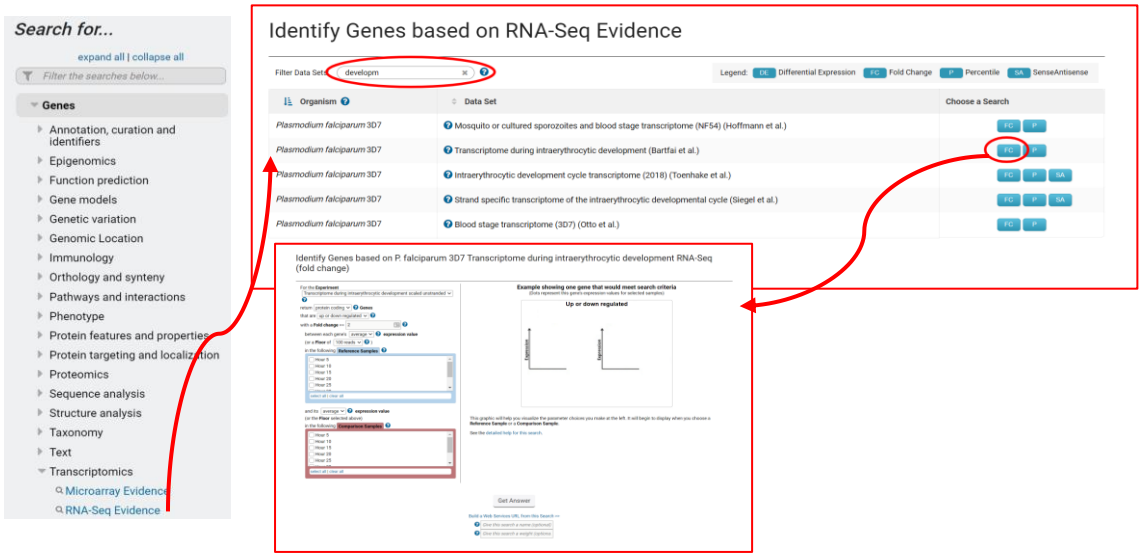
Exploring Transcriptomic data

1. Exploring RNA sequence data in *Plasmodium falciparum.*
   Note: For this exercise use http://www.plasmodb.org

a. Find all genes in *P. falciparum* that are up-regulated during the later stages of the intraerythrocytic cycle.

- Use the fold change search for the data set "**Transcriptome during intraerythrocytic development (Bartfai *et al.*)".** For this data set, synchronized Pf3D7 parasites were assayed by RNA-seq at 8 time-points during the iRBC cycle. We want to find genes that are up-regulated in the later time points (30, 35, 40 hours) using the early time points (5, 10, 15, 20, 25 hours) as reference.



- There are a number of parameters to manipulate in this search. As you modify parameters on the left side note the dynamic help on the right side. See screenshots.

- **Direction**: the direction of change in expression. Choose up-regulated.

- **Fold Change>=** the intensity of difference in expression needed before a gene is returned by the search. Choose 12 but feel free to modify this.

- **Reference Sample**: the samples that will serve as the reference when comparing expression between samples. choose 5, 10, 15, 20, 25

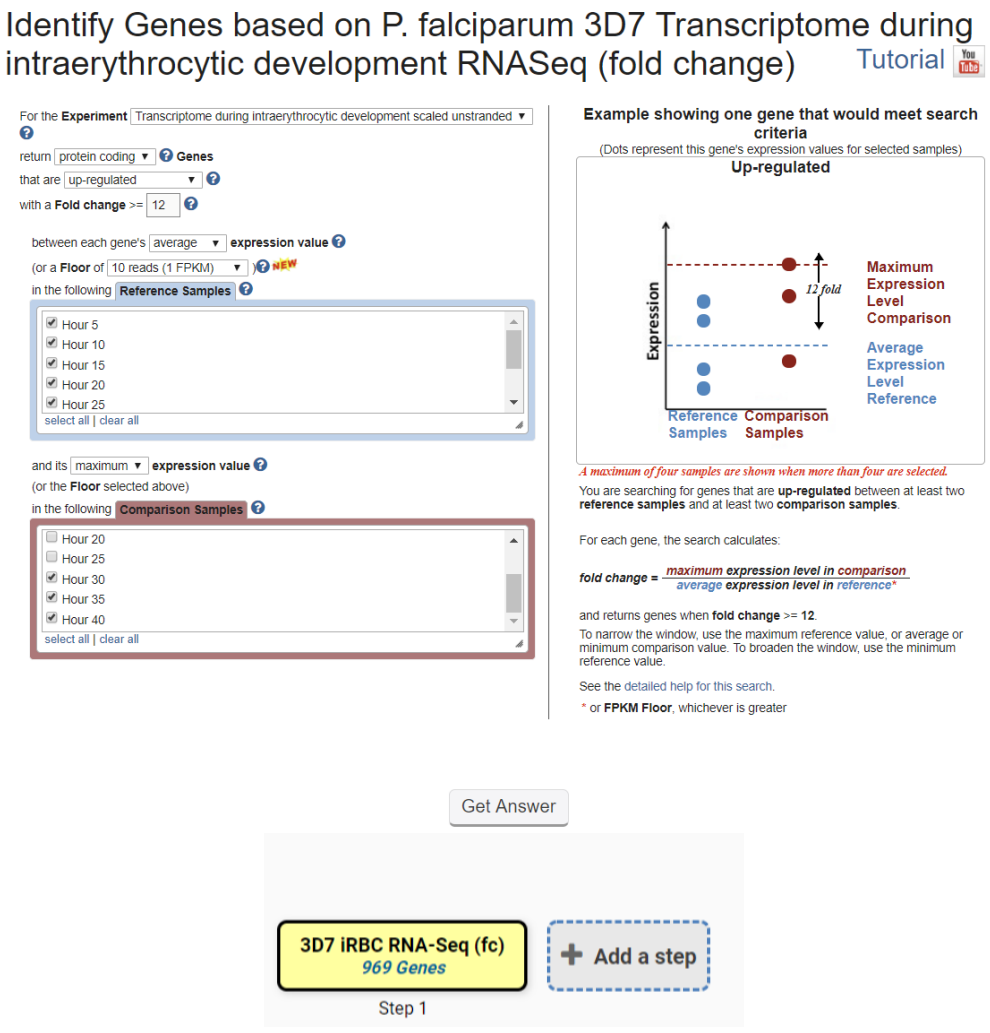
- **Between each gene's AVERAGE expression value:** This parameter appears once you have chosen two Reference Samples and defines the operation applied to reference samples.

Fold change is calculated as the ratio of two values (upregulated ratio = expression in comparison)/(expression in reference). When you choose multiple samples to serve as reference, we generate one number for the fold change calculation by using the minimum, maximum, or average. Choose average

- (or a Floor of 10 reads): This parameter defines a lower limit of aligned reads for a gene to avoid unreliable fold change calculations. (Low numbers of aligned reads means low expression but the low values may be may be technically inaccurate. Dividing by small numbers creates large numbers. 2000FPKM/10 = 200; 2000/0.1 = 20,000) If a gene has fewer than 10 aligned reads, it is assigned 10 reads before the fold change calculation is made. Leave this as default at 10 reads.

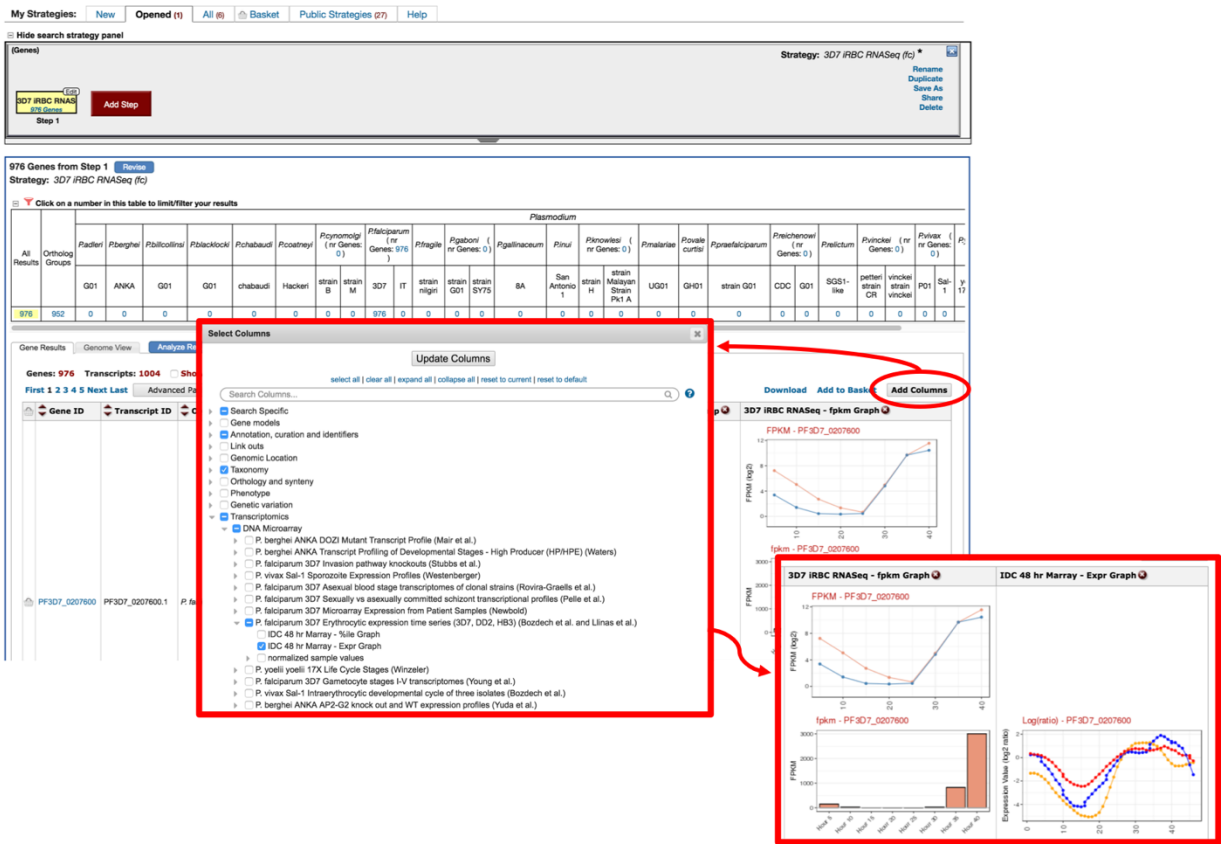
- Comparison Sample: the sample that you are comparing to the reference. In this case you are interested in genes that are up-regulated in later time points choose 30, 35, 40

- And its AVERAGE expression value: This parameter appears once you have chosen two Comparison Samples and defines the operation applied to comparison samples. See explanation above. Choose average

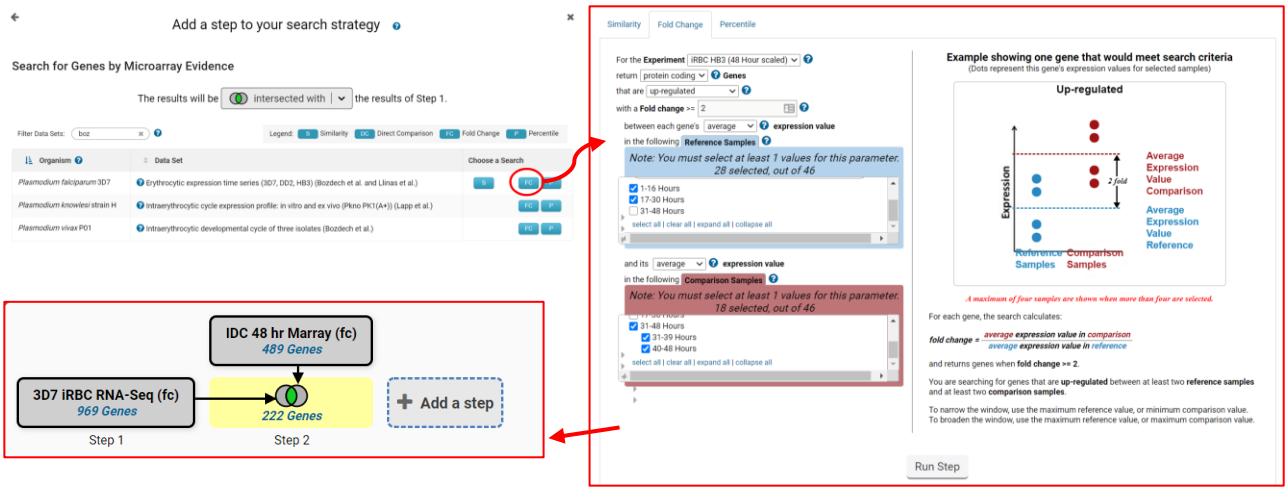


b. For the genes returned by the search, how does the RNA-sequence data compare to **microarray data**?

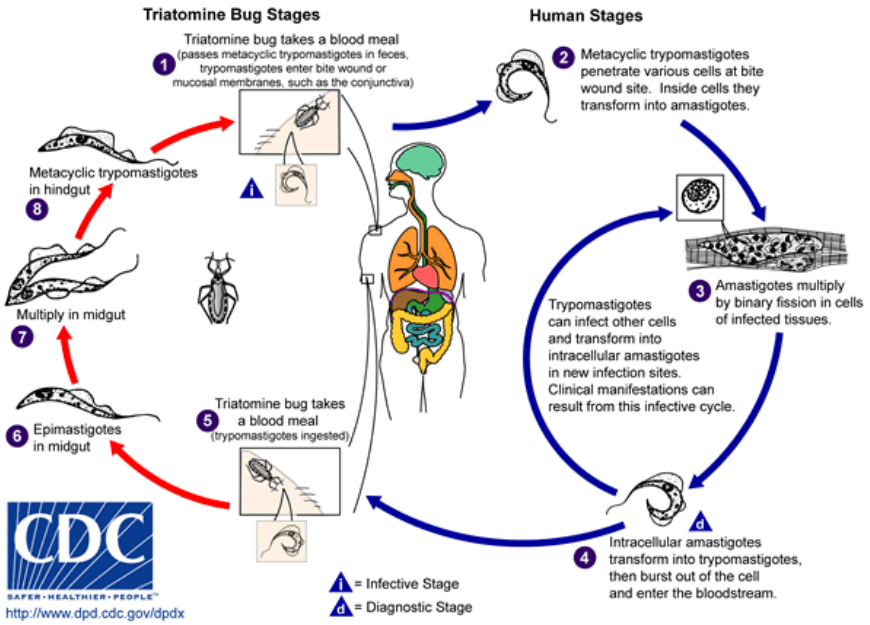
- Hint: PlasmoDB contains data from a similar experiment that was analyzed by microarray instead of RNA sequencing. This experiment is called: **Erythrocytic expression time series (3D7, DD2, HB3) (Bozdech et al. and Linas et al.). IDC 48 hr Marray – Expr Graph** shows normalized expression values. To directly compare the data for genes returned by the RNA-seq search that you just ran, add the column called "Pf-iRBC 48hr - Graph".



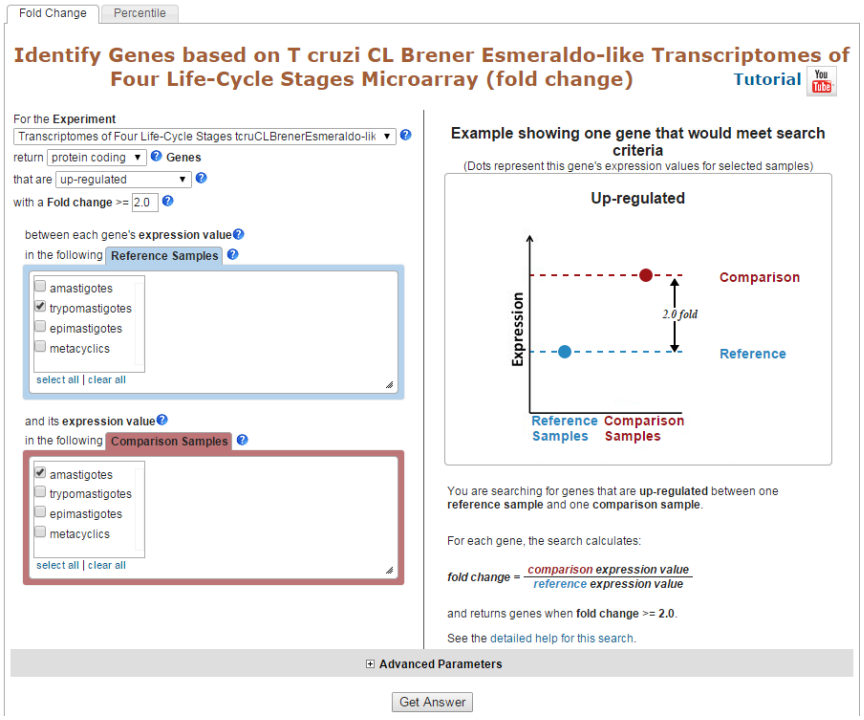
OPTIONAL: You can also run a fold change search using this experiment to compare results on a genome scale. Add a step to your strategy and intersect your current results (genes upregulated 12 fold in later IDC time periods) with a fold change search using the "Erythrocytic expression time series (3D7, Dd2, HB3) (Bozdech et al. and Linas et al.)" experiment (under microarray evidence). Configure it similarly to the RNA-seq experiment although you will probably need to make the fold change smaller (try 2 or 3) due to the decreased dynamic range of microarrays compared to RNA-seq.


Add a step to your search strategy
Search for Genes by Microarray Evidence
The results will be intersected with the results of Step 1.
Filter Data Sets: boz
Legend: S Similarity DC Direct Comparison FC Fold Change P Percentile
Organism
Data Set
Choose a Search
Plasmodium falciparum 3D7
Erythrocytic expression time series (3D7, DD2, HB3) (Bozdech et al. and Llinas et al.)
Plasmodium knowlesi strain H
Intraerythrocytic cycle expression profile: in vitro and ex vivo (Pkno PK1(A+)) (Lapp et al.)
Plasmodium vivax P01
Intraerythrocytic developmental cycle of three isolates (Bozdech et al.)
IDC 48 hr Marray (fc)
489 Genes
3D7 iRBC RNA-Seq (fc)
969 Genes
222 Genes
Add a step
Step 1
Step 2
Similarity
Fold Change
Percentile
For the Experiment iRBC HB3 (48 Hour scaled)
return protein coding Genes
that are up-regulated
with a Fold change >= 2
between each gene's average expression value
in the following Reference Samples
Note: You must select at least 1 values for this parameter.
28 selected, out of 46
1-16 Hours
17-30 Hours
31-48 Hours
select all | clear all | expand all | collapse all
and its average expression value
in the following Comparison Samples
Note: You must select at least 1 values for this parameter.
18 selected, out of 46
31-48 Hours
31-39 Hours
40-48 Hours
select all | clear all | expand all | collapse all
Example showing one gene that would meet search criteria
(Dots represent this gene's expression values for selected samples)
Up-regulated
Expression
2 fold
Average Expression Value Comparison
Average Expression Value Reference
Reference Samples
Comparison Samples
A maximum of four samples are shown when more than four are selected.
For each gene, the search calculates:
fold change = average expression value in comparison / average expression value in reference
and returns genes when fold change >= 2.
You are searching for genes that are up-regulated between at least two reference samples and at least two comparison samples.
To narrow the window, use the maximum reference value, or minimum comparison value.
To broaden the window, use the maximum reference value, or maximum comparison value.
Run Step

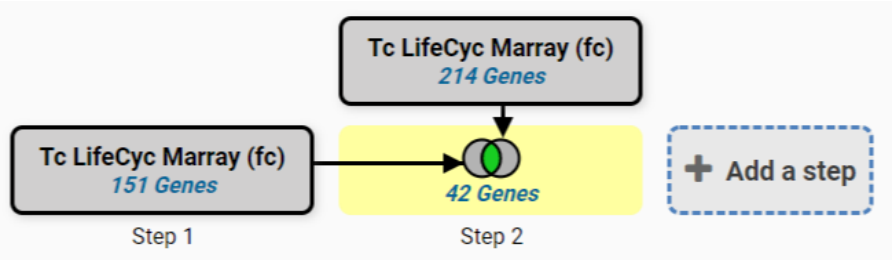
2. Exploring microarray data in TriTrypDB.
   Note: For this exercise use http://www.tritrypdb.org



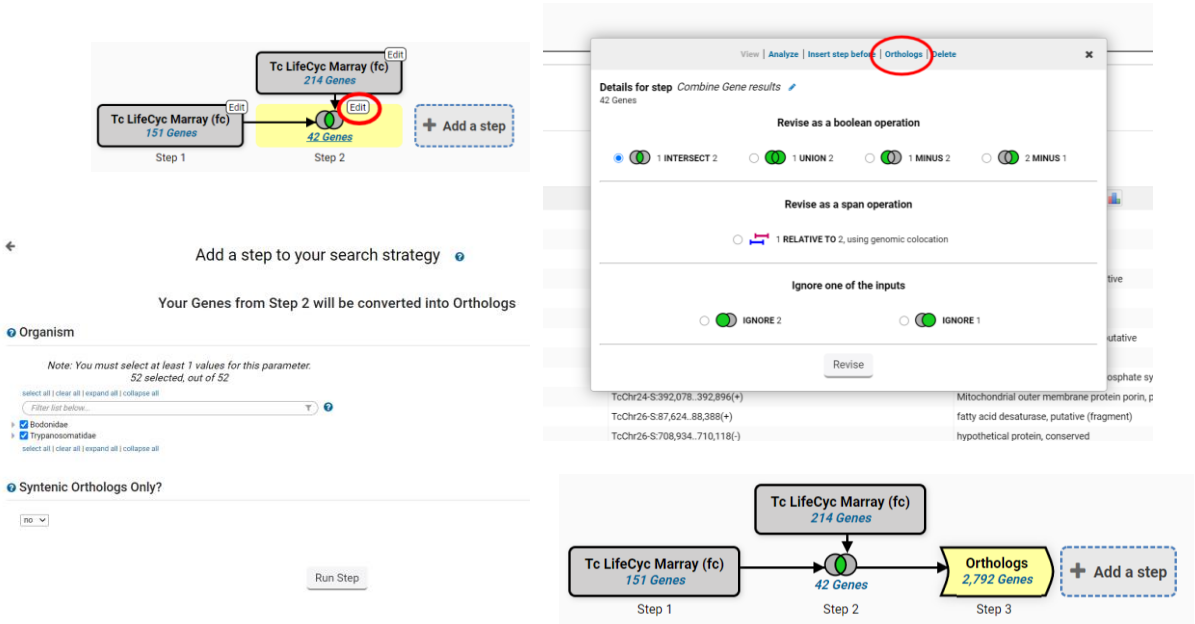
a. Find *T. cruzi* protein coding genes that are upregulated in amastigotes compared to trypomastigotes. Go to the transcript expression section then select **microarray**. Choose the fold change (FC) search for the data set called: **Transcriptomes of Four Life-Cycle Stages (Minning et al.)**.



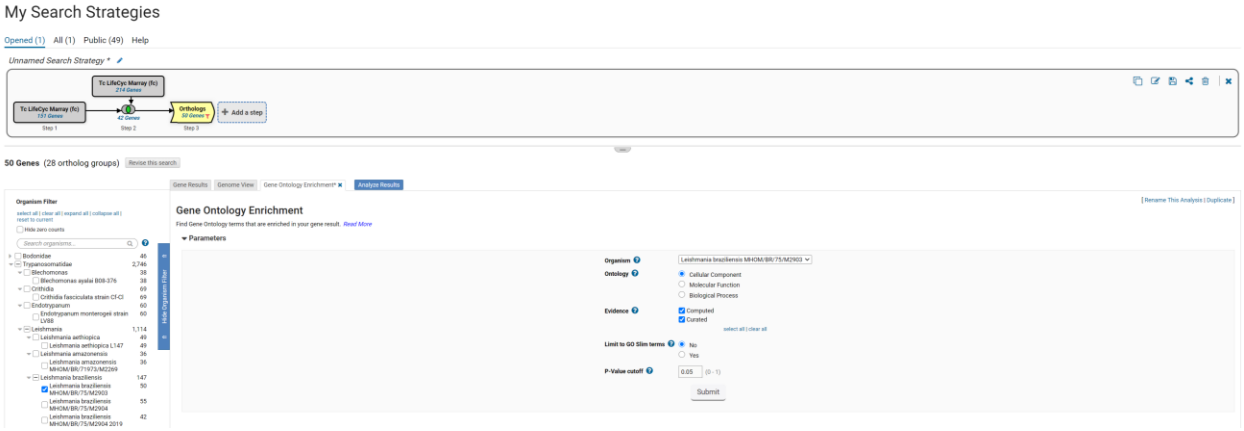
- Select the direction of regulation, your reference sample and your comparison sample. For the fold change keep the default value 2.
- How many genes did you find? Do the results seem plausible?
- Are any of these genes also up-regulated in the replicative insect stage compared to the transmissive insect stage? How can you find this out? (*Hint*: add a step and run a microarray search comparing expression of epimastigotes to metacyclics).



- Do these genes have orthologs in other kinetoplastids? Transform your results into orthologs in all other organisms in TriTrypDB. This can be done by adding a step, or by editing a step, as shown in the screenshots.

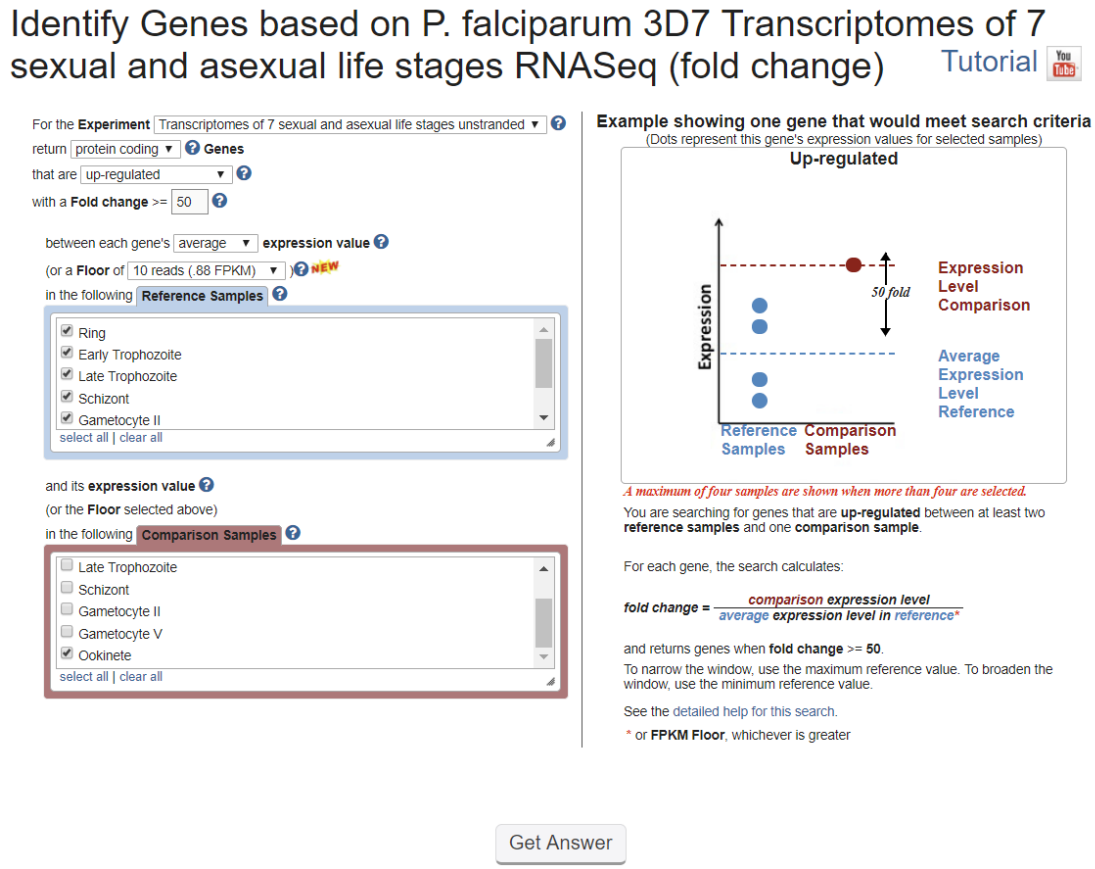


- How many orthologs exist in *L. braziliensis* MHOM/BR/75/M2903? (*Hint*: look at the organism filter to the left of your result list. Click on the check box next to a species, then click apply to view results from a specific species). Explore your results. Scan the product descriptions for this list of genes. Did you find anything interesting? Perhaps a GO enrichment analysis would support your ideas.



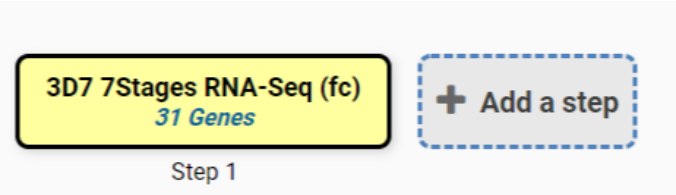
3. Finding genes based on RNAseq evidence and inferring function of hypothetical genes. Note: Use http://plasmodb.org for this exercise.

a. Find all genes in *P. falciparum* that are up-regulated at least 50-fold in ookinetes compared to other stages: "Transcriptomes of 7 sexual and asexual life stages (Lopez-Barragan et al.)". For this search select "average" for the operation applied on the reference samples.

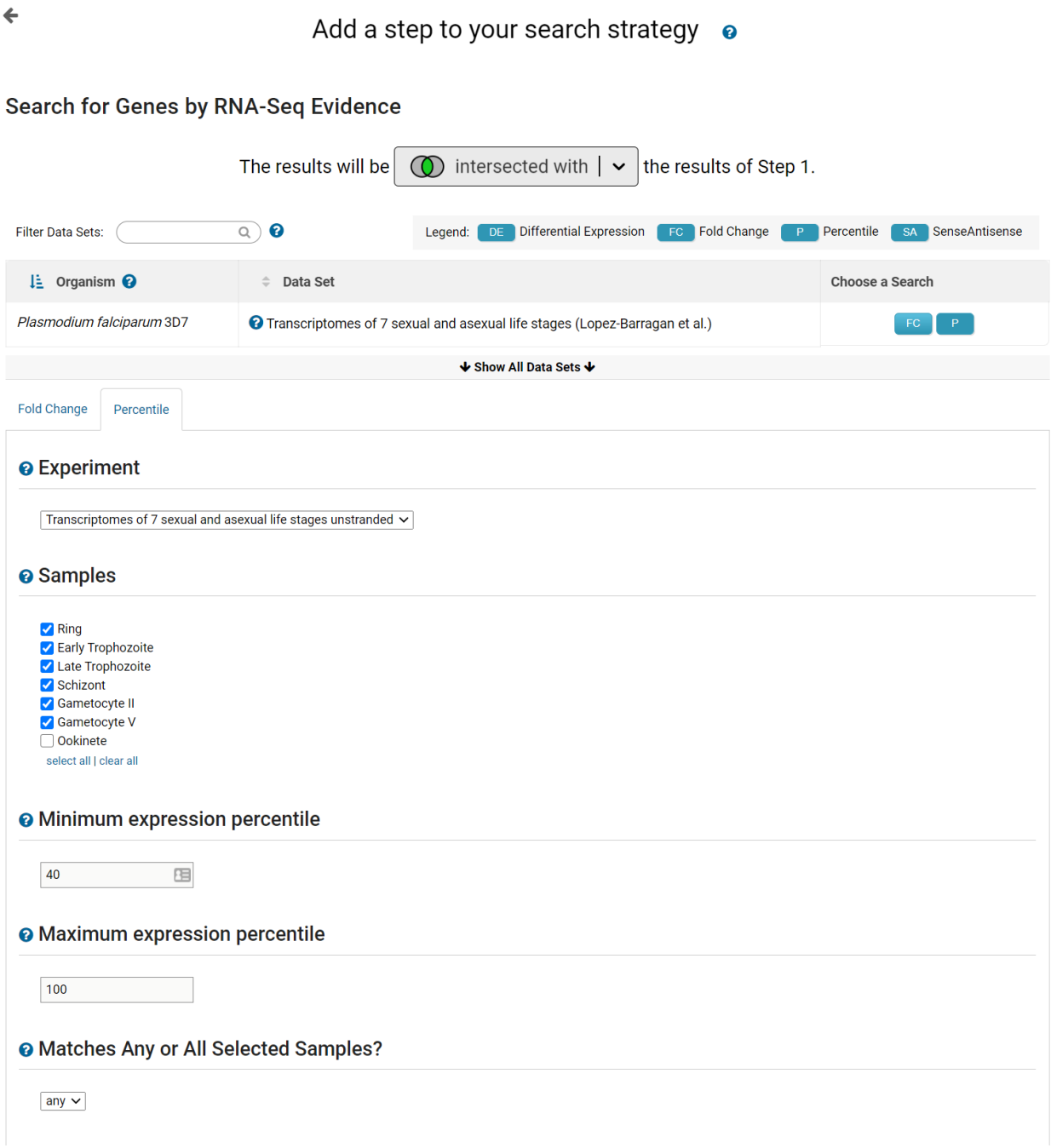


Get Answer

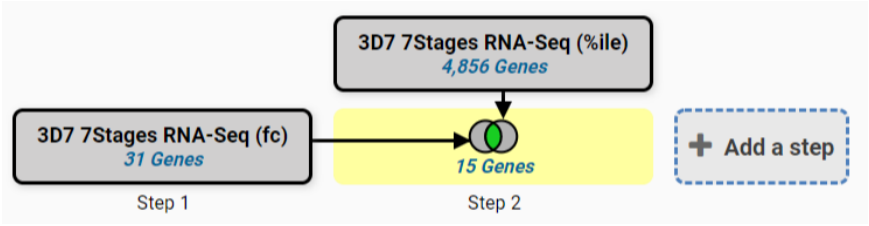
b. The above search will give you all genes that are up-regulated by 50 fold in ookinetes compared to the average expression level of other stages. Despite the high fold change, some genes in the list may be highly expressed in the other stages. How can you remove genes from the list that are highly expressed in the other stages?



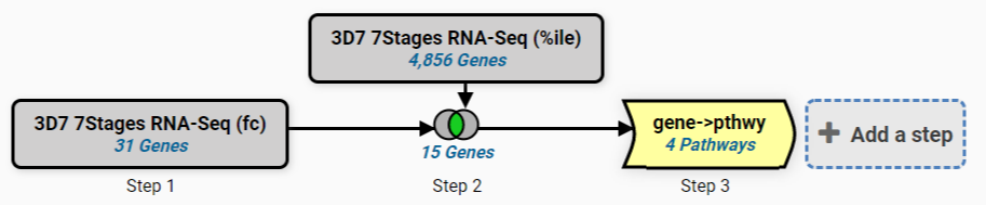
- *Hint: Add a search for genes based on RNA Seq evidence from the same experiment, but this time select the percentile search: P.f. seven stages - RNA Seq (percentile). What minimal percentile values should you choose? 40 – 100%? How does setting the any / all samples impact the result …. Which would be better in this case?*



- *Hint II: Try changing the operator from average to maximum for the set of non-ookinete stages in your initial fold change search. What does this do? How do the resulting genes compare with the two step strategy you generated in the first hint? Which hint do you think works better?*

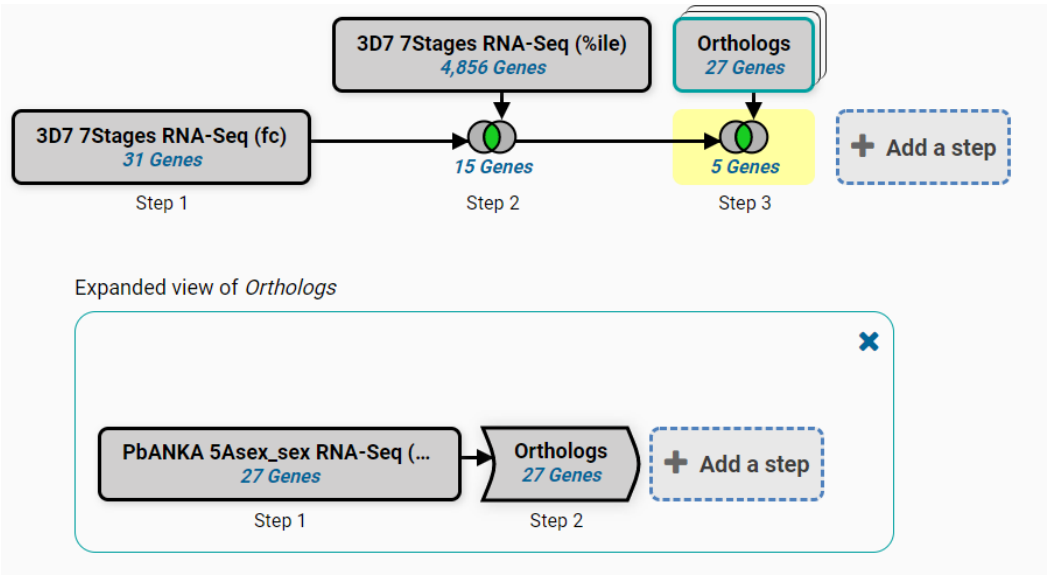


c. Which metabolic pathways are represented in this gene list? *Hint: add a step and transform results to pathways*. How does this result compare to running a pathways enrichment on step 2?



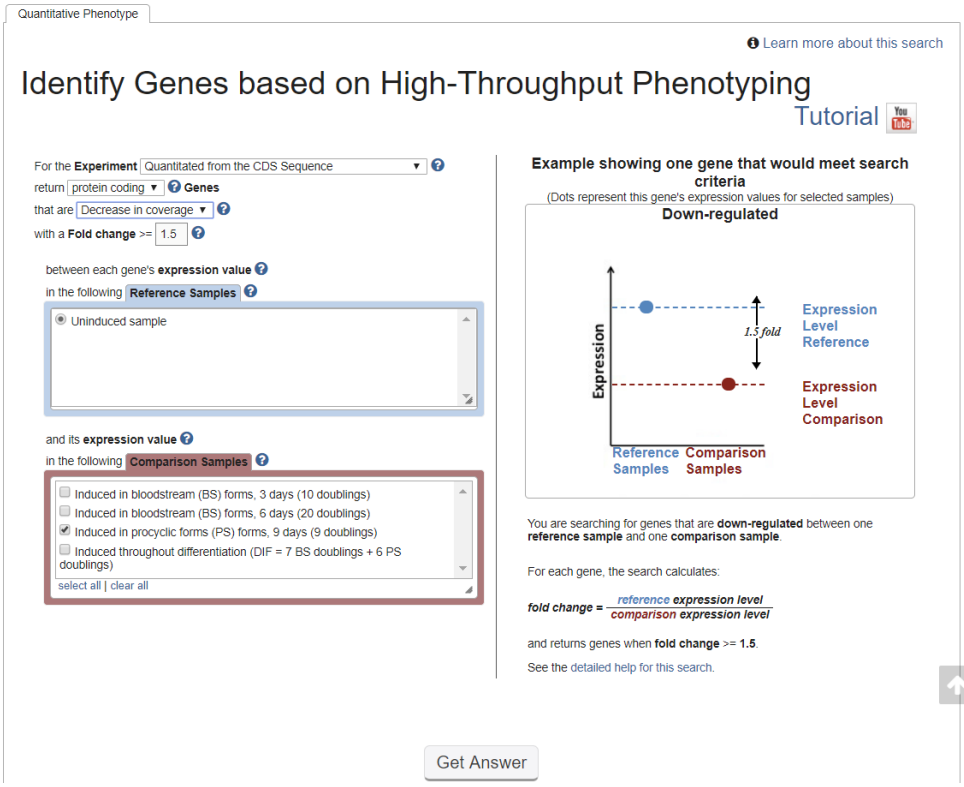
d. What happens if you revise the first step and modify the fold difference to a lower value - 10 for example? Compare results when you also modify the "between each genes" parameter. What happens if you set this to maximum? Which value do you think is most stringent for ensuring at 10 fold up regulation compared to the other samples?

e. PlasmoDB also has an experiment examining gene expression during sexual development in *Plasmodium berghei* (rodent malaria). Can you determine if there are genes that are up-regulated in both human and rodent ookinetes (compared to all other stages)? *Hint:* start by deleting the last step you added in this exercise (transform to pathways). To do this click on edit then delete in the popup. Next, add steps for the *P. berghei* experiments "P berghei ANKA 5 asexual and sexual stage transcriptomes RNASeq". Note that you will have to use a nested strategy or by running a separate strategy then combining both strategies.

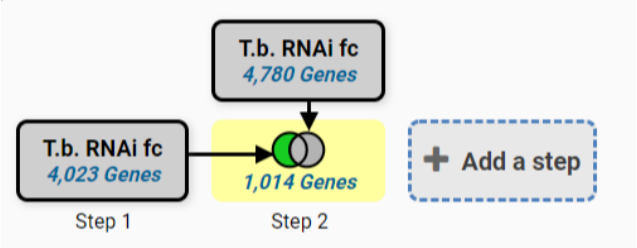


4. Find genes that are essential in procyclics but not in blood form *T. brucei*.
Note: for this exercise use http://TriTrypDB.org.

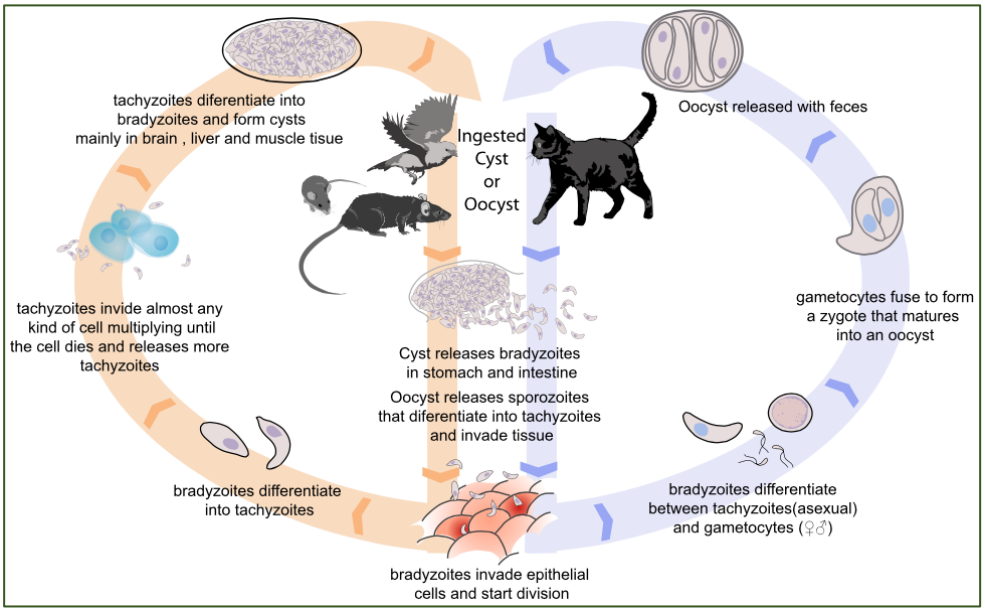
- Find the query for High Throughput Phenotyping. Think about how to set up this query (*Hint*: you will have to set up a two-step strategy). Remember you can play around with the parameters but there is no one correct way of setting them up –



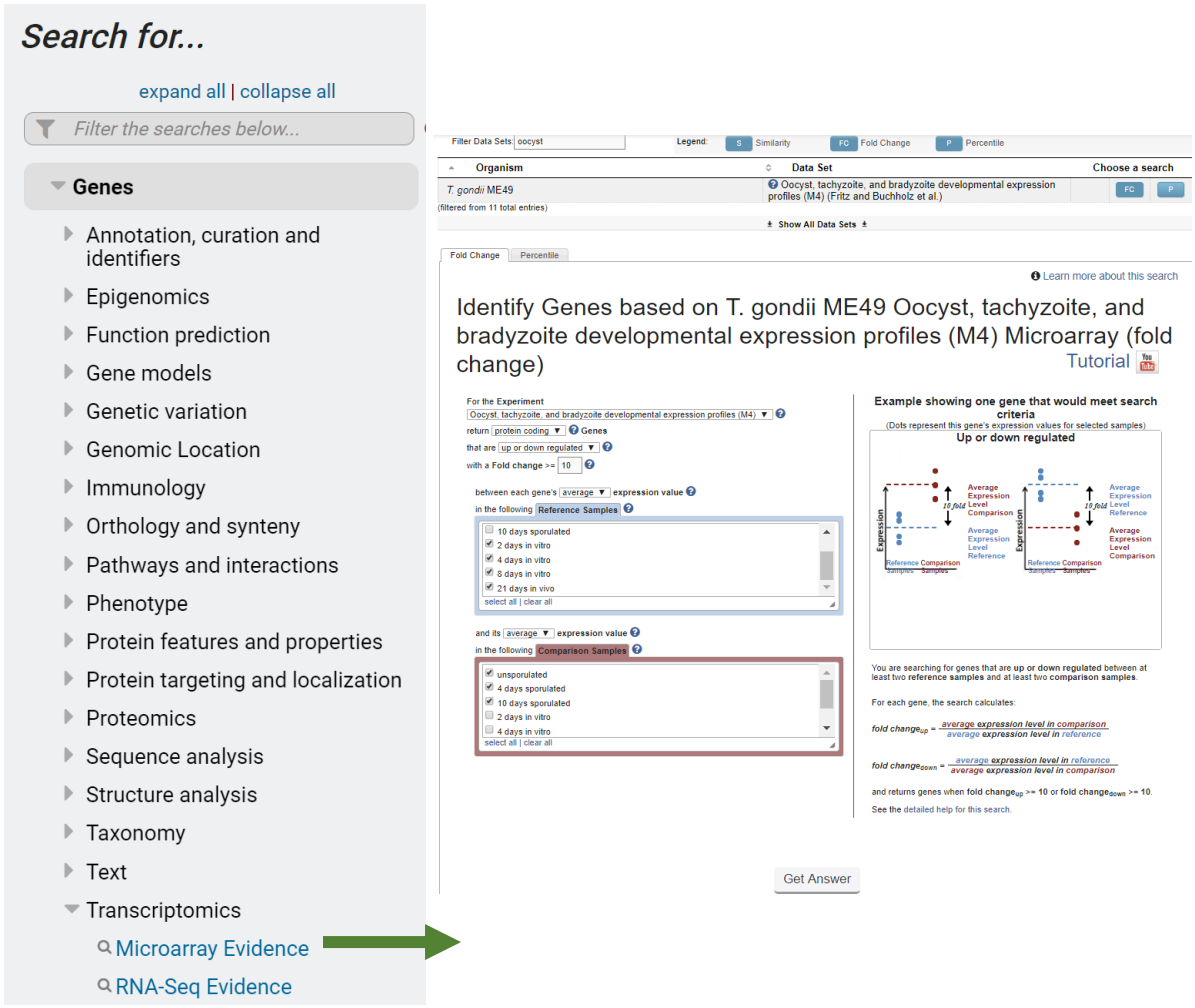
- Next add a step and run the same search except this time select the “induced bloodstream form” samples.
- How did you combine the results? Remember you want to find genes that are essential in procyclics and not in blood form.



5. Finding oocyst expressed genes in *T. gondii* based on microarray evidence.
Note: For this exercise use http://toxodb.org

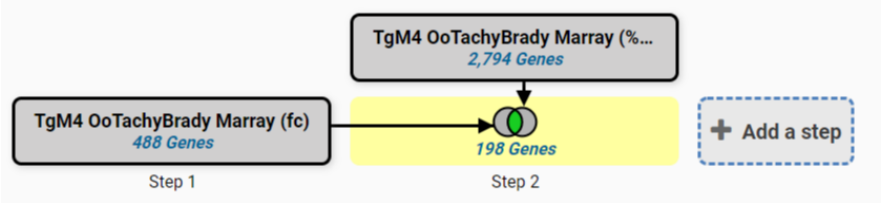


a. Find genes that are expressed at 10 fold higher levels in one of the oocyst stages than in any other stage in the "**Oocyst, tachyzoite, and bradyzoite developmental expression profiles (M4) (Fritz and Buchholz)**" microarray experiment.

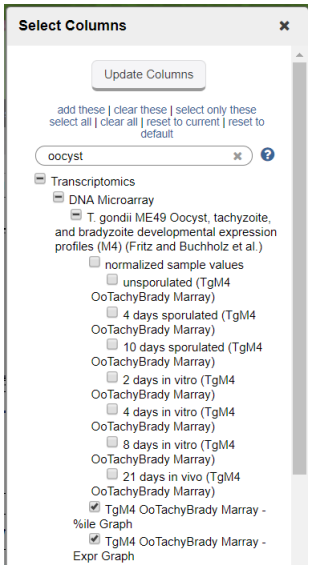


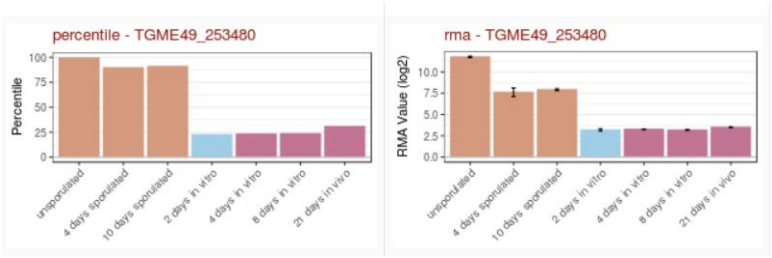
b. Add a step to limit this set of genes to only those for which all the non-oocyst stages are expressed below 50th percentile … i.e., likely not expressed at those stages. *(Hint*: after you click on add step find the same experiment under microarray expression and chose the percentile search).

- Select the 4 **non-oocyst** samples.
- We want all to have less than 50th percentile so set ***minimum percentile*** to 0 and ***maximum percentile*** to 50.
- Since we want all of them to be in this range, choose ALL in the "***Matches Any or All Selected Samples***".



- To view the graphs in the final result table, turn on the columns called “TgM4 OoTachyBrady Marray - Expr Graph” and “TgM4 OoTachyBrady Marray - %ile Graph” (inside the “T. gondii ME49 Oocyst, tachyzoite, and bradyzoite developmental expression profiles (M4) (Fritz and Buchholz et al.)” Microarray).



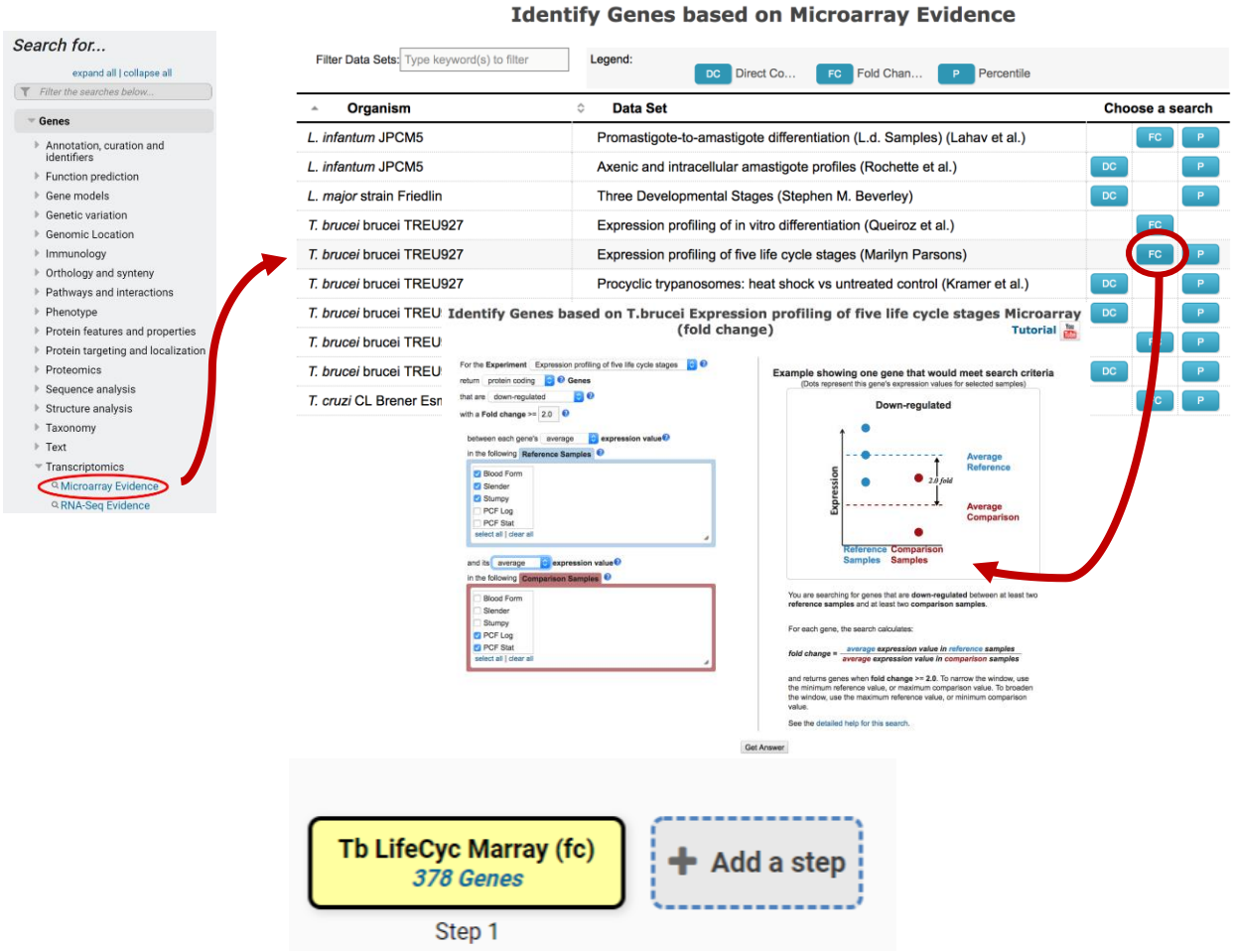


6. Comparing RNA abundance and Protein abundance data.

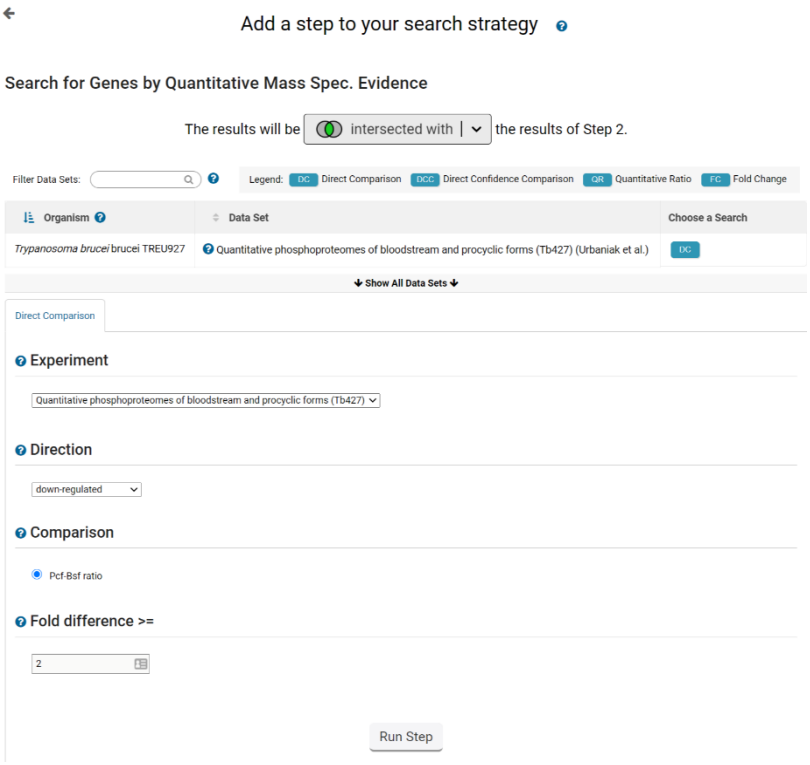
Note: for this exercise use http://TriTrypDB.org.

In this exercise we will compare genes that show differential RNA abundance levels between procyclic and blood form stages in *T. brucei* with genes that show differential protein abundance in these same stages.

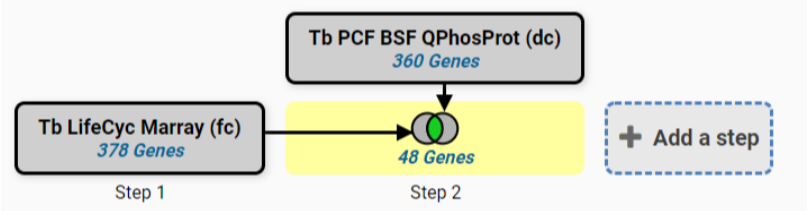
a. Find genes that are down-regulated 2-fold in procyclic form cells. Go to the search page for Genes by Microarray Evidence and select the fold change search for the "Expression profiling of five life cycle stages (Marilyn Parsons)" experiment and configure the search to return protein-coding genes that are down-regulated 2 fold in procyclic form (PCF) relative to the Blood Form reference sample. Since there are two PCF samples, it is reasonable to choose both and average them.



b. Add a step to compare with quantitative protein expression. Select protein expression then “Quantitative Mass Spec Evidence” and the "Quantitative phosphoproteomes of bloodstream and procyclic forms (Tb427) (Urbaniak et al.)" experiment. Configure this search to return genes that are down-regulated in procyclic form relative to blood form.



c. How many genes are in the intersection? Does this make sense? Make certain that you set the directions correctly.



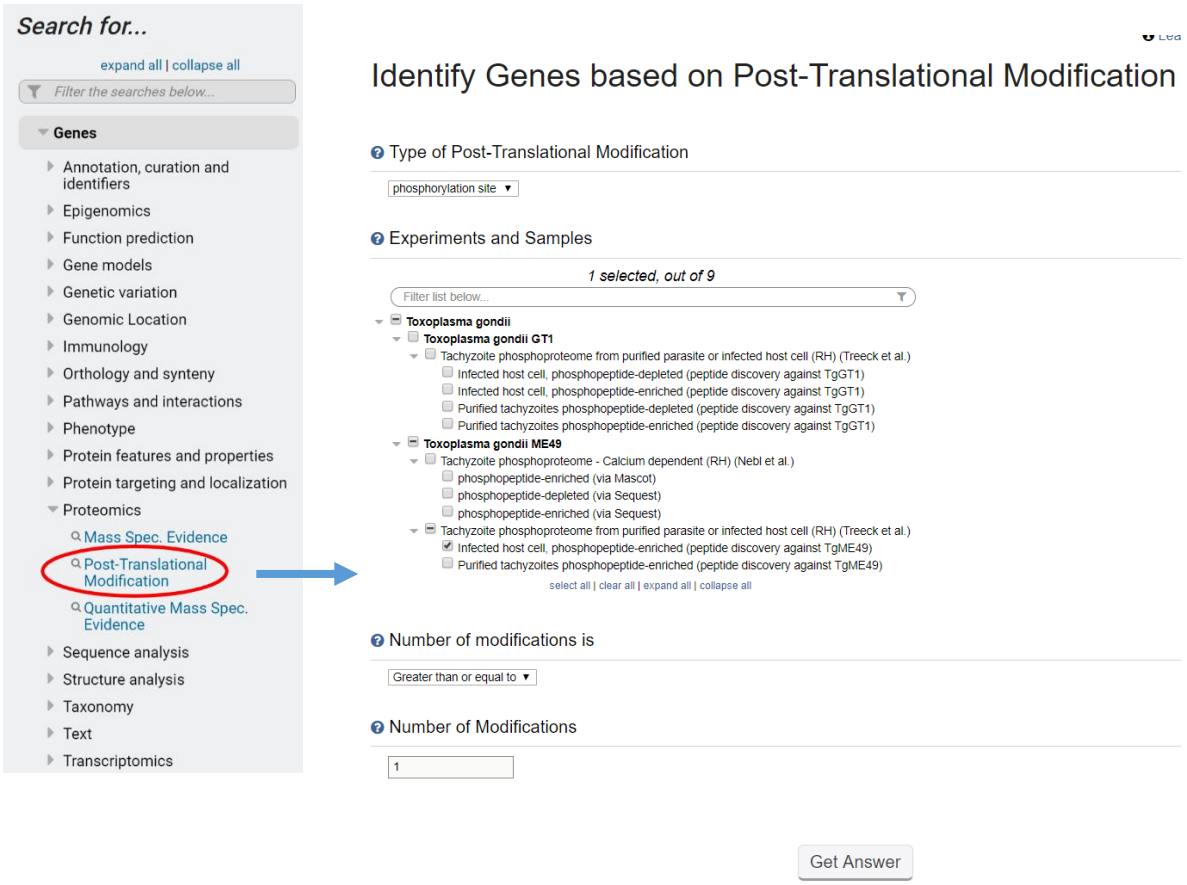
d. Try changing directions and compare up-regulated genes/proteins. (*Hint:* revise the existing strategy … you might want to duplicate it so you can keep both). When you change one of the steps but not the other do you have any genes in the intersection? Why might this be?

e. Can you think of ways to provide more confidence (or cast a broader net) in the microarray step? (*Hint:* you could insert steps to restrict based on percentile or add a RNA Sequencing step that has the same samples).

7. Find genes with evidence of protein phosphorylation in intracellular *Toxoplasma* tachyzoites.
For this exercise use http://www.toxodb.org

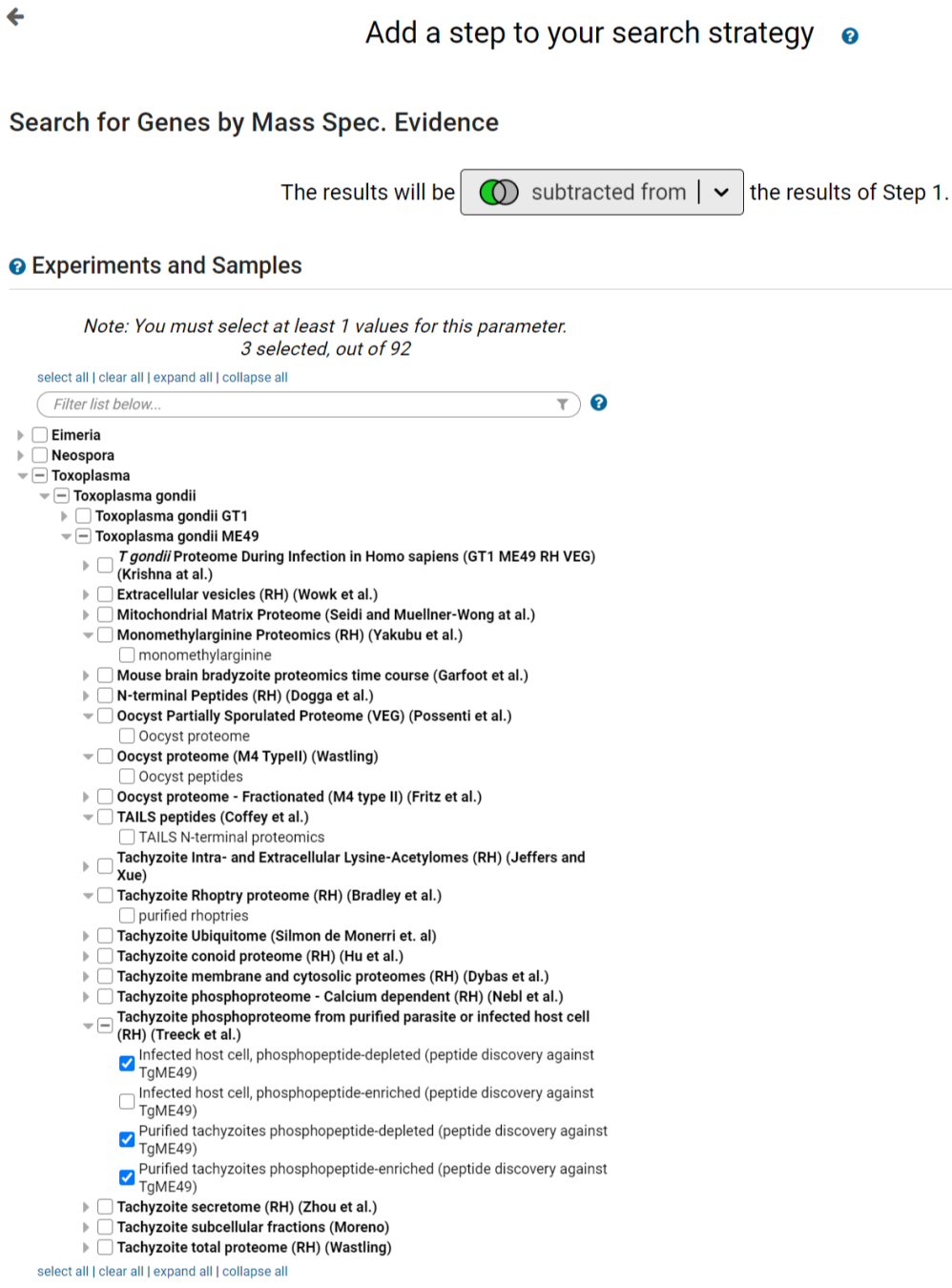
Phosphorylated peptides can be identified by searching the appropriate experiments in the Mass Spec Evidence search page.

7a. Find all genes with evidence of protein phosphorylation in intracellular tachyzoites. Navigate to the Post-Translational Modification search. Select the "**Infected host cell, phosphopeptide-enriched** (peptide discovery against **TgME49**)" sample under the experiment called "**Tachyzoite phosphoproteome from purified parasite or infected host cell (RH) (Treeck et al.)**"



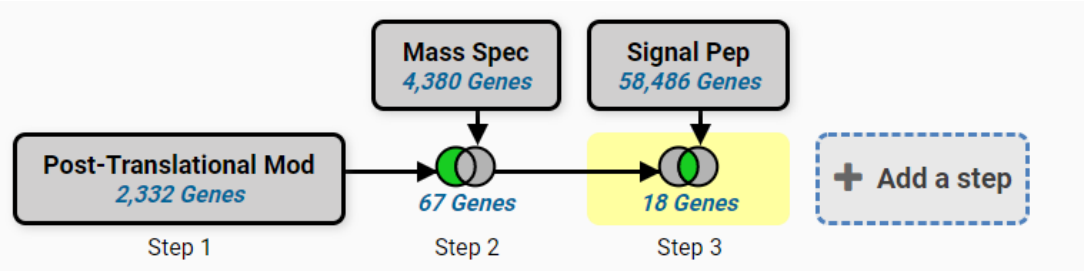
7b. Remove all genes with phosphorylation evidence from purified tachyzoites and the phosphopeptide depleted fractions.

Hint: Use the Mass Spec Evidence search to access the tachyzoite and depleted fractions. Subtract (1 minus 2) these results from your first search.



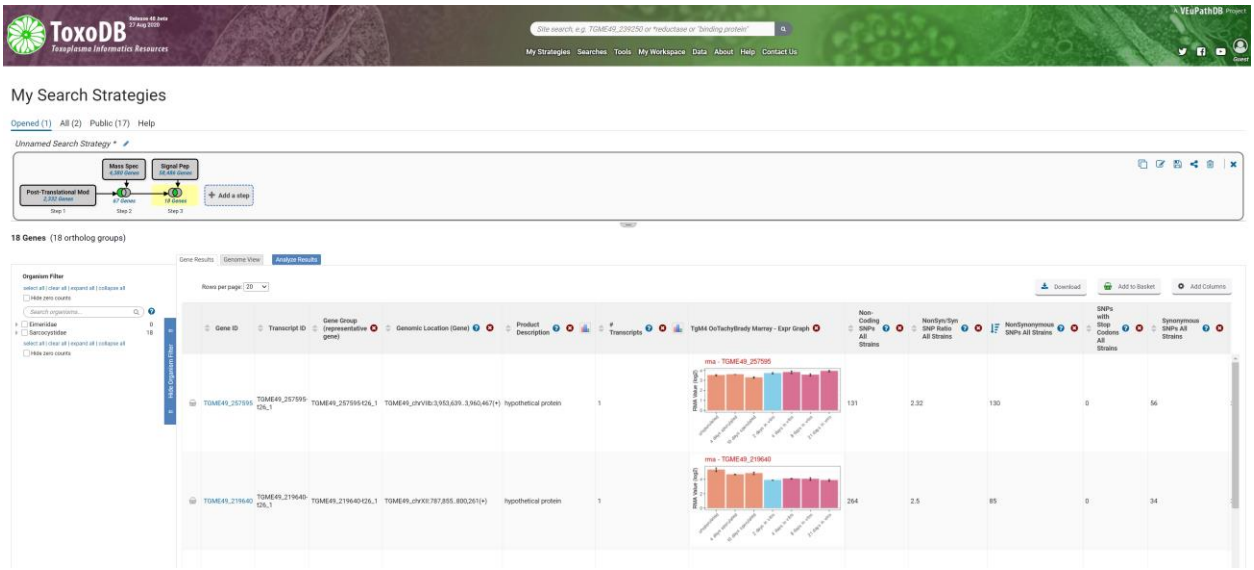
7d. Explore your results. What kinds of genes did you find? *Hint: use the Product description word column or perform a GO enrichment analysis of your results.*

7e. Are any of these genes likely to be secreted? Hint: add a step searching for genes with secretory signal peptides.



7f. Pick one or two of the hypothetical genes in your results and visit their gene pages. Can you infer anything about their function? Hint: explore the protein and expression sections.

7g. What about polymorphism data? Go back to your strategy and add columns for SNP data found under the population biology section. Explore the gene page for the gene that has the most number of non-synonymous SNPs. Hint: you can sort the columns by clicking on the up/down arrows next to the column names.



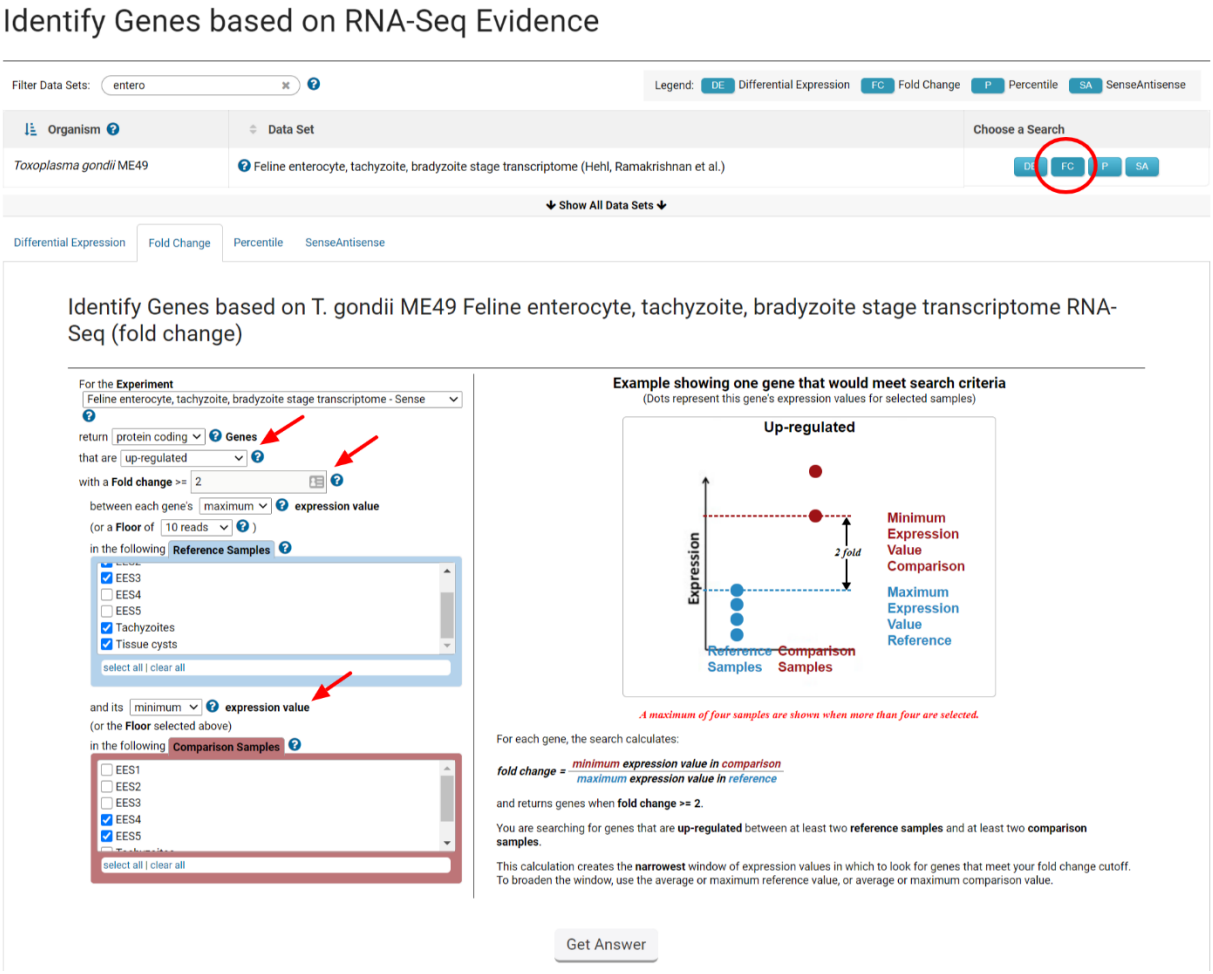
8. Find *T. gondii* genes expressed in late enteroepithelial stages

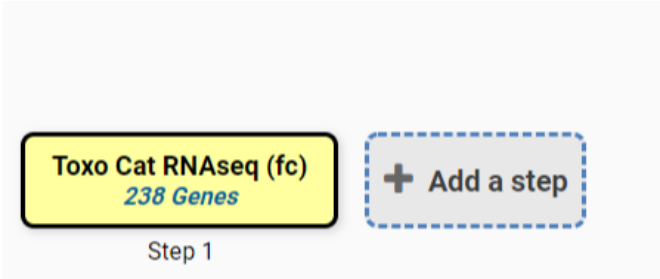
Toxoplasma gondii is a zoonotic pathogen for which felids serve as definitive hosts. In cats, the parasite undergoes several rounds of asexual replication before entering the sexual cycle which gives rise to oocysts that are shed into the environment. These then sporulate and become infective to humans and livestock. To understand the genes involved in the parasite development in the felid host and identify potential intervention targets, we designed a transcriptomic approach to compare the cat intestinal stages with the well characterized tachyzoites that mediate acute infection and tissue cysts that are responsible for chronic infection. Cats were infected with *T. gondii* CZ clone H3 tissue cysts from mouse brain and the intestinal stages were sampled at day 3, 5 and 7 post infection. As an input sample, we also collected tissue cysts from mouse brain. In vitro cultivated tachyzoites were also harvested. Total RNA was extracted, enriched for mRNA and used for cDNA synthesis. RNA-Seq was then performed to describe the transcriptomic repertoire of each developmental stage. RNA-seq datasets from each time point post inoculation with bradyzoites in kittens were subjected to cluster analysis and assigned to five enteroepithelial developmental stages (EES) according to their profile.

Cat enteroepithelial stages:

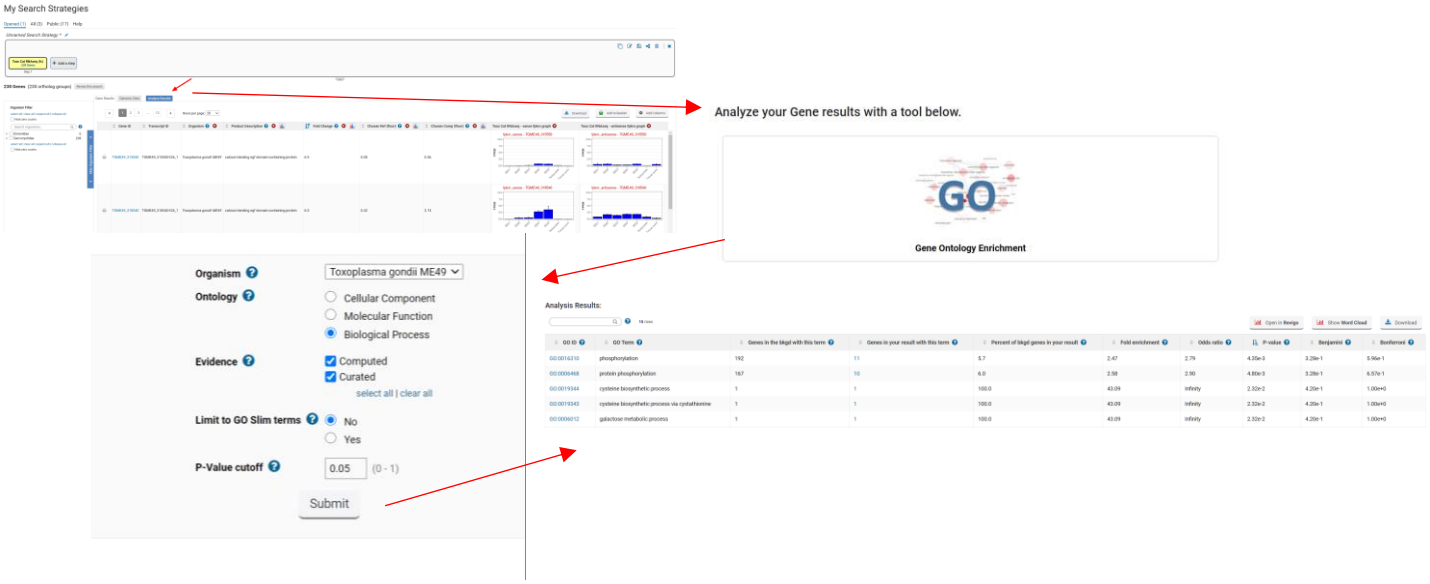
- EES1 = very early enteroepithelial stages
- EES2 = early enteroepithelial stages
- EES3 = mixed enteroepithelial stages
- EES4 = late enteroepithelial stages
- EES5 = very late enteroepithelial stages

- Navigate to the RNAseq searches and identify the experiment of cat enterocyte stages. Configure the search to identify call *T. gondii* genes that are upregulated by at least 2-fold in late and very late enteroepithelial stages (EES4 and EES5) compared to all other stages available from this experiment.





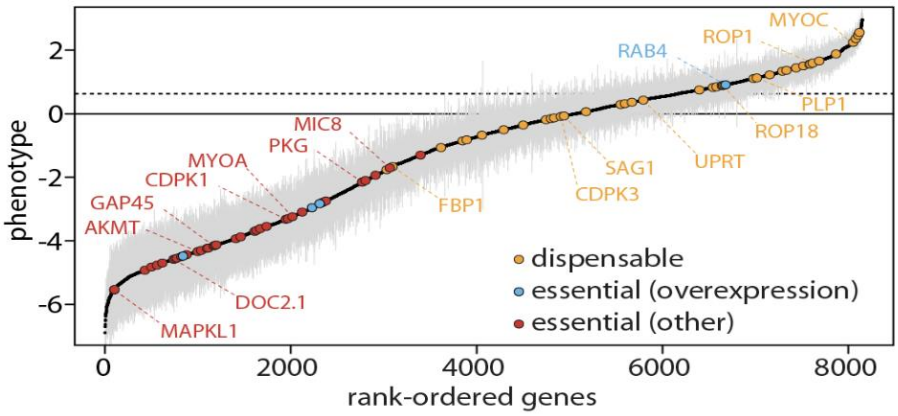
- What kinds of genes did this search identify? How can you determine if your results are enriched for a particular function? Try clicking on Analyze Results and explore the GO enrichment tool.

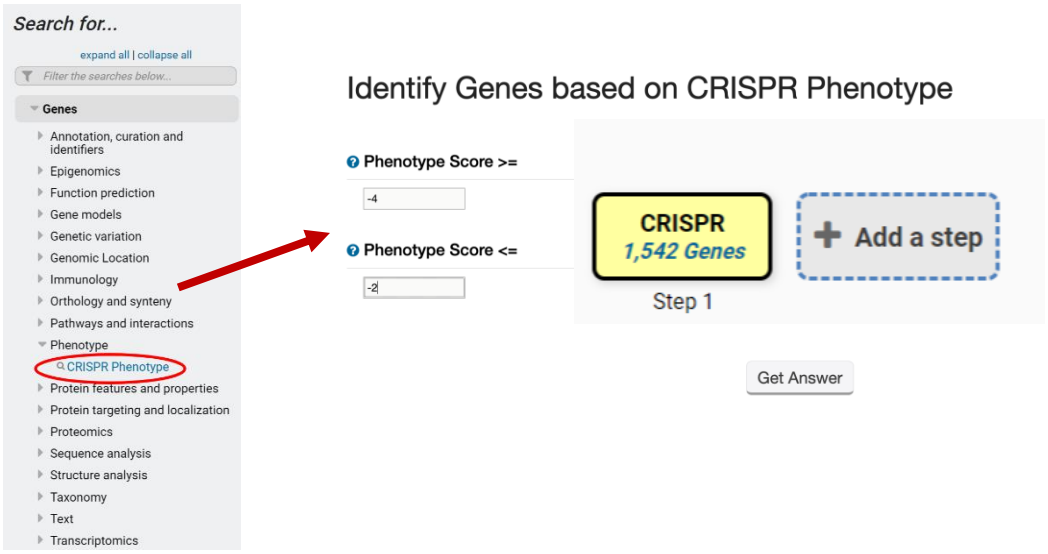

My Search Strategies
Analyze your Gene results with a tool below.
Gene Ontology Enrichment
Organism
Toxoplasma gondii ME49
Ontology
Cellular Component
Molecular Function
Biological Process
Evidence
Computed
Curated
select all | clear all
Limit to GO Slim terms
No
Yes
P-Value cutoff
0.05
(0 - 1)
Submit
Analysis Results:

9. Finding genes based on high throughput mutagenesis and fitness analysis.

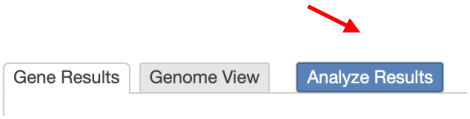
In EuPathDB we have a variety of studies where genome scale phenotypic analyses were carried out. In this exercise we'll use ToxoDB.org and look at fitness following CRISPR mutagenesis. You could also explore phenotyping studies in PlasmoDB or FungiDB if you prefer, the principles are the same.

- Navigate to the CRISPR phenotype search. Note that this search form is quite simple just requiring a range of fitness values. The defaults return all genes not limiting the search at all. This is only useful in as much as it tells you which genes were assayed which is nearly the entire genome. The tricky bit is deciding where to make the cutoffs. Again, the description on the search form is very helpful in this regard (as is the link to the paper … remember these phenotypes were assayed under specific conditions so just because a particular gene doesn't show a phenotype doesn't mean it wouldn't in other conditions (or infecting an actual host). The plot showing the phenotype score (fitness) is particularly useful. Red points along the plot are genes known to be essential under these conditions while yellow are known to be expendable. This will help you determine where to set the values. The last essential gene has a fitness score just >= than -4 so setting the phenotype score <= -2 would provide a pretty stringent search but still return more than 1000 genes. Try it. Do you get the expected results based on the number of genes returned?

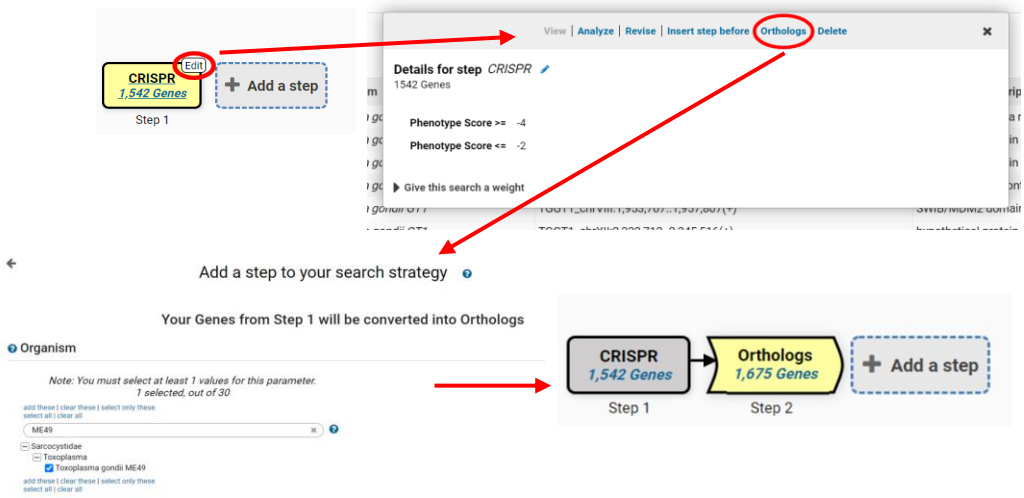




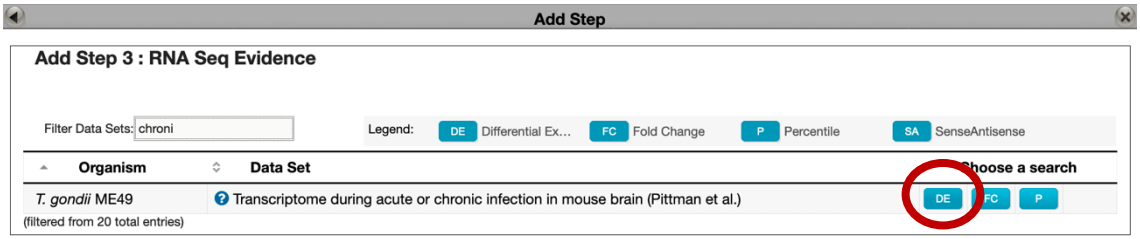
- Can you find additional evidence that these genes are essential? One way is to use the analysis tools to assess biological process and go function. Are the results what you would expect?



- Try adding columns to show additional data or intersecting these results with other queries, perhaps expression queries, to further assess this list. NOTE: this experiment was done in GT1 while all *T. gondii* functional data in ToxoDB is mapped to ME49 so an ortholog transform to ME49 is required before adding any additional functional studies.
- To do this, click on add step and select the Transform to orthologs option and select *T. gondii* ME49 to transform to.



- How many of these genes are upregulated in *in vivo* chronic stages of *T. gondii*?
    - Click on add step
    - Select the RNAseq searches under the Transcriptomics category
    - Find the experiment with chronic stages and run a search based on differentially expressed genes (DE).



- Intersect genes that are 2-fold upregulated in chronic stages compared to acute stages.

- What do these results look like? Do you find any interesting genes?