approximations are needed in conjunction with raw count data. To submit this information to IRIS-EDA, **you will need to provide ID data that follows the proceeding criteria**:

```
id        id_len_kbp
gene001          1.4
gene002          5.6
gene003          0.8
...              ...
```

Note: This file must be in a comma seperated value (CSV) format, where column 1 is the ID (e.g. gene, transcript, etc.) and column 2 is the approximation of length in **kilobase pairs**.

S3.2: A method to obtain this data

There are many ways to obtain these values. A common procedure would be to parse the respective general fearture format version 3 (GFF3) file and determine the length through calculating the difference between the start and end locations.

If you need help parsing this information, a primitive `R` function has been made, which you can find here. To run this function, you will need to install and load 3 packages into your R library:

- `ape`
- `stringr`
- `dplyr`

All of these packages can be found on the CRAN repository, or by using the `install.packages()` function provided in the base package of `R`.

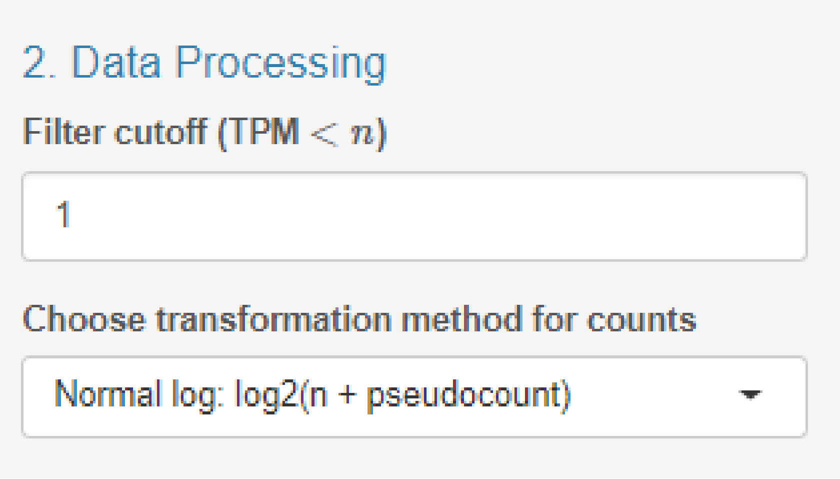
This function has four parameters:

- `gff`: the location to the GFF3 file on your computer
- `cts`: your raw count matrix that you will provide to IRIS-EDA. **Note**: this object must be a `matrix` type and have the same format that is found in the first section of this walkthrough (*A note about input data*).
- `type`: the GFF3 type column identifier (e.g. gene, transcript, exon, CDS, etc.)
- `attribute`: the GFF3 attribute (e.g. `ID`, `gene_id`, `transcript_id`, `gene_name`, or `transcript_name`)

Depending on the size of this file, this may take some time. This function will return a `tibble` data frame, so make sure you assign it to an object before you use `write.csv()`. After you perform this task, the file can successfully be submitted to IRIS-EDA.

S3.3: TPM filtration

Once you have submitted the data, you will notice that the `Filter cutoff` changes from `count data row sums` to `TPM`:



2. Data Processing

Filter cutoff (TPM < n)

1

Choose transformation method for counts

Normal log: log2(n + pseudocount)

The default is set to a value of `1`, however, this can be changed at the user's discretion.

Note (1): this value corresponds to which rowsums (i.e. ID sums) will be filtered out if they have a value that is less than the user parameter.

Note (2): "For users with 10X scRNA-Seq data or other data type with expected low expression across all genes, we have a submission option that changes default parameterizations to account for this. If submitting 10X scRNA data, please use the `scRNA - 10X Genomics` option.

S4: A note about input data

IRIS-EDA requires two pieces of information for analysis. The first is an expression estimation matrix, also referred to as a count matrix, displaying the gene expression estimates for each sample. The format requires a CSV file with the row names to list the gen IDs and column names to list the sample IDs. The second required input is a condition matrix, wherein the factor levels for each sample are provided. This file requires a CSV format and row names to be the sample IDs matching the sample IDs from the expression estimation matrix and the column names to be the condition factors.

The data used for this tutorial are derived from 28 *Vitis vinifera* (grape) samples with three distinct factors (Rootstock, row, and block). This data can viewed as the "big" example data set found under the `Submit and QC` tab under `1. Submission Parameters`.

S4.1: Expression matrices

Typically, an expression matrix, also known as count data or a count matrix refers to data where every i-th row and j-th column refer to how many reads are assigned to gene (ID) i in sample j. For example, if we have simplified count data for 4 samples and three genes, the `R` output will look something like this:

```
        sample1 sample2 sample3 sample4
gene001      23       3      45       2
```

```
gene002       6       7       7       8
gene003       0      34       3      42
```

Note^1: When loading count data into IRIS-EDA, make sure that the first column is your gene IDs and that sample names are short, concise, and avoid the use of mathematical operators (`+`, `-`, `/`, `*`, `^`, etc.) and spaces between words. If a space is necessary for legibility, please consider using an underscore (`_`)

Note^2: For sample names, **avoid starting any entries with numerical elements** (e.g. `1sample`, `2_human`, `42_amf`, etc.). This may potentially cause unexpected errors in downstream analyses!

S4.2: Condition matrices

Condition matrices, also known as metadata, details the design of your experiment. In this type of matrix, every i-th row and j-th column refer to factor levels assigned to sample i and factor j. For example, if were to look at the samples given in the count data section, the metadata `R` output will look something like this:

```
        condition time
sample1   treated   0h
sample2 untreated   0h
sample3   treated  24h
sample4 untreated  24h
```

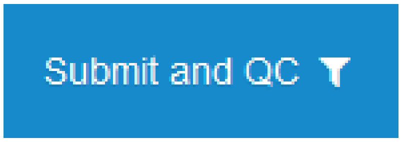
Note^1: When loading metadata into IRIS-EDA, make sure that the first column is your sample names and that column names and treatment levels are short, concise, and avoid the use of mathematical operators (`+`, `-`, `/`, `*`, `^`, etc.) and spaces between words. If a space is necessary for legibility, please consider using an underscore (`_`).

Note^2: For this data, **avoid starting any entries with numerical elements** (e.g. 1, 2, 42, etc.). This may potentially cause unexpected errors in downstream analyses!

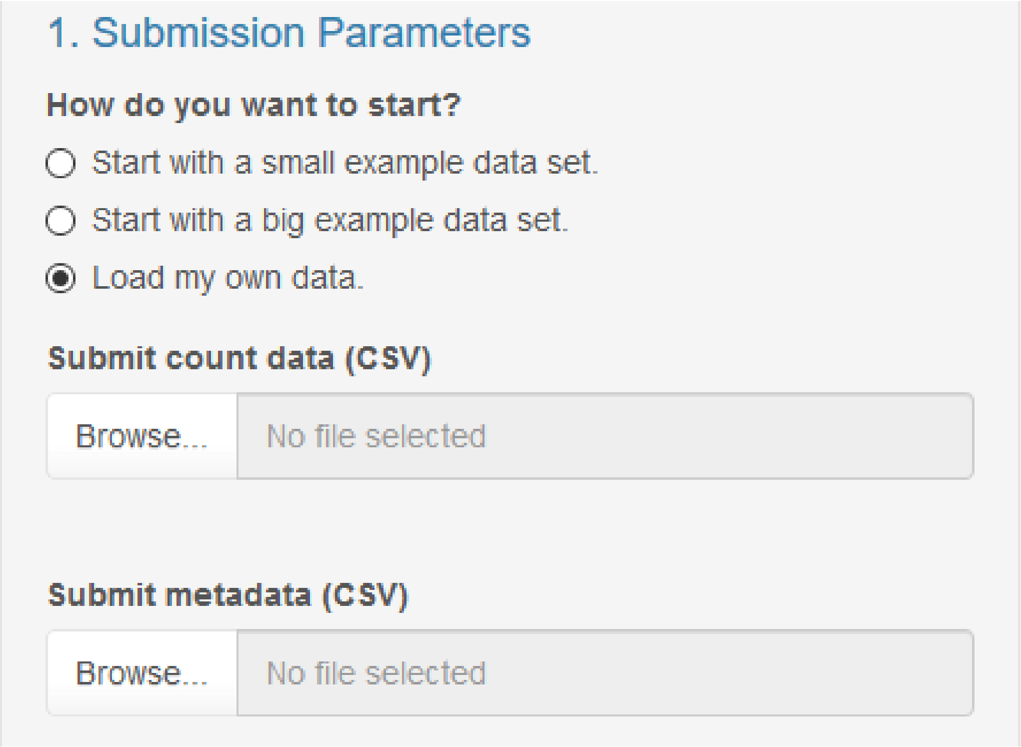
Note^3: Metadata can be expanded to fit the nature of your experiment (i.e. multiple factors can be added). The only thing that must remain consistent between these two matrices, is the sample information. Column names in count data **must** be the same as row names in the metadata.

S5: Quality Control

1. Click on the `Submit and QC` tab near the top-left corner of the application:



2. Under `1. Submission Paramters`, select either `Start with a small example data set`, `Start with a big example data set`, or `Load my own data` to upload user data. **Note:** User data requires one count matrix and one condition matrix:



1. Submission Parameters

How do you want to start?

○ Start with a small example data set.

○ Start with a big example data set.

● Load my own data.

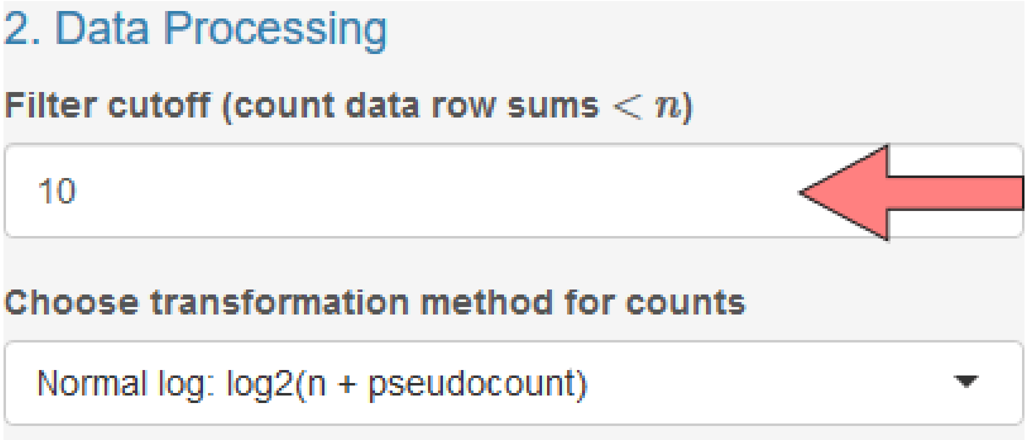
Submit count data (CSV)

Browse... No file selected

Submit metadata (CSV)

Browse... No file selected

2. Under `2. Data Processing`, select a filter cutoff to simplify and expedite computations by eliminating any rows that have below specified expression totals. The default argument for our application is `10`:



2. Data Processing

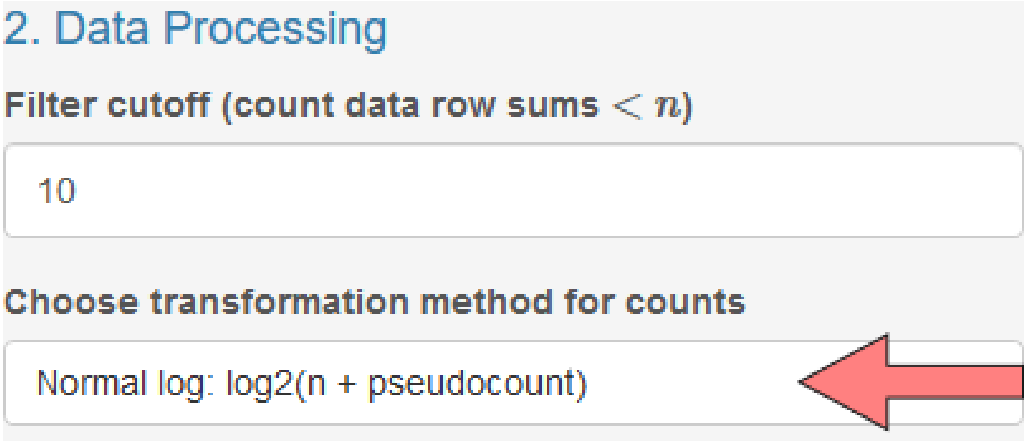
Filter cutoff (count data row sums $< n$)

10

Choose transformation method for counts

Normal log: log2(n + pseudocount)

3. Right below the filter cutoff parameter, select a transformation method for the count data for displaying the expression estimate. **Note:** For more information about any of these topics, take a look at the *FAQ* section at the bottom of this document:



2. Data Processing

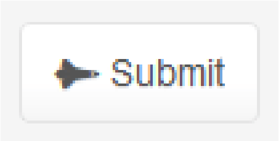
Filter cutoff (count data row sums < n)

10

Choose transformation method for counts

Normal log: log2(n + pseudocount)

4. Click `Submit` to load the data under `3. Launch Overview`:

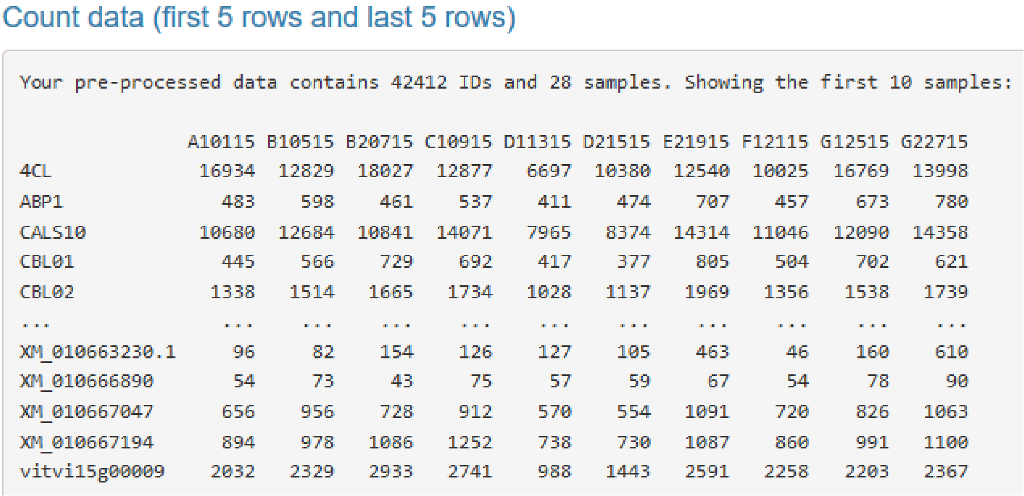


5. After you click `Submit`, the main page will now be populated with several pieces of information in two sub-tabs, `File Summary` and `Count Summary`.

S5.1: File Summary

`File Summary` provides a glimpse into the submitted data. This subtab includes three main components.

1. The `count data` section will detail the first and last five rows of the count data and also include the total number of IDs and samples:

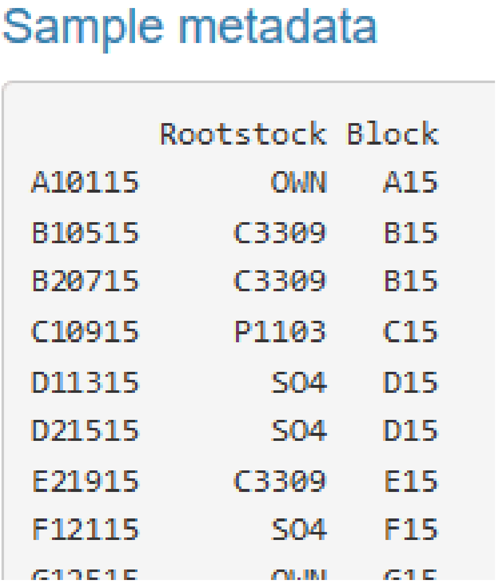


Count data (first 5 rows and last 5 rows)

```
Your pre-processed data contains 42412 IDs and 28 samples. Showing the first 10 samples:

               A10115 B10515 B20715 C10915 D11315 D21515 E21915 F12115 G12515 G22715
4CL             16934  12829  18027  12877   6697  10380  12540  10025  16769  13998
ABP1              483    598    461    537    411    474    707    457    673    780
CALS10          10680  12684  10841  14071   7965   8374  14314  11046  12090  14358
CBL01             445    566    729    692    417    377    805    504    702    621
CBL02            1338   1514   1665   1734   1028   1137   1969   1356   1538   1739
...               ...    ...    ...    ...    ...    ...    ...    ...    ...    ...
XM_010663230.1     96     82    154    126    127    105    463     46    160    610
XM_010666890       54     73     43     75     57     59     67     54     78     90
XM_010667047      656    956    728    912    570    554   1091    720    826   1063
XM_010667194      894    978   1086   1252    738    730   1087    860    991   1100
vitvi15g00009    2032   2329   2933   2741    988   1443   2591   2258   2203   2367
```

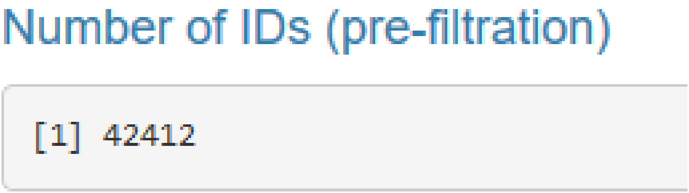
2. The second portion includes an overview of the condition data this is simply an `R` console-based output of this submitted CSV file:



Sample metadata

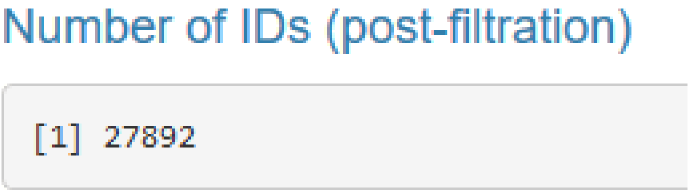
```
        Rootstock Block
A10115        OWN   A15
B10515      C3309   B15
B20715      C3309   B15
C10915      P1103   C15
D11315        SO4   D15
D21515        SO4   D15
E21915      C3309   E15
F12115        SO4   F15
G12515        OWN   G15
```

3. Finally, pre- and post-filtered gene ID counts gives a "before-and-after" count of the number of IDs that were filtered using the filter cutoff parameter under `2. Data Processing`:



Number of IDs (pre-filtration)

```
[1] 42412
```

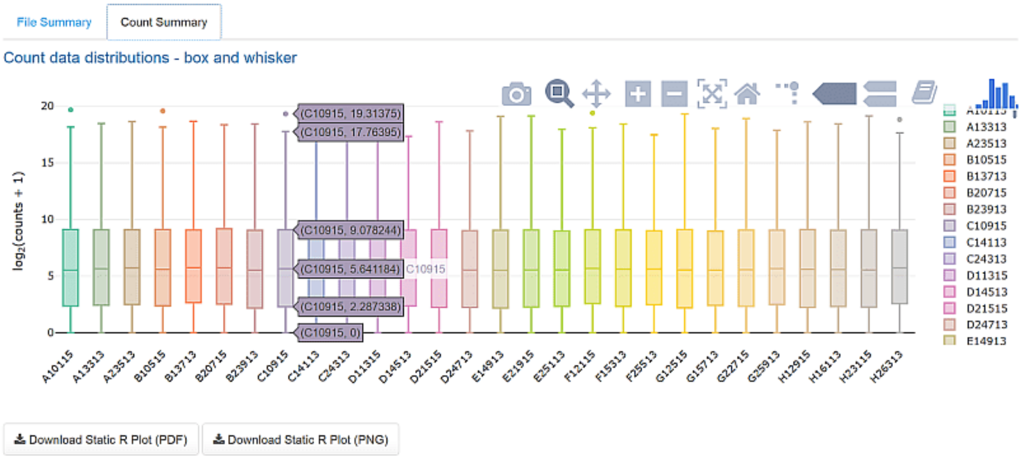


Number of IDs (post-filtration)

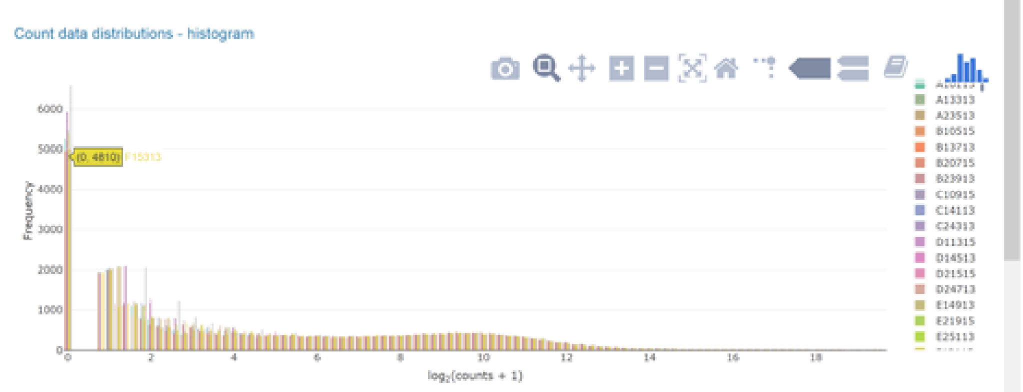
```
[1] 27892
```

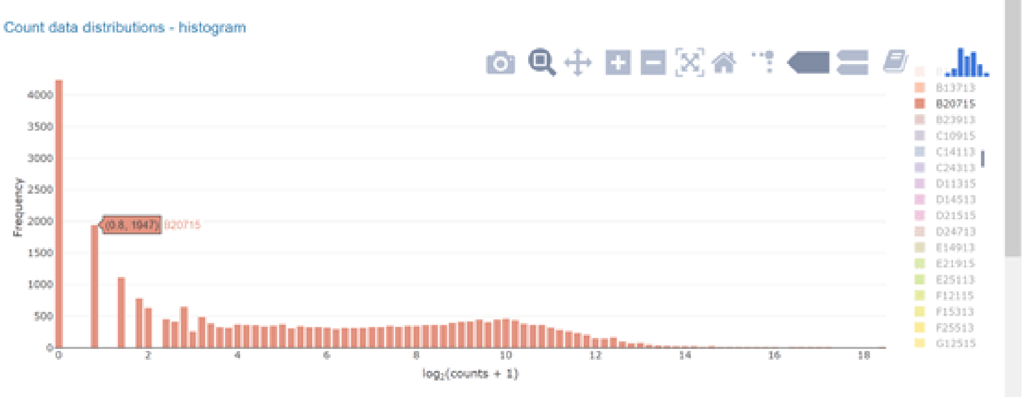
## S5.2: Count Summary `Count summary` provides three interactive, downloadable visualizations based on the file for each sample.

1. Box-and-Whisker Plot for transformed read counts by sample with interactivity showing the quartiles, maximum, and minimum count. *With the example data, it appears that the box-and-whisker plot for each sample is similar to the other samples. If one sample had a plot varying greatly from the others, it would indicate some required investigation into that specific sample in terms of the number of raw reads provided and proportion of reads aligned.*

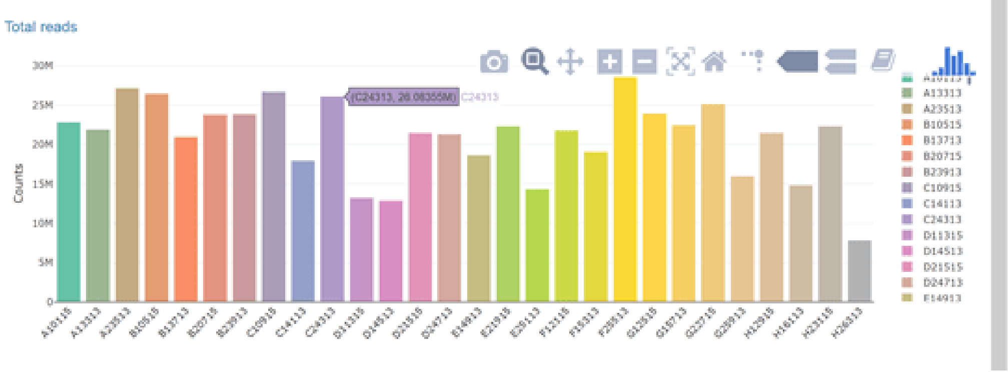


2. Count Data Distribution plot showing the frequency of transformed count data by sample with interactivity displaying the value and frequency. Additionally, double-clicking a sample ID in the legend isolates just that sample's histogram. Additional sample IDs can be select for more specific comparisons also. *With the example data, the histograms appear similar for each sample, indicating no significant derivation. Similar to the box-and-whisker plots, a single sample varying greatly from other samples may indicate a required investigation into the raw read counts or read alignment statistics.*





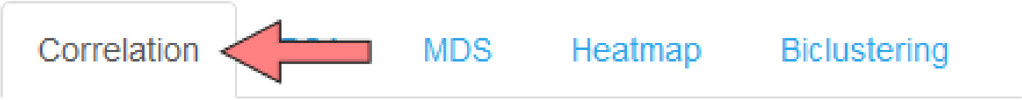
3. Total Reads displays a histogram of total reads by sample with interactivity for displaying actual total read counts for each sample. Double-clicking on a sample ID in the legend isolates that sample's read count histogram and allows for selecting of specific adjacent sample IDs for comparison. Total reads counts for individual samples that vary greatly from the other total read counts may indicate some issues with data preparation (sequencing) or read alignment. *Here, sample H26313 has a much lower total reads count than the other samples. This may be reflected in further comparative analyses.*



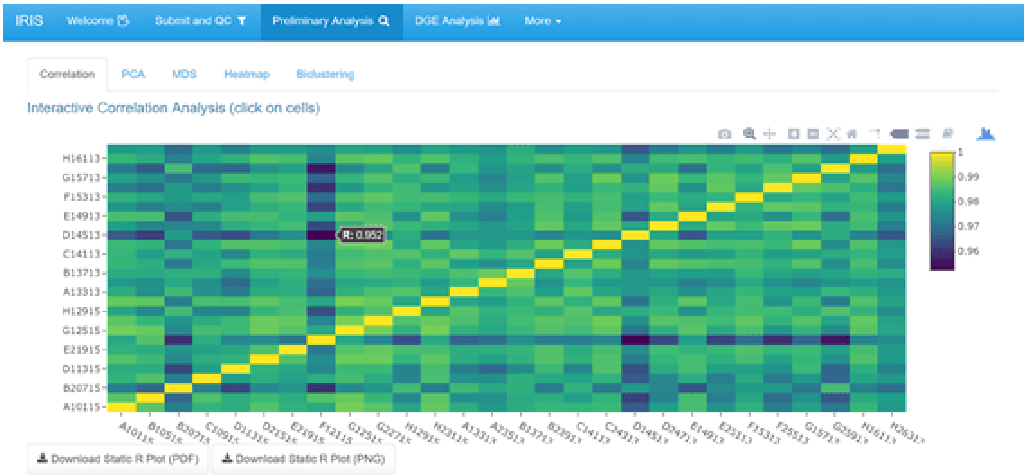
S6: Discovery-Driven Analyses

1. After examining your results on the `Submit and QC` tab, you may proceed to the `Discovery-Driven Analyses` tab:

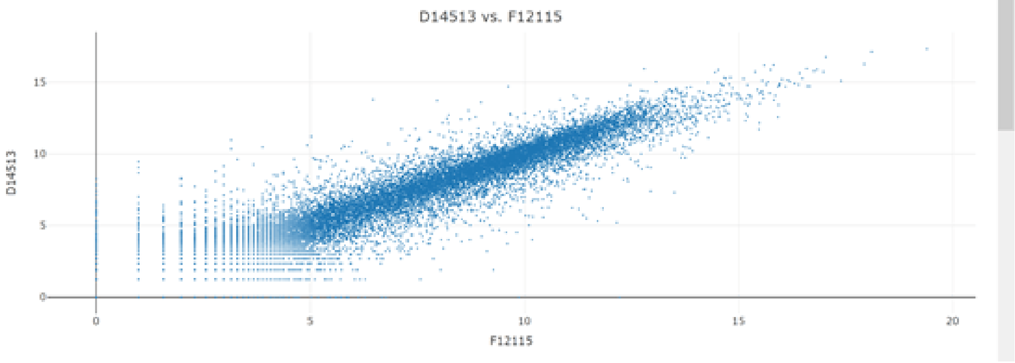
2. The `Discovery-Driven Analyses` tab will be populated with several subtabs, similar to the `Submit and QC` tab. The first subtab you will see is the `Correlation` tab. This tab provides correlation analysis of the expression matrix by sample:



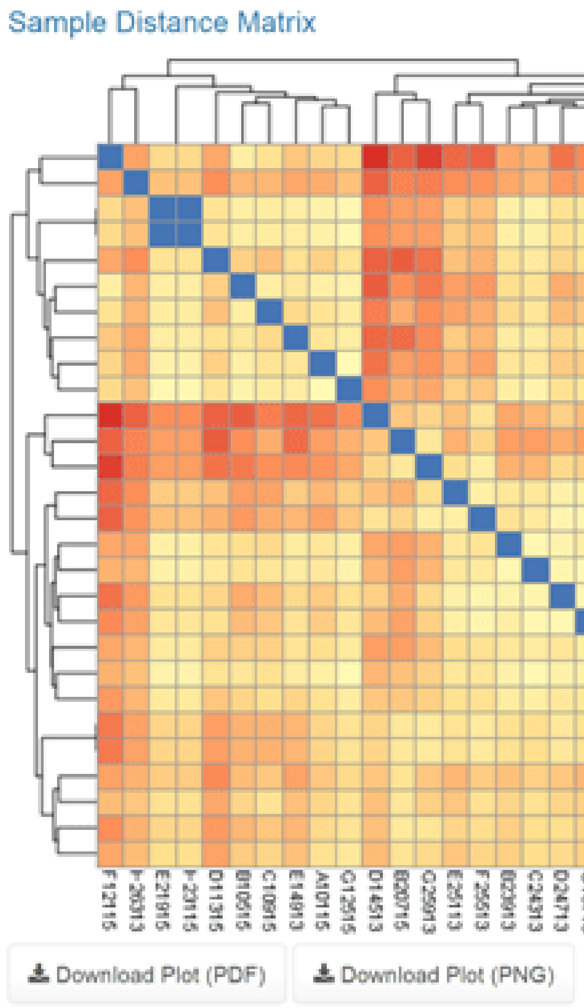
3. Under the `Correlation` subtab you will see several visualizations. `Interactive Correlation Analysis` displays a heatmap of the correlation between samples with interactivity showing the actual correlation between the two intersecting samples. *The example data shows most sample-sample correlations of 0.95 or larger, indicating relatively high correlation. The darker cells here signify less similar samples, which may yield more interesting differential expression results. This graph can indicate comparisons of interest in future analyses. In most cases, the high number of gene IDs with similar or identical expression estimates will cause correlations to be large, even for dissimilar genetic expression profiles:*



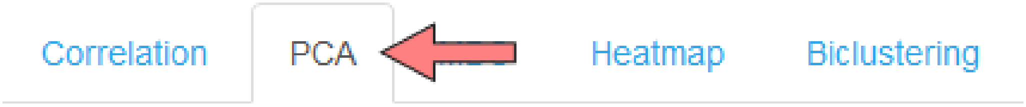
4. Clicking on a cell will provided a scatterplot of the two intersecting samples with each data point representing one gene ID. A scatterplot with points falling more closely along the diagonal indicates samples with more similar genetic expression profiles. *This scatterplot shows a clear trend of data points (gene IDs) falling along or close to the diagonal. That means these two samples have very similar genetic expression profiles. Data points that fall far from the diagonal line represent genes with dissimilar expression levels between the select samples:*



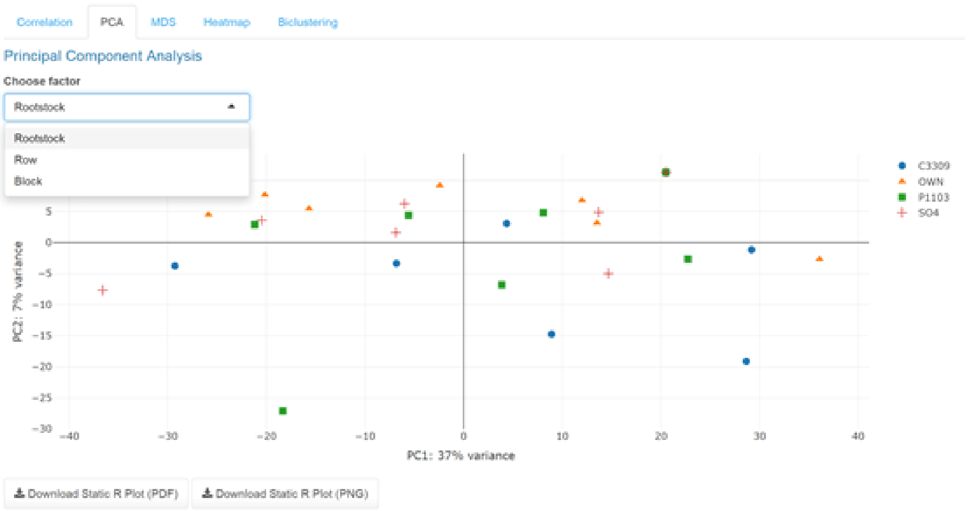
5. The Sample Distance Matrix provides a heatmap of the Euclidean distance between the gene expression vectors for each sample pair. The larger distance (darker red color) indicates samples with the most dissimilar genetic expression profiles. This matrix also includes a clustering of the samples based on the vectorized expression profiles. *With the example data, two distinct clusters can be observed through the first branching of the dendrogram. Additionally, as with the correlation heatmap, specific cells with a darker color indicate a more dissimilar pair of samples based on genetic expression:*



6. The next visualization will be under the `PCA` subtab. This subtab provides Principal Component Analysis (PCA) for the expression estimation matrix:



7. This analysis has the option of selecting a factor of interest. *With the example data, selecting "Rootstock" as the factor of interest provides a visualization of the first two components for each sample. In this application, PCA is a linear transformation of the gene expression levels, with the first component representing the transformed dimension with the most variability, and each subsequent component decreasing in variability. This analysis has the potential to isolate samples based on expression levels. Here, there does not appear to be any specific rootstock that separates from the others. If there were, it could help develop directions for further analysis. The axis labels indicate the first principal component accounts for 37% of the variance between samples, whereas the second principal component accounts for 7%:*

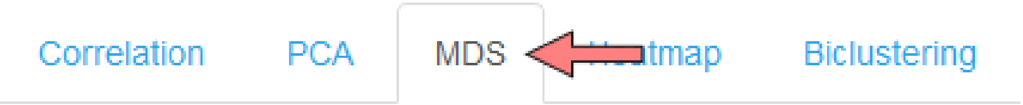


Principal Component Analysis

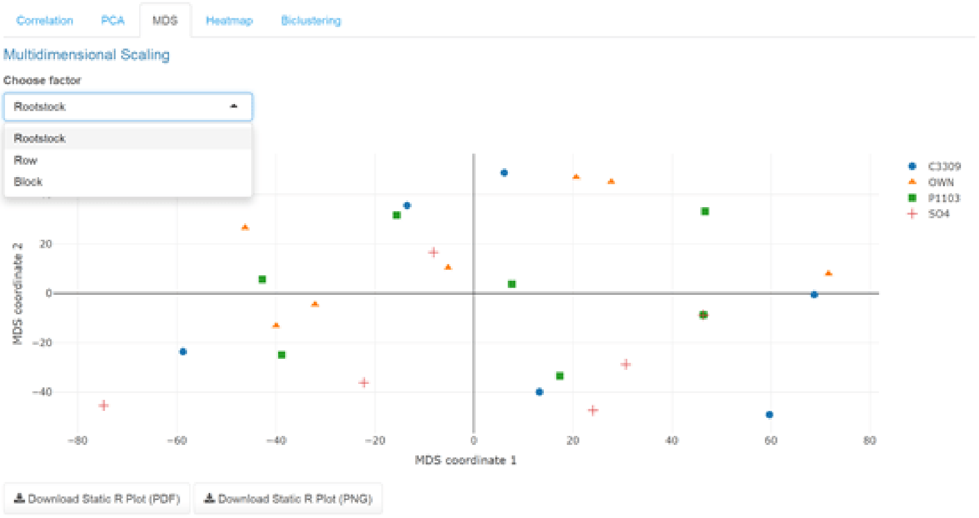
Choose factor

Rootstock

8. The next visualization will be under the `MDS` subtab. This subtab provides multi dimensional scaling for the expression estimation matrix. This is similar to PCA except is develops components through non-linear transformations of the gene expressions:



9. *Looking back at our sample data, we observe similar results to that of the PCA results, with similar potential interpretations if any sample or groups of samples were to differentiate from the others:*

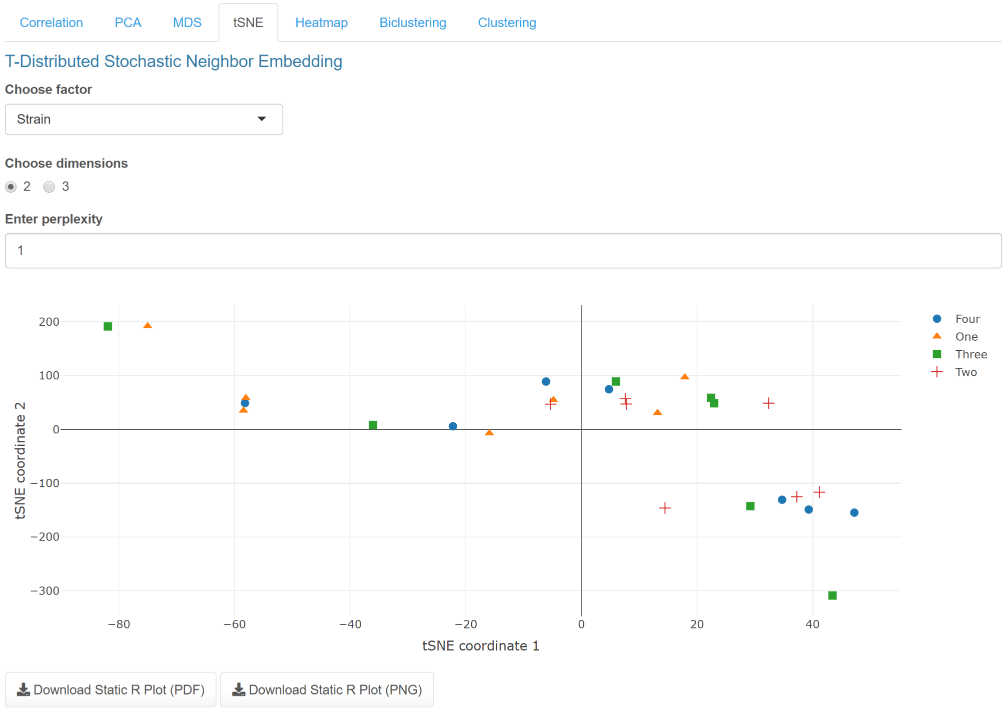


Multidimensional Scaling

Choose factor

Rootstock

10. t-SNE allows for visualization of results in two and three dimensions. The three-dimensional figure is also interactive, allowing users to move the axes to gain a better understanding of the three-dimensional layout of the sample points.



T-Distributed Stochastic Neighbor Embedding

Choose factor

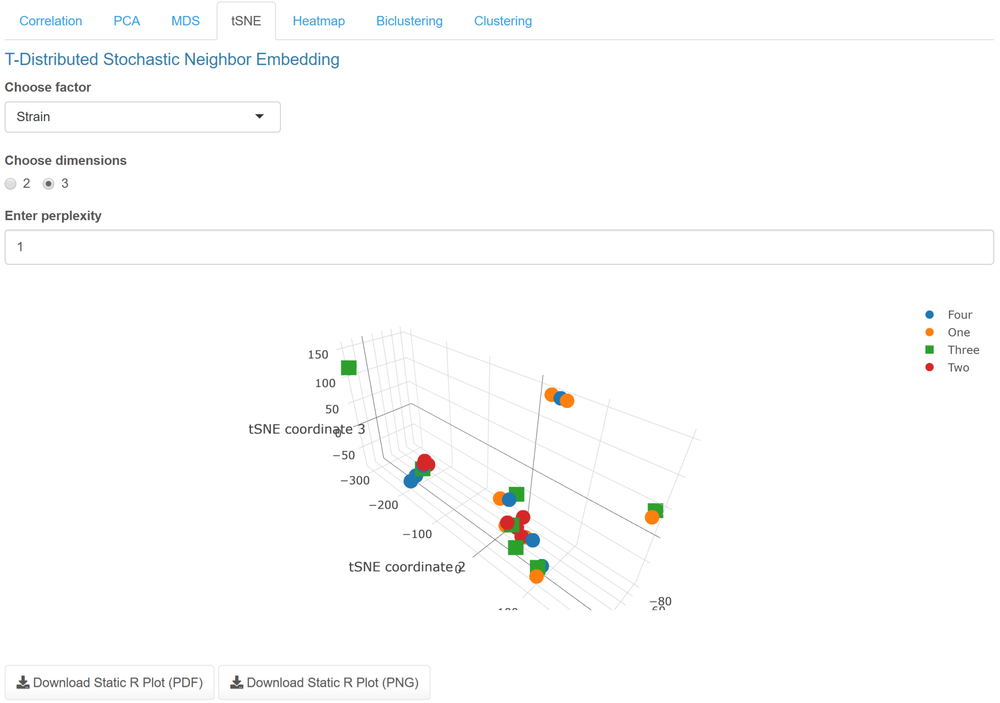
Strain

Choose dimensions

● 2 ○ 3

Enter perplexity

1



T-Distributed Stochastic Neighbor Embedding

Choose factor

Strain

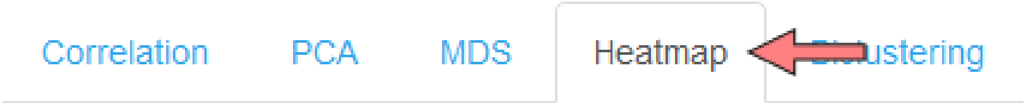
Choose dimensions

○ 2 ● 3

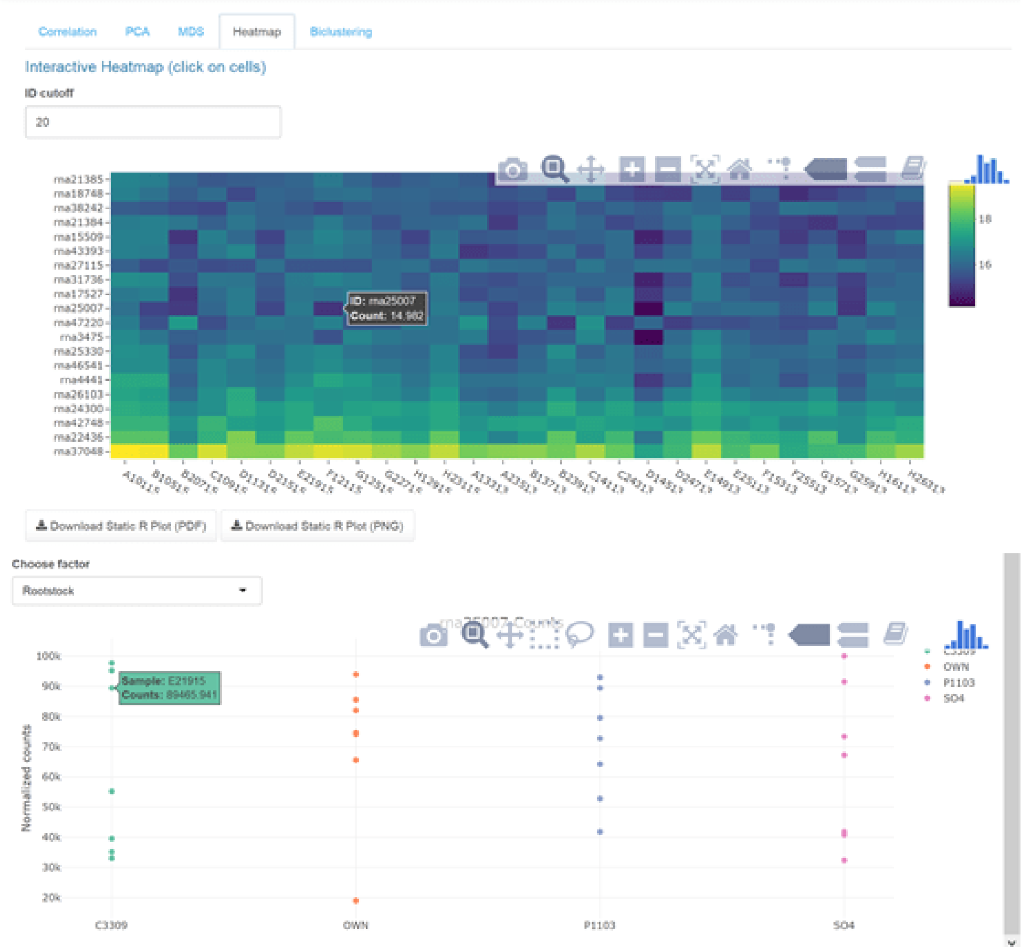
Enter perplexity

1

11. The next visualization will be under the `Heatmap` subtab. This subtab provides an interactive heatmap with rows representing the gene IDs and columns representing sample IDs:



12. This heatmap requires an ID cutoff to select the indicated number of gene IDs with the highest mean expression values for display. Selecting a cell displays a plot showing the total read counts for that specific gene ID by the selected factor. *With the example data, the 20 most variable gene IDs are displayed. The yellow color indicates gene IDs with a higher expression level for that sample, and the darker blue color represents a low expression level for that sample. Selecting ID: rna25007 shows the read counts for that ID by rootstock factor. This shows the "OWN" rootstock seems to have a higher expression level for that ID, with the exception of one sample:*



Interactive Heatmap (click on cells)

ID cutoff

20

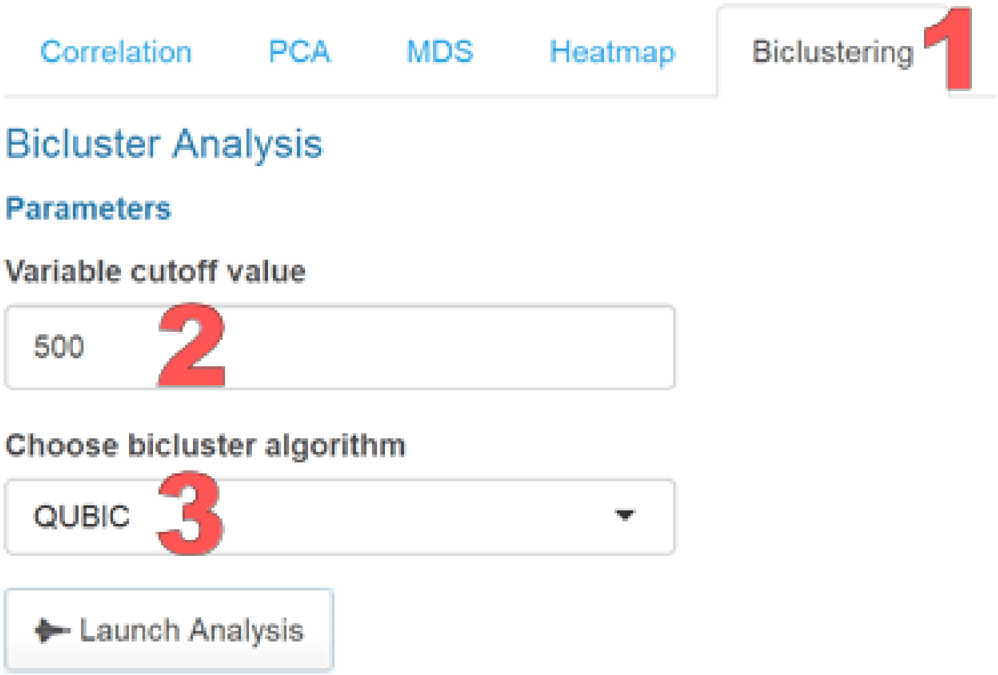
ID: rna25007
Count: 14.982

Choose factor

Rootstock

Sample: E21915
Counts: 89465.941

13. On the next subtab (1), `Biclustering` performs a biclustering analysis using one of the selected biclustering tools (3) with a maximum bicluster size of the indicated cutoff value (2):



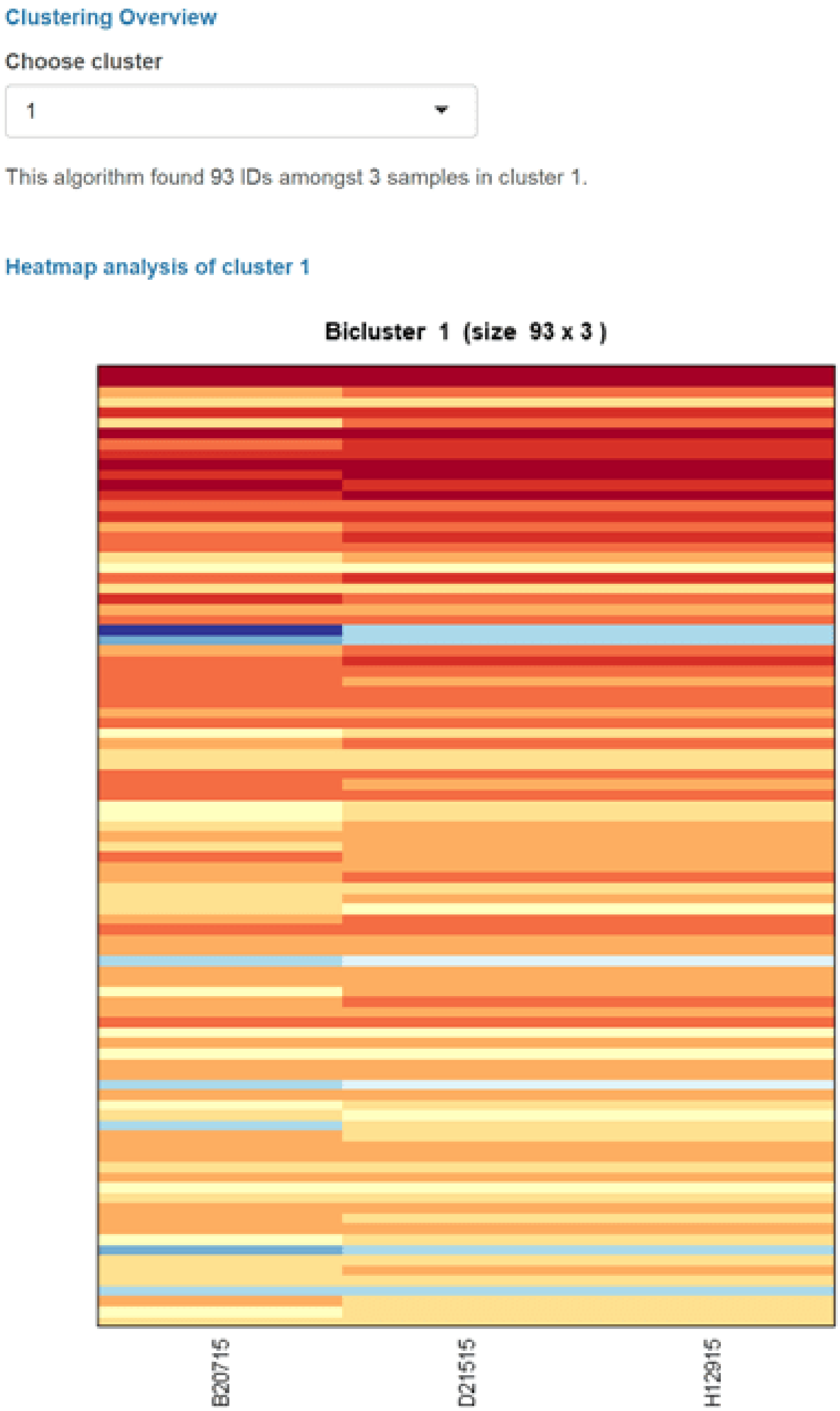
Variable cutoff value

500

Choose bicluster algorithm

QUBIC

14. Launching the analysis results in display of the first bicluster. Alternative clusters can be selected from the dropdown menu and the IDs and plot for each bicluster can be downloaded using the button below the visualization. *With the sample data, the biclusters can help select the samples under which certain gene IDs are similarly expressed. Since gene expression levels can vary greatly over all samples and conditions, a biclustering approach can isolate similar expression patterns on a level where traditional clustering may miss. The first cluster for the example data shows that samples B20715, D21515, and H12915 are expressed similarly under the isolated gene IDs. Interpretations can be made similarly for each subsequent bicluster:*



Clustering Overview

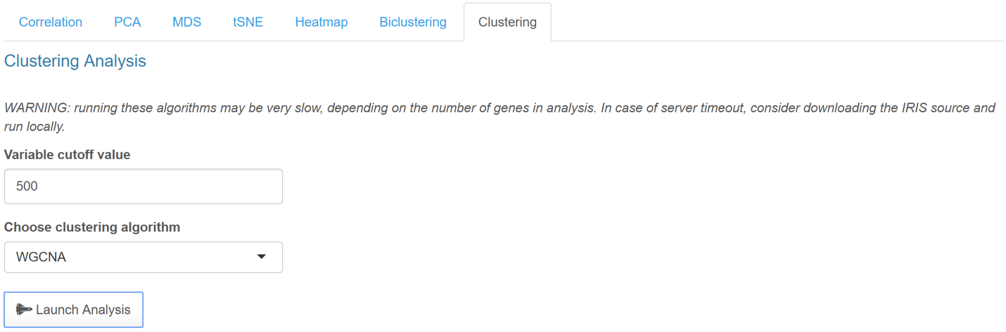
Choose cluster

1 ▾

This algorithm found 93 IDs amongst 3 samples in cluster 1.

Heatmap analysis of cluster 1

15. The `Clustering` tab allows for users to select one of three clustering methods respectively representing hierarchical, representative, and graph-based clustering: Weighted Gene Co-expression Network Analysis (WGCNA), k-medoids, and the Markov Clustering Algorithm (MCL). While all three methods have demonstrated high performance related to module detection, the default is WGCNA due to it being the best of the three. Here, users should be careful with large datasets, as these methods can take large amounts of time to run. Setting the variable cutoff option lower will reduce the time by selecting fewer of the most differentially expressed genes to use in clustering.



Clustering Analysis

WARNING: running these algorithms may be very slow, depending on the number of genes in analysis. In case of server timeout, consider downloading the IRIS source and run locally.

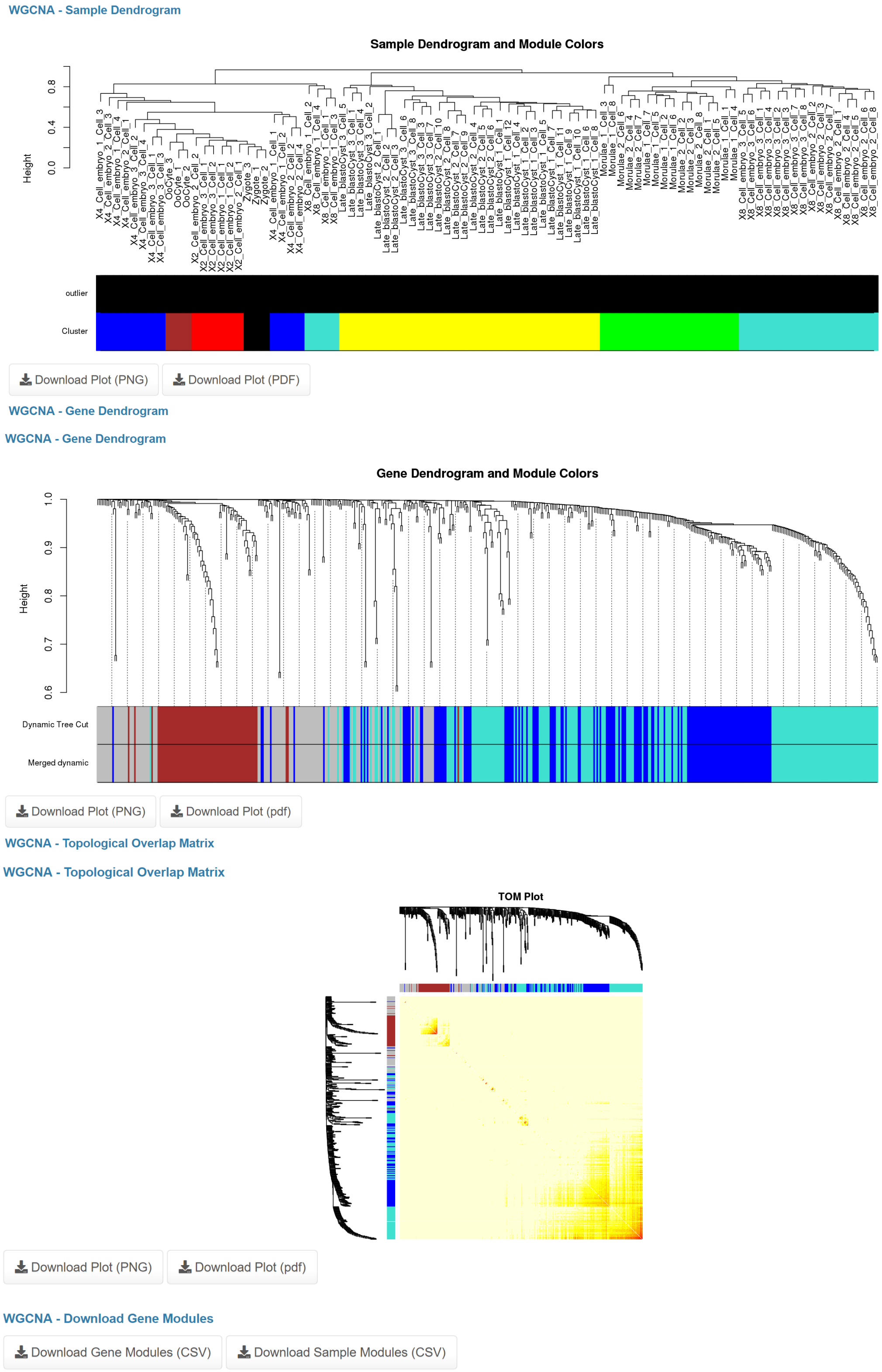
Variable cutoff value

500

Choose clustering algorithm

WGCNA

16. The WGCNA method generates figures related to sample and gene dendrograms, as well as a topological overlap matrix based on the genes. Additionally, users can download lists of the genes and samples with respective cluster assignments.



WGCNA - Sample Dendrogram

WGCNA - Gene Dendrogram

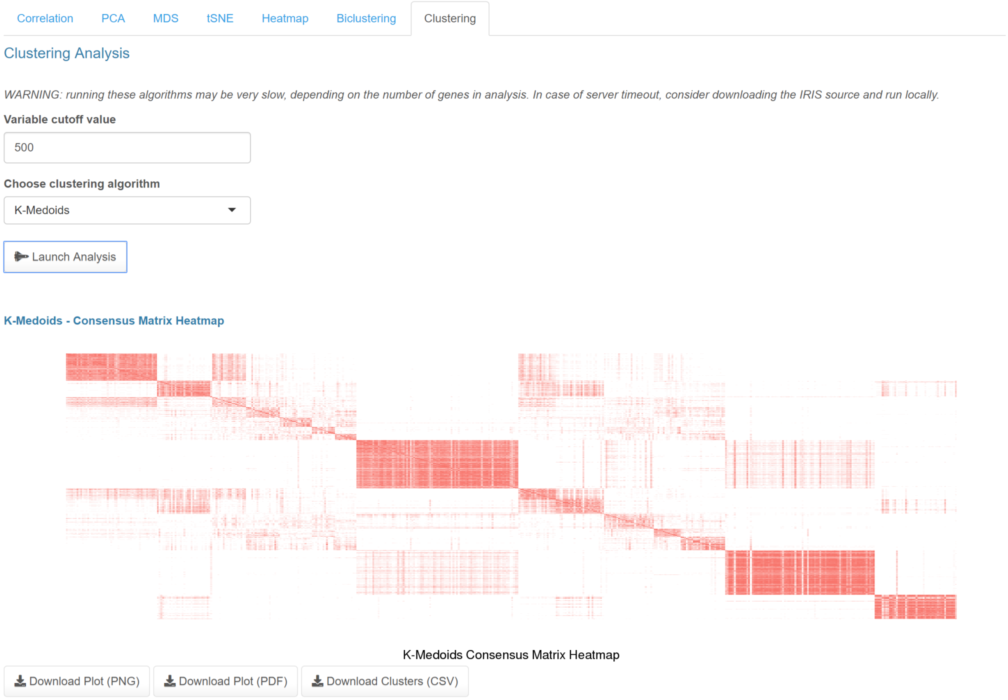
WGCNA - Gene Dendrogram

WGCNA - Topological Overlap Matrix

WGCNA - Topological Overlap Matrix

WGCNA - Download Gene Modules

17. The k-medoids method produces a downloadable consensus matrix heatmap. Additionally, the generated clusters from this method are downloadable.



Clustering Analysis

WARNING: running these algorithms may be very slow, depending on the number of genes in analysis. In case of server timeout, consider downloading the IRIS source and run locally.

Variable cutoff value

500

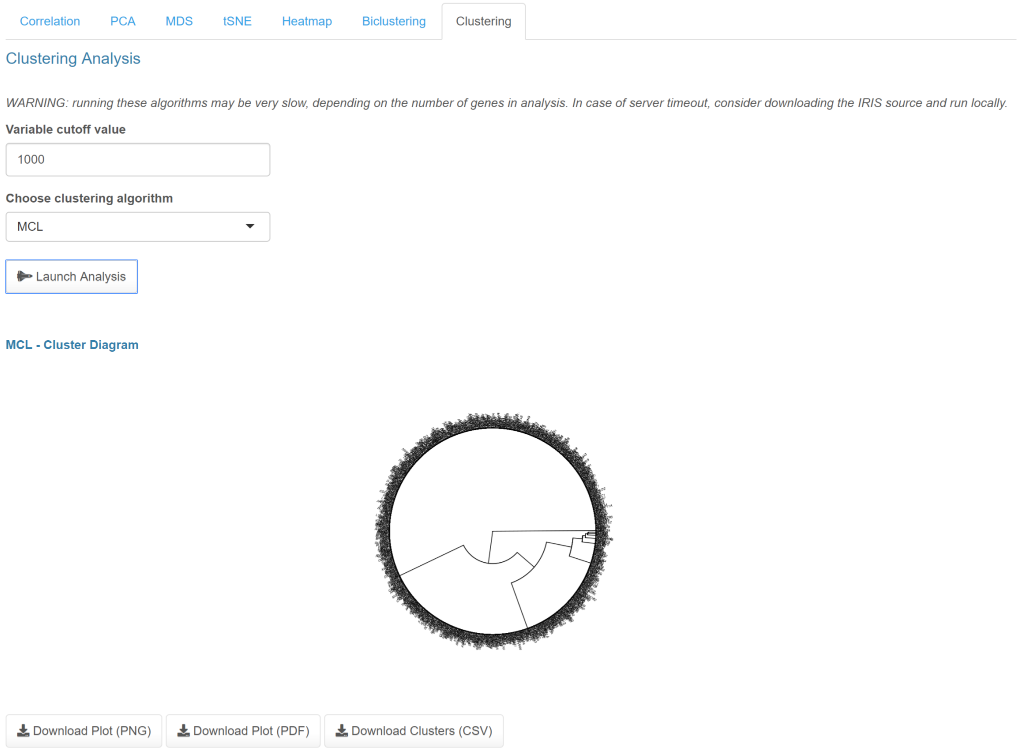
Choose clustering algorithm

K-Medoids

K-Medoids - Consensus Matrix Heatmap

K-Medoids Consensus Matrix Heatmap

18. The MCL method generates a cluster diagram, along with downloadable cluster compositions.



Clustering Analysis

WARNING: running these algorithms may be very slow, depending on the number of genes in analysis. In case of server timeout, consider downloading the IRIS source and run locally.

Variable cutoff value

1000

Choose clustering algorithm

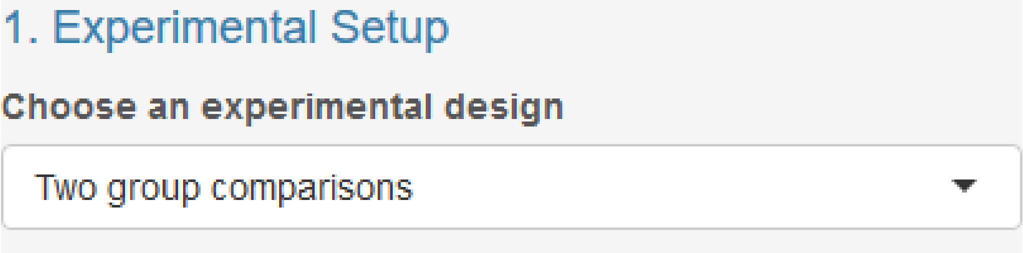
MCL

MCL - Cluster Diagram

S7: Differential Gene Expression

1. After uploading the user data or selecting the example data, users can go directly to the `DGE Analysis` tab, preceding the `Discovery-Driven Analyses` functions:

2. Once you are on this tab, you will be greeted with several options. `1. Experimental Setup` will allow users to select an experimental design for their DGE analysis. Options are Two-group comparison, Multiple factor comparisons, Classical interaction design, Additive models (paired or blocking designs), Main effects, Main effects with grouping factors, or Custom design:



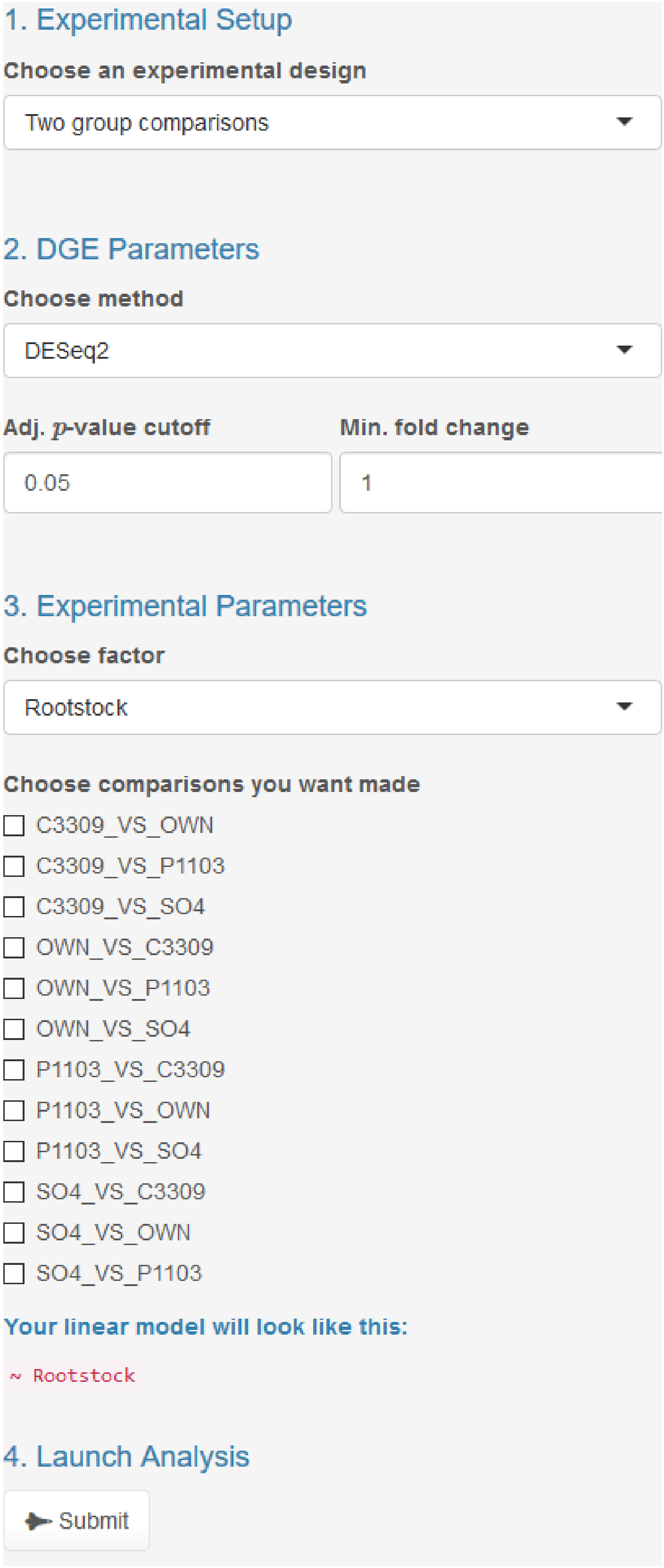
1. Experimental Setup

Choose an experimental design

Two group comparisons

S7.1: An overview of experimental designs

1. The `Two-group comparisons` options is the traditional approach for DGE and compares two factors levels for the selected factor type. *With the example data, selecting "Two group comparisons" for the experimental design and "Rootstock" for the factor allows for specific pairwise comparisons of Rootstock factor levels. Here, we can select specific comparisons of interest from the permutations of all pairwise comparisons. Selecting all comparison options will provide inverse duplications, so specific selections may be needed. Below, all unique pairwise combinations are selected. The linear model is also displayed for users interested in the model used for comparisons:*



1. Experimental Setup

Choose an experimental design

Two group comparisons

2. DGE Parameters

Choose method

DESeq2

Adj. p-value cutoff: 0.05

Min. fold change: 1

3. Experimental Parameters

Choose factor

Rootstock

Choose comparisons you want made

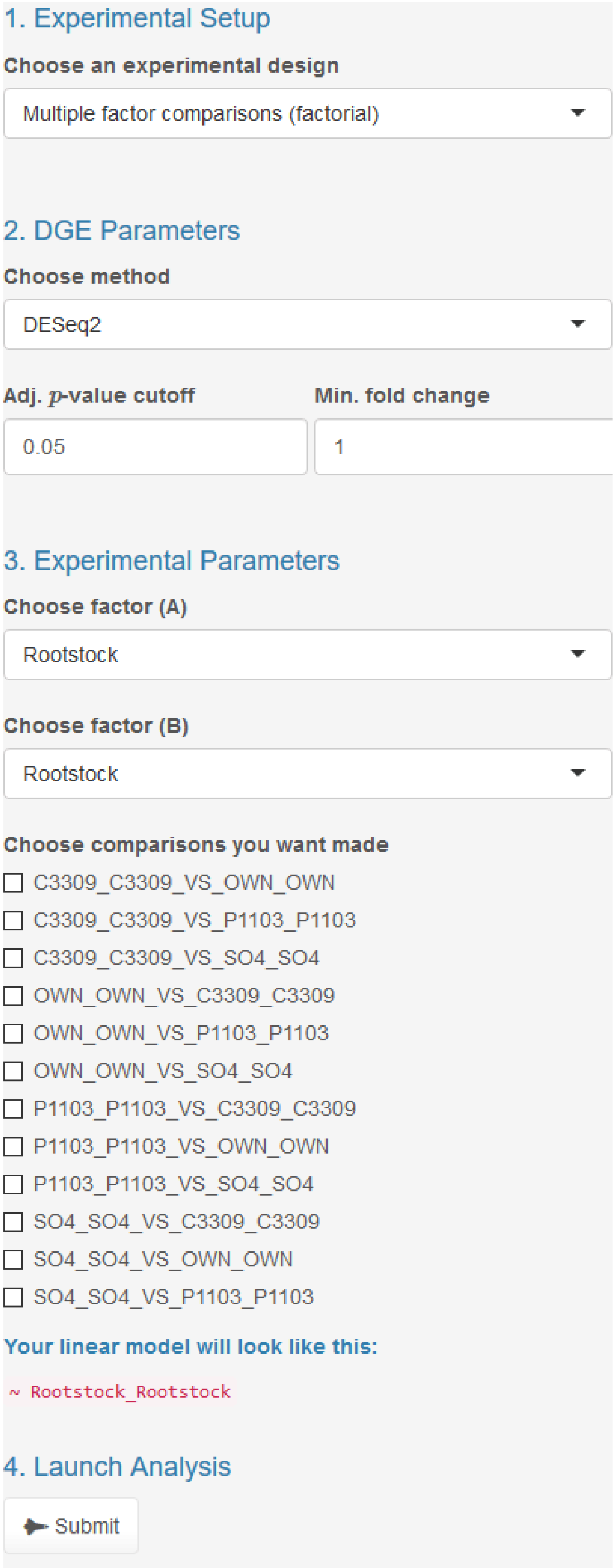
- □ C3309_VS_OWN
- □ C3309_VS_P1103
- □ C3309_VS_SO4
- □ OWN_VS_C3309
- □ OWN_VS_P1103
- □ OWN_VS_SO4
- □ P1103_VS_C3309
- □ P1103_VS_OWN
- □ P1103_VS_SO4
- □ SO4_VS_C3309
- □ SO4_VS_OWN
- □ SO4_VS_P1103

Your linear model will look like this:

```
~ Rootstock
```

4. Launch Analysis

2. The `Multiple factor comparisons` design has users select two factor levels and performs all crosswise comparisons for the two chosen factor levels. *With the example data, the Multiple factor comparisons design with Rootstock and Block selected as the two factors provides optional comparisons for each rootstock separated by block. In this situation, as with the other designs, the user selects which comparisons are of interest. Selecting C3309_B_VS_C3309_E allows for a comparisons of gene expression levels for the same rootstock in two different blocks. This provides insight into the locations and possibly time (due to time requirements for sampling) for this specific rootstock:*



1. Experimental Setup

Choose an experimental design

Multiple factor comparisons (factorial)

2. DGE Parameters

Choose method

DESeq2

Adj. p-value cutoff: 0.05

Min. fold change: 1

3. Experimental Parameters

Choose factor (A)

Rootstock

Choose factor (B)

Rootstock

Choose comparisons you want made

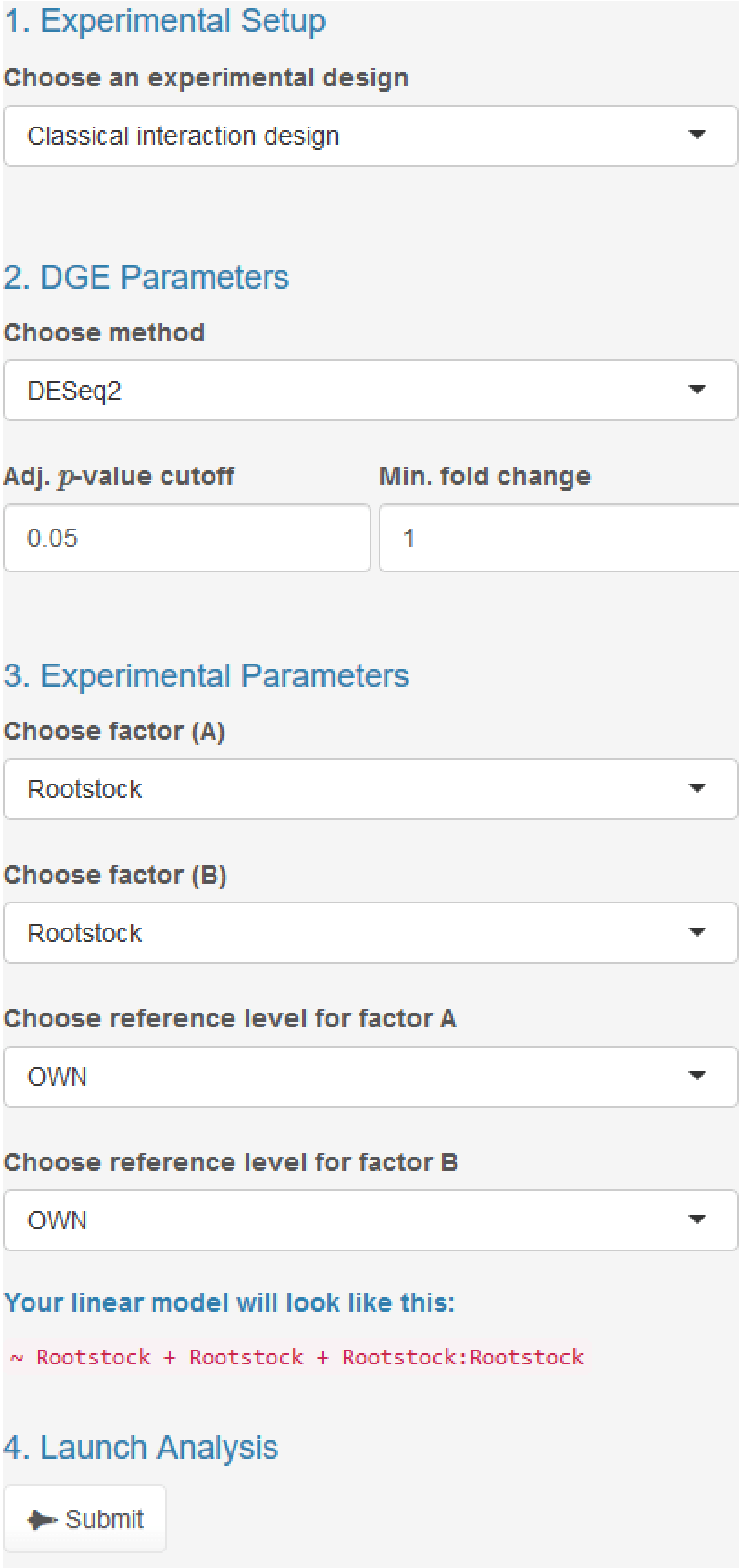
- ☐ C3309_C3309_VS_OWN_OWN
- ☐ C3309_C3309_VS_P1103_P1103
- ☐ C3309_C3309_VS_SO4_SO4
- ☐ OWN_OWN_VS_C3309_C3309
- ☐ OWN_OWN_VS_P1103_P1103
- ☐ OWN_OWN_VS_SO4_SO4
- ☐ P1103_P1103_VS_C3309_C3309
- ☐ P1103_P1103_VS_OWN_OWN
- ☐ P1103_P1103_VS_SO4_SO4
- ☐ SO4_SO4_VS_C3309_C3309
- ☐ SO4_SO4_VS_OWN_OWN
- ☐ SO4_SO4_VS_P1103_P1103

Your linear model will look like this:

```
~ Rootstock_Rootstock
```

4. Launch Analysis

3. The `Classical interaction design` allows the user to select two factor levels and one reference level for each of the selected factors. *With the experimental data, the Classic interaction design allows for selection of two factors and a reference for each. In this case, the Rootstock and Row are the chosen factors and OWN and 15 are the selected reference levels for comparison. The contrast levels selected upon submission will provide DEGs with respect to these two levels:*



1. Experimental Setup

Choose an experimental design

Classical interaction design

2. DGE Parameters

Choose method

DESeq2

Adj. p-value cutoff

0.05

Min. fold change

1

3. Experimental Parameters

Choose factor (A)

Rootstock

Choose factor (B)

Rootstock

Choose reference level for factor A

OWN

Choose reference level for factor B

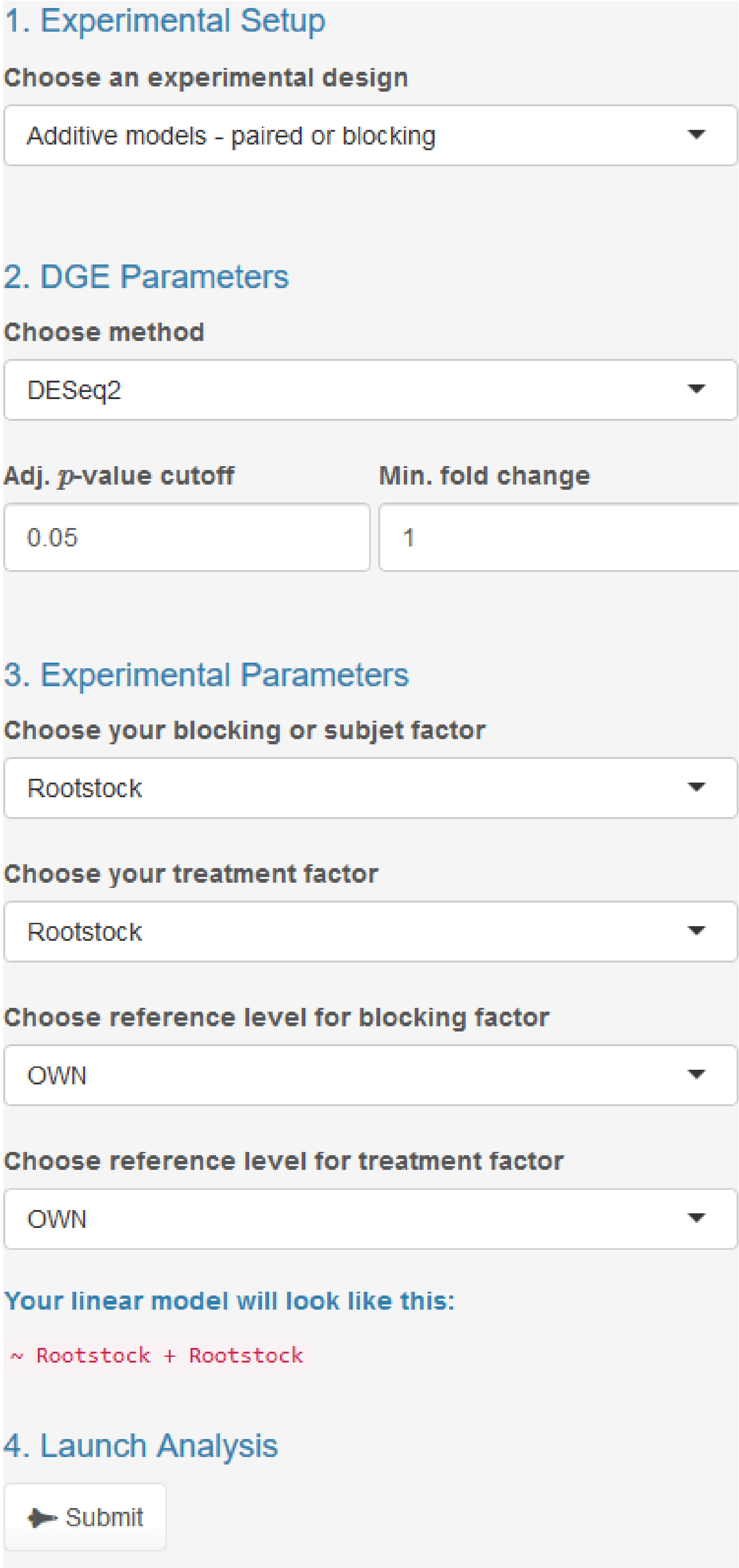
OWN

Your linear model will look like this:

```
~ Rootstock + Rootstock + Rootstock:Rootstock
```

4. Launch Analysis

4. The `Additive models` design is useful when samples are paired or blocked into distinct groupings. This format requires selected factors, one for the pairing or blocking factor and the other for the treatment factor. Additionally, a reference level for each selected factor is required. *In the example data, the Rootstock factor can be considered for grouping and the treatment factor is Block. OWN is selected as the reference level for the blocking factor and the A15 Block is selected for the treatment reference level:*



1. Experimental Setup

Choose an experimental design

Additive models - paired or blocking

2. DGE Parameters

Choose method

DESeq2

Adj. *p*-value cutoff

0.05

Min. fold change

1

3. Experimental Parameters

Choose your blocking or subjet factor

Rootstock

Choose your treatment factor

Rootstock

Choose reference level for blocking factor

OWN

Choose reference level for treatment factor

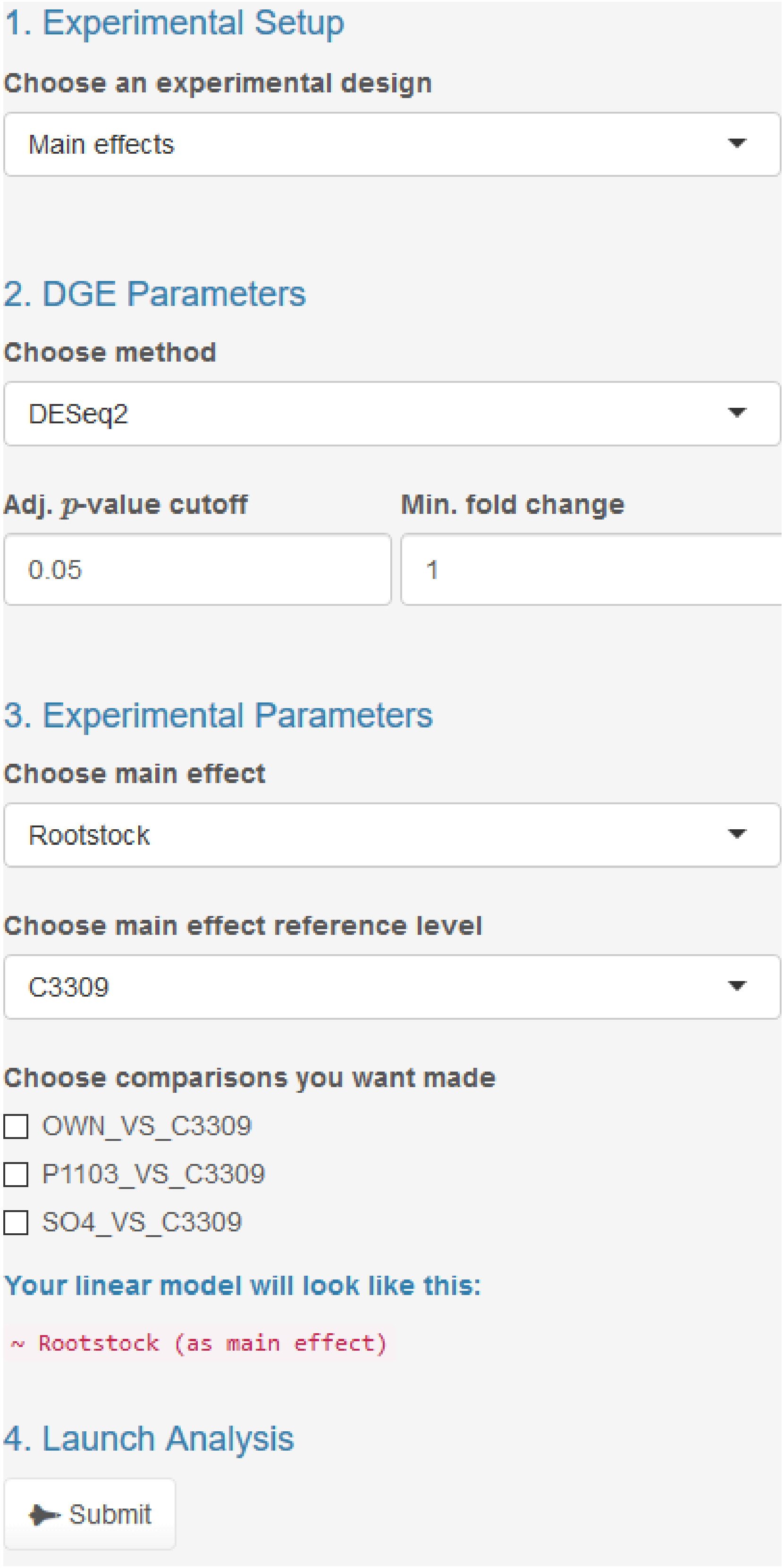
OWN

Your linear model will look like this:

```
~ Rootstock + Rootstock
```

4. Launch Analysis

5. `Main Effects` experimental design allows for testing the significance of a factor across multiple factor levels. In this situation, any significant deviation from an intercept would result in a significantly differential expressed gene. Using this approach is most useful when users want to test the significance of a factor that has more than two levels. This design requires indication of which factor to test as the main effect. Additionally, users must specify a factor reference level for the lfc and for corresponding visualizations. The p-values, adjusted p-values, and related differentially expressed genes are not affected by the selected reference level. *In the example data, the Rootstock factor is selected, which will test whether Rootstock has a significant impact on the genetic expression across all Rootstock factor levels. Selection of the C3309 Rootstock factor level as a reference will provide lfc values and visualizations relative to this level, while p-values and adjusted p-values will be calculated with respect to the main effect of the chosen factor:*



1. Experimental Setup

Choose an experimental design

Main effects

2. DGE Parameters

Choose method

DESeq2

Adj. p-value cutoff

0.05

Min. fold change

1

3. Experimental Parameters

Choose main effect

Rootstock

Choose main effect reference level

C3309

Choose comparisons you want made

□ OWN_VS_C3309

□ P1103_VS_C3309

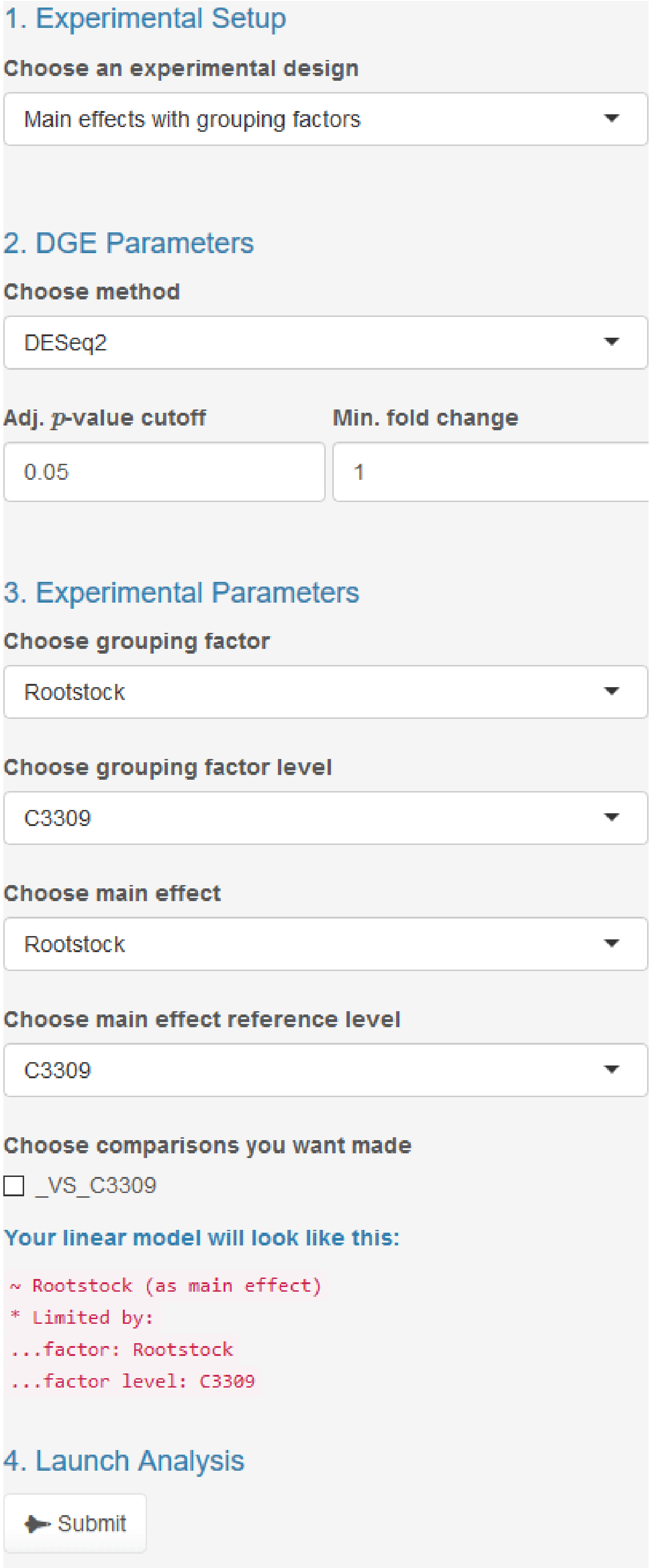
□ SO4_VS_C3309

Your linear model will look like this:

```
~ Rootstock (as main effect)
```

4. Launch Analysis

6. `Main effects with grouping factors` design allows for a more detailed main effects testing to be performed. This design tests the indicated main effect when the data is subset by a user-indicated factor. Requirements for this design are indication of main effect to test, factor for grouping, and grouping factor level for which to provide results. As with the main effects design, the user also specifies a main effect reference level for lfc and visualizations. *In the example data, Rootstock is selected as the grouping factor, with C3309 selected as the grouping factor level for which the results table will be based on. The Block factor is selected to test the main effect, with B13 selected as the main effect reference level. This analysis will indicate which genes are differentially expressed when account for Block only for the C3309 samples:*



1. Experimental Setup

Choose an experimental design

Main effects with grouping factors

2. DGE Parameters

Choose method

DESeq2

Adj. p-value cutoff: 0.05

Min. fold change: 1

3. Experimental Parameters

Choose grouping factor

Rootstock

Choose grouping factor level

C3309

Choose main effect

Rootstock

Choose main effect reference level

C3309

Choose comparisons you want made

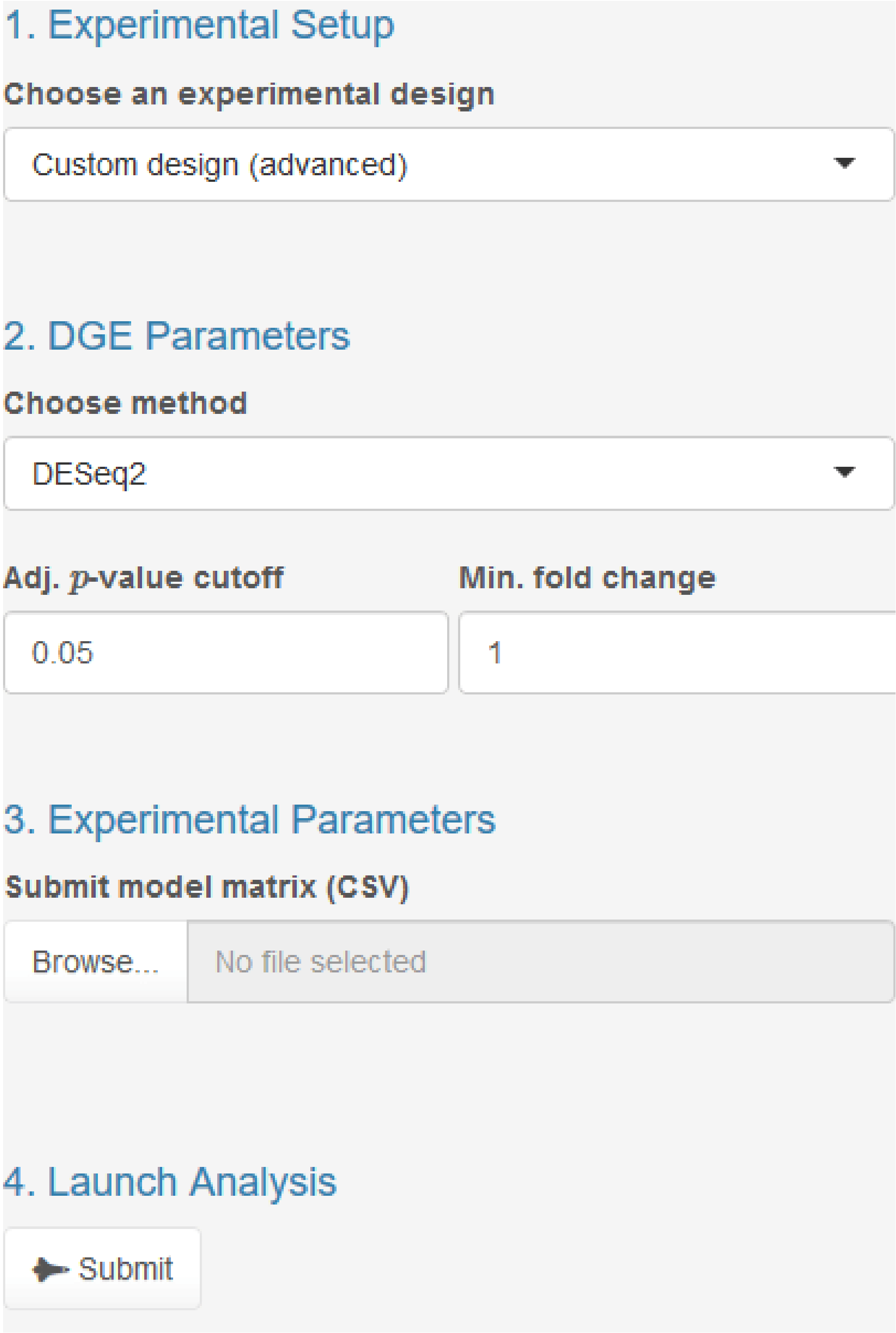
☐ _VS_C3309

Your linear model will look like this:

```
~ Rootstock (as main effect)
* Limited by:
...factor: Rootstock
...factor level: C3309
```

4. Launch Analysis

7. Finally, `Custom design` provides advanced users with a method for indicating their own design matrix. This method is provided so that users can perform more intricate DGE analyses beyond what IRIS-EDA already provides as experimental design options. It is only recommended that users with advanced knowledge of the modeling process to use this option, as the required design matrix may not be intuitive to use appropriately:



1. Experimental Setup

Choose an experimental design

Custom design (advanced)

2. DGE Parameters

Choose method

DESeq2

Adj. p-value cutoff: 0.05

Min. fold change: 1

3. Experimental Parameters

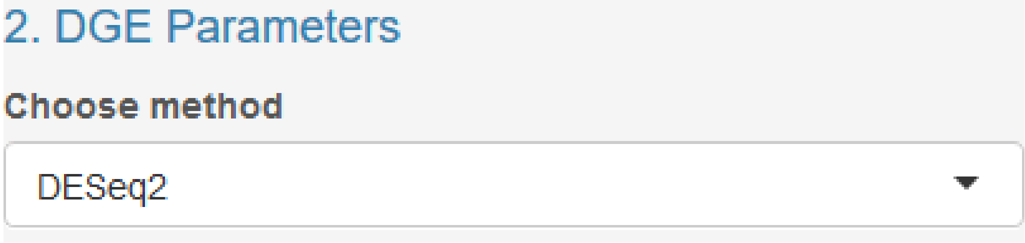
Submit model matrix (CSV)

No file selected

4. Launch Analysis

S7.2: Setting up an experiment

1. Following experimental design selection, the user can then select the desired tool for performing differential gene expression analysis. This will be found under `2. DGE Parameters`. Options for this purpose are `DESeq2`, `edgeR`, and `limma-voom`. If the user selects one of the main effects design options, the only options for DGE tools are `DESeq2` and `edgeR`:

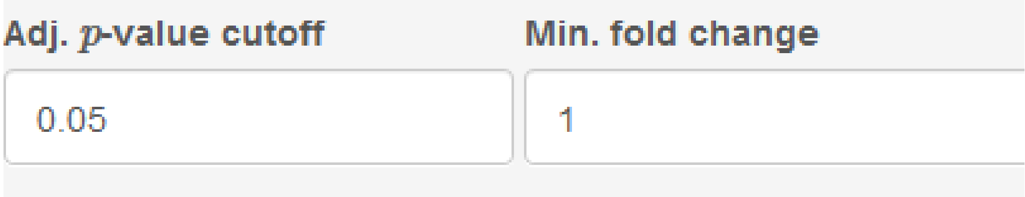


2. DGE Parameters

Choose method

DESeq2

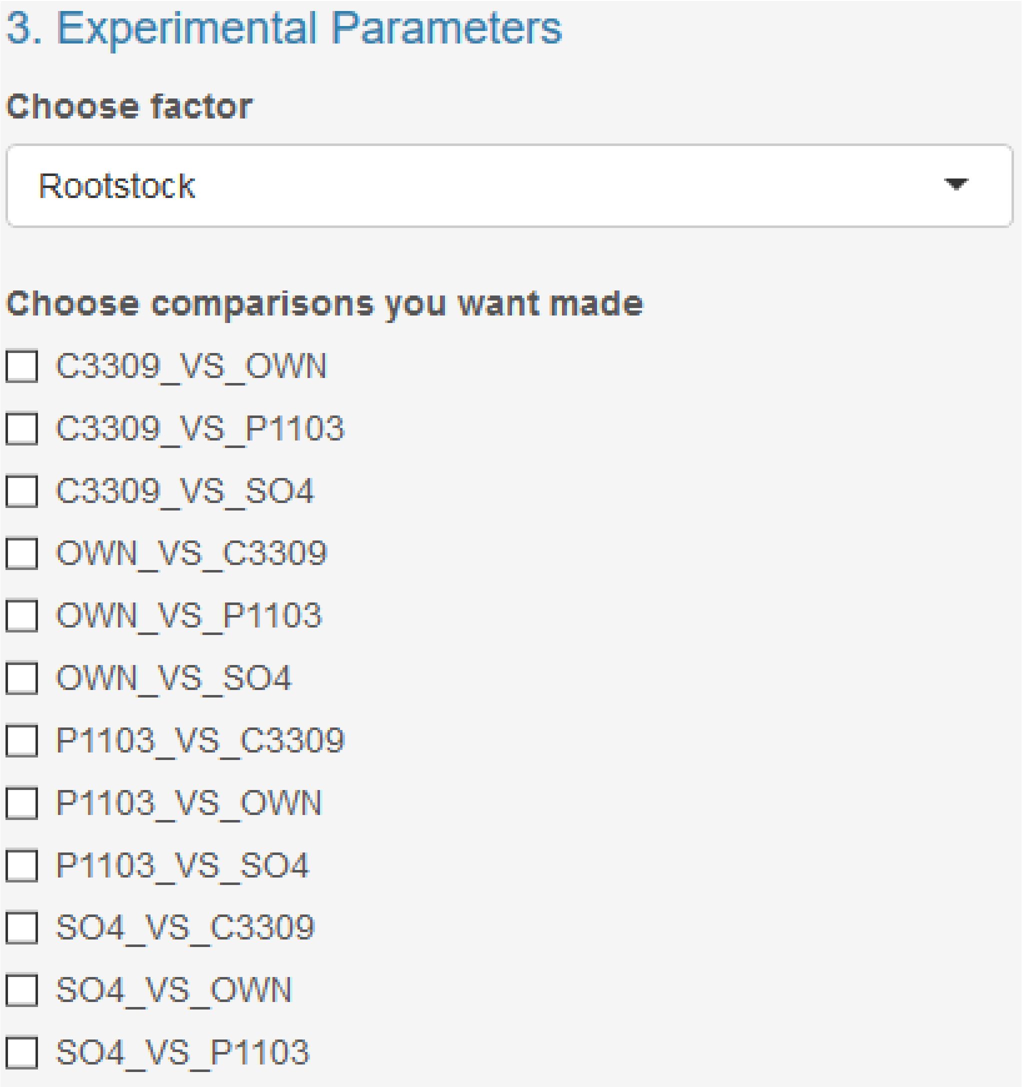
2. Also under `2. DGE Parameters`, adjusted p-value and log-fold change cutoffs can be specified to filter the results provided. The default values are 0.05 for adjusted p-value and 1 for the minimum log-fold change. To provide all gene comparisons, select 1 for adjusted p-value cutoff and 0 for the minimum log-fold change:



Adj. p-value cutoff: 0.05

Min. fold change: 1

3. Upon indication of the experimental design, DGE tool and cutoffs, the parameters and comparisons for the specified analysis must be provided. Such parameters can be found under `3. Experimental Parameters`. Depending on the selected experimental design, the required parameters and comparisons will vary:



3. Experimental Parameters

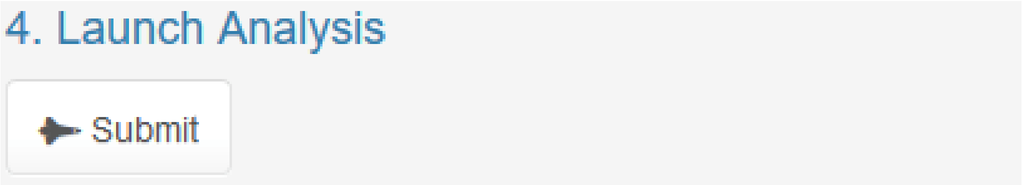
Choose factor

Rootstock

Choose comparisons you want made

□ C3309_VS_OWN
□ C3309_VS_P1103
□ C3309_VS_SO4
□ OWN_VS_C3309
□ OWN_VS_P1103
□ OWN_VS_SO4
□ P1103_VS_C3309
□ P1103_VS_OWN
□ P1103_VS_SO4
□ SO4_VS_C3309
□ SO4_VS_OWN
□ SO4_VS_P1103

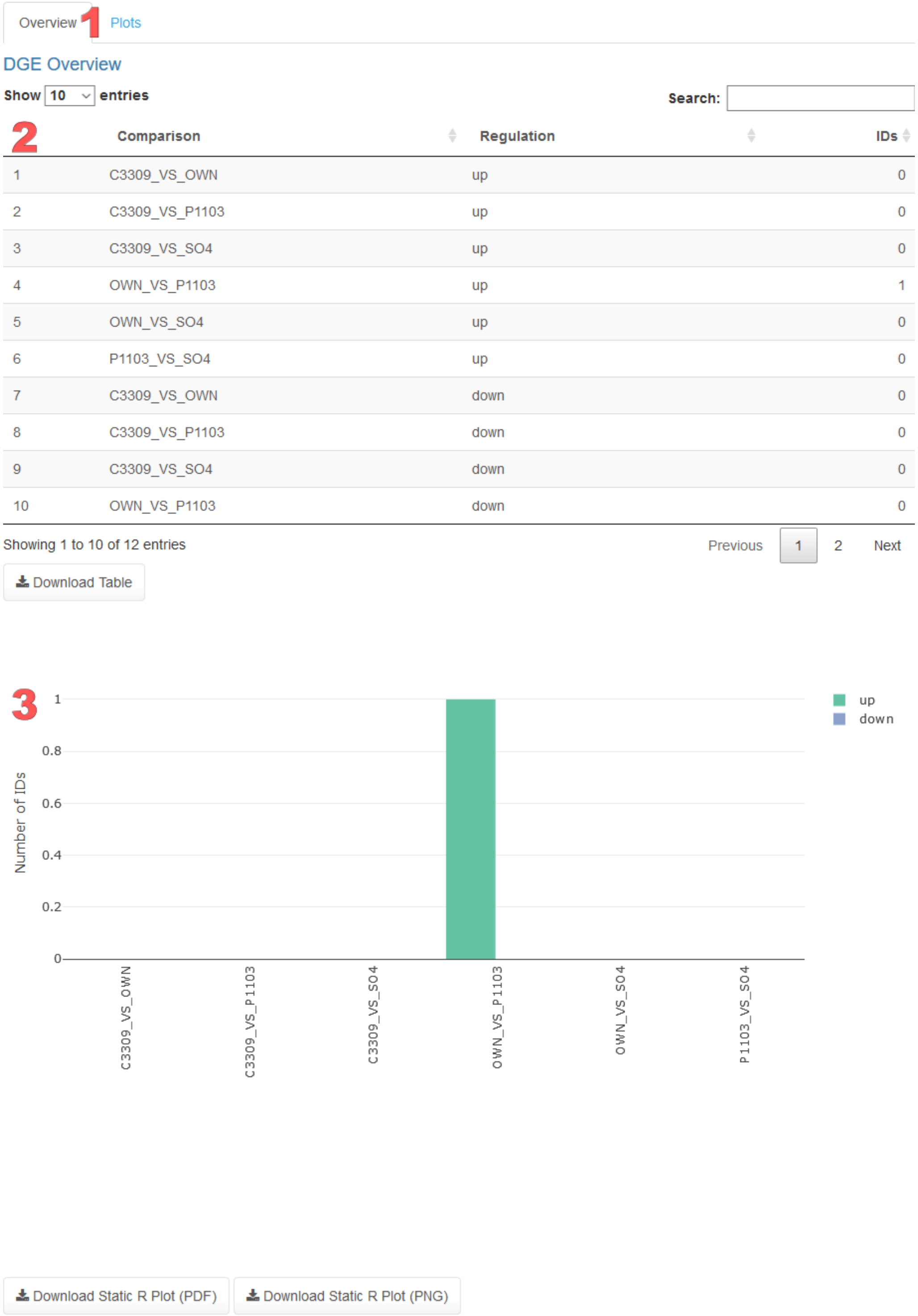
4. Launching the analysis will perform the DGE analysis and provide results. This is found under `4. Launch Analysis`. **Note:** Be mindful that this process may take more time than previous steps. Adjusting the cutoff values after submission will automatically update the generated visualizations and results table:



4. Launch Analysis

5. If you haven't crashed your computer or our server, the main page will now be populated with data in the two subtabs: `Overview` and `Plots`.

6. The `Overview` subtab (1) will display information based on the number of differentially expressed genes (DEGs), including a table of significantly up- and down-regulated gene ID counts by selected comparisons (2) and an interactive barplot representing this data (3). *With the example data, the results show only one gene is differentially expressed in either direction from the two-group comparison based on the Rootstock factor. This information seems to follow with the previously investigated figures showing limited clustering of samples from the PCA and MDS and high correlation values between samples:*



DGE Overview

Show 10 entries

Search:

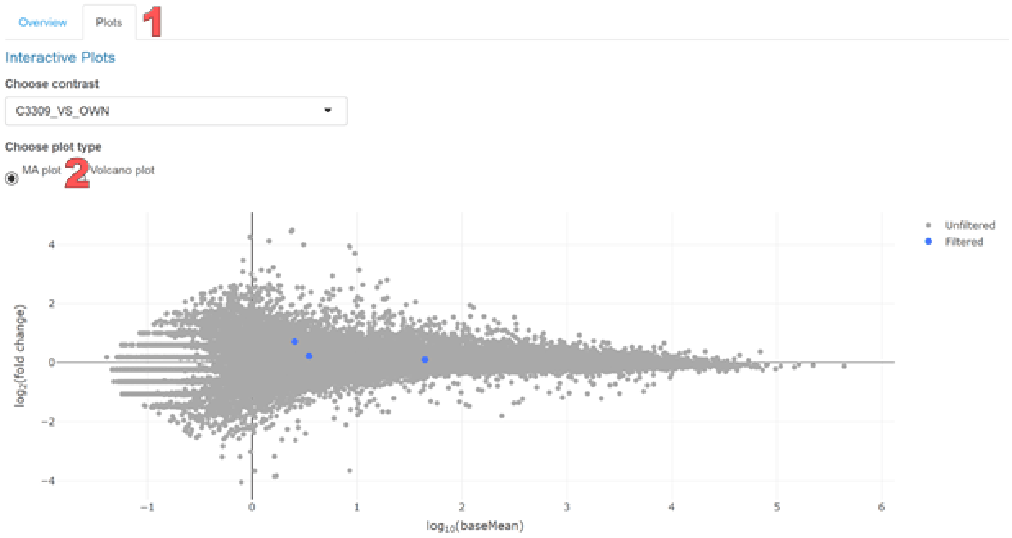
	Comparison	Regulation	IDs
1	C3309_VS_OWN	up	0
2	C3309_VS_P1103	up	0
3	C3309_VS_SO4	up	0
4	OWN_VS_P1103	up	1
5	OWN_VS_SO4	up	0
6	P1103_VS_SO4	up	0
7	C3309_VS_OWN	down	0
8	C3309_VS_P1103	down	0
9	C3309_VS_SO4	down	0
10	OWN_VS_P1103	down	0

Showing 1 to 10 of 12 entries

Previous 1 2 Next

7. The `Plots` subtab (1) will provides interactive visualizations based on the differential expression analysis as well as the actual results file from the previously-selected tool based on the selected comparison. The MA plot (2) shows the transformed log-fold change compared to

the transformed base mean for each gene ID. Specific points can be selected, highlighting both the point and the corresponding row in the results table found below the figure. Additionally, the gene ID can be selected from the results table, which will highlight the location of that gene ID on the figure:



Interactive Plots

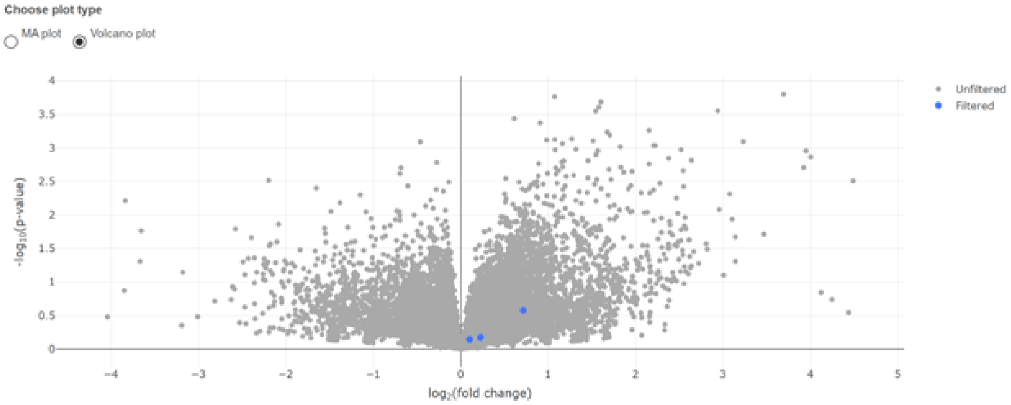
Choose contrast

C3309_VS_OWN

Choose plot type

● MA plot ○ Volcano plot

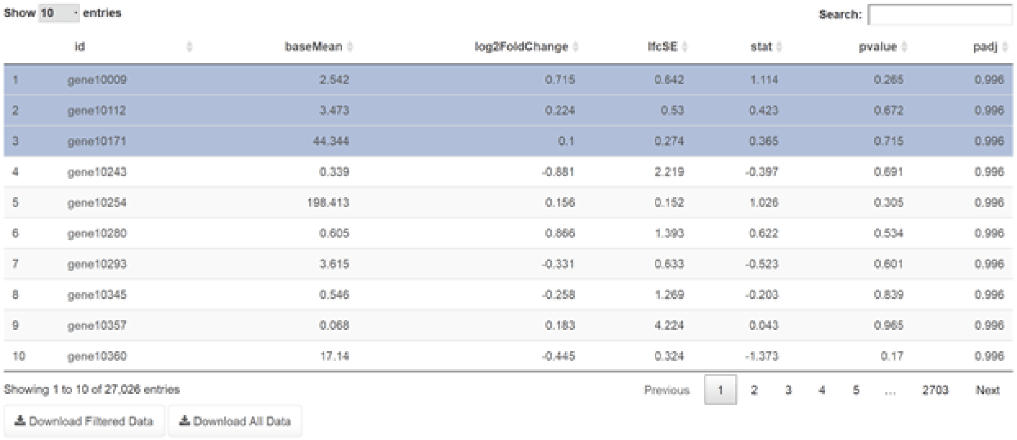
8. The Volcano plot shows a comparison of the transformed p-value compared with the transformed log-fold change. As with the MA plot, the Volcano plot is interactively connected with the results table found below the figure:



Choose plot type

○ MA plot ● Volcano plot

9. Finally, the results table is generated in coordination with the above plots and displays the output file of the selected tool's differential gene expression analysis. This table can be sorted increasingly or decreasingly by any of column. The integrated search feature also allows for specific gene IDs to be found in the table. This results file can be exported in a filtered or unfiltered format. *With the example data, sorting by adjusted p-value (padj) shows that there are no differentially expressed genes for this specific comparison (C3309 vs. OWN):*



	id	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
1	gene10009	2.542	0.715	0.642	1.114	0.265	0.996
2	gene10112	3.473	0.224	0.53	0.423	0.672	0.996
3	gene10171	44.344	0.1	0.274	0.365	0.715	0.996
4	gene10243	0.339	-0.881	2.219	-0.397	0.691	0.996
5	gene10254	198.413	0.156	0.152	1.026	0.305	0.996
6	gene10280	0.605	0.866	1.393	0.622	0.534	0.996
7	gene10293	3.615	-0.331	0.633	-0.523	0.601	0.996
8	gene10345	0.546	-0.258	1.269	-0.203	0.839	0.996
9	gene10357	0.068	0.183	4.224	0.043	0.965	0.996
10	gene10360	17.14	-0.445	0.324	-1.373	0.17	0.996

Showing 1 to 10 of 27,026 entries

S8: GEO Usage

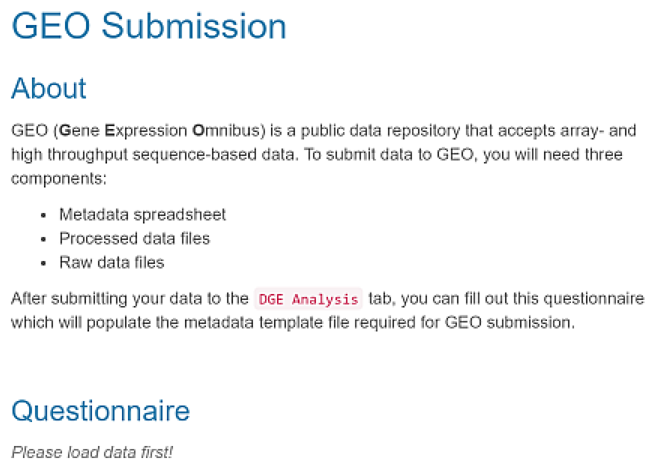
S8.1: Overview

GEO (**G**ene **E**xpression **O**mnibus) is a public data repository that accepts array- and high throughput sequence-based data. To submit data to GEO, you will need three components:

- Metadata spreadsheet
- Processed data files
- Raw data files

The `GEO` section of this web server will aid in the production of two of the prior components: **metadata and processed data generation.** This works by having the user fill out a dynamic questionnaire which follows the template foundation located on NCBI's servers shown here. While the user fills out this form, IRIS-EDA will automatically parse your raw count data into seperate text files based on the number of samples found in your raw count matrix. This can be downloaded via ZIP file. Additionally, the information provided by the column data matrix will be used to populate several components of the metadata, including sample information, characteristics, and MD5 checksum generation.

To get started, you *must* load both the raw count data and sample information on the `Submit and QC` tab. If you do not and head directly to the `GEO` tab, you will be greeted with a page that looks like this:



GEO Submission

About

GEO (**G**ene **E**xpression **O**mnibus) is a public data repository that accepts array- and high throughput sequence-based data. To submit data to GEO, you will need three components:

- Metadata spreadsheet
- Processed data files
- Raw data files

After submitting your data to the `DGE Analysis` tab, you can fill out this questionnaire which will populate the metadata template file required for GEO submission.

Questionnaire

Please load data first!

## S8.2: Series After you have successfully submitted your sample data and sample information, the first section that you will need to fill out is the **Series** section. This section describes the overall experiment. Specifically, there are six major components:

1. **Title**: a unique title (*less than 255 characters*) that describes the overall study.
2. **Summary**: a *thorough* description of the goals and objectives of this study. The abstract from the associated publication may be suitabe. You may provide as much text here as necessary.
3. **Overall design**: Indicate how many samples are analyzed, if replicates are included, are there control and/or reference Samples, etc.
4. **Contributor(s)**: Who contributed to this study? You must provide the information in the following format (`Firstname,Initial,Lastname`). Examples of this format:

```
* "Brandon,T,Monier"
* "Jane,Doe"
```

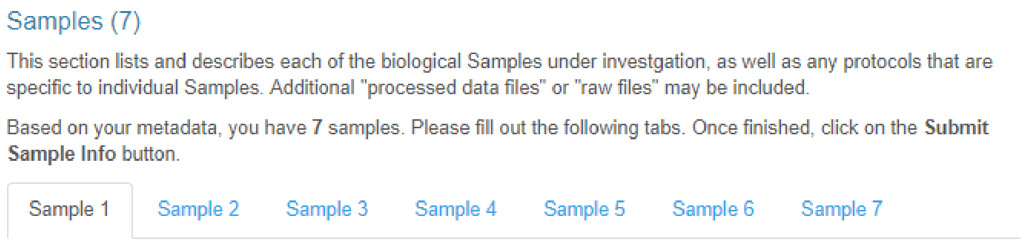
   This component is dynamic in this framework. If there was more than one contributor, click the `Add Contributor` button to add as many individuals who aided in this endeavor.

5. **Supplementary file**: If you want to submit your raw count matrix provided earlier, include the file name here (**optional**).
6. **SRA center name code**: Only enter a value if your institute already has a `Center_Name` code registered with SRA. Otherwise, leave empty (**optional**).

S8.3: Samples

This section lists and describes each of the biological Samples under investgation, as well as any protocols that are specific to individual Samples. Additional “processed data files” or “raw files” may be included.

Additionally, this section is dynamically generated. The tabs that will populate this section will be based on the number of samples you have submitted for analysis. For example, the “small example data set” has seven samples, therefore the number of tabs that would have to be filled out would be seven:



Samples (7)

This section lists and describes each of the biological Samples under investgation, as well as any protocols that are specific to individual Samples. Additional "processed data files" or "raw files" may be included.

Based on your metadata, you have 7 samples. Please fill out the following tabs. Once finished, click on the **Submit Sample Info** button.

It is mandatory that you fill out all samples. In order for the spreadsheet to properly be formatted, all of these sample tabs need to be filled out. Each sample has the following criteria:

1. **Sample name**: This will automatically be filled out for you. This provides an unique identifier for each sample that will not affect any downstream naming schemes.
2. **Title**: A unique title that describes the sample.
3. **Source name**: Briefly identfiy the biological material. (e.g. vastus lateralis muscle)
4. **Organism**: Identify the organism(s) from which the sequences were derived.
5. **Molecule**: The type of molecule that was extracted from the biological material. Choose from one of the preselected items from the dropdown input.
6. **Description**: Additional information not provided in the other fields, or paste in broad descriptions that cannot be easily dissected into the other fields.
7. **Processed data file**: The name of the file containing the processed data. **Note (1)**: This step has partially been completed for you. This will be the individual sample text file of raw counts. If you have more than one processed data file that you would like to contribute to GEO, click the `Add Proc. Data File` button to add as many entries you need.
8. **Raw file**: The name of the file containing the raw data. Similar to the processed data, additional raw data can be provided by clicking the `Add Raw Data File` button.

Note (2): If you have taken a look at the metadata template before, you will also notice that this section contains a characteristic column. This column is automatically generated for you based on your sample information provided in conjuntion with your raw count matrix. For example, the "small example data set" contains the following columns of conditions:

- condition
- type

Therefore, **two** characteristics columns will be generated in the spreadsheet. Each of the rows in these columns will based on the factor levels for each condition.

S8.4: Protocols

This section provides the public with information about what protocols were used to conduct this experiment. This section has the following criteria:

1. **Growth protocol**: Describe the conditions that were used to grow or maintain organisms or cells prior to extract preparation (**optional**).
2. **Treatment protocol**: Describe the treatments applied to the biological material prior to extract preparation.

3. **Extract prototocol**: Describe the protocols used to extract and prepare the material to be sequenced.
4. **Library construction protocol**: Describe the library construction protocol.
5. **Library strategy**: A Short Read Archive-specific field that describes the sequencing technique for this library. Choose from one of the preselected items from the dropdown input.

S8.5: Data processing pipeline

This section includes steps for various data processing techniques. Base-calling, alignment, filtering, peak-calling, generation of normalized, abundance measurements, etc.

1. **Data processing step**: Provide details of how processed data were generated. This can include procedures described in the paragraph above. Similar to prior steps, this section is dynamic. To add more steps, click the `Add Data Proc. Step` butto.
2. **Genome build**: UCSC or NCBI genome build number (e.g. hg18, mm9, etc.) or reference sequence used for read alignment.
3. **Processed data files format and content**: For each processed data file type, provide a description of the format and content.

S8.6: Processed data files

For each file that you provided additional processed data in the `Samples` section, you will need to provide additional information about these files below:

1. **File type**: The type of processed file. Examples include, peak, wig, bed, gff, bigWig, etc.
2. **MD5 file checksum**: MD5 checksum of the file. This helps GEO verify that the file transfer was complete and didn't corrupt your file.

S8.7: Raw files

Similar to the `Processed data files` section above, for each file listed in the "raw files" of the `Samples` section, provide additional information about these files below:

1. **File name**: The name of the file provided in the `Samples` section.
2. **File type**: The type of processed file. Examples include, peak, wig, bed, gff, bigWig, etc.
3. **MD5 file checksum**: MD5 checksum of the file. This helps GEO verify that the file transfer was complete and didn't corrupt your file.
4. **Instrument model**: Include the instrument make and model used to sequence the samples. Choose from one of the preselected items from the dropdown input.
5. **Read length**: The number of bases expected in each raw sequence. If you are using variable read length technology (e.g. 454, PacBio, Ion Torrent), put `0` for variable read length.

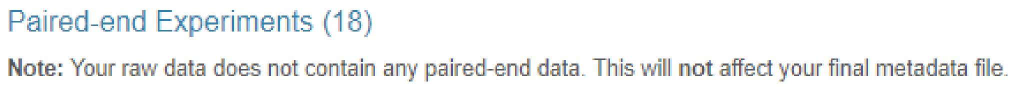
6. **Single or paired-end**: Choose either if your samples were sequenced with single reads or paired-end reads. If you used SOLiD technology, choose `SOLiD` in the options. **This is critical for the final section**.

S8.8: Paired-end experiments / SOLiD data

This section will only need to be filled out if your raw files used paired-end or SOLiD-based sequencing technologies. For paired-end experiments, list the 2 associated raw files, along with some additional information:

1. **File name 1**: Paired-end read 1.
2. **File name 2**: Paired-end read 2.
3. **Average instert size**: Average size of the insert for paired-end reads.
4. **Standard deviation**: Standard deviation of insert size. This is typically around 10% of the insert size stated.

Note (1): The previously mentioned components are for standard paired-end read sets only. If your raw reads used SOLiD technology, this section will may appear void of any information (see example):



Paired-end Experiments (18)

Note: Your raw data does not contain any paired-end data. This will not affect your final metadata file.

Instead of 2 reads, you will need to provide 4 (due to the basis of this technology) In the final section before the downloads portion. Therfore, instead of 2 “File names”, You will be prompted to fill out 4; each corresponding to their respective read.

S8.9: Downloads

After you have submitted all of your information, you can download two items:

- An Excel spreadsheet of the metadata;
- A ZIP file of individual sample reads as text files.

S9: Using large expression matrices

For users interested in analyzing large expression matrices, commonly experienced in single-cell analyses, we have proposed the following best-practices.

First, users should locally load the IRIS-EDA tool. Using the webserver version with large datasets will frequently lead to disconnecting from the server due to time constraints. Instructions for how to run locally can be found in S2: Accessibility.

Second, certain functionalities may either take too long to run or will not generate usable information. Because of this, we recommend using only the following functionalities within IRIS-EDA for large expression matrices:

1. PCA

2. MDS
3. t-SNE
4. biclustering with QUBIC

Users with large datasets should expect significant wait times for each analysis, even when using the above list.

S10: References

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