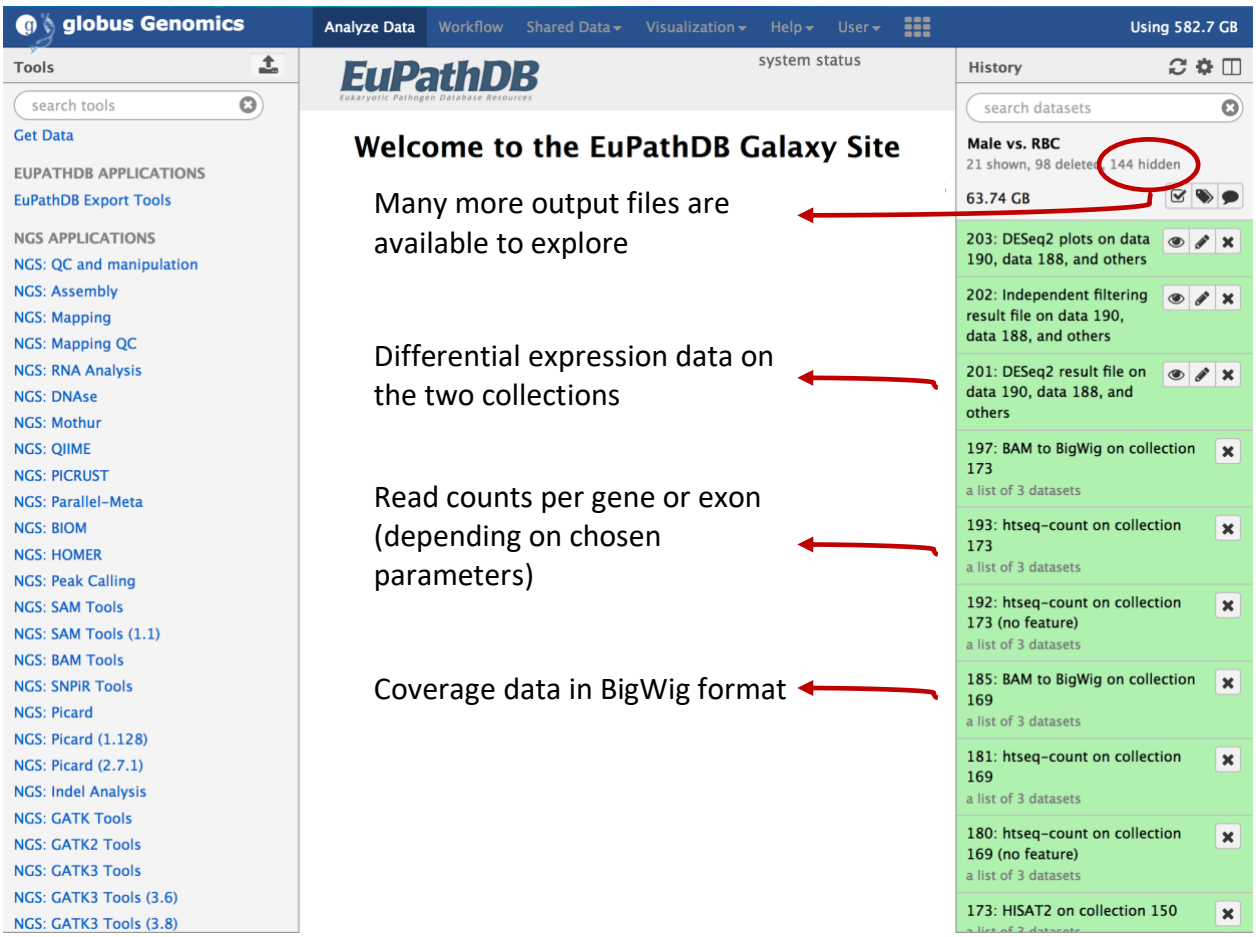
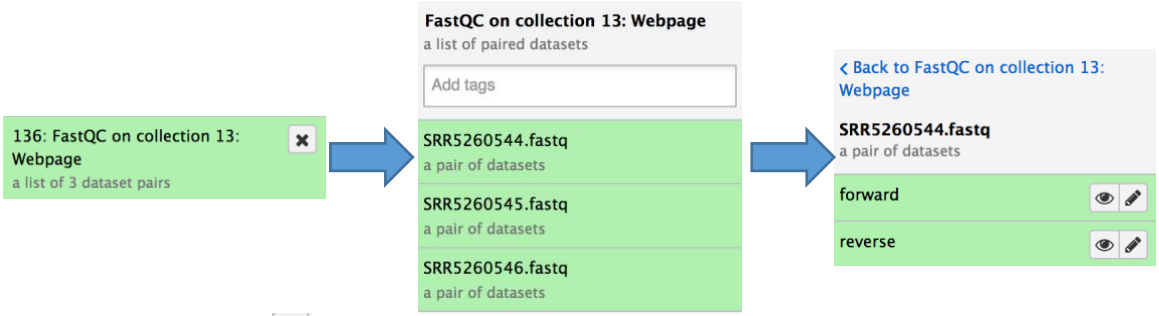
RNA sequence data analysis via Galaxy, Part II Uploading data and starting the workflow (Group Exercise)

The goal of this exercise is to examine the results from the Galaxy RNAseq analysis workflow that ran overnight. If everything worked out you should see a list of completed workflow steps (Green). The workflow generates many output files, however not all of the output files are visible. You can explore all the hidden files clicking on the word "hidden" (red circle) – this will reveal all hidden files.

Resources:
[FastQC Result Interpretation](https://workshop.eupathdb.org/athens/2019/exercises/fastqc_results-2.pdf)
(https://workshop.eupathdb.org/athens/2019/exercises/fastqc_results-2.pdf)
[Beginner DESeq2 guide](https://workshop.eupathdb.org/athens/2019/exercises/beginner_DeSeq2.pdf) (https://workshop.eupathdb.org/athens/2019/exercises/beginner_DeSeq2.pdf)
[FastQC output](https://workshop.eupathdb.org/athens/2019/exercises/fastqc_output.pdf) (https://workshop.eupathdb.org/athens/2019/exercises/fastqc_output.pdf)
[SNP Eff manual](http://snpeff.sourceforge.net/SnpEff_manual.html) (http://snpeff.sourceforge.net/SnpEff_manual.html)
[Trimmomatic Manual](http://www.usadellab.org/cms/uploads/supplementary/Trimmomatic/TrimmomaticManual_V0.32.pdf)
(http://www.usadellab.org/cms/uploads/supplementary/Trimmomatic/TrimmomaticManual_V0.32.pdf)



Step 1: Explore the FastQC results. To do this find the step called "FastQC on collection ##: Webpage". Click on the name this will open up the FastQ pairs, click on one of them then



click on view data icon ( ) on either forward or reverse. Note that each FastQ file will have its own FastQC results. An explanation of each of the FastQC results is provided as a link on the main workshop website or at the bottom of the FastQC results page.

SRR5260544_1.fastq.gz FastQC Report

FastQC Report
Tue 12 Jun 2018
SRR5260544_1.fastq.gz

Summary

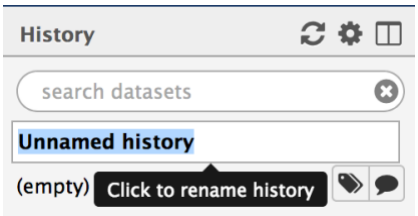
- Basic Statistics
- Per base sequence quality
- Per tile sequence quality
- Per sequence quality scores
- Per base sequence content
- Per sequence GC content
- Per base N content
- Sequence Length Distribution
- Sequence Duplication Levels
- Overrepresented sequences
- Adapter Content
- Kmer Content

Basic Statistics

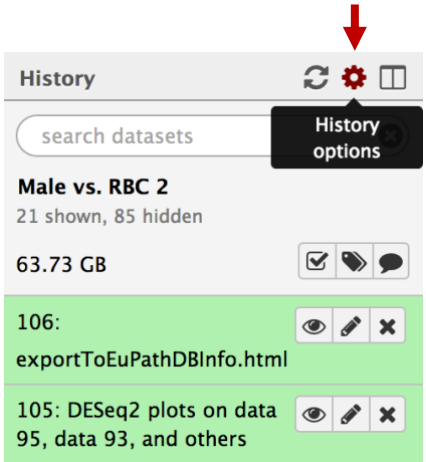
Measure	Value
Filename	SRR5260544_1.fastq.gz
File type	Conventional base calls

Step 2: Sharing histories with others:

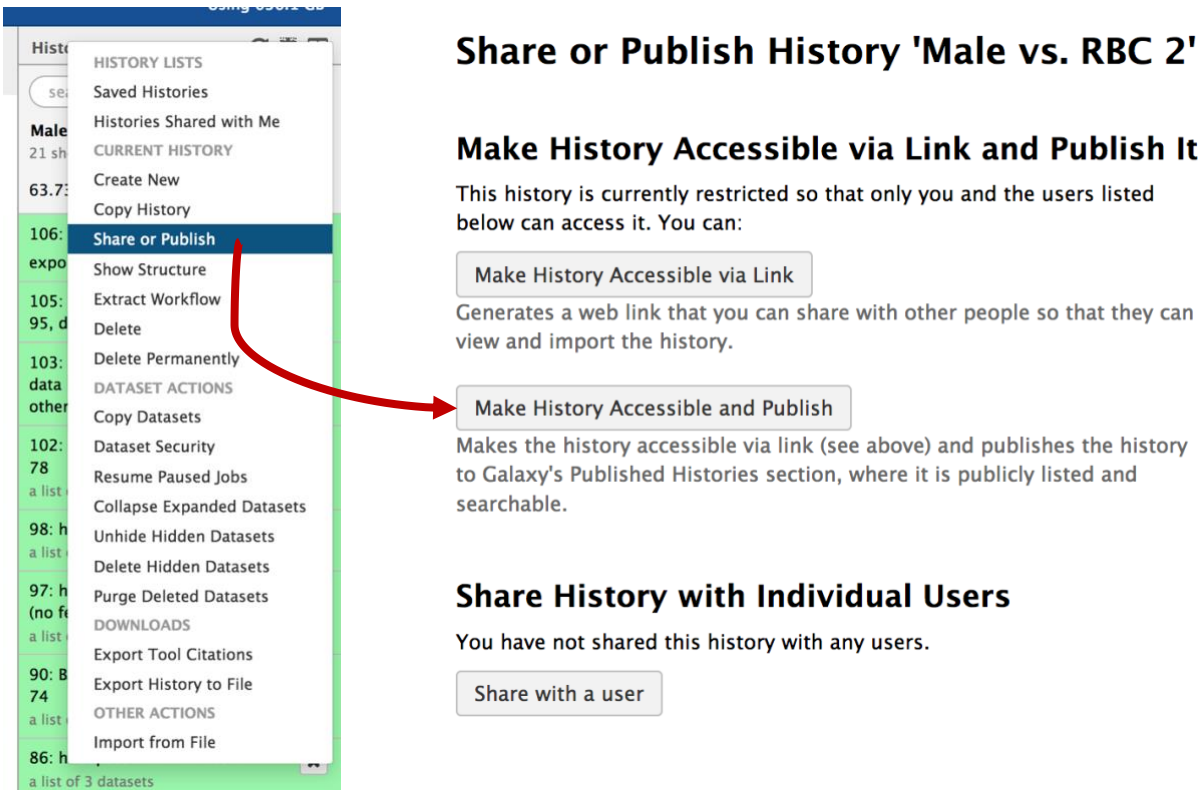
a. Make sure your history has a useful name – you can change the name by clicking on “unnamed history”



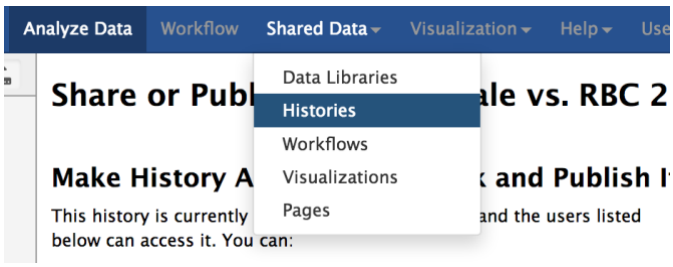
b. Click on the history options menu icon



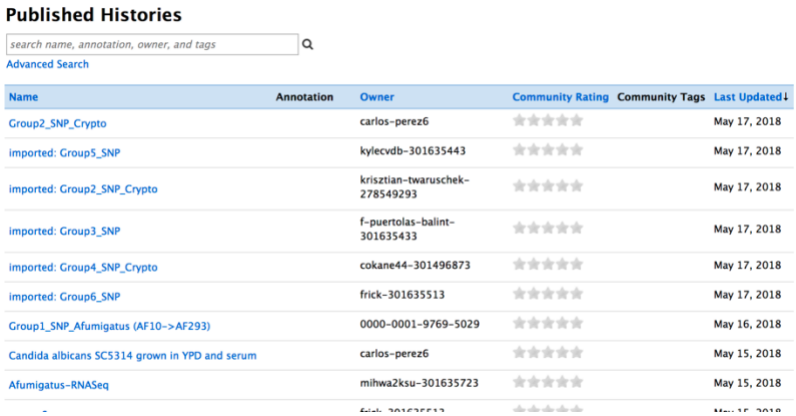
c. Select the “Share or Publish” option, the click on the “Make History Accessible and Publish” button in the center section.



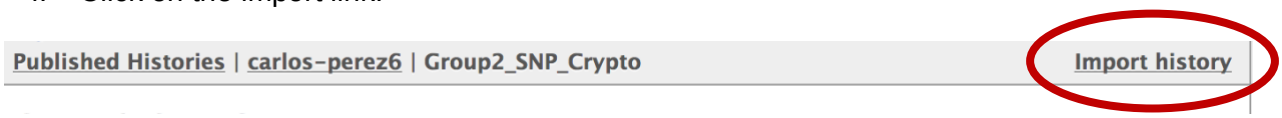
d. To import a shared history, go to the “histories” section (under the shared data menu item).



e. Find the history you would like to import and click on it.



f. Click on the import link.



Step 3: Explore the differential expression results:

DESeq2 is a package with essential estimates expression values and calculates differential expression. DESeq2 requires counts as input files. You can explore details of DESeq2 here: https://bioc.ism.ac.jp/packages/2.14/bioc/vignettes/DESeq2/inst/doc/beginner.pdf

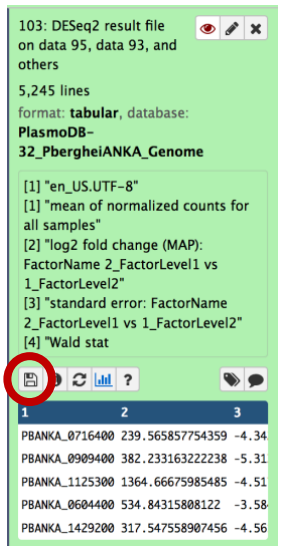
We will explore two output files:

A. DESeq2 Plots – you can view these directly in galaxy by clicking on the view icon. These plots give you an idea about the quality of the experiment. The link above includes a detailed description of the graphs.
B. DESeq2 results file – this is a table which contains the actual differential expression results. These can be viewed within galaxy but it will be more useful to download this table and open in Excel so you can sort results and big genes of interest.

The tabular file contains 7 columns:

COLUMN	DESCRIPTION
1	Gene Identifiers
2	mean normalized counts, averaged over all samples from both conditions
3	the logarithm (to basis 2) of the fold change (See the note in inputs section)
4	standard error estimate for the log2 fold change estimate
5	Wald statistic
6	p value for the statistical significance of this change
7	p value adjusted for multiple testing with the Benjamini-Hochberg procedure which controls false discovery rate (FDR)

C. To download the table, click on the step then click on the save icon.



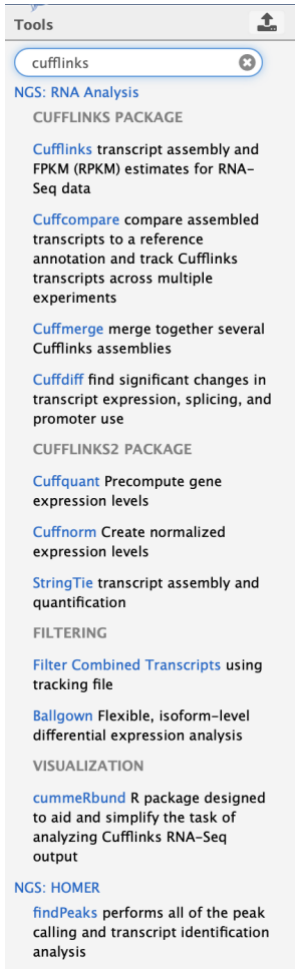
*** **important: the file name ends with the extension .tabular – change this to .txt then open the file in Excel.**

D. Explore the results in Excel. For example, sort them based on the log2 fold change – column 3.
E. Pick a list of gene IDs from column 3 that are up-regulated with a good corrected P value (column 7) and load then into PlasmoDB using the Gene by ID search. You can then analyze these results by GO enrichment for example. Do the same for down-regulated genes.
F. Compare results from the other groups. Can you find genes are that are uniquely up or down regulated in the conditions tested?

Exporting data to EuPathDB

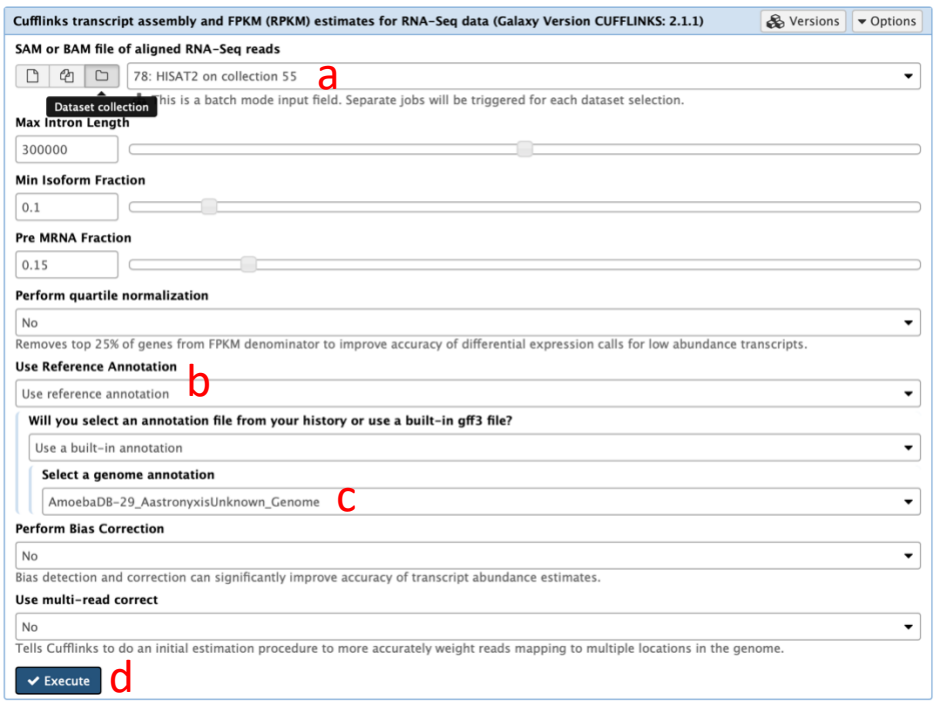
The EuPathDB RNAseq export tool provides a mechanism to query your RNAseq results (FPKM values) using EuPathDB search tools.

However, to use this feature you need to generate FPKM values for genes in you datasets. To this you need a tool called Cufflinks and read alignment files – BAM files. Our workflow from yesterday generated BAM alignment files from a tool called HISAT2.



Follow these steps to generate FPKM values:

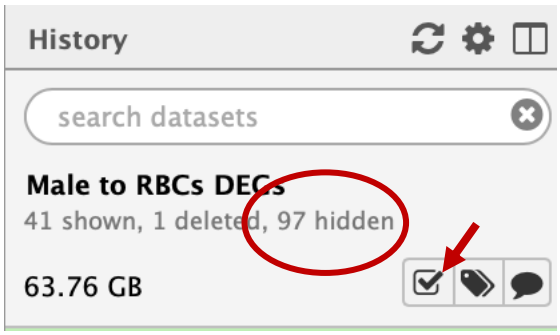
1. Find the tool called Cufflinks by typing the word cufflinks in the tool search box on the left-hand side.
2. Click on the tool to access its parameters.
3. Modify the cufflinks parameters
    - Change the input file to collection and select one of the HISAT2 collections
    - Change the Use Reference Annotation from "No" to "use reference annotation'
    - Select the appropriate reference genome from the drop down list
    - Click on execute.

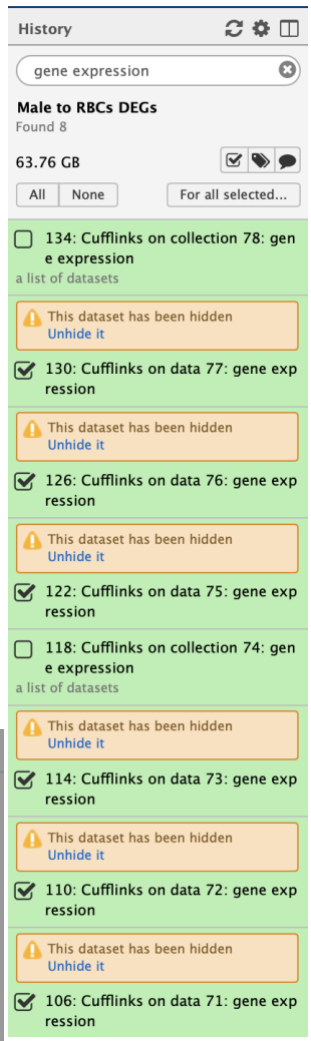


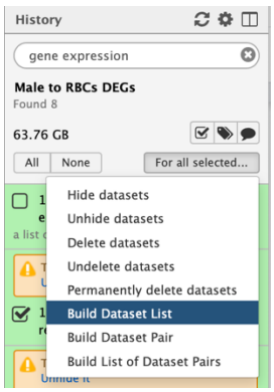
After Cufflinks is done running, the next step is to take the FPKM output files from the collection outputs and put them into a single collection. Notice that cufflinks generates three types of FPKM files (or collections in this case): 1. Gene expression 2. Transcript

expression 3. Assembled transcripts. We will only worry about the gene expression files for this section.

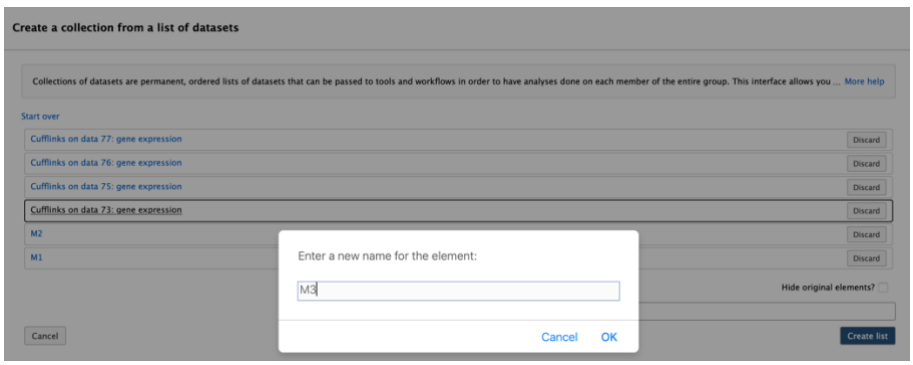
- Since we have collections of output files we will need to show all hidden files so we can generate the single collection. To do this, click on the word hidden in the upper right-hand side of the screen
- This will expose all hidden files.
- Click on the check box to perform an operation on multiple datasets (arrow in above image)
- Find all files containing the words "gene expression" and select all the cufflinks files (**NOT** the collections)
- Build a dataset list by clicking on the "for all selected" button and select "Build dataset list".
- Rename each of the datasets in the list and give this collection a meaningful name.



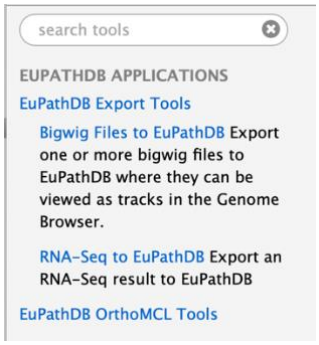




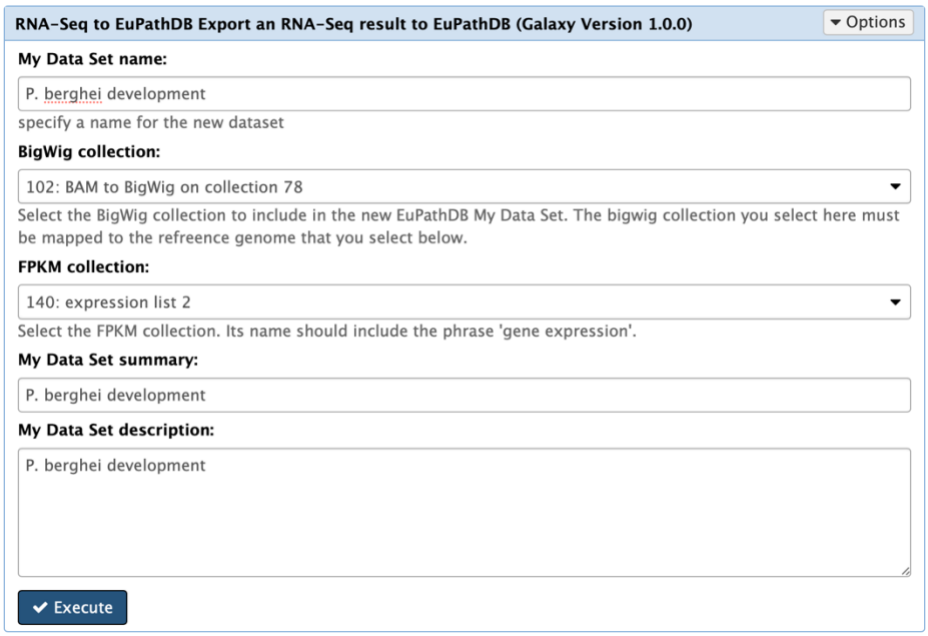
Step 4: Export Expression files to EuPathDB



1. Click on "EuPathDB Export Tools" in the left-hand panel.
2. Click on the tool called "RNA-Seq to EuPathDB"



3. Fill up the export tool and select the correct files to export.



4. Click on the “My Datasets” link in the grey menu bar. You should see the dataset you exported from galaxy in this list. Click on it and explore the dataset page.
5. Click on Execute and wait for the export step to complete.
6. When export is complete, go to the EuPathDB website with the genomes for this data, e.g PlasmoDB.
7. Click on the available search and explore this page. Can you run a search to identify genes differentially expressed between the two conditions you analyzed in Galaxy. How do these compare to the results you got from DEseq2?