# BS222 Practical 2. Autumn 2018.

# Next Generation Sequencing (NGS) Analysis in Galaxy

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There are two ways of doing computational analysis of Next Generation Sequencing (NGS) data: the professional way and the easy way☺. The professional way is to work in the command-line UNIX environment on a computer cluster, because the files are usually so large that it is not possible to operate with them on a personal computer. The easy way is to use an online software tool called Galaxy. Today we will be exploring the latter possibility. This "easy" way is sometimes also a very proper way, in particular if a friendly IT systems administrator has spent weeks to adjust this software, and your lecturers have double-checked that the computational tasks are doable during the practical ☺. Galaxy is intended to be the software of choice for learning and understanding how NGS analysis works, but it may have some glitches. If you encounter a glitch please keep patient and don't panic – just wait for the lecturer who will save you. Importantly, the aim of the practical is to understand the main NGS concepts, so please try to see the see the forest for the trees.

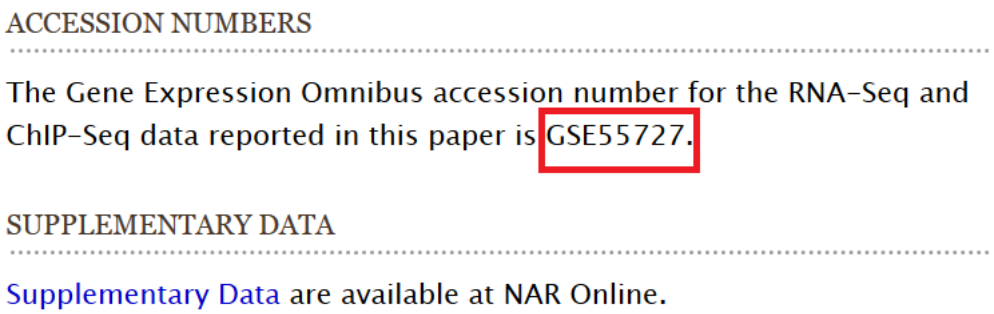
*It is also worth noting, that in this practical you will be working with real NGS data, and you can in principle make real scientific discoveries, in which case don't forget to document them. If this does not interest you then I don't know what else can make you interested in this practical. Oh, wait; may be also the fact that this is the only practical this year where you can get experience with NGS analysis to boost your employability and add "NGS data analysis skills" to your CV? ☺*

**Introduction.** Our practical will be based on the data reported in the study entitled "Integrative genomic analysis reveals widespread enhancer regulation by p53 in response to DNA damage" (Younger et al. (2015) *Nucleic Acids Res.* 43 (9): 4447-4462). The full text of this article is available at http://nar.oxfordjournals.org/content/43/9/4447.long. This paper is about chromatin binding of the tumour suppressor protein p53. The authors determine genome-wide p53 binding profiles in human and mouse cells. Their main finding is that p53 binding occurs predominantly within transcriptional enhancers. The authors report both human and mouse ChIP-seq datasets, but mostly analyse the human data in the paper. Today we will perform analysis based on their mouse data. In this practical we will determine, where in the genome our protein of interest, called p53, is binding – because where it is binding determined which genes it is regulating. In the second practical we will be using this information to answer real biomedical questions, such as what happens with these cells as they respond to the anticancer drugs.

Plan of this practical:

1. Understand where to get NGS data online – follow the lecturer
2. Understand Galaxy – an online platform for NGS analysis – follow the lecturer
3. Understand ChIP-seq data formats – follow the lecturer
4. Understand how to map reads to the target genome in Galaxy (do not do the mapping!)
   *(You do not need to perform the mapping step because I did the mapping for you already)*
5. Find peaks of p53 ChIP-seq (p53 binding sites) using MACS2 in Galaxy
6. Task 6. Compare the peaks that we determined with the peaks reported by Younger et al.
7. Intersect p53 peaks with enhancers and promoters using BedTools in Galaxy
8. Find enrichment of p53 binding at enhancers and promoters using BedTools in Galaxy

**Task 1. Understand where to get NGS data (GEO) – follow the lecturer.** After carefully reading the paper's abstract we scroll down the bottom of the manuscript to find where the authors have deposited their data. We find the following:



ACCESSION NUMBERS

The Gene Expression Omnibus accession number for the RNA-Seq and ChIP-Seq data reported in this paper is GSE55727.

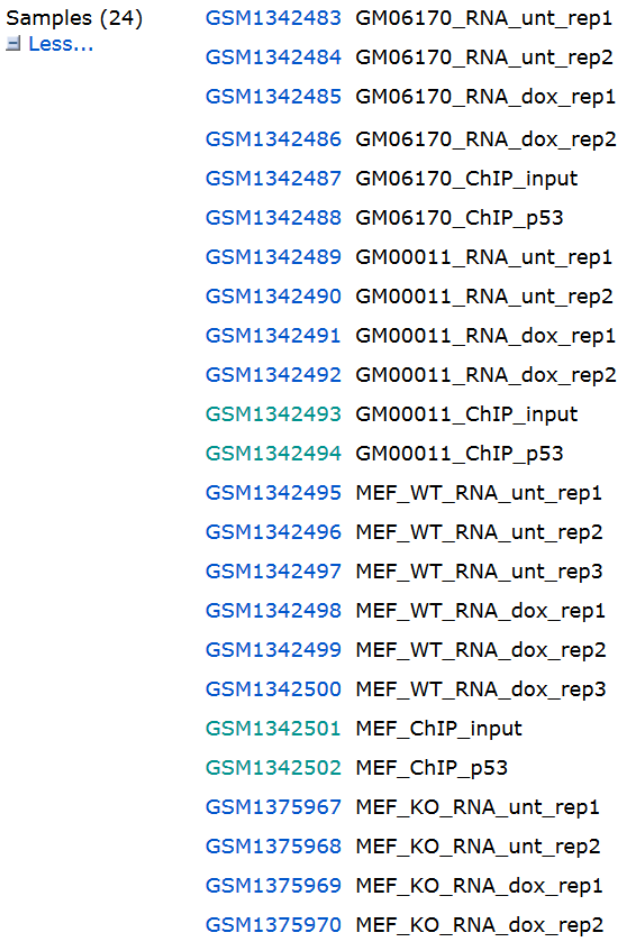
SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

Using the Gene Expression Omnibus (GEO) accession number GSE557227 reported by the authors, we find their data at the following link:

https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55727

Opening this link in the browser, we can see the complete description of the experimental details of this study, and the list of the samples which they have deposited (you have to click on "more" next to the sample list):



Samples (24)
Less...

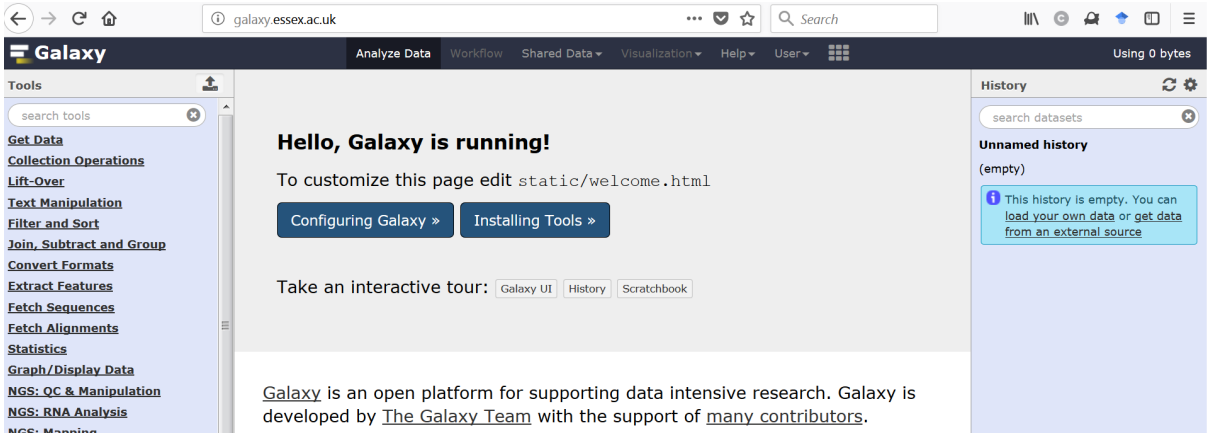
GSM1342483 GM06170_RNA_unt_rep1
GSM1342484 GM06170_RNA_unt_rep2
GSM1342485 GM06170_RNA_dox_rep1
GSM1342486 GM06170_RNA_dox_rep2
GSM1342487 GM06170_ChIP_input
GSM1342488 GM06170_ChIP_p53
GSM1342489 GM00011_RNA_unt_rep1
GSM1342490 GM00011_RNA_unt_rep2
GSM1342491 GM00011_RNA_dox_rep1
GSM1342492 GM00011_RNA_dox_rep2
GSM1342493 GM00011_ChIP_input
GSM1342494 GM00011_ChIP_p53
GSM1342495 MEF_WT_RNA_unt_rep1
GSM1342496 MEF_WT_RNA_unt_rep2
GSM1342497 MEF_WT_RNA_unt_rep3
GSM1342498 MEF_WT_RNA_dox_rep1
GSM1342499 MEF_WT_RNA_dox_rep2
GSM1342500 MEF_WT_RNA_dox_rep3
GSM1342501 MEF_ChIP_input
GSM1342502 MEF_ChIP_p53
GSM1375967 MEF_KO_RNA_unt_rep1
GSM1375968 MEF_KO_RNA_unt_rep2
GSM1375969 MEF_KO_RNA_dox_rep1
GSM1375970 MEF_KO_RNA_dox_rep2

We will be working with the samples MEF_ChIP_p53 and MEF_ChIP_Input. “MEF” stands for mouse embryonic fibroblasts. “p53” stands for the sample which has undergone ChIP-seq with antibody against p53 protein, and “Input” is the same sample, but sequenced without antibody. Our task for this practical will be to analyse these data: check whether the conclusions of the authors of the paper are correct (or may be suggest new scientific conclusions and make a scientific discovery!)

**Task 2. Understand Galaxy – an online platform for NGS analysis – follow the lecturer.**

Galaxy is open-source software arising from a large international project that aims to provide a user-friendly environment for all kinds of NGS analysis. Galaxy provides a web server that can be installed locally (we have a local version at http://galaxy.essex.ac.uk), and then the systems administrator has to take care of this server and install all the required software. Almost any software tool that exists as a command line tool for UNIX can be also installed on Galaxy, where users do not need to struggle with the “unfriendly” UNIX environment. Some serous programmers, however, still work in UNIX. The teaching materials about Galaxy are available here: https://galaxyproject.org/learn/

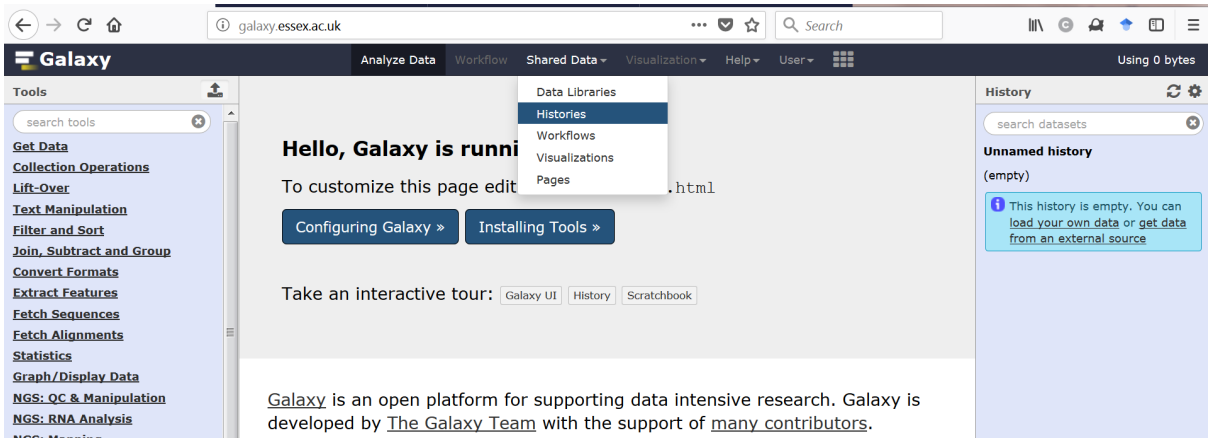
2.1. Let us open our Galaxy. Open an internet browser and type this address: **galaxy.essex.ac.uk**:



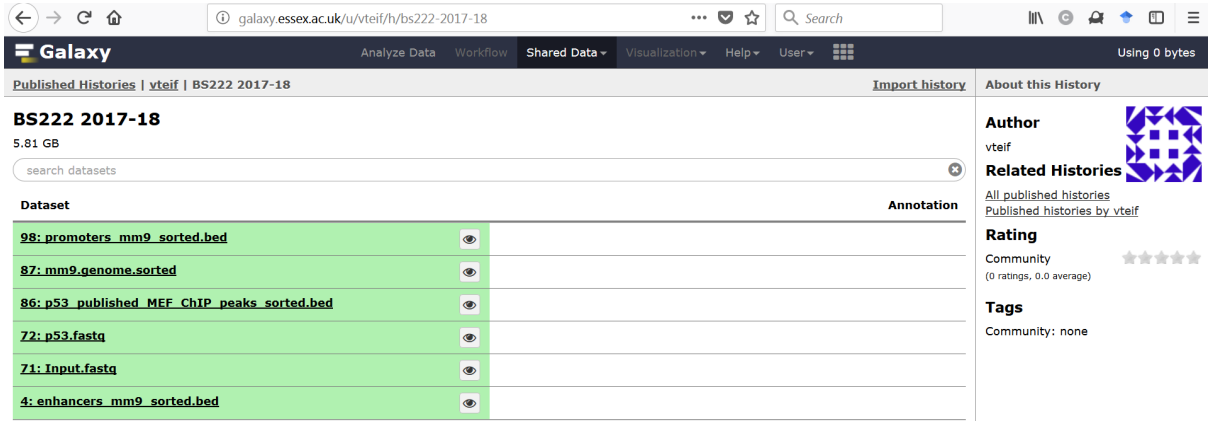

2.2. Create your account on Galaxy. Go to User > Register, and register an account using your university email. Then login as this user, and continue working under the same user name today.

Now we need to load to the Galaxy our data. Since the files that we want to use are quite large (about 6Gb), there is no need that each of us upload such files to the Galaxy. It is enough that the files have been uploaded once, and then we all can use them. In Galaxy there are several ways to share files between different users. One was is to share “history”. The history is what you see on your right side of the Galaxy. Currently you history is empty. I have previously uploaded to Galaxy all the files that we need today, and shared this history with everyone. You now have to find my shared history and import it so that it will become your history. Let us do this.

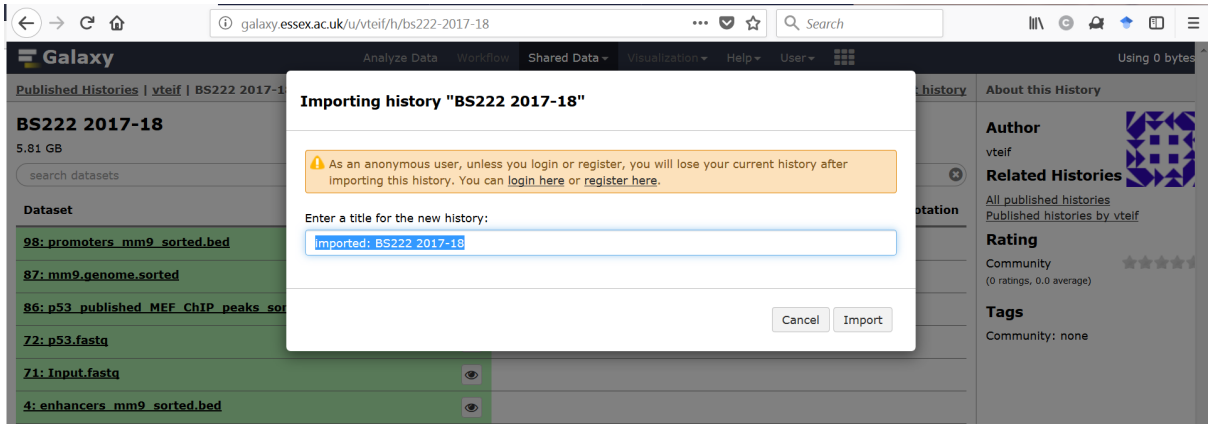
2.3. Click Shared data/Histories:



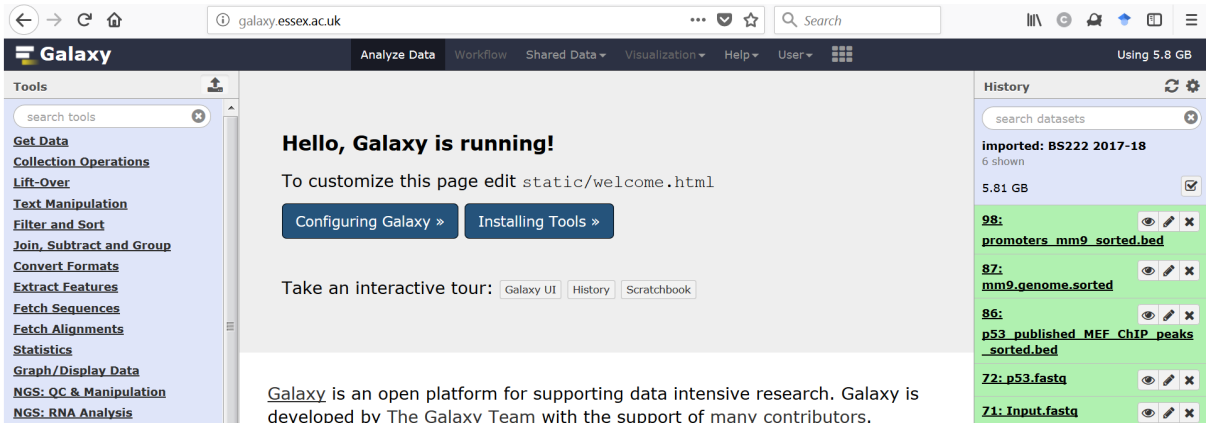

2.4. Then select the history called BS222 2017-18:




2.5. Then click “Import history”:

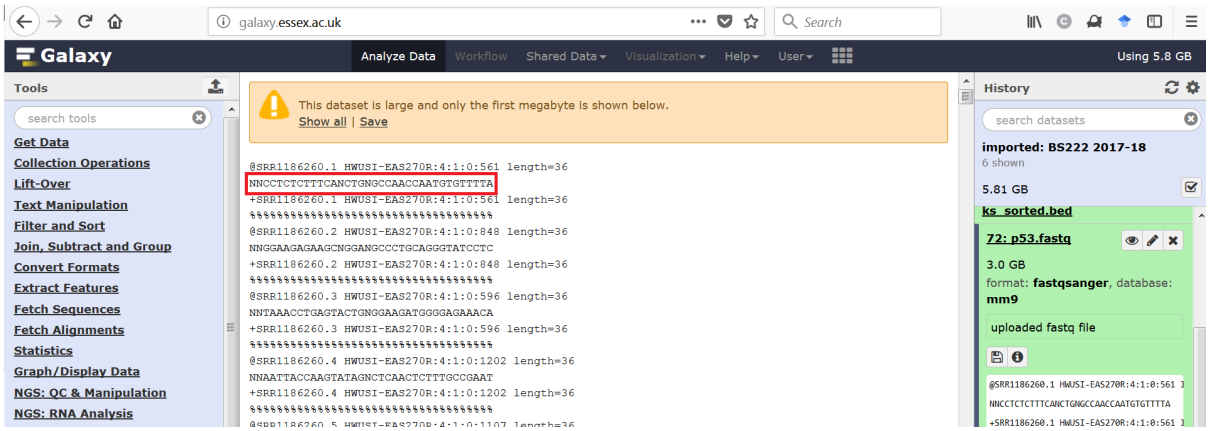



2.6. Click “Import”. This will replace your empty history with the history “Imported BS222 2017-18”:




## Task 3. Understand ChIP-seq data formats – follow the lecturer

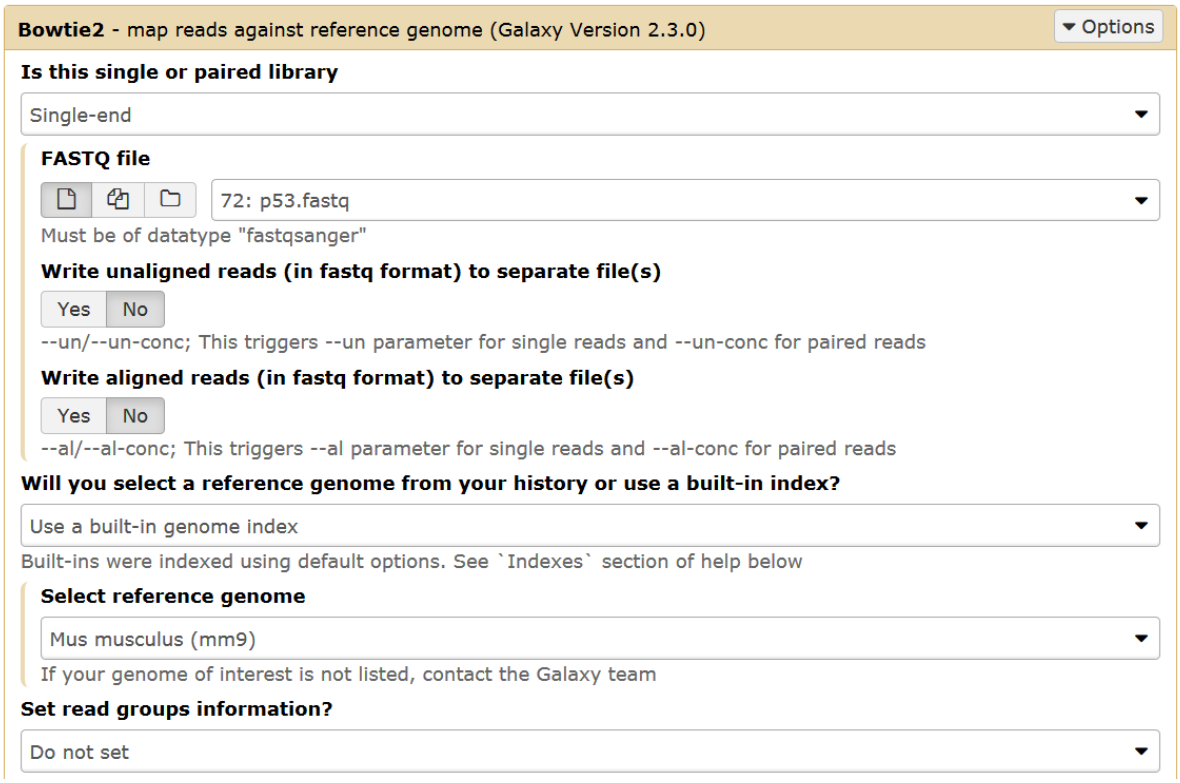
As you can see, your new history in Galaxy now includes several files. The most important are p53.fastq and Input.fastq – they contain the initial raw data as they were received from the sequencing machine. Let's look at each of them and understand how they are structured. You can click on the eye pictogram to look inside each of these file. For example, here we have opened the p53.fastq file – this file contains the sequences of all DNA fragments determined in the ChIP-seq experiment with p53 antibody. The red rectangle shows an example of one read. In this case each read is 36 nucleotide long:




## Task 4. Understand how to map reads to the target genome in Galaxy.

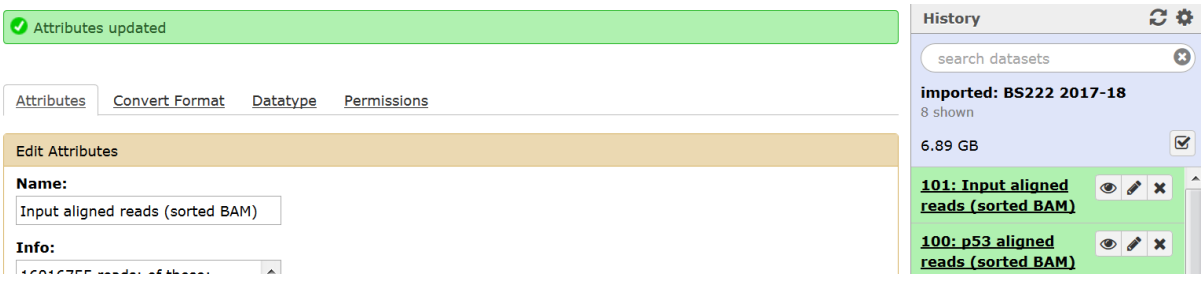
The first step in ChIP-seq analysis is mapping (also known as "alignment") of the reads. This is usually the most time-consuming and computationally demanding task in NGS analysis. Therefore, in order to save your time I have already performed this task for you – you do not need to perform the mapping, but you need to understand how the mapping was done by me. Before the mapping is performed we only know the DNA sequence of each read, but do not know yet where each read is positioned in the genome. After the mapping is performed, we know for each read its location in the genome (for some reads there could be potentially several locations – the lecturer will discuss this).

4.1. Locate in the left panel software Bowtie2, and click on it:




4.2. Select library type “single-end”, file name “p53.fastq”, and reference genome “Mus musculus (mm9)”. Do NOT click “execute” (do NOT start the mapping), because if we all submit jobs for mapping we all will have to wait very long and there will be not enough space on the cluster to keep all our files. There is no need to repeat the mapping because I have already did the mapping for you, and you have the results of the mapping in the history that you have already imported. So please do not click the “Execute” button, it would take too long to wait to map it again.

The history that I have shared with you contains files “p53 aligned reads (sorted BAM)” and “Input aligned reads (sorted BAM)”. These are the mapped data based on p53 and its control experiment “Input” correspondingly.




4.3. Click on the files “p53 aligned reads (sorted BAM)” and “Input aligned reads (sorted BAM)”. Hint: Do not click on the eye pictogram; click directly on the file name. How many reads are mapped in p53? How many reads mapped in Input? How many reads did not map in p53? How many reads did not map in Input?

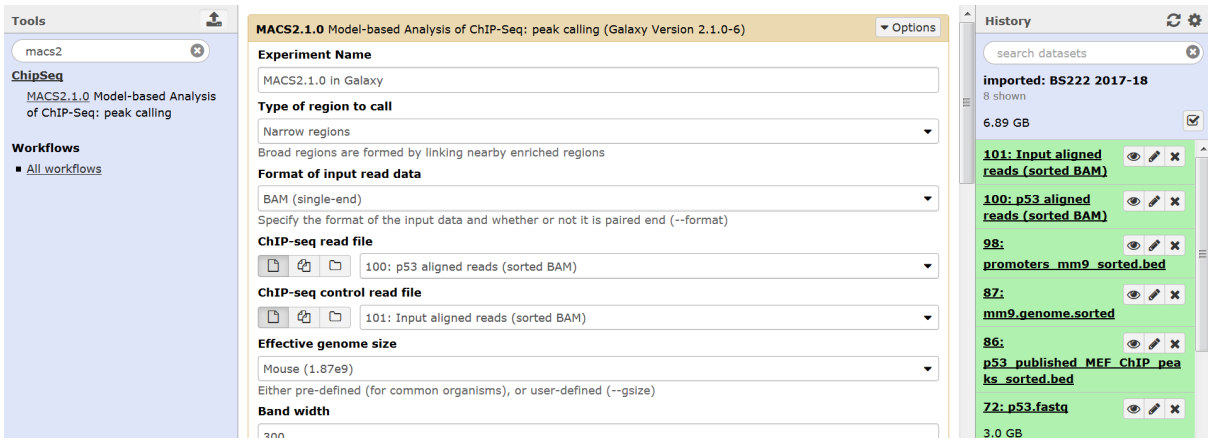
**Task 5. Find peaks of p53 binding using MACS2 in Galaxy.**

Now let us do some calculations. Please switch to the history named "BS222 Data 2018".

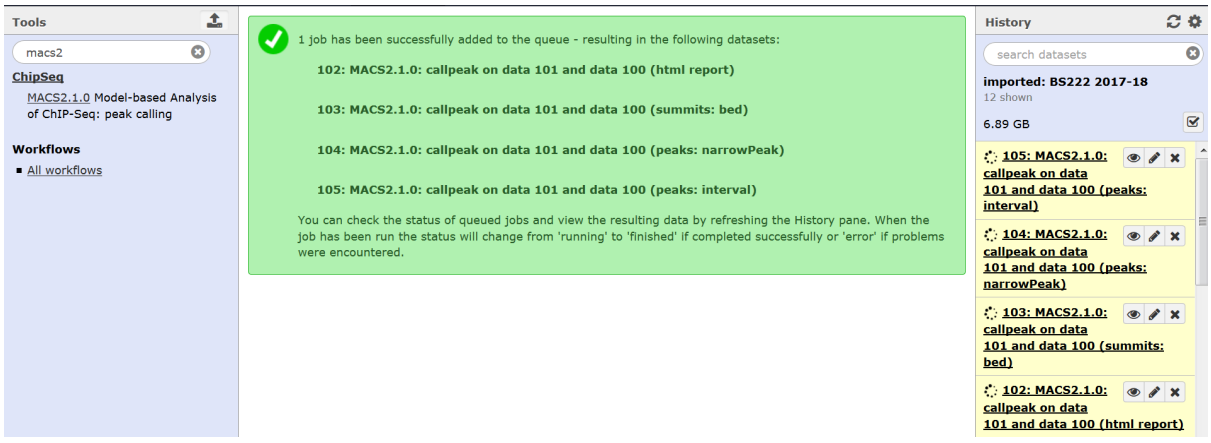
**[If our local Galaxy installation at Essex is overloaded we will do the next steps at the central Galaxy installation at https://usegalaxy.org. In this case we will jump to Task 5* on page 9]**

Let us determine the locations of bound p53 genome-wide. If you remember the lecture about NGS analysis, locations which are bound by proteins a visually seen as peaks on the protein binding occupancy landscape. We now need to locate the positions of all these peaks. At this step we will need both the p53 and Input mapped reads. Why? Because we only need the peaks that appeared in the ChIP-seq experiments using antibody against p53 and not the peaks which appeared in the control experiment.

5.1. Locate on Galaxy the software called MACS2, and click on it:



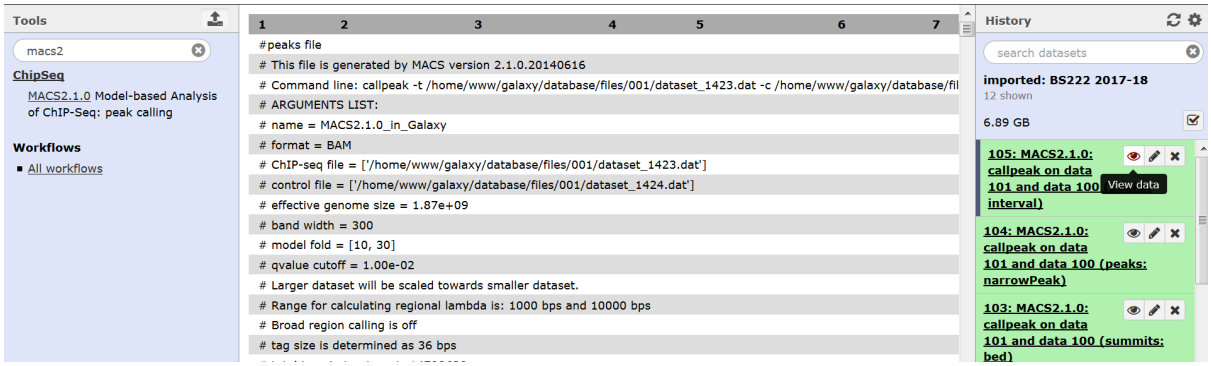

5.2. Select Type of regions to call "Narrow regions", Format of input read data "BAM (single-end)", ChIP-seq read file "p53 aligned reads", ChIP-seq control read file "Input aligned reads", and Effective genome size "Mouse". Then click "execute" at the bottom of the page:




This calculation will take about 15-20 minutes (if our server is in a good mood☺)

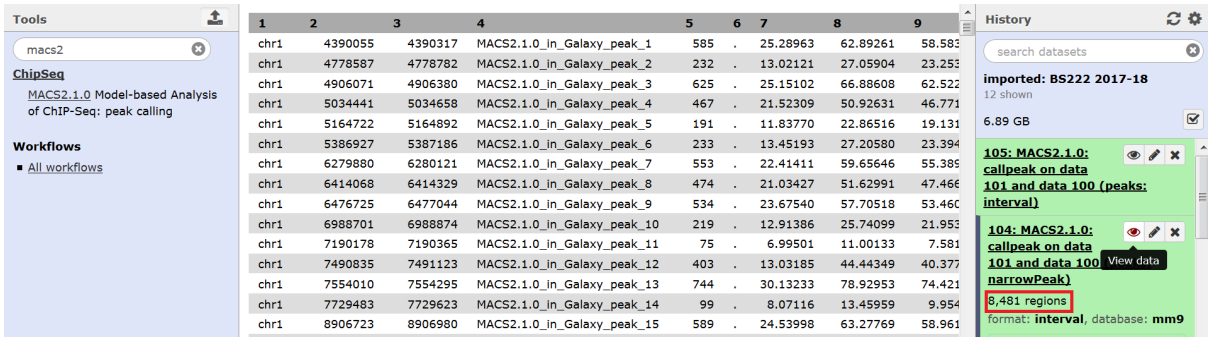
5.3. While the job is being executed listen to the lecturer's explanations about the algorithm of peak calling, and read about the parameters of MACS2 at its Galaxy page, as well as at its own web page: https://github.com/taoliu/MACS

5.4. When the calculation is finished we can have a look at each of the four new files that are created:




Which parameters determine the number of regions that are reported as peaks? How can we change these parameters to get more/less peaks?

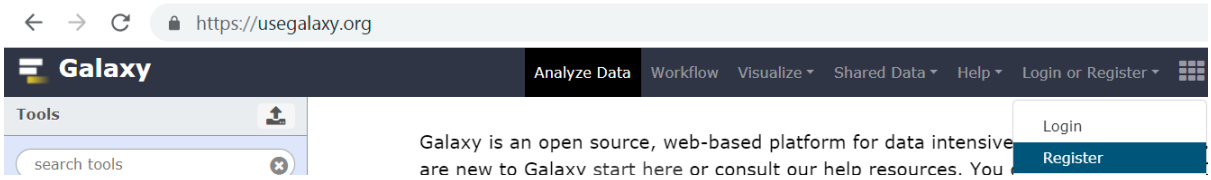
5.5. How many regions did we get as p53 binding peaks?



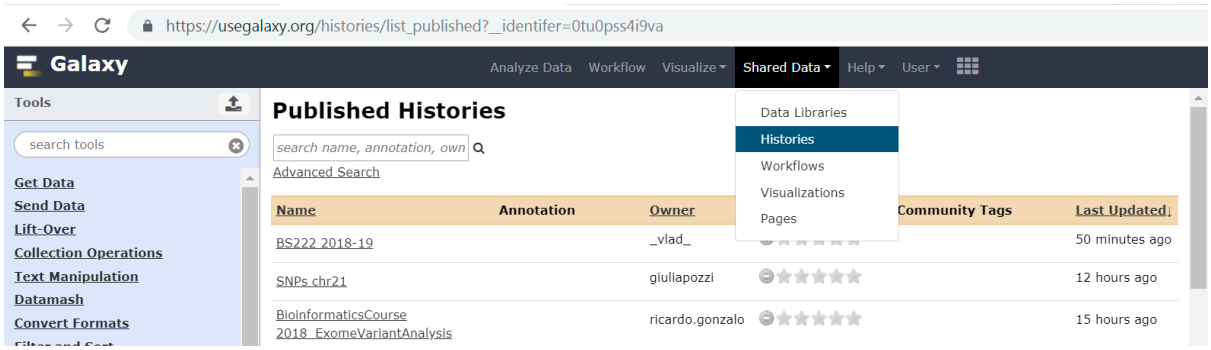
**Task 5*. If our local Galaxy installation at Essex is overloaded we will do step 5 at the central Galaxy installation at https://usegalaxy.org. as described below.**

Let us now explore the central installation of Galaxy at https://usegalaxy.org, which is available for anyone, not just for students of our university. In fact, you can use it in your future projects.

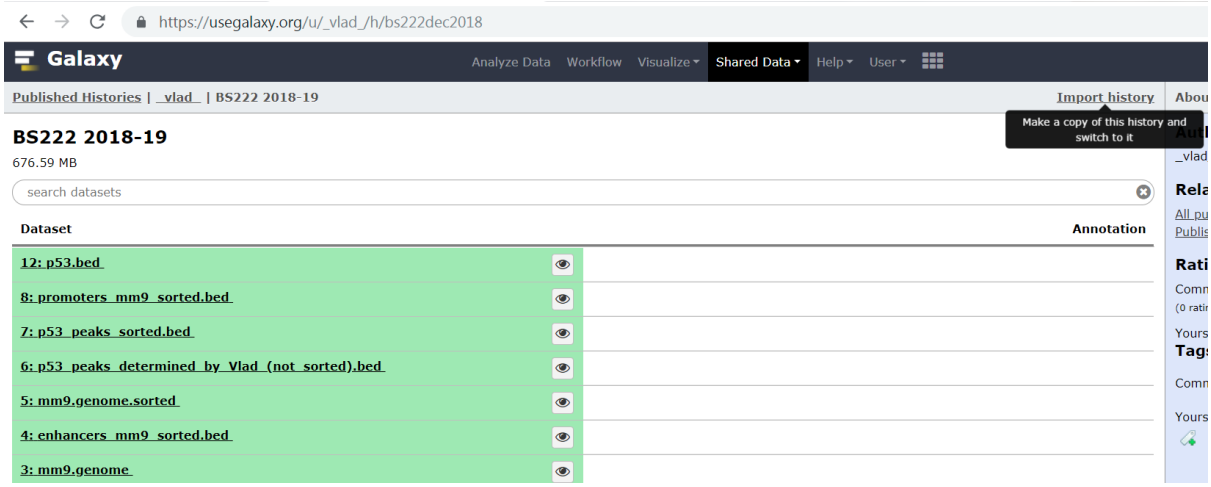
Go to https://usegalaxy.org and register an account in the same way as you previously did at our local Galaxy installation:



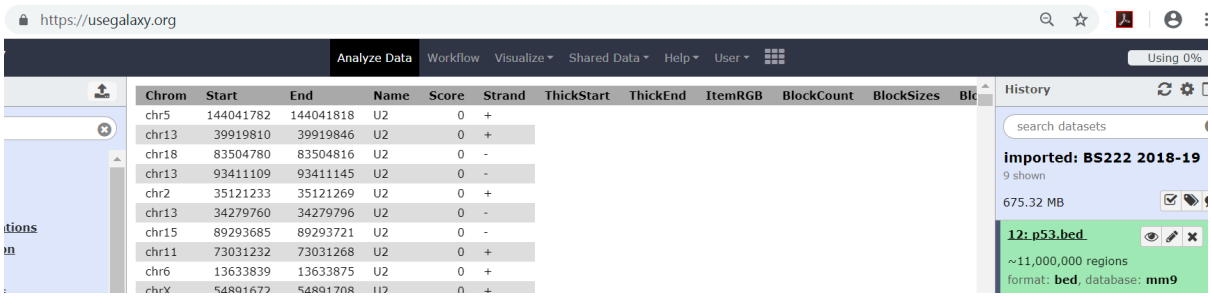

Then go to menu "Shared data"> "Histories", and select history named "BS222 2018-19":



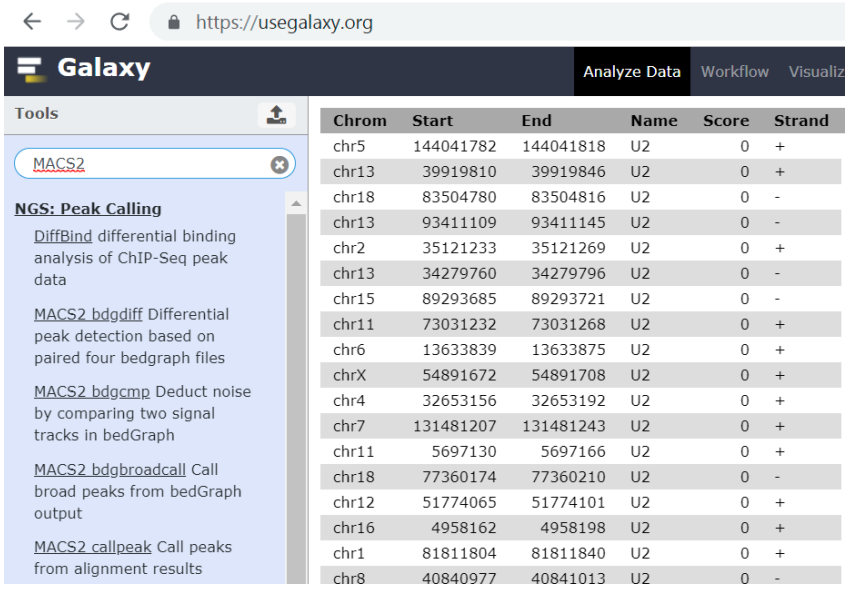

Then open the history "BS222 2018-19" and import it to your current history:




This history contains files with the same names as in our local Galaxy installation. In addition, there are mapped DNA reads in a more compact BED format. In this format, only the genomic coordinates are provided. You can see these files by clicking on the eye icon on the files "p53.bed" and "Input.bed":




Now let's determine p53 binding peaks (or in other words, do peak calling), using these two files "p53.bed" and "Input.bed". To do so, let's locate a program MACS2 that is doing peak calling. For example, we can find it by entering the name "MACS2" in the search field as shown below:

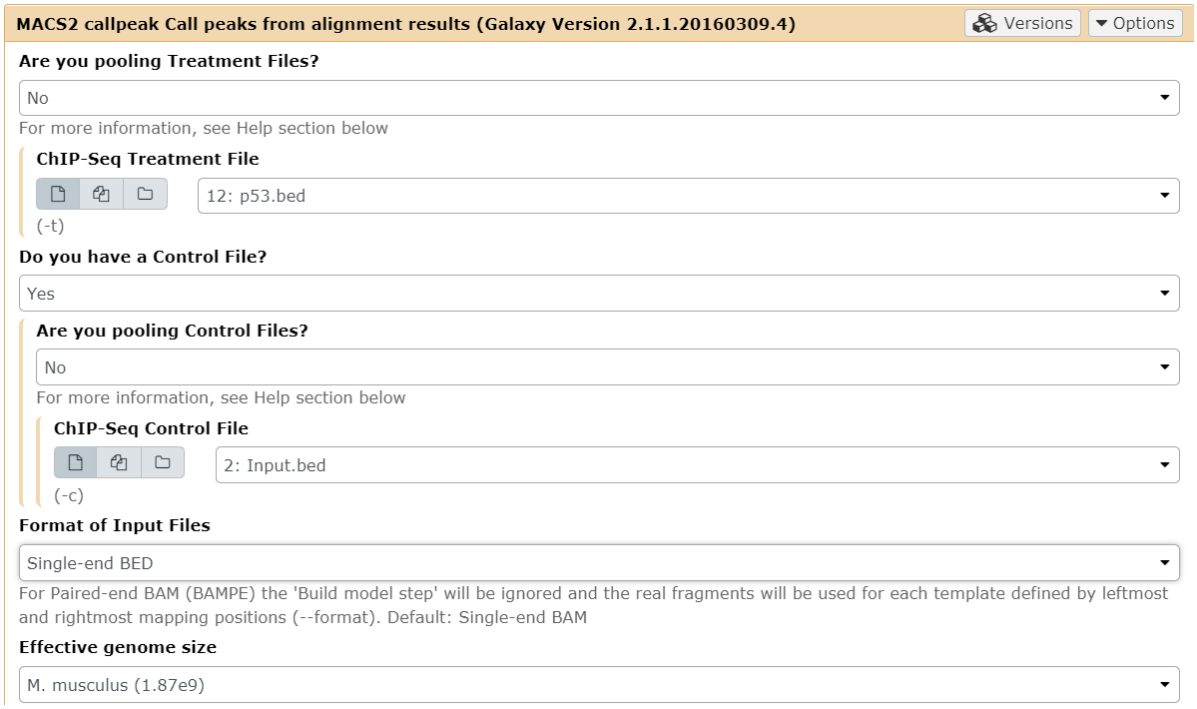



Then let's select "MACS2 callpeaks" (Call peaks from alignment results). In the MACS2 menu let us select the following options:

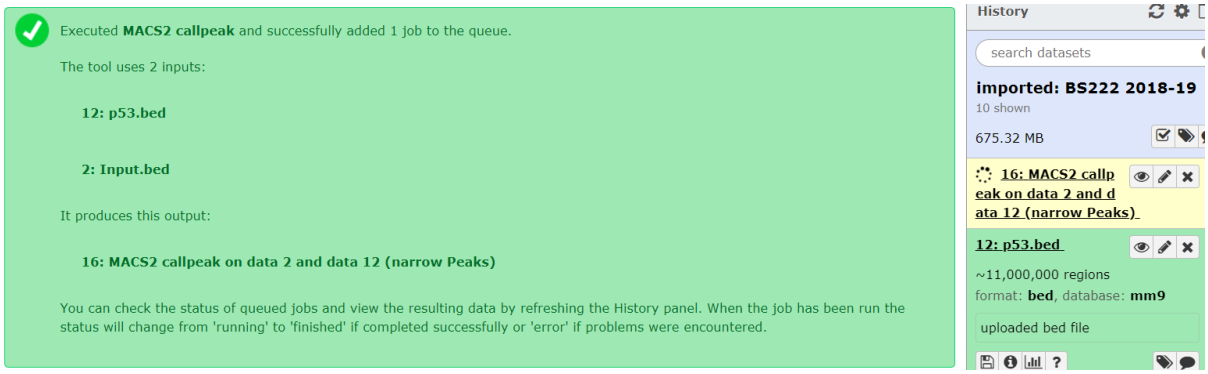
- ChIP-seq treatment file: p53.bed
- Do you have a control file: yes
- ChIP-seq control file: Input.bed
- Format of input files: single-end BED
- Effective genome size: m. musculus

Keep the rest parameters as they are by default (do not change).

Scroll to the end of the page and click the "Execute" button:

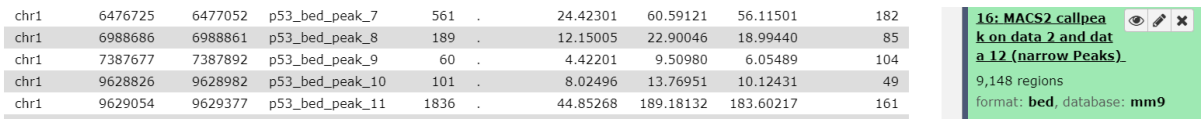



If you did everything correct, the following kind of screen appears:




The new dataset with peaks that is being created will be first grey (which means it is standing in a queue), then it will turn yellow (which means that this job is running), and finally when it will turn green the calculation will be finished. In my experience this calculation took about 10 minutes, but it may be very different if all of us will submit our jobs to the same server at the same time, so be prepared for longer waiting times. It is also a good time to go get a cap of tea ☺

When this calculation is finished you can have a look at the resulting file:



| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| chr1 | 6476725 | 6477052 | p53_bed_peak_7 | 561 | . | 24.42301 | 60.59121 | 56.11501 | 182 |
| chr1 | 6988686 | 6988861 | p53_bed_peak_8 | 189 | . | 12.15005 | 22.90046 | 18.99440 | 85 |
| chr1 | 7387677 | 7387892 | p53_bed_peak_9 | 60 | . | 4.42201 | 9.50980 | 6.05489 | 104 |
| chr1 | 9628826 | 9628982 | p53_bed_peak_10 | 101 | . | 8.02496 | 13.76951 | 10.12431 | 49 |
| chr1 | 9629054 | 9629377 | p53_bed_peak_11 | 1836 | . | 44.85268 | 189.18132 | 183.60217 | 161 |



The number of peaks that you obtain may be different from me if you changes some parameters (in which case you need to be able to explain what you changed and how it affected your peak calling ☺)

In the example above, the resulting file has 9.148 regions. Each region corresponds to one ChIP-seq peak, or in other words, to one p53-bound genomic location.

**Task 6. Compare the peaks that we determined with the peaks reported by Younger et al.**

Now let's have a look at the peaks that have been reported by the authors of this study. Remember where the data came from? We can look in the GEO database, were we took the data from https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55727). At the bottom of the entry, we can see the following:

**Relations**

| | |
|---|---|
| BioProject | PRJNA240784 |
| SRA | SRP039598 |

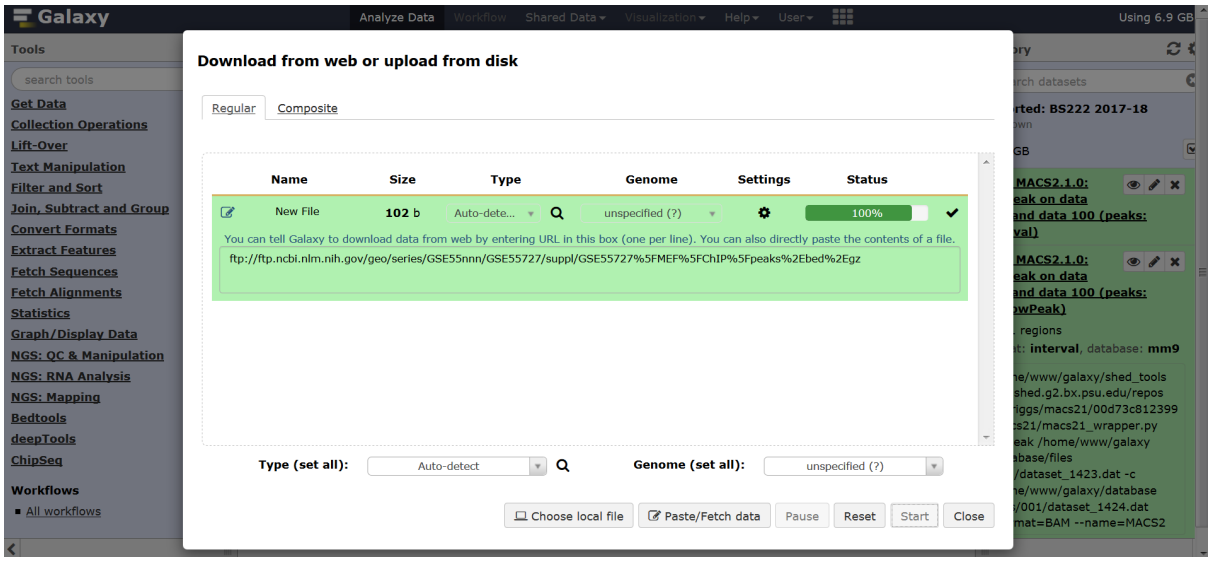
| Download family | Format |
|---|---|
| SOFT formatted family file(s) | SOFT |
| MINiML formatted family file(s) | MINiML |
| Series Matrix File(s) | TXT |

| Supplementary file | Size | Download | File type/resource |
|---|---|---|---|
| SRP/SRP039/SRP039598 | | (ftp) | SRA Study |
| GSE55727_Human_ChIP_peaks.bed.gz | 24.2 Kb | (ftp)(http) | BED |
| GSE55727_Human_RNA_Expression_Matrix.txt.gz | 1000.0 Kb | (ftp)(http) | TXT |
| GSE55727_MEF_ChIP_peaks.bed.gz | 27.6 Kb | (ftp)(http) | BED |
| GSE55727_MEF_KO_RNA_Expression_Matrix.txt.gz | 570.5 Kb | (ftp)(http) | TXT |
| GSE55727_MEF_WT_RNA_Expression_Matrix.txt.gz | 784.4 Kb | (ftp)(http) | TXT |

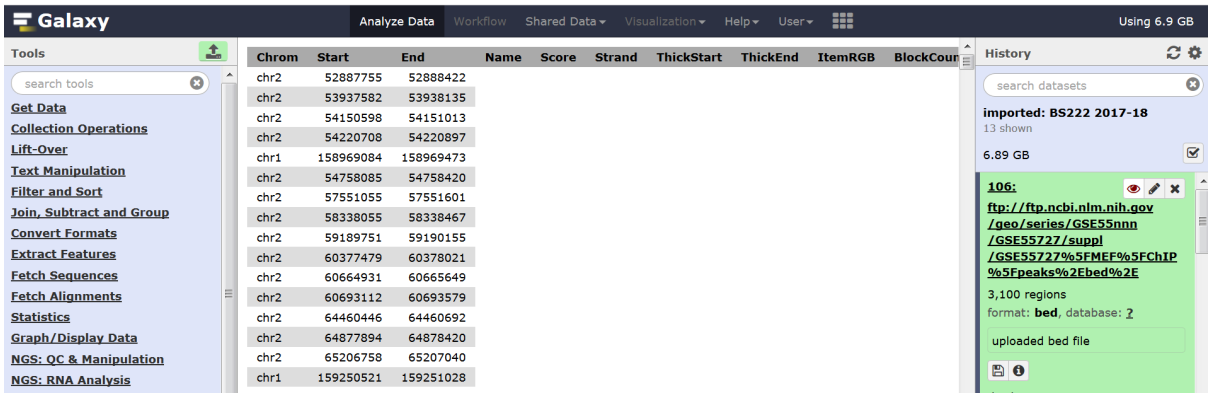
*Raw data provided as supplementary file*

*Processed data is available on Series record*

We are particularly interested in the file "GSE55727_MEF_ChIP_peaks.bed.gz". This is the file with the peaks determined by the authors. I have already copied it to the Galaxy history shared with you so you need not to download it from the Internet. But if you wish to do so you can do this by selecting Get Data from the left menu and following the screenshot below that shows you how I did this:




In the Galaxy history shared with you I have renamed this file to "p53 peaks sorted". We can view this file. We are mostly interested in the question how many regions (ChIP-seq peaks) are there.




There is one peak per line (or "per region"). How many regions are there in this file?

Why is the number of peaks that we have found different from the number of peaks determined by the authors of this paper? Discuss.

### Task 7. Intersect p53 peaks with enhancers and promoters using BedTools in Galaxy

[If our local Galaxy installation at Essex is overloaded we will do this step at the central Galaxy installation at https://usegalaxy.org. All other instructions apply. Just instead of the history "BS222 2017-18" on our local server http://galaxy.essex.ac.uk you will be using the history "BS222 2018-19" on the central Galaxy server https://usegalaxy.org]

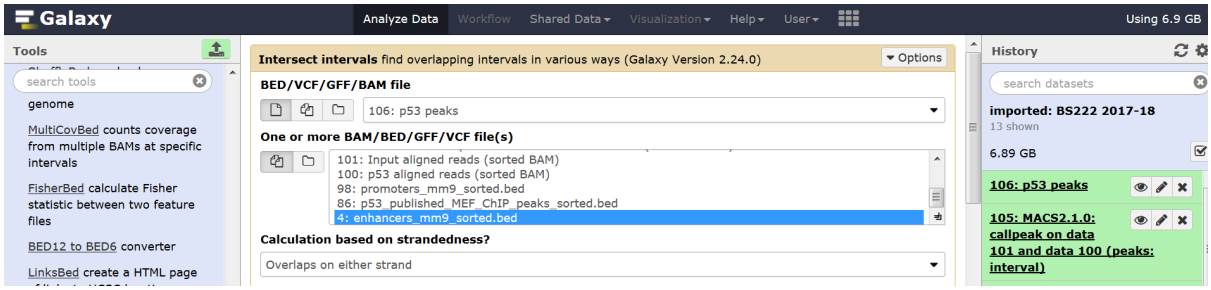
Peaks are genomic regions (defined by the chromosome, region start, region end, etc). In the BED format (the format typically used to store genomic regions after peak calling), we have columns in exactly this order (chromosome, region start, region end).

In the next task we want to intersect the genomic regions which are identified as p53 binding sites by the authors of the original paper with the regions corresponding to mouse enhancers and promoters. Here is a schematic picture which explains the "intersection" between two sets of genomic regions:

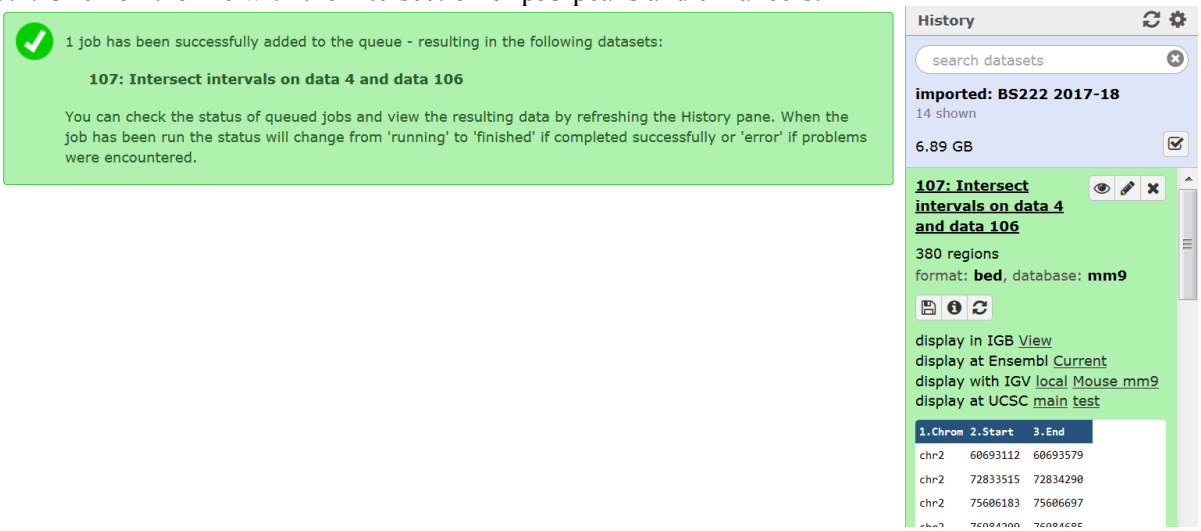

Intersection is one of the main concepts in ChIP-seq analysis. To do this we will use command Intersect Intervals from the software package BedTools.

A detailed description of all parameters of this command is provided at the following link: http://bedtools.readthedocs.io/en/latest/content/tools/intersect.html

7.1. Locate on Galaxy software "BedTools", and inside BedTools select "Intersect Intervals". Open it. Then select the names of two files with genomic regions that you want to intersect. Select as the first file *"p53 peaks sorted"*, and as the second file "enhancers_mm9.sorted" (this is the file with mouse enhancers which I have prepared for you). Then click "Execute" at the bottom of the page. This calculations will take just several seconds is there is no queue on the server.




7.2. Click on the file with the intersection of p53 peaks and enhancers:




How many regions are there in this file? Do you remember how many regions there were in total in the file with *"p53 peaks sorted"* reported by the authors that you have used in this intersection? Are there many regions intersecting with enhancers? How did you decide that this is "many"?

7.3. Repeat step 6.2., but now intersect p53 peaks with promoters (select for the intersection files *"p53 peaks sorted"* and "promoters_mm9_sorted.bed". How many p53 peaks intersects with promoters? Is it a lot? How do you know that this is a lot or not?

## 8. Finding enrichment of p53 binding at enhancers and promoters using BedTools in Galaxy

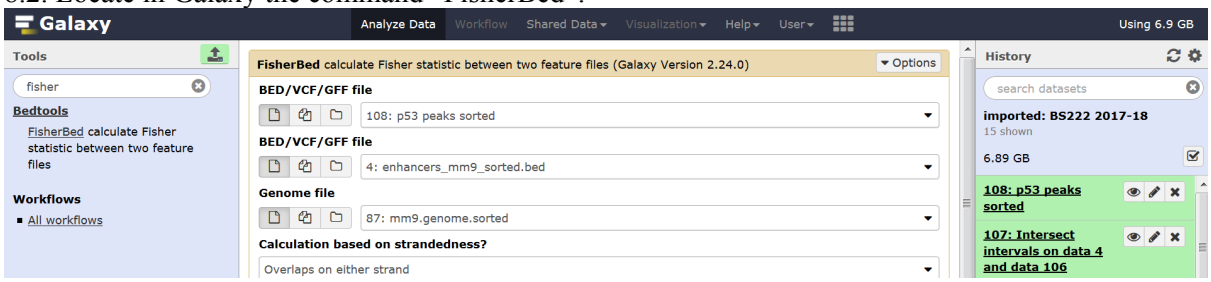
[If our local Galaxy installation at Essex is overloaded we will do this step at the central Galaxy installation at https://usegalaxy.org. All other instructions apply. The only difference is that instead of the history "BS222 2017-18" on our local server galaxy.essex.ac.uk you will be using the history "BS222 2018-19" on the central Galaxy server https://usegalaxy.org]

At the previous steps you have noticed that the pure knowledge of how many p53 peaks intersects with promoters or enhancers does not tell us whether this is a lot or not. Indeed, the critical thing that we do not know is how many regions would intersect with promoters of enhancers by chance if we would randomly select the same number of regions as in the set of p53 peaks, just with random genomic locations. Basically, if our peaks have a higher proportion of regions that intersect with enhancers than what oen would expect by chance, then we can say that p53 peaks are statistically enriched in enhancers. There are different ways to check for this statistical hypothesis. One of the simplest possibilities is to perform the Fisher test (remember the introduction to statistics from Year 1?) The Fisher test will give us a quantitative measure of the statistical significance of our hypothesis (our hypothesis is that p53 peaks are enriched in enhancers). The Fisher test will calculate for us a P-value, which is the probability that the same situation happens just by chance (randomly). Obviously, if it can happen by chance randomly, this is not a real biological effect. Only if our biological finding

has a very low probability to happen by chance (low P-value), only then our finding is statistically significant. So let us test the conclusion of the authors of the paper that p53 likes to bind in enhancers.

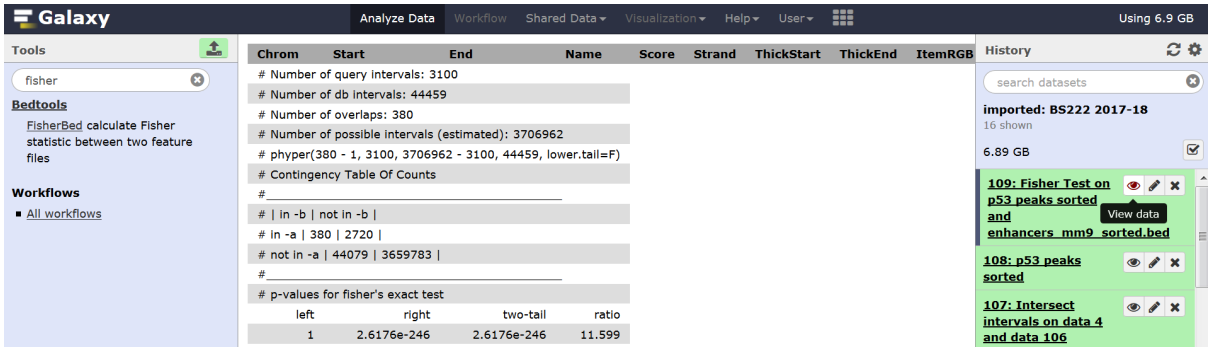
8.1. The Fisher text that is available on the Galaxy will only work on the sorted data, so we have to sort our peaks first. I did it for you already for the peaks reported by the authors of this paper, which are in the file named *"p53 peaks sorted"* (this is why is has the word sorted in its name). If you want to do it yourself later you can use the command "SortBed" in Galaxy to sort any BED-format file.

8.2. Locate in Galaxy the command "FisherBed":




8.3. Select the names of the two files for which we want to perform the Fisher test: "p53 peaks sorted", and "enhancers_mm9_sorted". For the Genome file select "mm9.genome.sorted" (this is the file that contains the lengths of all mouse chromosomes – this information is needed to perform the statistical significance test). Then click "execute":

8.4. The results of the Fisher test are reported in the following way:




In the table above, we need to look at the two-tail P-value. If the P-value is smaller than 0.05 the results are usually considered as significant. Are our results significant? The value indicated as "ratio" shows the enrichment of p53 peaks with enhancers. In the case above, for example, ratio=11.599. This means that p53 binding sites are more than 11-fold enriched with enhancers in comparison with what would be expected by chance.

Hint: Some of these numbers will be needed for filling in your coursework☺

8.5. Determine p53 enrichment at promoters using the file promoters_mm9_sorted.bed and following the steps 7.2-7.4.

Is p53 also enriched at promoters? Where is it enriched stronger, at promoters or enhancers? Discuss.

This is the end of our first computational practical. Please keep all your notes as these will be helpful for your next practical, as well as for answering the questions in the coursework. If you will forget any numbers obtained during this practical you will be able to see them again on the Galaxy. You will be also able to play with Galaxy later. Your Galaxy account will be retained for you.