# ChromAcS – User Manual

Version: 1.0.0

Authors: ChromAcS Development Team

Project Link: https://github.com/epigen-bioinfolab/CHROMACS

## Introduction

ChromAcS (**Chrom**atin **Ac**cessibility Analysis **S**uite) is a user-friendly, GUI-based, end-to-end pipeline for analyzing paired-end ATAC-seq data. ChromAcS integrates best-practice tools into a single cohesive workflow to perform automated quality control, adapter trimming, read alignment, coverage track generation, peak calling, genomic peak annotation, and differential accessibility analysis. It further incorporates motif enrichment and regulatory network analysis, motif footprinting, peak overlap comparison, and integration with gene expression datasets, enabling versatile downstream multi-omic exploration. The pipeline supports reference genome handling across multiple species, peak annotation, and multiple modes of differential accessibility analysis, including both replicate-based and no-replicate scenarios.

ChromAcS is a GUI-based ATAC-seq analysis platform with multi-species support, enabling real-time progress monitoring, flexible re-execution of downstream steps without restarting the workflow, and easy deployment in a Linux environment using our automated setup script for installing all required dependencies.

## Installation Guide

ChromAcS is primarily developed and tested for Linux-based platforms (e.g., Ubuntu, Fedora, Debian), which are recommended for full compatibility and optimal performance. While Linux is the officially supported environment, ChromAcS may also function on other Unix-like systems such as macOS, provided the necessary dependencies are met. Use on Windows is not officially supported, but advanced users may attempt installation via WSL (Windows Subsystem for Linux) or a virtual machine running a Linux distribution.

To ensure reproducibility and to prevent conflicts with other software, ChromAcS is designed to operate within an isolated Conda environment. This simplifies installation and management of the required packages and dependencies, without clashing with other tools.

The installation process is straightforward and involves the following steps:

**Step 1: (Clone ChromAcS repository or download as a ZIP):**

To clone the repository and navigate to it, just paste the following after opening the terminal-

```
git clone https://github.com/epigen-bioinfolab/CHROMACS.git
cd CHROMACS
```

Alternatively, you can download the zip file-

1. Go to https://github.com/epigen-bioinfolab/CHROMACS
2. Click the green **<> Code button** → Download ZIP
3. Extract the ZIP file. The folder will be named CHROMACS-main. Configure to the CHROMACS-main-

   ```
   cd CHROMACS-main
   ```

**Step 2: Create and activate the environment :**

If you have followed the above steps, you should find yourself inside the CHROMACS (or, CHROMACS-main) directory on the terminal (be certain that you are indeed inside such a directory; if not, you can manually click to search and go inside the directory). In your terminal, paste the following-

```
conda env create -f environment.yml
conda activate chromacs
```

***Note (a prerequisite for Step 2):***

*Step 2 might take several minutes to set up the conda environment. However, please ensure that you have Conda installed on your system. For example, you can install Miniconda as follows-*

```
# Download the Miniconda installer (64-bit)
wget https://repo.anaconda.com/miniconda/Miniconda3-latest-Linux-x86_64.sh

# Run the installer
bash Miniconda3-latest-Linux-x86_64.sh

# Follow the prompts and restart your terminal (or run 'source ~/.bashrc')
```

**Step 3: Install the tool:**

Paste this on your terminal (ensure that you are indeed inside the CHROMACS environment setup in conda)-

```
pip install .   # DO NOT FORGET THE DOT (.) at the end “pip install .”
```

### Step 4: Launch the tool:

Just type the following in the terminal to launch the base tool

```
Chromacs
```

### Step 4a (Add-on): Launch the ChromAcS-AddOn: Additional Analysis Toolkit :

```
chromacs-addon
```

### Extra guides:-

### How to relaunch the tool once you close it?

After installing and running the tool, you may shut the tool. Now, to relaunch it, the steps are even easier, just open your terminal anywhere (you do not need to be inside the CHROMACS directory now) and activate conda (type the following in your terminal)-

```
conda activate chromacs
chromacs
```

### How to uninstall the tool?

If you wish to uninstall ChromAcS, you need to navigate to the CHROMACS directory, then type in the following in your terminal-

```
pip uninstall chromacs -y
```

Remove the associated Conda environment-

```
conda env remove -n chromacs
```

## Navigating inside ChromAcS

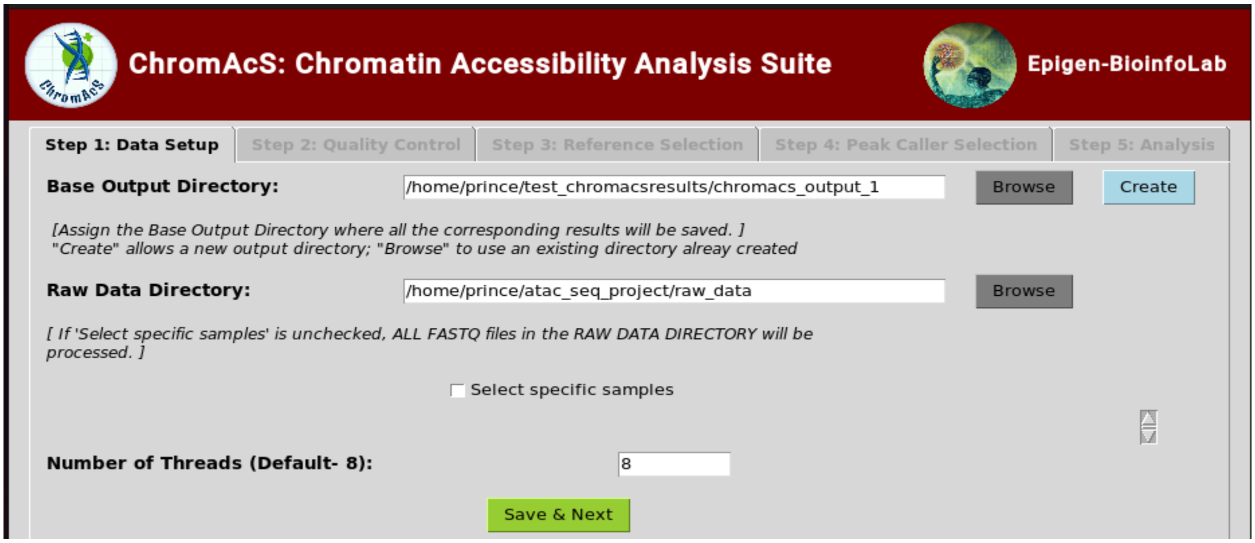
ChromAcS provides a simple and straightforward navigation for the users, so that they can easily set up the different parameters accordingly. Here, we provide a step-by-step guide on how we used ChromAcS to process ATAC-seq data from two related NCBI GEO BioProjects- GSE100750 (BioProject: PRJNA392915) and GSE73874 (BioProject: PRJNA301678), both published by Guler *et al* (https://pubmed.ncbi.nlm.nih.gov/28781121). These datasets profile the chromatin accessibility in PC9 lung cancer cell lines and their drug-tolerant persister (DTP) derivatives under various treatment conditions. We select two experimental groups to simulate a two-condition ATAC-seq differential accessibility study, where condition 1 consists of the untreated parental PC9 cells, and condition 2 consists of DTPs (these are drug-tolerant subpopulations of cells that survived erlotinib, an EGFR inhibitor, treatment).

| Conditions | Sample IDs | Control Sample IDs |
|---|---|---|
| Condition 1 (untreated) | SRR5800657 (GSM2692559) | SRR2927084 (GSM1904729) |
|  | SRR5800658 (GSM2692560) |  |
|  | SRR5800659 (GSM2692561) |  |
| Condition 2 (treated/DTPs) | SRR5800660 (GSM2692562) | SRR2927085 (GSM1904730) |
|  | SRR5800661 (GSM2692563) |  |
|  | SRR5800662 (GSM2692564) |  |

The three samples in condition 1 and condition 2 are biological replicates of parental PC9 cells and DTPS, respectively.

### Step 1: Setting the base output directory and the raw data directory

You need to put the raw paired-end ATAC-seq data in a single directory and make a new directory where all the outputs generated by ChromAcS will be saved. Then inside ChromAcS, you need to browse inside those-


ChromAcS: Chromatin Accessibility Analysis Suite
Epigen-BioinfoLab
Step 1: Data Setup
Step 2: Quality Control
Step 3: Reference Selection
Step 4: Peak Caller Selection
Step 5: Analysis
Browse
Create
Browse
Save & Next

**Base Output Directory:** /home/prince/test_chromacsresults/chromacs_output_1

*[Assign the Base Output Directory where all the corresponding results will be saved. ]*
*"Create" allows a new output directory; "Browse" to use an existing directory alreay created*

**Raw Data Directory:** /home/prince/atac_seq_project/raw_data

*[ If 'Select specific samples' is unchecked, ALL FASTQ files in the RAW DATA DIRECTORY will be processed. ]*

□ Select specific samples

**Number of Threads (Default- 8):** 8

#### *How to browse it right*

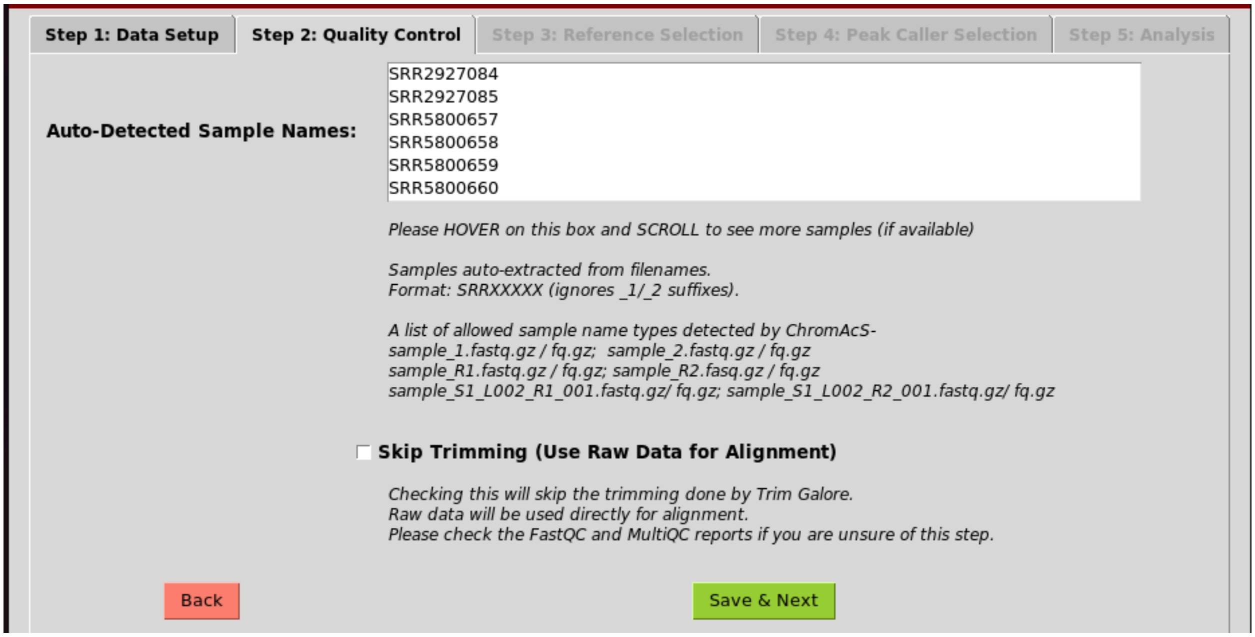
Here, you must remain careful that while browsing the required directories, you must navigate inside the given directory. For example, here our raw data is saved inside the “home/prince/atac_seq_project/raw_data”, so while browsing, we must go inside the raw_data itself (users may often mistakenly just move inside the atac_seq_project directory and see the raw_data, but not click inside it. But you MUST click inside the directory itself).

***Thread setup***

Another parameter to set up here is the number of threads that would be assigned to several of the dependencies inside ChromAcS. We would recommend keeping it to the default of 8 if you have 16 GB of memory (RAM). If you have lower RAM, you may decrease the thread count.

***Processing all the samples?***

Note that, if you keep the “Select specific samples” UNCHECKED, all the paired-end files inside the “Raw Data Directory” will be processed in the downstream step as you can check in the next step (Step 2)-



**Auto-Detected Sample Names:**

```
SRR2927084
SRR2927085
SRR5800657
SRR5800658
SRR5800659
SRR5800660
```

*Please HOVER on this box and SCROLL to see more samples (if available)*

*Samples auto-extracted from filenames.*
*Format: SRRXXXXX (ignores _1/_2 suffixes).*

*A list of allowed sample name types detected by ChromAcS-*
*sample_1.fastq.gz / fq.gz;  sample_2.fastq.gz / fq.gz*
*sample_R1.fastq.gz / fq.gz; sample_R2.fasq.gz / fq.gz*
*sample_S1_L002_R1_001.fastq.gz/ fq.gz; sample_S1_L002_R2_001.fastq.gz/ fq.gz*

□ **Skip Trimming (Use Raw Data for Alignment)**

*Checking this will skip the trimming done by Trim Galore.*
*Raw data will be used directly for alignment.*
*Please check the FastQC and MultiQC reports if you are unsure of this step.*

As you can see, the auto-detected sample names detected all the samples (here, you see only six of the sample names; scrolling down in that box will show the other two samples as well.) We had a total of 8 samples, that is, 16 reads. Now, there are some prerequisites for the file names of samples, and we have tried to add most of the naming conventions typically used.

***Which names will be valid for a successful downstream processing in ChromAcS?***

sample_1.fastq.gz / fq.gz;  sample_2.fastq.gz / fq.gz

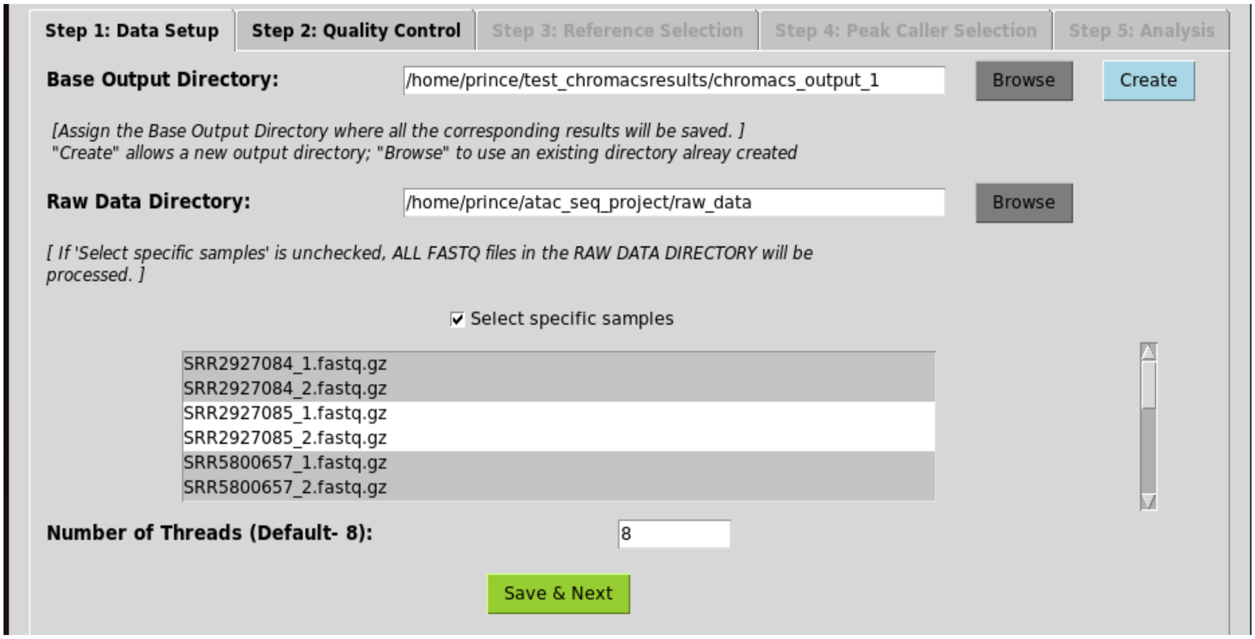
sample_R1.fastq.gz / fq.gz; sample_R2.fasq.gz / fq.gz

sample_S1_L002_R1_001.fastq.gz/ fq.gz; sample_S1_L002_R2_001.fastq.gz/ fq.gz

Here, the first part is the forward reads and the second part (after a semi-colon) is the reverse reads.

***Processing Selective samples?***

This can be done simply by CHECKING the “Select specific samples” in step 1-



**Base Output Directory:** /home/prince/test_chromacsresults/chromacs_output_1

*[Assign the Base Output Directory where all the corresponding results will be saved. ]*
*"Create" allows a new output directory; "Browse" to use an existing directory alreay created*

**Raw Data Directory:** /home/prince/atac_seq_project/raw_data

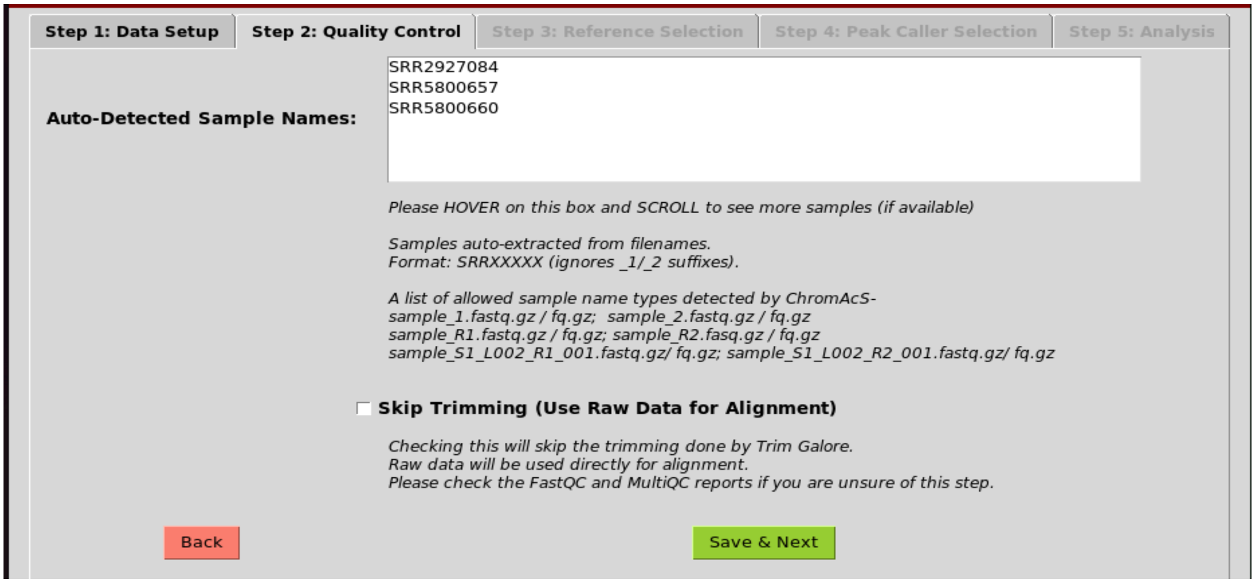
*[ If 'Select specific samples' is unchecked, ALL FASTQ files in the RAW DATA DIRECTORY will be processed. ]*

☑ Select specific samples

SRR2927084_1.fastq.gz
SRR2927084_2.fastq.gz
SRR2927085_1.fastq.gz
SRR2927085_2.fastq.gz
SRR5800657_1.fastq.gz
SRR5800657_2.fastq.gz

**Number of Threads (Default- 8):** 8

Once you CHECK the “Select specific samples”, a new text widget appears, showing the samples. You can click on the required samples to select. However, be certain to include both reads of the paired-end samples. Once you are done selecting the specific samples, you can verify those again in step 2-



**Auto-Detected Sample Names:**

SRR2927084
SRR5800657
SRR5800660

*Please HOVER on this box and SCROLL to see more samples (if available)*

*Samples auto-extracted from filenames.*
*Format: SRRXXXXX (ignores _1/_2 suffixes).*

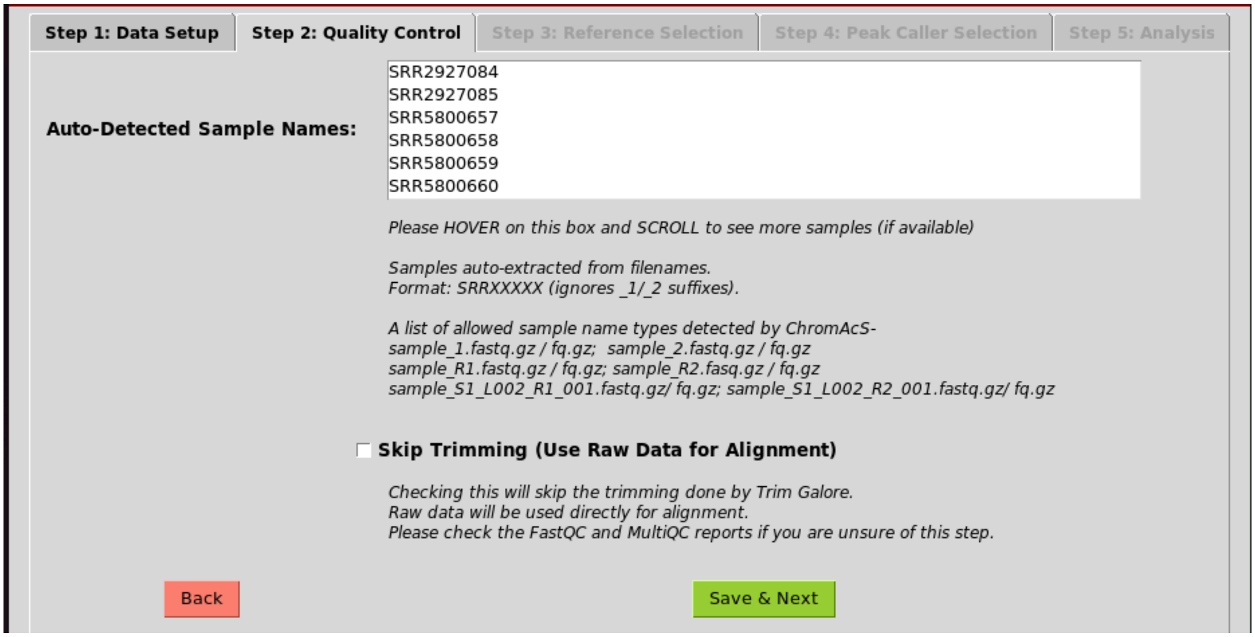
*A list of allowed sample name types detected by ChromAcS-*
*sample_1.fastq.gz / fq.gz;  sample_2.fastq.gz / fq.gz*
*sample_R1.fastq.gz / fq.gz; sample_R2.fasq.gz / fq.gz*
*sample_S1_L002_R1_001.fastq.gz/ fq.gz; sample_S1_L002_R2_001.fastq.gz/ fq.gz*

☐ **Skip Trimming (Use Raw Data for Alignment)**

*Checking this will skip the trimming done by Trim Galore.*
*Raw data will be used directly for alignment.*
*Please check the FastQC and MultiQC reports if you are unsure of this step.*

### Step 2: Verifying the selected samples and choosing trimming

This step is pretty straightforward, as you verify here the samples detected by ChromAcS inside your assigned Raw_data_directory. You can also decide in this step whether you want to allow ChromAcS to perform the trimming operation or not. ChromAcS pipeline uses Trim Galore for trimming the reads.



**Auto-Detected Sample Names:**

```
SRR2927084
SRR2927085
SRR5800657
SRR5800658
SRR5800659
SRR5800660
```

*Please HOVER on this box and SCROLL to see more samples (if available)*

*Samples auto-extracted from filenames.*
*Format: SRRXXXXX (ignores _1/_2 suffixes).*

*A list of allowed sample name types detected by ChromAcS-*
*sample_1.fastq.gz / fq.gz; sample_2.fastq.gz / fq.gz*
*sample_R1.fastq.gz / fq.gz; sample_R2.fasq.gz / fq.gz*
*sample_S1_L002_R1_001.fastq.gz/ fq.gz; sample_S1_L002_R2_001.fastq.gz/ fq.gz*

□ **Skip Trimming (Use Raw Data for Alignment)**

*Checking this will skip the trimming done by Trim Galore.*
*Raw data will be used directly for alignment.*
*Please check the FastQC and MultiQC reports if you are unsure of this step.*

***What happens if you skip trimming?***

If you CHECK “Skip Trimming (Use Raw Data for Alignment)”, then ChromAcS will align the raw Fastq reads in the subsequent steps. This will skip the trimming steps by Trim Galore.

***Can you use your own trimmed reads?***

Yes, you can use your own trimmed reads, that is, if you prefer to trim your data with a separate trim tool (like trimmomatic, BBDuk, etc.), then you can rename the output with the naming conventions suggested in the tool. This you just simply use this reads in the raw data directory, and MUST CHECK “Skip Trimming (Use Raw Data for Alignment)”. In that way, you would be using your own trimmed data, if you are unsure to use our default Trim Galore trimming.

### Step 3: Selecting the Desired Organism/ Reference Genome

In this step, you need to assign the organism you are studying. Currently, ChromAcS support the following list of organisms-

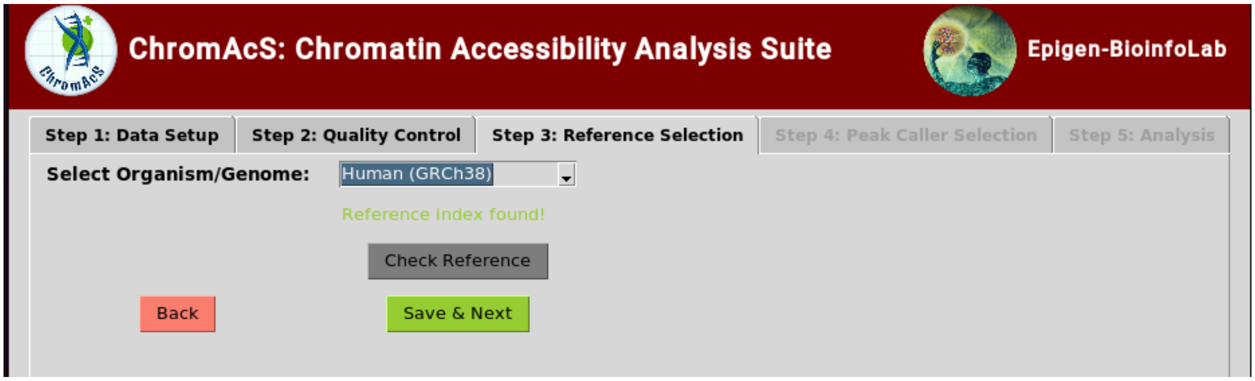
***Table 1. List of organisms supported***

| Common Name | Scientific Name | Genome Version |
|---|---|---|
| Human | *Homo sapiens* | GRCh38 |
| Mouse | *Mus musculus* | GRCm39 |
| Rat | *Rattus norvegicus* | mRatBN7.2 |
| Cow | *Bos taurus* | ARS-UCD1.3 |
| Pig | *Sus scrofa* | Sscrofa11.1 |
| Chicken | *Gallus gallus* | bGalGal1.mat.broiler.GRCg7b |
| Chimpanzee | *Pan troglodytes* | Pan_tro_3.0 |
| Dog | *Canis lupus familiaris* | ROS_Cfam_1.0 |
| Goat | *Capra hircus* | ARS1 |
| Rabbit | *Oryctolagus cuniculus* | OryCun2.0 |
| Gorilla | *Gorilla gorilla* | gorGor4 |
| Rhesus Macaque | *Macaca mulatta* | Mmul_10 |
| Zebrafish | *Danio rerio* | GRCz11 |
| Frog | *Xenopus tropicalis* | UCB_Xtro_10.0 |
| Indian Cobra | *Naja naja* | Nana_v5 |
| Atlantic Salmon | *Salmo salar* | Ssal_v3.1 |
| C. elegans | *Caenorhabditis elegans* | WBcel235 |
| Drosophila | *Drosophila melanogaster* | BDGP6 |

This step is crucial as it determines which reference genome to build and index, which GTF file to download and process in the later steps, and also very crucial for the annotation steps.

***Does ChromAcS build and index the reference genome on every run?***

No, it only does it for the first run for a given reference genome (thus, it takes some time on the first run for a given reference genome, around 90-120 minutes extra depending on thread setup and internet connection speed). But once you have already run ChromAcS for a given reference genome, it saves the Bowtie2 built indices in a separate directory within the "chromacs" conda environment, along with the corresponding GTF file, the reference TSS file, the TxDB object, and the org.db file (used during annotation later). This makes the next runs smoother and saves a significant amount of time. You can check whether the reference genome has already been built or not by clicking "Check Reference" in step 3.

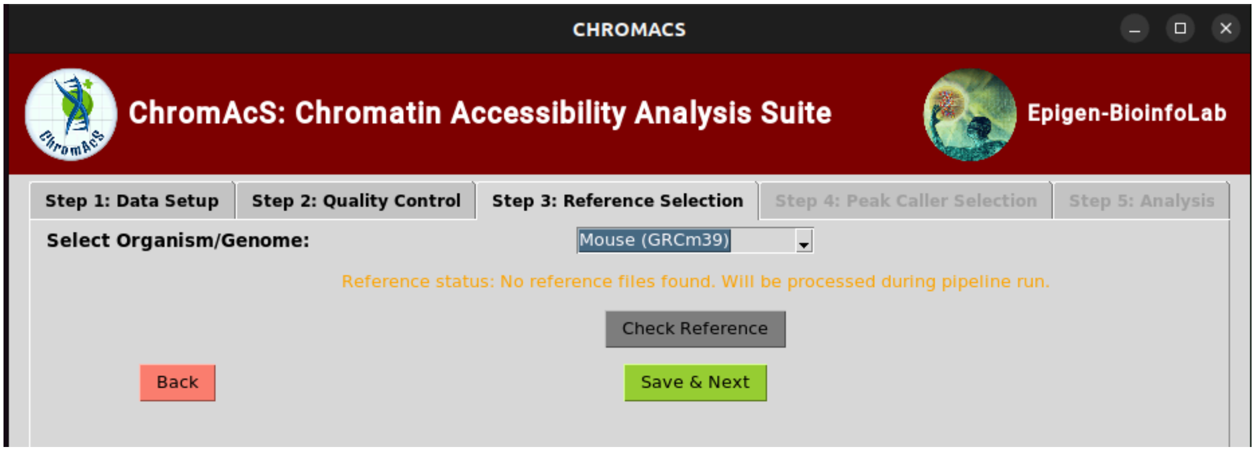

ChromAcS: Chromatin Accessibility Analysis Suite
Epigen-BioinfoLab
Step 1: Data Setup
Step 2: Quality Control
Step 3: Reference Selection
Step 4: Peak Caller Selection
Step 5: Analysis
Check Reference
Back
Save & Next

Select Organism/Genome: Human (GRCh38)

Reference index found!

As you can see here, the message says "Reference index found!", suggesting that the user has already run the tool once for Human (GRCh38). And thus, during the current run, the building will be avoided.

If it was not run, and for the first run, a following message will be shown on clicking the "Check Reference"-

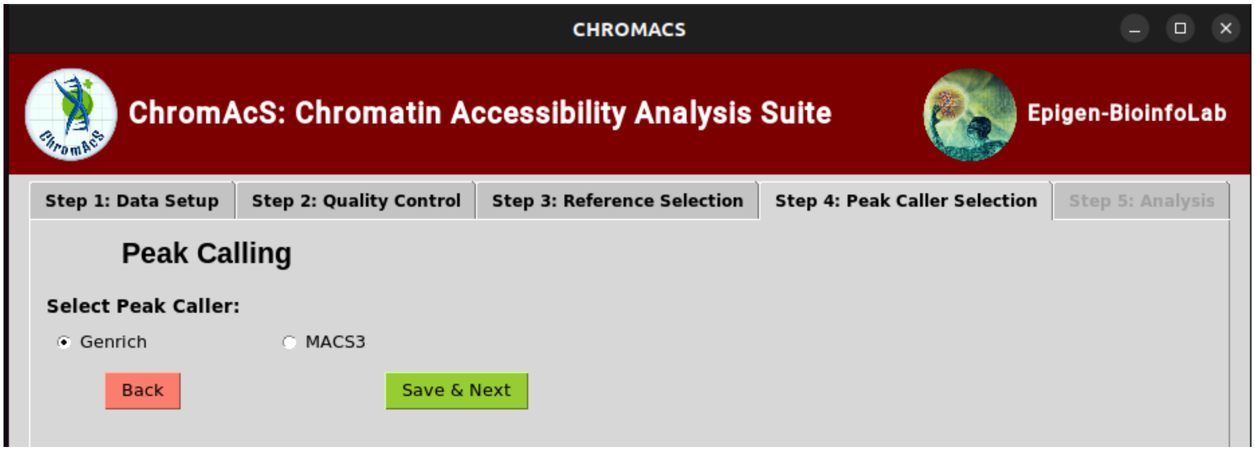


Select Organism/Genome: Mouse (GRCm39)

Reference status: No reference files found. Will be processed during pipeline run.

## Step 4: Selection of Peak Caller and Adjusting the Parameters

This is one of the most crucial steps, where the user needs to select a desired peak caller from two options: a) Genrich or b) MACS3.

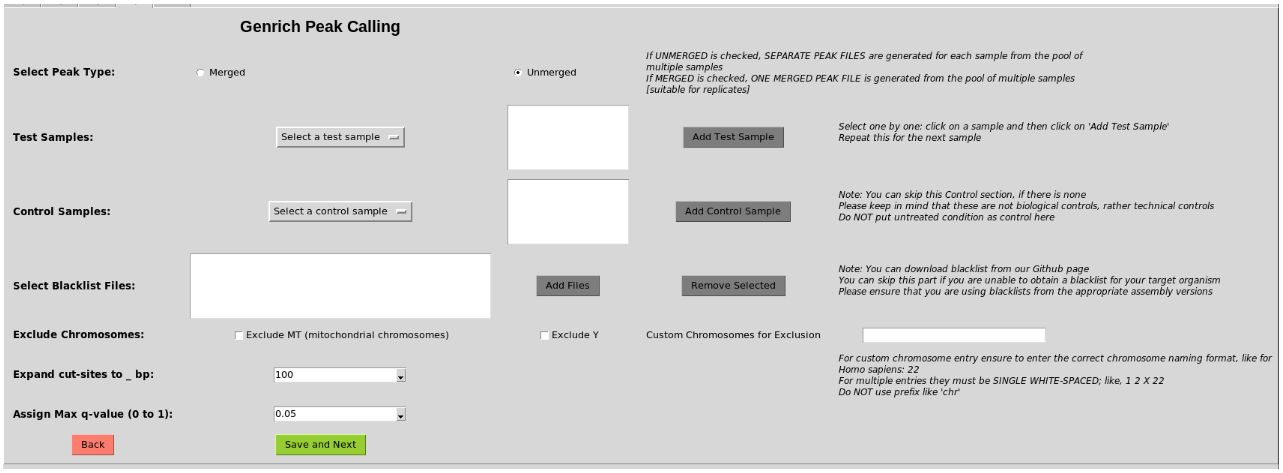

CHROMACS
ChromAcS: Chromatin Accessibility Analysis Suite
Epigen-BioinfoLab
Step 1: Data Setup
Step 2: Quality Control
Step 3: Reference Selection
Step 4: Peak Caller Selection
Step 5: Analysis
Back
Save & Next

Peak Calling

Select Peak Caller:

● Genrich ○ MACS3

Once you have selected a peak caller, it takes you to the parameter setup, where you can adjust several parameters. The parameters are more or less similar for both peak callers-



Genrich Peak Calling

Select Peak Type: ○ Merged ● Unmerged

*If UNMERGED is checked, SEPARATE PEAK FILES are generated for each sample from the pool of multiple samples*
*If MERGED is checked, ONE MERGED PEAK FILE is generated from the pool of multiple samples [suitable for replicates]*

Test Samples: Select a test sample

*Select one by one: click on a sample and then click on 'Add Test Sample'*
*Repeat this for the next sample*

Control Samples: Select a control sample

*Note: You can skip this Control section, if there is none*
*Please keep in mind that these are not biological controls, rather technical controls*
*Do NOT put untreated condition as control here*

Select Blacklist Files:

*Note: You can download blacklist from our Github page*
*You can skip this part if you are unable to obtain a blacklist for your target organism*
*Please ensure that you are using blacklists from the appropriate assembly versions*

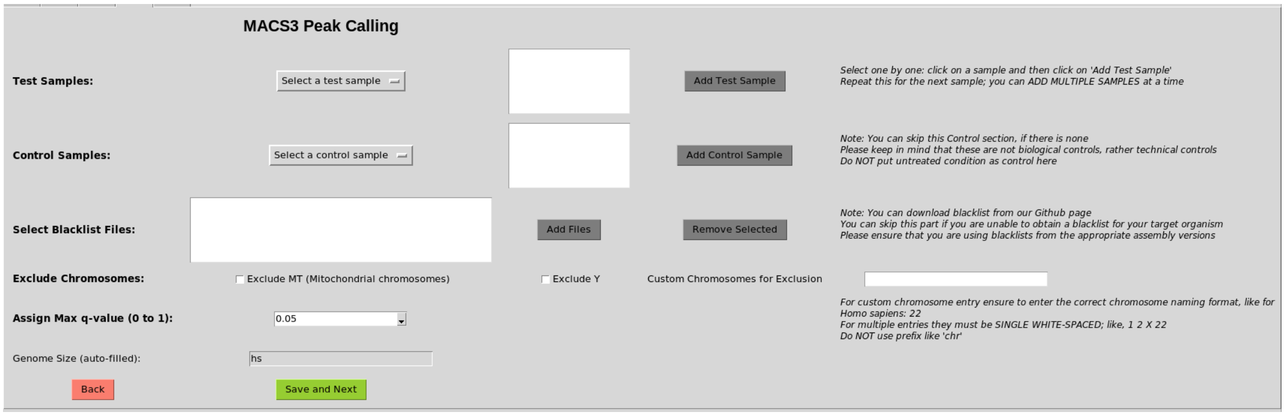
Exclude Chromosomes: □ Exclude MT (mitochondrial chromosomes) □ Exclude Y Custom Chromosomes for Exclusion

*For custom chromosome entry ensure to enter the correct chromosome naming format, like for Homo sapiens: 22*
*For multiple entries they must be SINGLE WHITE-SPACED; like, 1 2 X 22*
*Do NOT use prefix like 'chr'*

Expand cut-sites to _ bp: 100

Assign Max q-value (0 to 1): 0.05

(Genrich parameter setups)



Select one by one: click on a sample and then click on 'Add Test Sample'
Repeat this for the next sample; you can ADD MULTIPLE SAMPLES at a time

Note: You can skip this Control section, if there is none
Please keep in mind that these are not biological controls, rather technical controls
Do NOT put untreated condition as control here

Note: You can download blacklist from our Github page
You can skip this part if you are unable to obtain a blacklist for your target organism
Please ensure that you are using blacklists from the appropriate assembly versions

For custom chromosome entry ensure to enter the correct chromosome naming format, like for Homo sapiens: 22
For multiple entries they must be SINGLE WHITE-SPACED; like, 1 2 X 22
Do NOT use prefix like 'chr'

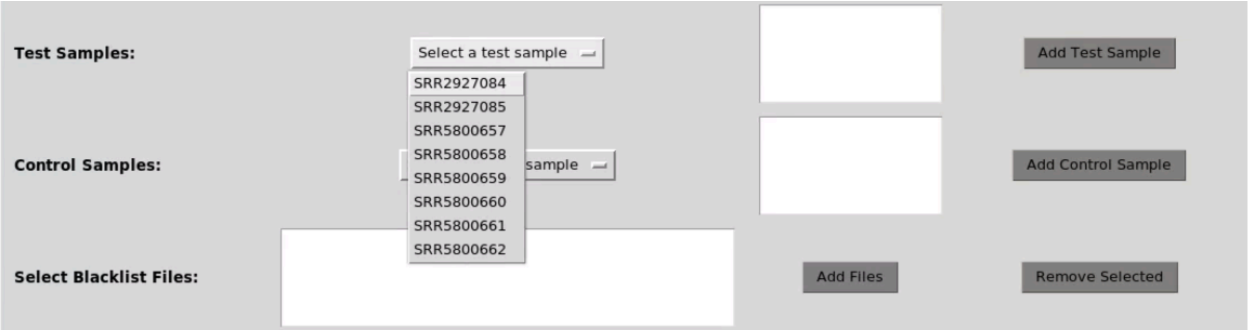
Assign Max q-value (0 to 1): 0.05

Genome Size (auto-filled): hs

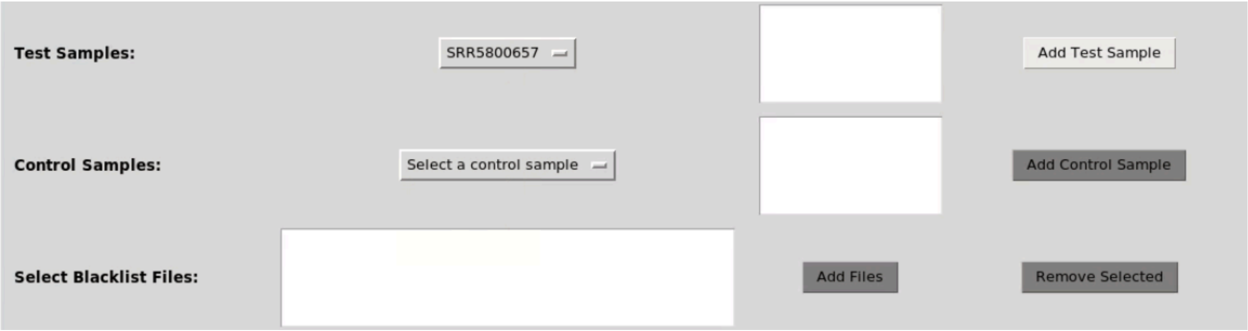
(MACS3 parameter setups)

### *How to set up the test samples?*

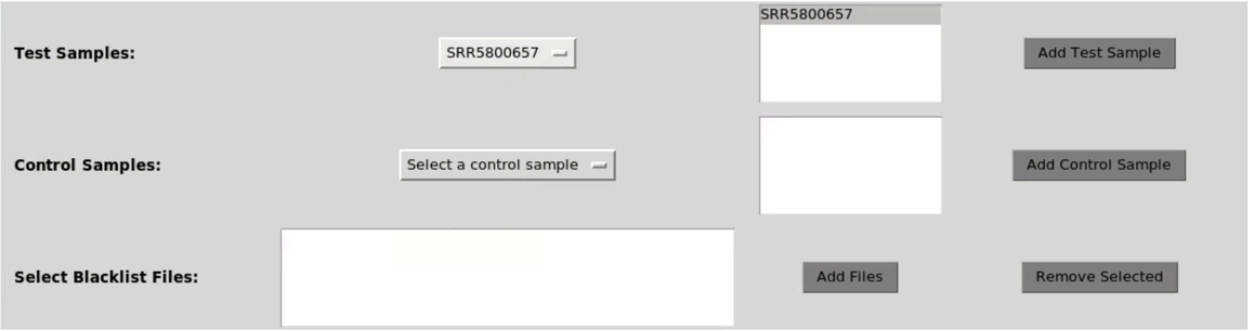
You need to add the test sample one by one; that is, click on the “Select a test sample” and the test samples will appear. From there, you need to click on one test sample and then click on the “Add Test Sample”. You need to repeat this for multiple test samples-



(When you click on the test sample, a drop-down menu appears with all the samples, from which you click on one sample at a time.)



(Once you have clicked on a desired sample, it appears on the test sample, but it has not been added to the list to be processed yet for peak calling.)



Test Samples: SRR5800657

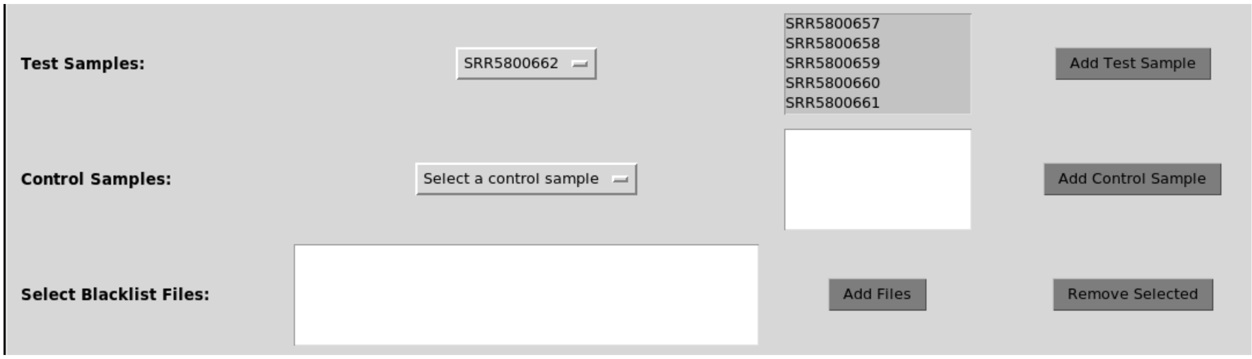
SRR5800657

Control Samples: Select a control sample

Select Blacklist Files:

(When you click on the “Add Test Sample”, the desired sample will appear in the list box to be used during peak calling.)

You need to repeat this step for all the test samples you need to process, and for both conditions. For instance, in our case study, we added all six test samples for both conditions - treated and untreated in these test samples.



Test Samples: SRR5800662

SRR5800657
SRR5800658
SRR5800659
SRR5800660
SRR5800661

Control Samples: Select a control sample

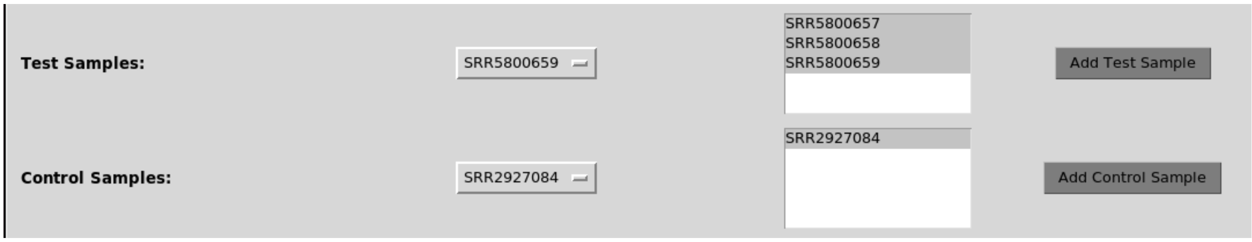
Select Blacklist Files:

(All six test samples have been added; here you see only five, another one can be seen by scrolling down by hovering the mouse over that particular list box)

***How to set up the control samples?***

The user needs to keep in mind that the control samples here mean the technical controls used by the peak callers to clear some of the background signals during peak calling. So, they are not the conventional controls in the biological sense, like we should not assign our “untreated” condition samples as control samples here. Rather, our control samples were SRR2927084 for the untreated test samples, and SRR2927085 for the treated/DTP samples.

In such designs where we have two different technical controls for the two conditions, the peak calling parameters need to be adjusted accordingly-



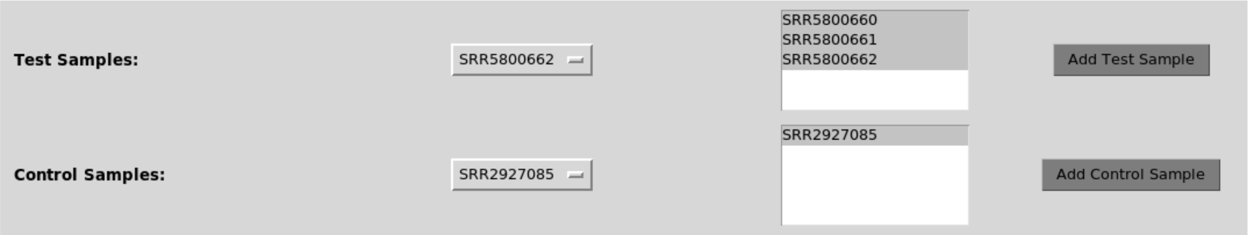
**Test Samples:** SRR5800659

SRR5800657<br>SRR5800658<br>SRR5800659

**Control Samples:** SRR2927084

SRR2927084

(Setting up the untreated condition test samples and the required technical control. With this design, we need to run the entire pipeline once. Then we repeat the process for the treated condition test samples.



**Test Samples:** SRR5800662

SRR5800660<br>SRR5800661<br>SRR5800662

**Control Samples:** SRR2927085

SRR2927085

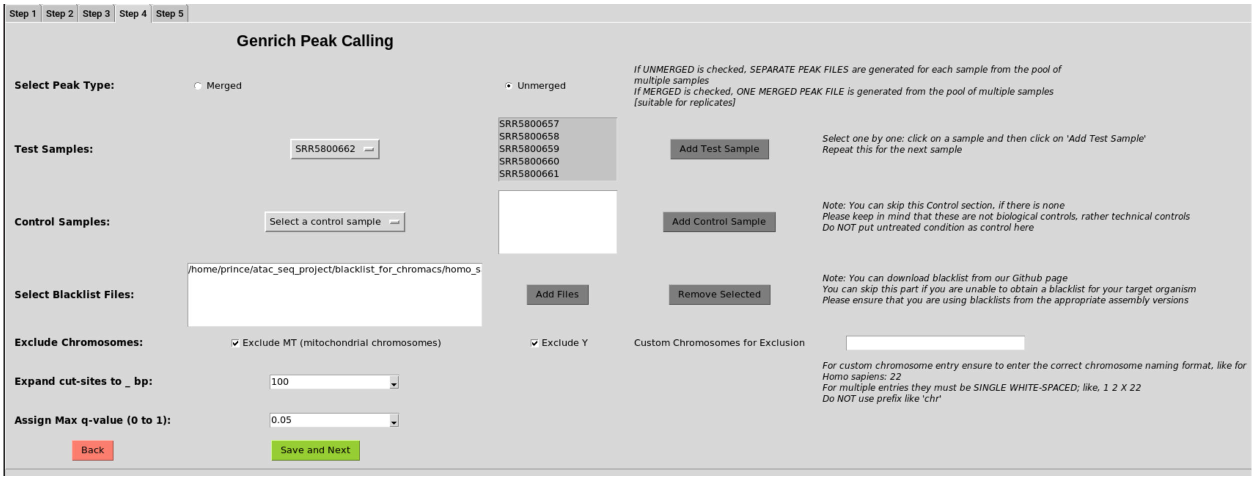
(setting up the treated condition test samples, and the required technical control. So we can not run ChromAcS with the different technical controls for both conditions at a time; rather, in such designs, we need to run the tool twice, selectively for both conditions.

***What if you do not have the technical controls in your study design?***

You can run the tool regardless of technical controls; it is optional. We would suggest running without the technical controls, as the technical controls may limit/ exclude many of the relevant biological peaks due to stringency.

***Setting up the miscellaneous parameters***

The rest of the parameters can be set up by the user according to their preference, for example, setting up the q-value, the d-value (exclusive in Genrich), Blacklist regions, and the Exclusion regions.



Genrich Peak Calling

Select Peak Type: ○ Merged ● Unmerged

*If UNMERGED is checked, SEPARATE PEAK FILES are generated for each sample from the pool of multiple samples*
*If MERGED is checked, ONE MERGED PEAK FILE is generated from the pool of multiple samples [suitable for replicates]*

Test Samples: SRR5800662

SRR5800657
SRR5800658
SRR5800659
SRR5800660
SRR5800661

*Select one by one: click on a sample and then click on 'Add Test Sample'*
*Repeat this for the next sample*

Control Samples: Select a control sample

*Note: You can skip this Control section, if there is none*
*Please keep in mind that these are not biological controls, rather technical controls*
*Do NOT put untreated condition as control here*

Select Blacklist Files: /home/prince/atac_seq_project/blacklist_for_chromacs/homo_s

*Note: You can download blacklist from our Github page*
*You can skip this part if you are unable to obtain a blacklist for your target organism*
*Please ensure that you are using blacklists from the appropriate assembly versions*

Exclude Chromosomes: ☑ Exclude MT (mitochondrial chromosomes) ☑ Exclude Y Custom Chromosomes for Exclusion

*For custom chromosome entry ensure to enter the correct chromosome naming format, like for Homo sapiens: 22*
*For multiple entries they must be SINGLE WHITE-SPACED; like, 1 2 X 22*
*Do NOT use prefix like 'chr'*

Expand cut-sites to _ bp: 100

Assign Max q-value (0 to 1): 0.05

For Blacklist Files, we would suggest only those BED files where the chromosome naming is in Ensembl formats, that is, like 1, 2, 3, 12, 21, X, Y (not, chr1, chr2, chrX). If you do not find relevant blacklist files, it is fine to keep this empty.

For the other exclusion regions, you can select the default MT exclusion, Y chromosome exclusion (we added these two exclusively since in most studies it is relevant to exclude these two). However, you can type in a custom set of chromosomes in the text box for exclusion, which must be space-separated, like 1 2 22 12 (not like 1, 2, 22, 12 or etc.) This step is also optional.

For Genrich, you can mention the exact bp of expanding cut sites, that is, the d-value. You can select any relevant numbers from the drop-down menu, or you can even type in your own desired value. The default value is 100.

You can assign the q-value in a similar manner. The default value is 0.05.

***An important consideration for Blacklist files***

Since the blacklist exclusion is optional, you need to obtain the blacklist BED files from ENCODE and process them to remove the prefix of "chr". Or, you could download a blacklist from our GitHub page (link). But this does not contain the blacklists for all organisms, as for most organisms, the blacklists are still not well-curated.

Once you download the blacklists, you must keep those under a directory structure where the names of directories do not contain any white-spaces in them.

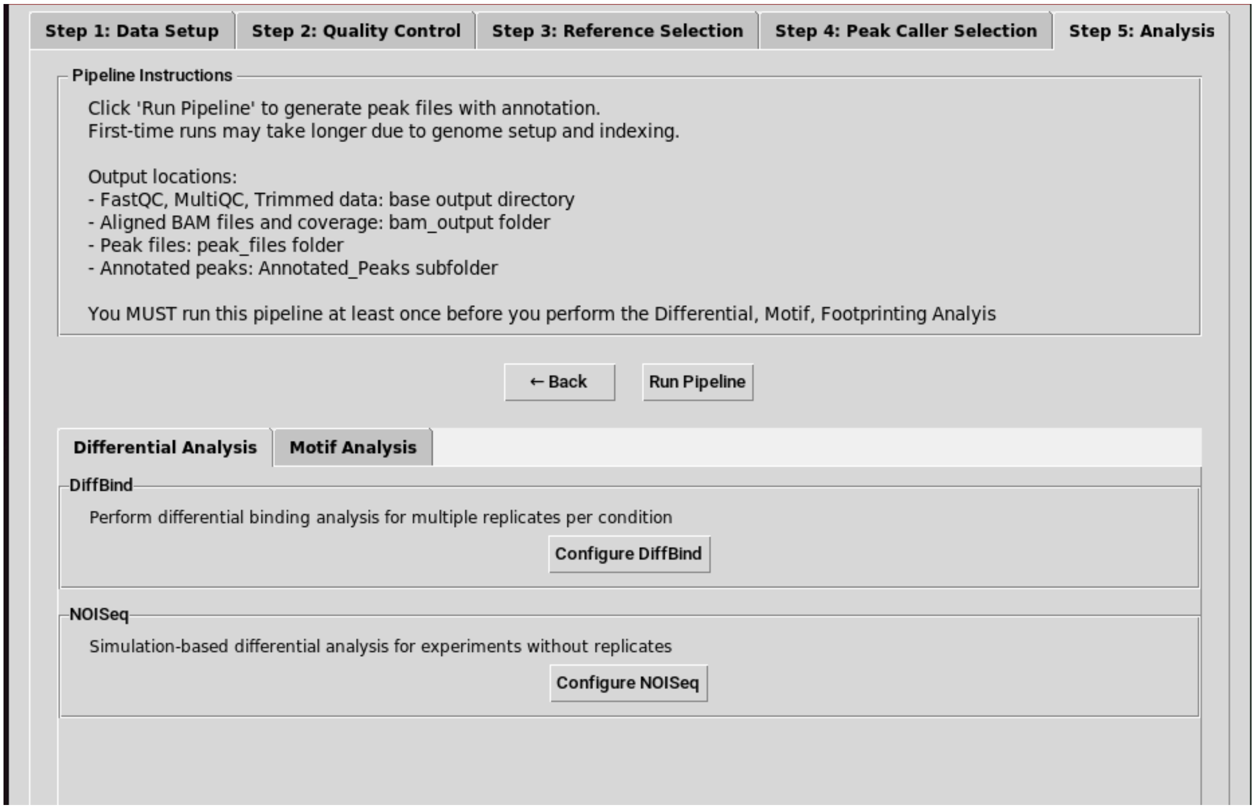
Like, they can be like this -
*/home/prince/atac_seq_project/blacklist_for_chromacs/homo_sapiens*

But not like this-
*/home/prince/atac_seq_project/blacklist_for_chromacs/homo sapiens*

Or not like this- */home/prince/atac seq project/blacklist_for_chromacs/homo_sapiens*
(note how these directory structures have white-spaces in their names. This will affect the Genrich input of blacklists, and is an inherent limitation of how Genrich takes in the blacklist input from a directory structure.)

**Step 5: Running the entire pipeline**

Once you are done with the parameter setup, in step 5, you just need to click on “Run Pipeline”. This begins the entire process in a modular manner, beginning with FastQC, MultiQC, trimming by Trim Galore (if not disabled), alignment and coverage studies, peak calling and then peak annotation.



Click 'Run Pipeline' to generate peak files with annotation.
First-time runs may take longer due to genome setup and indexing.

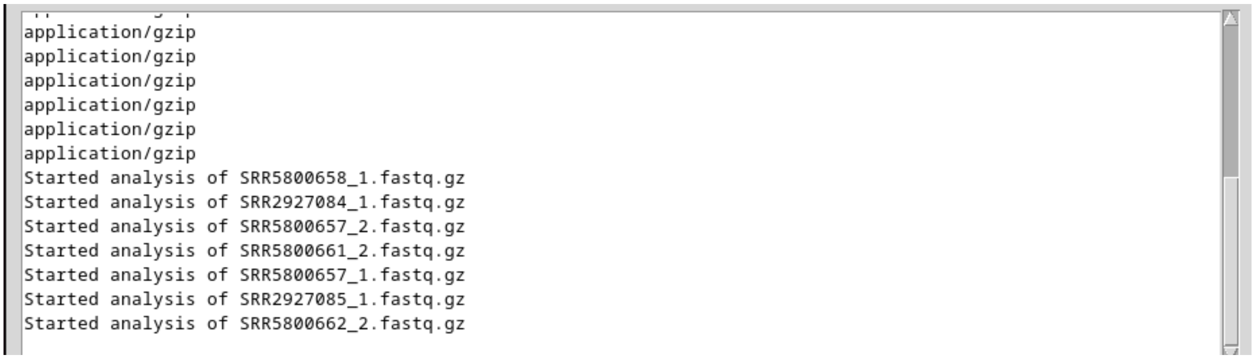
Output locations:
- FastQC, MultiQC, Trimmed data: base output directory
- Aligned BAM files and coverage: bam_output folder
- Peak files: peak_files folder
- Annotated peaks: Annotated_Peaks subfolder

You MUST run this pipeline at least once before you perform the Differential, Motif, Footprinting Analyis

Perform differential binding analysis for multiple replicates per condition

Simulation-based differential analysis for experiments without replicates

You can even track the real-time progress of the run while the pipeline is running.



```
application/gzip
application/gzip
application/gzip
application/gzip
application/gzip
application/gzip
Started analysis of SRR5800658_1.fastq.gz
Started analysis of SRR2927084_1.fastq.gz
Started analysis of SRR5800657_2.fastq.gz
Started analysis of SRR5800661_2.fastq.gz
Started analysis of SRR5800657_1.fastq.gz
Started analysis of SRR2927085_1.fastq.gz
Started analysis of SRR5800662_2.fastq.gz
```

You can even observe the outputs in the required directory inside the base_output_directory, which you assigned in step 1. But in order to know where to look for which result, an architecture of the output file design can help guide you. [link to result architecture]

### Step 5a: Running DiffBind

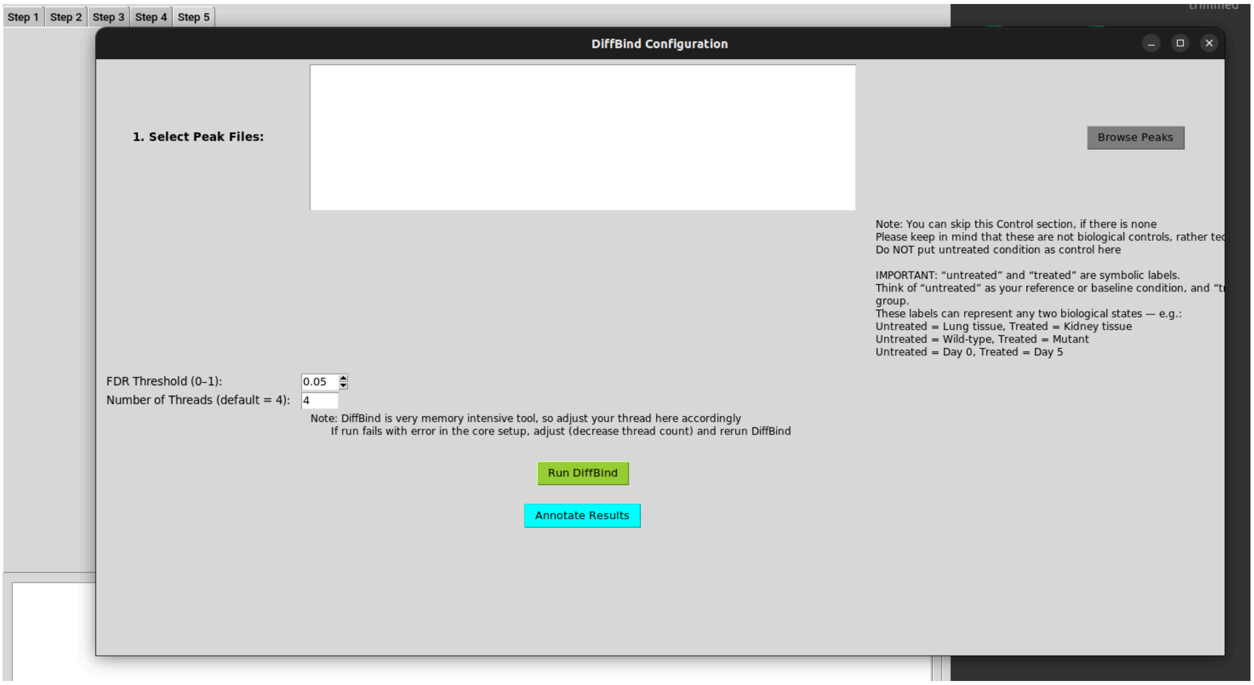
You will notice that ChromAcS step 5 has two Differential Analysis modules in step 5, which are assigned for differential binding analysis following peak annotation. When the peak calling is over, you can select either DiffBind or NOISeq for differential peak analysis.

#### *When do you select DiffBind?*

If your research design includes more than one biological replicate per condition, then we would recommend you to use the DiffBind module of ChromAcS. In our case study, there were three biological replicates per condition, that is, three replicates (SRR5800657, SRR5800658, SRR5800659) are untreated replicates, whereas, three replicates (SRR5800660, SRR5800661, SRR6800662) are treated (drug-tolerant persistors/ DTP) replicates.

#### *How to configure DiffBind parameters?*

When you click on “Configure DiffBind” a new tab will open, where you can configure the DiffBind parameters and set up the analysis-



1. Select Peak Files:

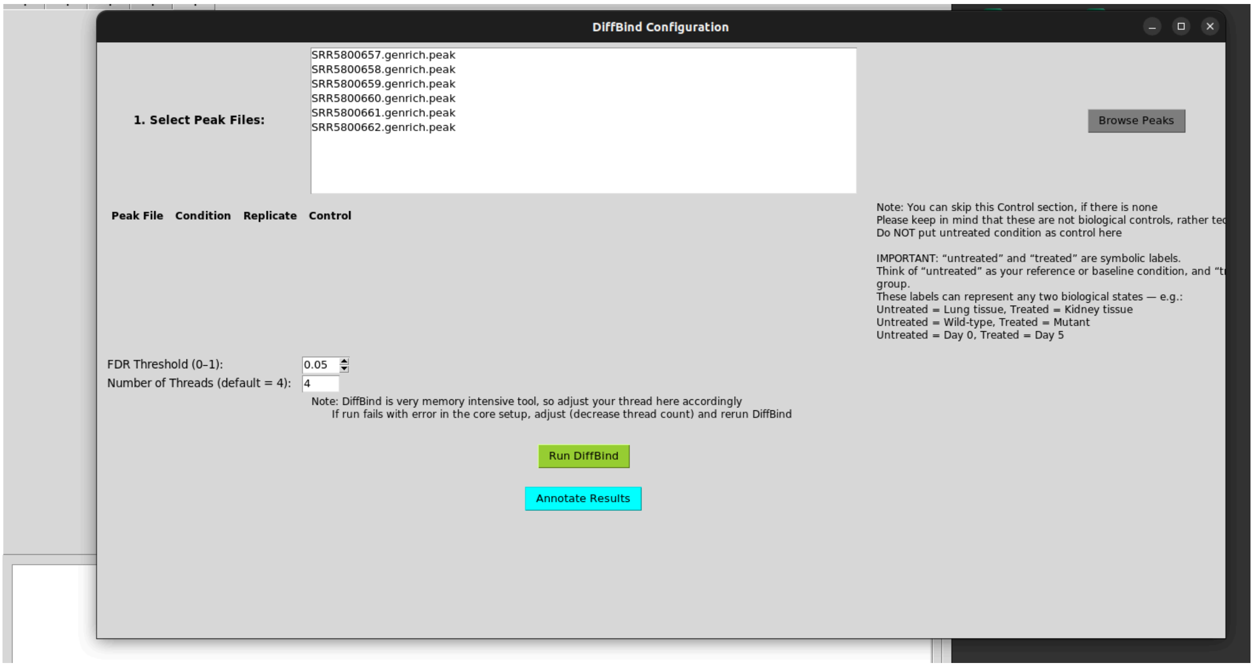
Note: You can skip this Control section, if there is none
Please keep in mind that these are not biological controls, rather tec
Do NOT put untreated condition as control here

IMPORTANT: “untreated” and “treated” are symbolic labels.
Think of “untreated” as your reference or baseline condition, and “t
group.
These labels can represent any two biological states — e.g.:
Untreated = Lung tissue, Treated = Kidney tissue
Untreated = Wild-type, Treated = Mutant
Untreated = Day 0, Treated = Day 5

FDR Threshold (0–1): 0.05
Number of Threads (default = 4): 4

Note: DiffBind is very memory intensive tool, so adjust your thread here accordingly
If run fails with error in the core setup, adjust (decrease thread count) and rerun DiffBind

(This DiffBind Configuration tab opens over the existing main tab, and on first look, it seems mostly empty. You need to click on the “Browse Peaks” to reveal the peak files you obtained after running the pipeline)



1. Select Peak Files:

SRR5800657.genrich.peak
SRR5800658.genrich.peak
SRR5800659.genrich.peak
SRR5800660.genrich.peak
SRR5800661.genrich.peak
SRR5800662.genrich.peak

Peak File Condition Replicate Control

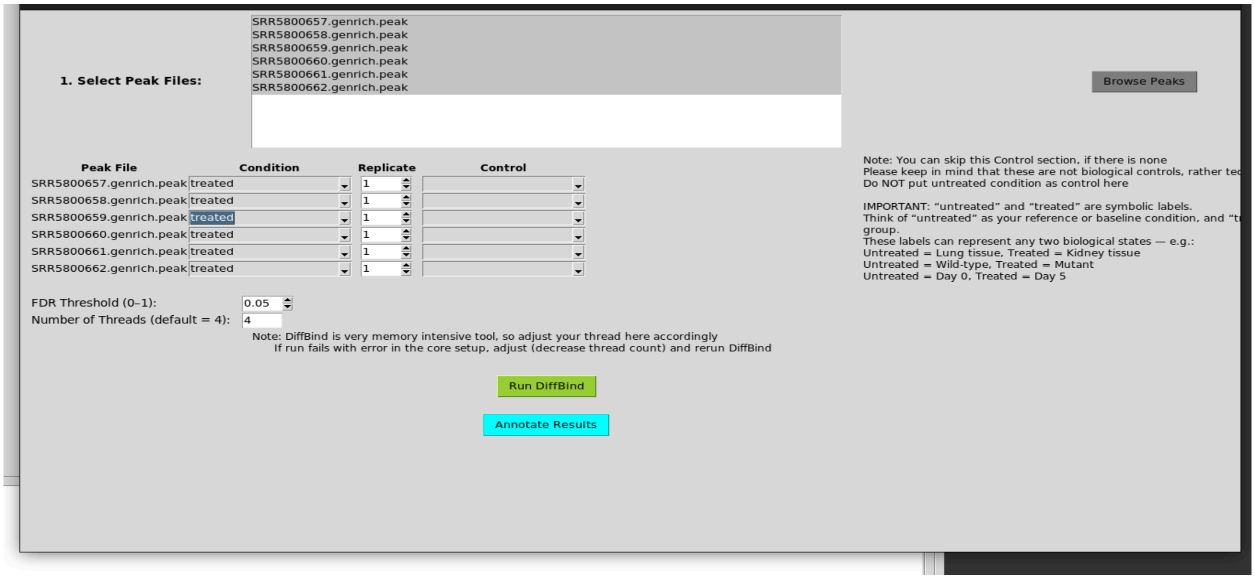
Note: You can skip this Control section, if there is none
Please keep in mind that these are not biological controls, rather tec
Do NOT put untreated condition as control here

IMPORTANT: “untreated” and “treated” are symbolic labels.
Think of “untreated” as your reference or baseline condition, and “tr
group.
These labels can represent any two biological states — e.g.:
Untreated = Lung tissue, Treated = Kidney tissue
Untreated = Wild-type, Treated = Mutant
Untreated = Day 0, Treated = Day 5

FDR Threshold (0–1): 0.05
Number of Threads (default = 4): 4

Note: DiffBind is very memory intensive tool, so adjust your thread here accordingly
If run fails with error in the core setup, adjust (decrease thread count) and rerun DiffBind

(Once you click on ”Browse Peaks”, the peak files will appear. Still, you won’t be able to assign the conditions and replicate values to those. For that, you need to select all the peak files you would like to include in the analysis. ChromAcS does this selection-based peak analysis in differential studies because it includes two different peak callers, and you might not want to mix the peaks called by different tools in one single comparison.



1. Select Peak Files:

SRR5800657.genrich.peak
SRR5800658.genrich.peak
SRR5800659.genrich.peak
SRR5800660.genrich.peak
SRR5800661.genrich.peak
SRR5800662.genrich.peak

| Peak File | Condition | Replicate | Control |
|---|---|---|---|
| SRR5800657.genrich.peak | treated | 1 | |
| SRR5800658.genrich.peak | treated | 1 | |
| SRR5800659.genrich.peak | treated | 1 | |
| SRR5800660.genrich.peak | treated | 1 | |
| SRR5800661.genrich.peak | treated | 1 | |
| SRR5800662.genrich.peak | treated | 1 | |

Note: You can skip this Control section, if there is none
Please keep in mind that these are not biological controls, rather tec
Do NOT put untreated condition as control here

IMPORTANT: “untreated” and “treated” are symbolic labels.
Think of “untreated” as your reference or baseline condition, and “t
group.
These labels can represent any two biological states — e.g.:
Untreated = Lung tissue, Treated = Kidney tissue
Untreated = Wild-type, Treated = Mutant
Untreated = Day 0, Treated = Day 5

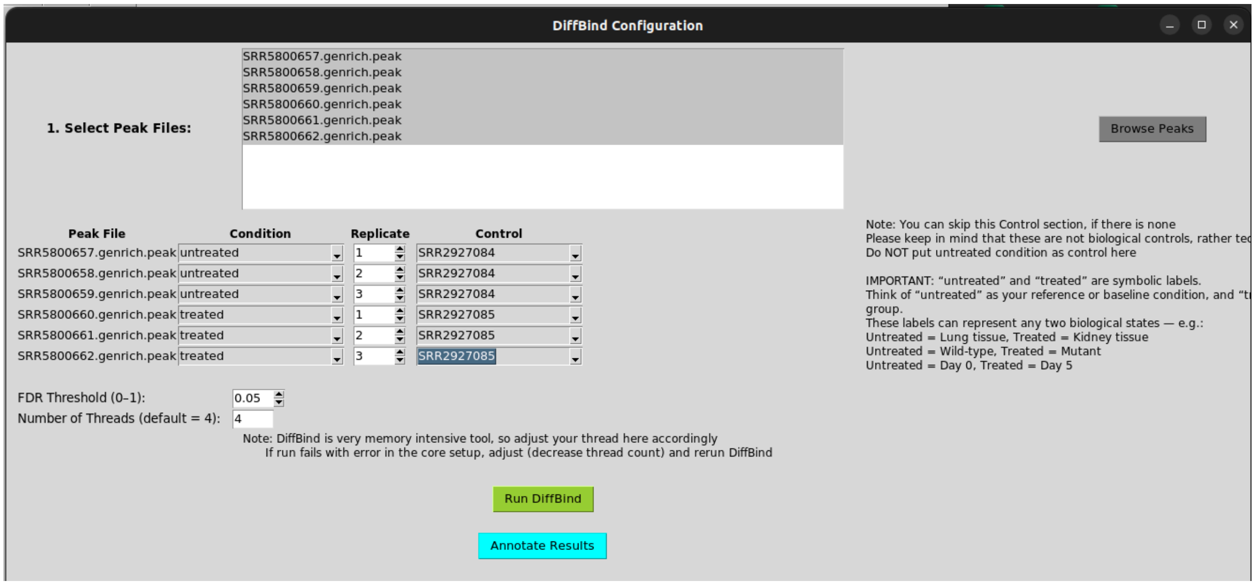
FDR Threshold (0–1): 0.05
Number of Threads (default = 4): 4

Note: DiffBind is very memory intensive tool, so adjust your thread here accordingly
If run fails with error in the core setup, adjust (decrease thread count) and rerun DiffBind

(Once you click on the peak files, they will appear in this format from which you can assign the different conditions, replicate number, and the control files; in the bottom part you can customize the FDR threshold, and assign the number of threads for DiffBind analysis.)

***How do we set up the conditions and replicates?***

In our case study, there were three biological replicates per condition; that is, three replicates (SRR5800657, SRR5800658, SRR5800659) are untreated replicates, whereas three replicates (SRR5800660, SRR5800661, SRR6800662) are treated (drug-tolerant persistors/ DTP) replicates. So we set up the conditions and replicates as follows-



1. Select Peak Files:

SRR5800657.genrich.peak
SRR5800658.genrich.peak
SRR5800659.genrich.peak
SRR5800660.genrich.peak
SRR5800661.genrich.peak
SRR5800662.genrich.peak

| Peak File | Condition | Replicate | Control |
|---|---|---|---|
| SRR5800657.genrich.peak | untreated | 1 | SRR2927084 |
| SRR5800658.genrich.peak | untreated | 2 | SRR2927084 |
| SRR5800659.genrich.peak | untreated | 3 | SRR2927084 |
| SRR5800660.genrich.peak | treated | 1 | SRR2927085 |
| SRR5800661.genrich.peak | treated | 2 | SRR2927085 |
| SRR5800662.genrich.peak | treated | 3 | SRR2927085 |

FDR Threshold (0–1): 0.05
Number of Threads (default = 4): 4

Note: DiffBind is very memory intensive tool, so adjust your thread here accordingly
If run fails with error in the core setup, adjust (decrease thread count) and rerun DiffBind

Note: You can skip this Control section, if there is none
Please keep in mind that these are not biological controls, rather tec
Do NOT put untreated condition as control here

IMPORTANT: “untreated” and “treated” are symbolic labels.
Think of “untreated” as your reference or baseline condition, and “tr
group.
These labels can represent any two biological states — e.g.:
Untreated = Lung tissue, Treated = Kidney tissue
Untreated = Wild-type, Treated = Mutant
Untreated = Day 0, Treated = Day 5

(Once you click on the condition box, a drop-down menu appears, from which you can assign either an untreated (baseline) or treated (test) condition. The replicate can be set up in a similar manner; the order does not matter, actually. The controls used here are technical controls, not the biological untreated condition as the control.)

***What does the condition represent here?***

In this condition parameter, you get two options: untreated and treated. Please keep in mind that these are **just symbolic labels**, which means-

**Untreated** ⇒ **baseline** condition ⇒ could be untreated samples, wild-type samples, day 0 of treatment, or even a different tissue you want to contrast against

**Treated** ⇒ **test condition** ⇒ could be treated samples, mutant samples, day 5 of treatment, or even another tissue you want to compare the former with

So, DiffBind will generate the differential sites and loss, gain sites accordingly. Which means that the logFoldChanges would be compared in terms of the baseline for those test conditions.

For instance, in our study, the ChromAcS DiffBind module will show the results of gain sites as those regions where peaks are enriched in the treated compared to the untreated, and the loss sites as the reverse.

***Does the order of the peak files or the condition matter?***

No, the order of the peak files or conditions does not matter at all. Just the name of the condition against the peak file matters. Be certain to put the correct condition against the desired peak files. Your peak files could be ordered like this and yet yield the correct result-

| Peak File | Condition | Replicate | Control |
|---|---|---|---|
| SRR5800661 | Treated (test) | 1 | SRR2927085 |
| SRR5800658 | Untreated (baseline) | 1 | SRR2927084 |
| SRR5800662 | Treated (test) | 2 | SRR2927085 |
| SRR5800660 | Treated (test) | 3 | SRR2927085 |
| SRR5800657 | Untreated (baseline) | 2 | SRR2927084 |
| SRR5800659 | Untreated (baseline) | 3 | SRR2927084 |

The results of ChromAcS DiffBind module will always consider the peak files under the untreated condition as baseline, regardless of any order your peak files are oriented in. Please do not assign the same replicate value to two different peaks.

***Does it work for only three replicates?***

No, the ChromAcS DiffBind module is not limited to triplicates studies. You can use this for anything greater than two per condition. This means that at least two samples per condition are essential to run this module.

***Does it work if I have a single sample per condition?***

No, in that case, you have to select the ChromAcS NOISeq module.

***Why do we need to set up the thread again here? We already set up in step 1?***

DiffBind can be very resource-intensive in terms of memory usage, and thus, the run may crash even with 8 threads if the RAM is limited. So, we would suggest using 4 threads in a 16 GB RAM system, even if the 8 threads worked fine for the main pipeline run.

***How do you annotate the results of ChromAcS DiffBind analysis?***

Simply by clicking the Annotate Results inside the DiffBind Configuration tab, once the DiffBind run is complete. Do NOT click Annotate Results until the DiffBind run is over.

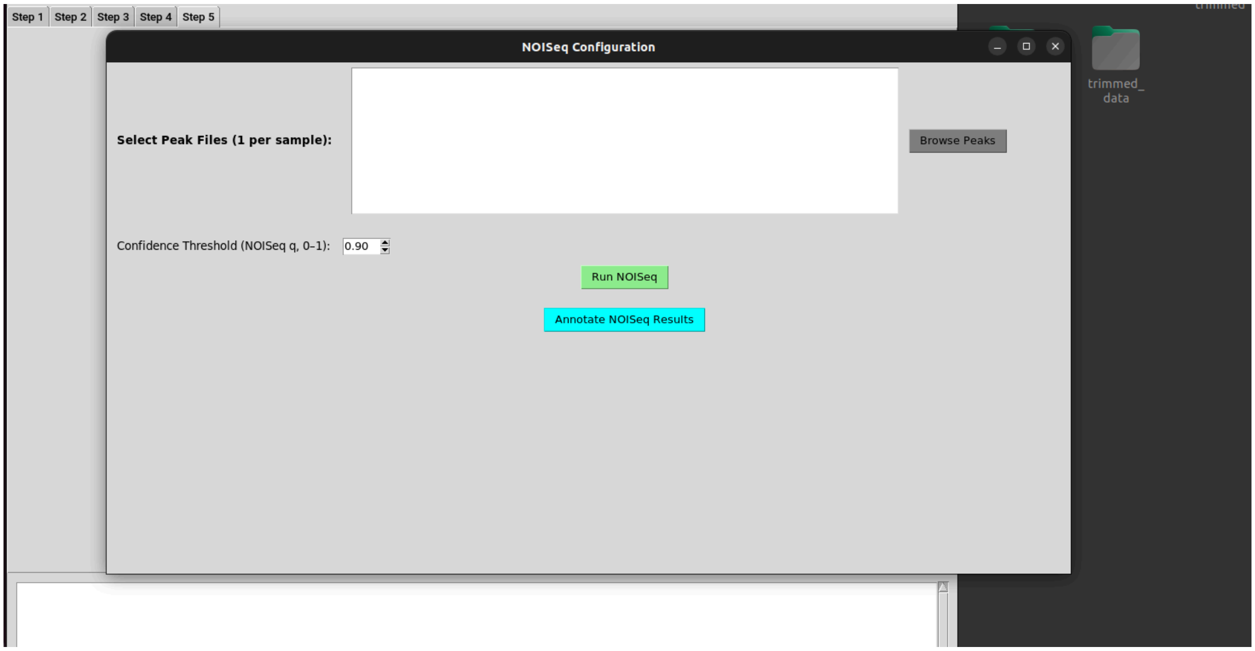
### Step 5b: Running NOISeq

#### *When do you select NOISeq?*

If your research design includes only one biological sample per condition, then we would recommend that you use the NOISeq module of ChromAcS.

#### *How to configure DiffBind parameters?*

When you click on “Configure NOISeq” a new tab will open, where you can configure the NOISeq parameters and set up the analysis-

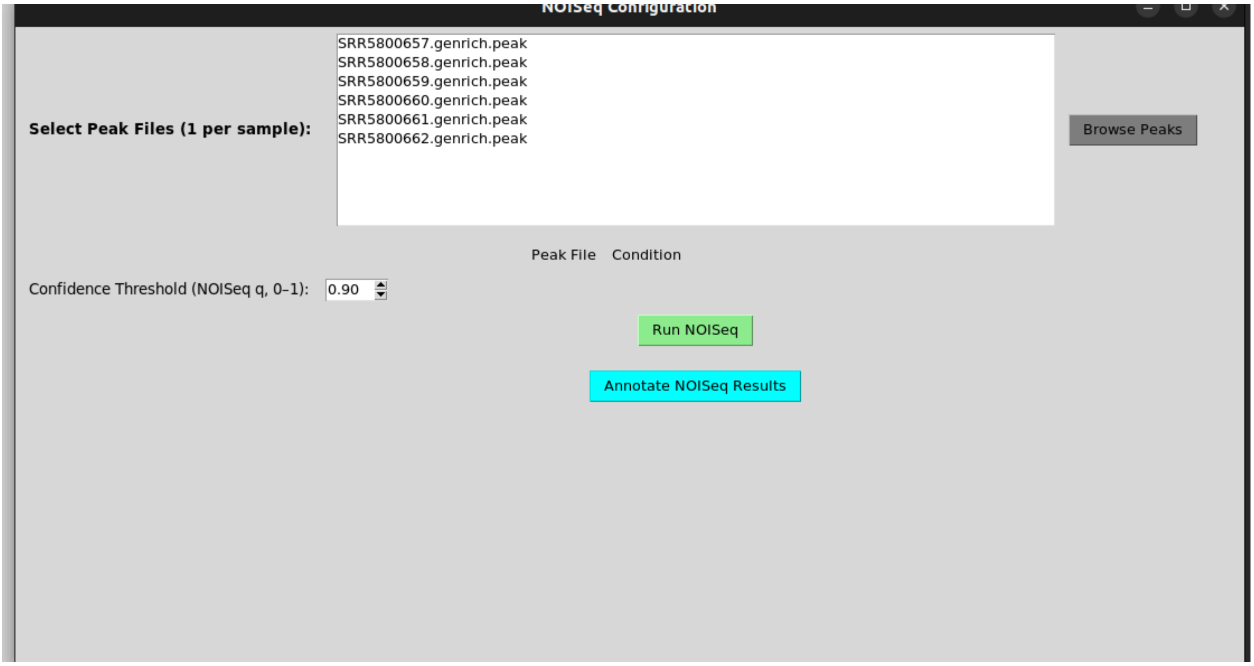


NOISeq Configuration

Select Peak Files (1 per sample):

Confidence Threshold (NOISeq q, 0–1): 0.90

(This NOISeq Configuration tab opens over the existing main tab, and on first look, it seems mostly empty. You need to click on the “Browse Peaks” to reveal the peak files you obtained after running the pipeline)



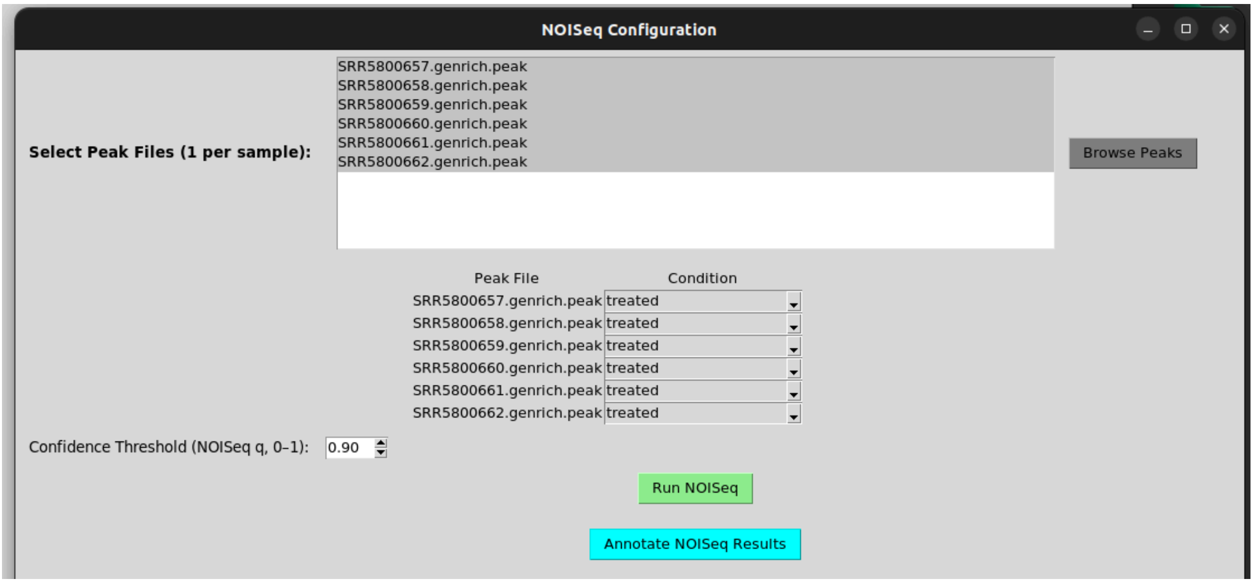
Select Peak Files (1 per sample):

```
SRR5800657.genrich.peak
SRR5800658.genrich.peak
SRR5800659.genrich.peak
SRR5800660.genrich.peak
SRR5800661.genrich.peak
SRR5800662.genrich.peak
```

Peak File Condition

Confidence Threshold (NOISeq q, 0–1): 0.90

(Once you click on ”Browse Peaks”, the peak files will appear. Still, you won’t be able to assign the conditions and replicate values to those. For that, you need to select all the peak files you would like to include in the analysis. ChromAcS does this selection-based peak analysis in differential studies because it includes two different peak callers, and you might not want to mix the peaks called by different tools in one single comparison)



Select Peak Files (1 per sample):

```
SRR5800657.genrich.peak
SRR5800658.genrich.peak
SRR5800659.genrich.peak
SRR5800660.genrich.peak
SRR5800661.genrich.peak
SRR5800662.genrich.peak
```

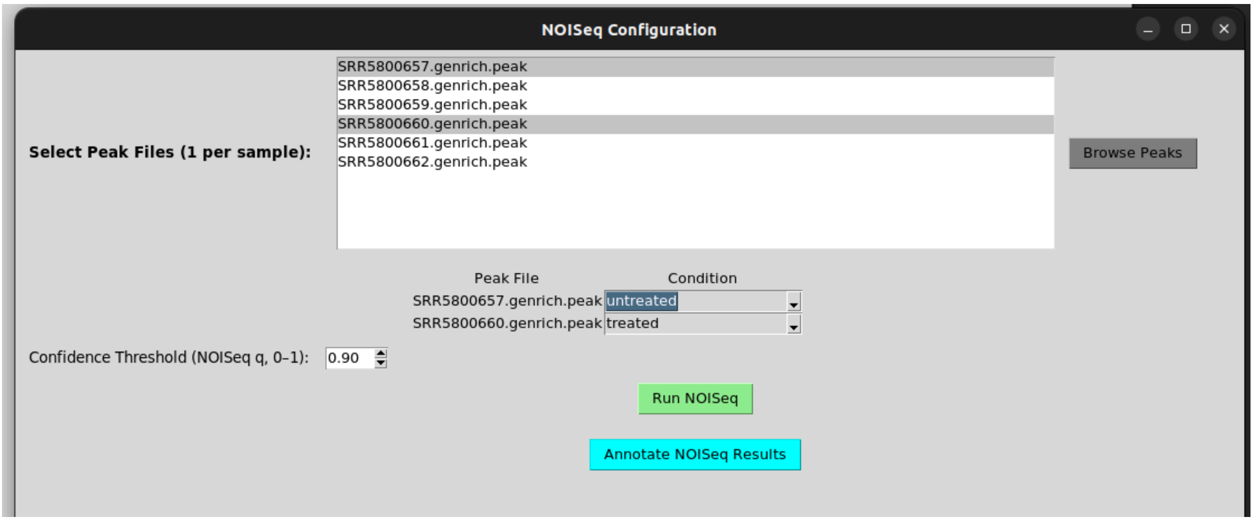
| Peak File | Condition |
|---|---|
| SRR5800657.genrich.peak | treated |
| SRR5800658.genrich.peak | treated |
| SRR5800659.genrich.peak | treated |
| SRR5800660.genrich.peak | treated |
| SRR5800661.genrich.peak | treated |
| SRR5800662.genrich.peak | treated |

Confidence Threshold (NOISeq q, 0–1): 0.90

(Once you click on the peak files, they will appear in this format from which you can assign the different conditions; NOISeq does not allow control files; in the bottom part, you can customize the NOISeq confidence threshold, which is actually quite different from typical FDR values. For instance, 0.9 would mean 90% confidence, thus a lower number of differential peaks at higher values.)

### *How do we set up the conditions?*

In our case study, there were three biological replicates per condition. But assuming that we had only one sample per condition, that is, SRR5800657 as the untreated condition and SRR5800660 as the treated condition, the configuration could be done as follows-



Select Peak Files (1 per sample):

SRR5800657.genrich.peak
SRR5800658.genrich.peak
SRR5800659.genrich.peak
SRR5800660.genrich.peak
SRR5800661.genrich.peak
SRR5800662.genrich.peak

| Peak File | Condition |
|---|---|
| SRR5800657.genrich.peak | untreated |
| SRR5800660.genrich.peak | treated |

Confidence Threshold (NOISeq q, 0–1): 0.90

(Once you click on the condition box, a drop-down menu appears, from which you can assign either an untreated or treated condition)

### *What does the condition represent here?*

In this condition parameter, you get two options: untreated and treated. Please keep in mind that these are **just symbolic labels**, which means-

**Untreated** ⇒ **baseline** condition ⇒ could be untreated samples, wild-type samples, day 0 of treatment, or even a different tissue you want to contrast against

**Treated** ⇒ **test condition** ⇒ could be treated samples, mutant samples, day 5 of treatment, or even another tissue you want to compare the former with

So, NOISeq will generate the differential sites and loss, gain sites accordingly. Which means that the logFoldChanges would be compared in terms of the baseline for those test conditions. For instance, in our study, the ChromAcS NOISeq module will show the results of gain sites as those regions where peaks are enriched in the treated compared to the untreated, and the loss sites as the reverse.

### *Does the order of the peak files or the condition matter?*

No, the order of the peak files or conditions does not matter at all. Just the name of the condition against the peak file matters. Be certain to put the correct condition against the desired peak files. Your peak files could be ordered like this and yet yield the correct result-

| Peak File | Condition |
|---|---|
| SRR5800660 | Treated (test) |
| SRR5800657 | Untreated (baseline) |

The results of the ChromAcS NOISeq module will always consider the peak files under the untreated condition as baseline, regardless of any order your peak files are oriented in.

***Does our NOISeq module work for studies with replicates?***

No, the ChromAcS NOISeq module is not optimized to work for replicates. If you have biological replicates in your study, please use the ChromAcs DiffBind module exclusively.

***Does it work if I have a single sample per condition?***

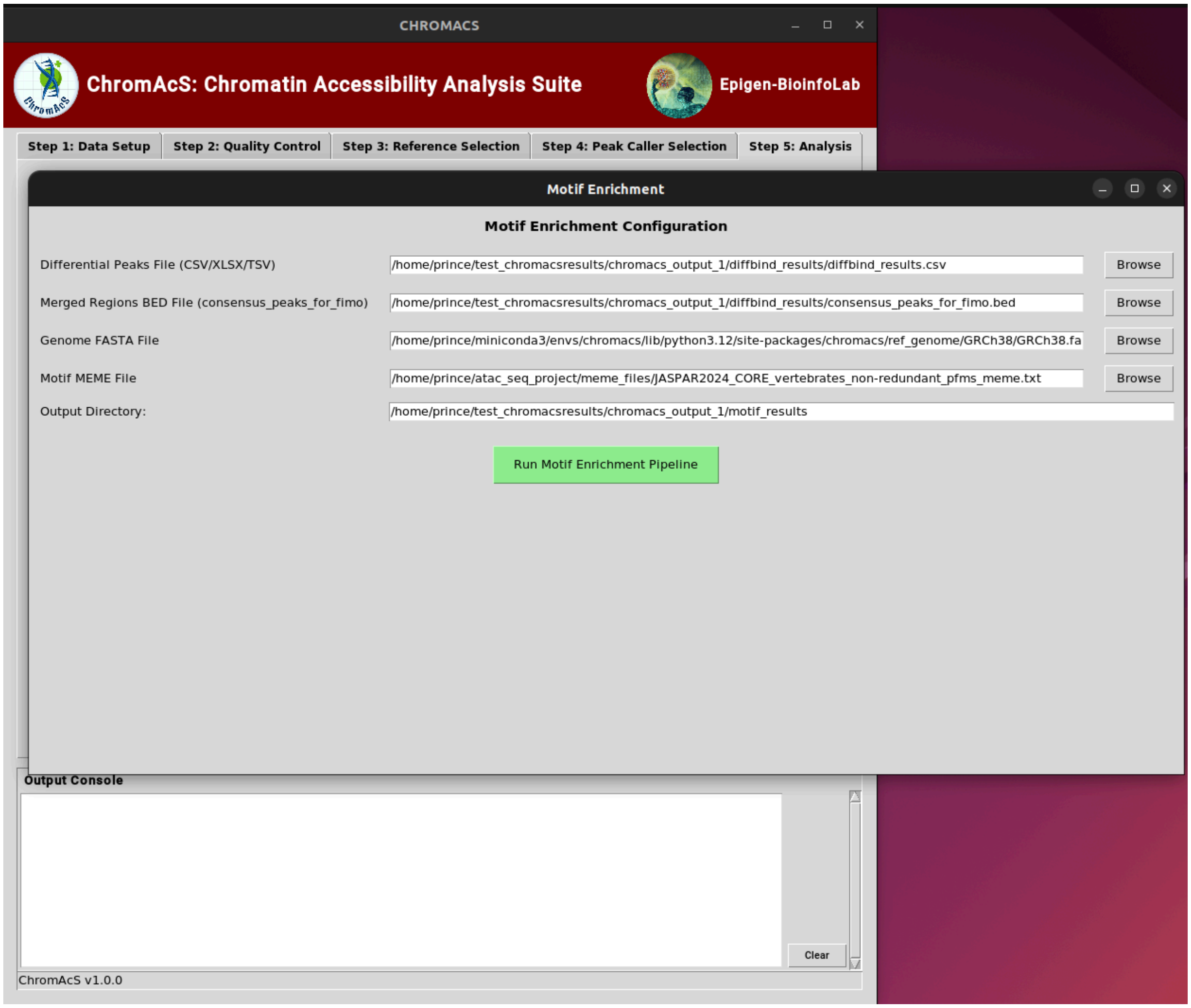
Yes, NOISeq is optimized for a single sample per condition and works on a noise-based assumption for differential peak analysis.

***How do you annotate the results of ChromAcS NOISeq analysis?***

Simply by clicking the Annotate Results inside the NOISeq Configuration tab, once the NOISeq run is complete. Do NOT click Annotate Results until the NOISeq run is over.

**Step 5c: Motif Enrichment and Footprinting**

Step 5 also includes two modules for Transcription factor motif analysis, one is motif enrichment involving mostly FIMO, and another is for motif footprinting involving TOBIAS.



Motif Enrichment Configuration

Differential Peaks File (CSV/XLSX/TSV) /home/prince/test_chromacsresults/chromacs_output_1/diffbind_results/diffbind_results.csv

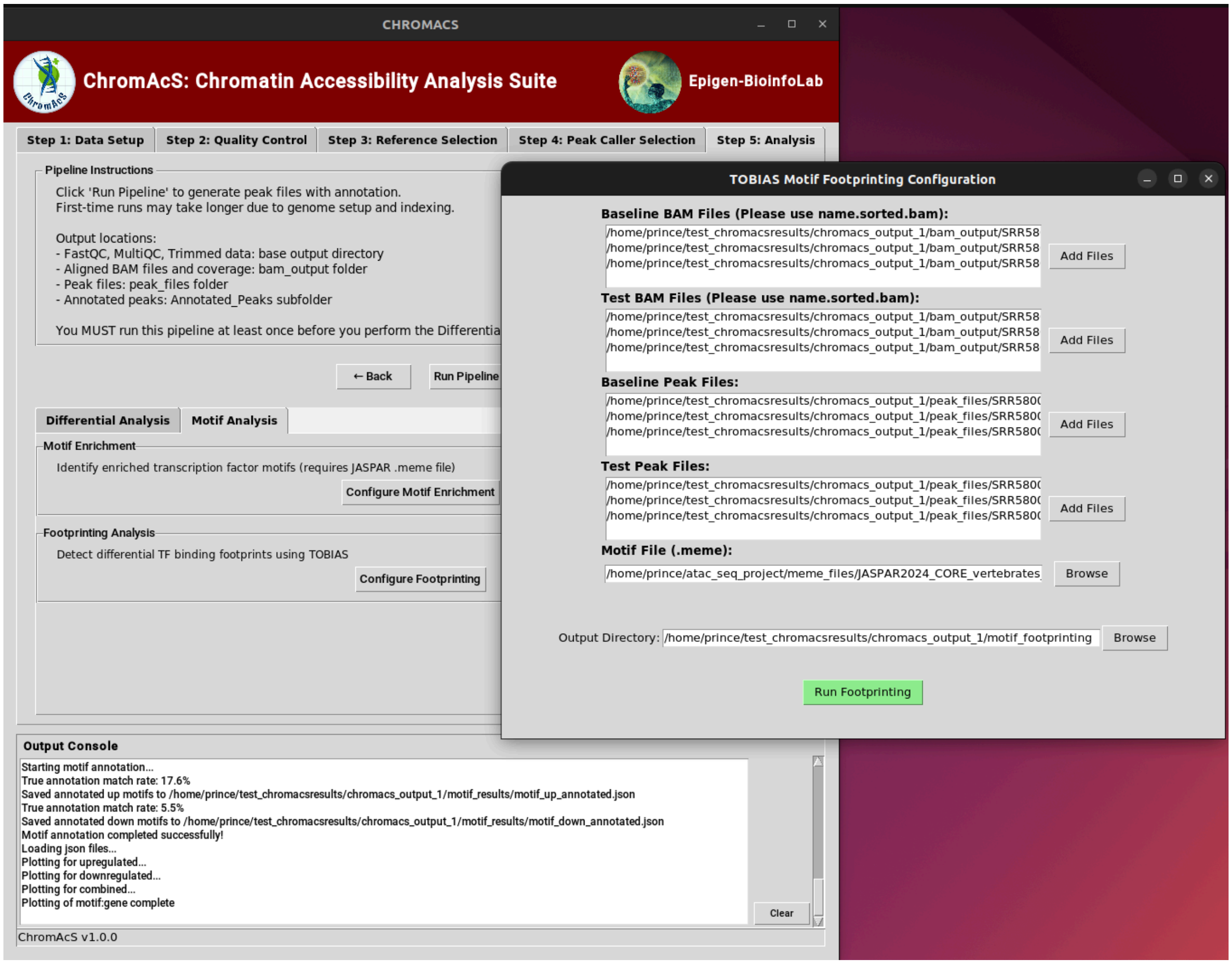
Merged Regions BED File (consensus_peaks_for_fimo) /home/prince/test_chromacsresults/chromacs_output_1/diffbind_results/consensus_peaks_for_fimo.bed

Genome FASTA File /home/prince/miniconda3/envs/chromacs/lib/python3.12/site-packages/chromacs/ref_genome/GRCh38/GRCh38.fa

Motif MEME File /home/prince/atac_seq_project/meme_files/JASPAR2024_CORE_vertebrates_non-redundant_pfms_meme.txt

Output Directory: /home/prince/test_chromacsresults/chromacs_output_1/motif_results

(Configuring across motif enrichment module is pretty straightforward, first you need to input the differential peak file obtained from step 5b, or step 5c [for DiffBind it is the “diffbind_results.csv”' for NOISeq it is the “noisq_results.xlsx”]), the second input is the consensus_peaks_for_fimo.bed which will also be present in the same directory if you had run either step 5b (DiffBind) or step 5c (NOISeq). The third field is usually auto-filled with the reference genome fasta sequence. For the fourth field, you must obtain a motif MEME file according to your choice and preferences. Here we have used https://jaspar.elixir.no/download/data/2024/CORE/JASPAR2024_CORE_non-redundant_pfms_jaspar.txt from JASPAR. The output directory will be auto-created. Just click Run Motif Enrichment Pipeline to begin the analysis. This may take some time.



Pipeline Instructions

Click 'Run Pipeline' to generate peak files with annotation.
First-time runs may take longer due to genome setup and indexing.

Output locations:
- FastQC, MultiQC, Trimmed data: base output directory
- Aligned BAM files and coverage: bam_output folder
- Peak files: peak_files folder
- Annotated peaks: Annotated_Peaks subfolder

You MUST run this pipeline at least once before you perform the Differentia

Motif Enrichment

Identify enriched transcription factor motifs (requires JASPAR .meme file)

Footprinting Analysis

Detect differential TF binding footprints using TOBIAS

TOBIAS Motif Footprinting Configuration

Baseline BAM Files (Please use name.sorted.bam):

```
/home/prince/test_chromacsresults/chromacs_output_1/bam_output/SRR58
/home/prince/test_chromacsresults/chromacs_output_1/bam_output/SRR58
/home/prince/test_chromacsresults/chromacs_output_1/bam_output/SRR58
```

Test BAM Files (Please use name.sorted.bam):

```
/home/prince/test_chromacsresults/chromacs_output_1/bam_output/SRR58
/home/prince/test_chromacsresults/chromacs_output_1/bam_output/SRR58
/home/prince/test_chromacsresults/chromacs_output_1/bam_output/SRR58
```

Baseline Peak Files:

```
/home/prince/test_chromacsresults/chromacs_output_1/peak_files/SRR580(
/home/prince/test_chromacsresults/chromacs_output_1/peak_files/SRR580(
/home/prince/test_chromacsresults/chromacs_output_1/peak_files/SRR580(
```

Test Peak Files:

```
/home/prince/test_chromacsresults/chromacs_output_1/peak_files/SRR580(
/home/prince/test_chromacsresults/chromacs_output_1/peak_files/SRR580(
/home/prince/test_chromacsresults/chromacs_output_1/peak_files/SRR580(
```

Motif File (.meme): /home/prince/atac_seq_project/meme_files/JASPAR2024_CORE_vertebrates

Output Directory: /home/prince/test_chromacsresults/chromacs_output_1/motif_footprinting

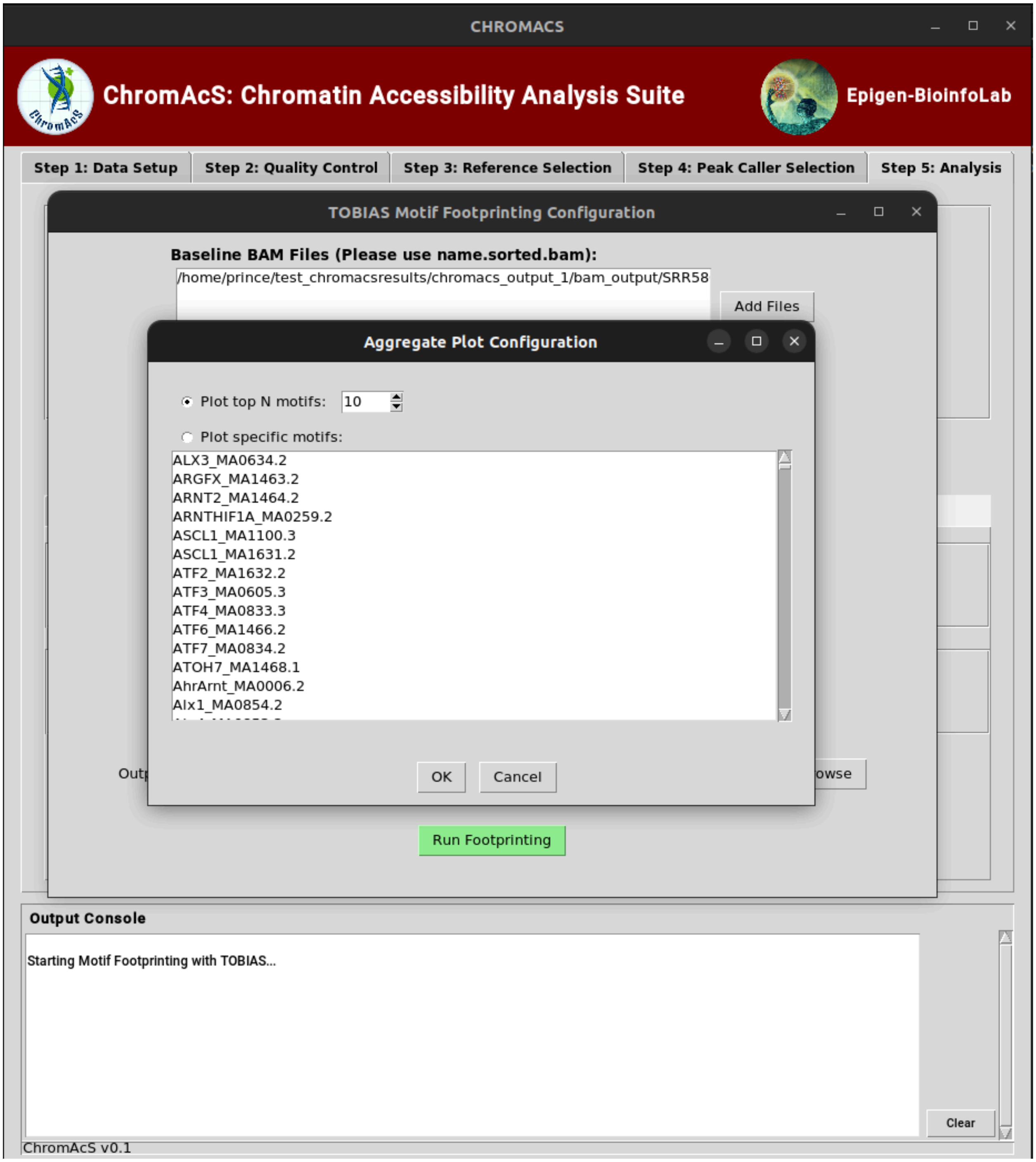
Output Console

```
Starting motif annotation...
True annotation match rate: 17.6%
Saved annotated up motifs to /home/prince/test_chromacsresults/chromacs_output_1/motif_results/motif_up_annotated.json
True annotation match rate: 5.5%
Saved annotated down motifs to /home/prince/test_chromacsresults/chromacs_output_1/motif_results/motif_down_annotated.json
Motif annotation completed successfully!
Loading json files...
Plotting for upregulated...
Plotting for downregulated...
Plotting for combined...
Plotting of motif:gene complete
```

ChromAcS v1.0.0

(ChromAcS configures Motif footprinting of TOBIAS and integrates it into a single pipeline and provides publication-ready outputs. This can be done very easily; if you have run the pipeline up to peak calling once, then you can perform motif footprinting here. When you browse baseline BAM files, it will automatically take you to the BAM_files directory, where you can find your desired BAM files. Be careful to choose the name_sorted_bam files. The same goes for the test BAM files. The baseline peaks are also selected in a similar manner, just be cautious to put the consistent peak files, avoiding mixing up Genrich or MACS3 peaks. For motif meme files, we used https://jaspar.elixir.no/download/data/2024/CORE/JASPAR2024_CORE_non-redundant_pfms_jaspar.txt; but you may choose any desired meme file.)

During the motif footprinting analysis, a dialogue box will appear mid-run and ask for the desired motifs or the top significant motifs you would like to see-


CHROMACS
ChromAcS: Chromatin Accessibility Analysis Suite
Epigen-BioinfoLab
Step 1: Data Setup
Step 2: Quality Control
Step 3: Reference Selection
Step 4: Peak Caller Selection
Step 5: Analysis
TOBIAS Motif Footprinting Configuration
Add Files
Aggregate Plot Configuration
OK
Cancel
Outp
owse
Run Footprinting
Output Console
Clear
ChromAcS v0.1

Baseline BAM Files (Please use name.sorted.bam):

/home/prince/test_chromacsresults/chromacs_output_1/bam_output/SRR58

● Plot top N motifs: 10

○ Plot specific motifs:

ALX3_MA0634.2
ARGFX_MA1463.2
ARNT2_MA1464.2
ARNTHIF1A_MA0259.2
ASCL1_MA1100.3
ASCL1_MA1631.2
ATF2_MA1632.2
ATF3_MA0605.3
ATF4_MA0833.3
ATF6_MA1466.2
ATF7_MA0834.2
ATOH7_MA1468.1
AhrArnt_MA0006.2
Alx1_MA0854.2

```
Starting Motif Footprinting with TOBIAS...
```

***Does our motif analysis work for studies without replicates?***

Yes, if you provide single samples per condition, they will be analyzed. For multiple replicates per condition, ChromAcS generates merged bam and merged peaks per condition, and process them.

# Random Navigation “Hacks” in ChromAcS

ChromAcS is designed to be modular in nature, and thus, if by any chance it crashes on any local device, the user can rerun it on the same base output directory and proceed from where it crashed. But in order to track everything and outputs generated, the user needs to have a detailed overview of the output directory architecture, that is, how the outputs are saved and in which directories and sub-directories.

A typical structure of the output files would look like this-

```
base_output_directory/

├── fastqc_raw/              → Raw FastQC reports

├── multiqc_raw/             → MultiQC summary (raw)

├── trimmed_data/            → Trimmed FASTQs + reports

├── fastqc_trimmed/          → FastQC reports of trimmed data

├── multiqc_trimmed/         → MultiQC summary of trimmed data

├── bam_output/              → Sorted BAMs (name-sorted and coordinate-sorted) + index
                               (of sorted BAM files) + flagstat results

├── normalized_coverage/   → BigWig files of the sorted BAM
    └── Coverage_profiles/ → TSS enrichment heatmap based on the BIgWIG files

├── peak_files/              → Peak files from Genrich/MACS3
    └── Annotated_Peaks/   → Annotated peaks + peak statistics summary

├── diffbind_results/        → DiffBind differential analysis results
   └── Annotated_DiffBind/   → Annotated files + statistics summary

├── noisq_results/           → NOISeq differential analysis results
    └── Annotated_NoiSeq/  → Annotated files + statistics summary

├── motif_results/                → Motif enrichment analysis results + plots
    └── motif_gene_analysis/  → motif-to-gene association + plots

├── motif_footprinting/             → Motif footprinting results
    └── bindetect_results/  → all on differential motif footprinting across two conditions
    └── tobias_aggregate_plots/  → shows the differential footprints as plots
```

Here, all these directories are auto-generated once ChromAcS begins to run (i.e., when the user clicks run pipeline, however, diffbind_results and noisq_results would be generated later when the user runs those modules. Some of the directories contain sub-directories within which the relevant results of the files processed in the directory are saved (the sub-directories are shown here under the main directories).

***Can I check the results once one step is done but ChromAcS is still running?***

Yes, you can absolutely check the results in the directories while ChromAcS is still running. For instance, if the MultiQC step is shown to be complete within the real-time text widget update on the ChromAcS screen, you can navigate to the relevant directory and check the outputs while trimming is still running inside ChromAcS.

***How is ChromAcS modular, and how can I exploit this?***

ChromAcS proceeds the pipeline beginning from FastQC till peak calling and annotation seamlessly, after which the user can proceed for differential peak analysis using DiffBind or NOISeq ChromAcS module. So, it may seem that only the differential peak analysis is modular, and the rest of the steps are not. But in reality, ChromAcS can recognize which steps have been performed earlier within the main pipeline and continue to run the pipeline from the subsequent steps. Let’s say that for some unexpected reason, ChromAcS crashed in the local device during the trimming of samples. Now, if the user reruns ChromAcS and, while navigating, assigns the same base output directory, it will be able to continue from trimming. Even if the trimming fails during, let’s say the third out of eight samples we ran, ChromAcS will proceed to run trimming during the rerun from the third sample.

***How is the modular nature of ChromAcS helpful during parameter optimization?***

The modular nature of ChromAcS can be significantly effective during parameter optimization of the analysis. Let’s assume that the user needs to observe which d-value of Genrich can be most relevant to achieve the best biological rationale of their results, or which p-value they want for their analysis.

In that case, the user can run Genrich with one d-value (for example, 100) during the first run. The output peak files will be saved in the peak files directory. Now the user can check those peak files and perform differential analysis through the DiffBind or NOISeq modules.

Now, for a different d-value set up (for example, 200) during their second run, the user first needs to cut the peak files directory only from their base output directory and paste it to a different directory (if they wish to keep this process non-destructive). And then rerun ChromAcS with their desired d-value of 200, and during this second run, ChromAcS will begin from the peak calling directly due to its being modular in nature. This significantly helps to reduce time during parameter optimization.

Users can employ a similar approach to rerun ChromAcS for different peak callers (for example, trying with Genrich for the first run, and then MACS3 for another run), for differential analysis (using DiffBind with different thresholds of confidence), and several other combinations of parameters.

# ChromAcS-AddOn: Additional Analysis Tool - User Manual

## Tab 1: Peak Overlap Analysis

This module checks whether ATAC-seq peaks (from ChromAcS or DiffBind) overlap with genomic regions such as histone marks or enhancer annotations.

**Inputs:**
- One ATAC-seq peak file (ATAC-seq peak, Differential ATAC-seq file, or annotated file). This file should have the first three columns: chromosome, start, and end. Only tab-separated text file with header.
- Upload up to 5 additional BED/peak files. Only tab-separated text file with no header (or, add a hast tag # at the beginning if your tabular file has a header). This file should have the minimum first four columns in the order: chromosome, start, end, name. Rest columns can be in any order. Example:

Example-1 (BED, no header):

```
chr1    1500    1800    H3K27ac_peak1
chr1    3500    3800    H3K27ac_peak2
```

Example-1 (Peak, # tag header):

```
#chromosome start    end       name
chr1    100036623    100037420    H3K4me1
chr1    100047999    100048530    H3K4me1
chr1    100049270    100049895    H3K4me1
```

- Set Window size in bp (e.g., 200). This will extend the ATAC-seq peaks on both sides for this base-pair to find overlap.

**Steps:**
1. Click 'Browse' to upload the ATAC-seq peak file.
2. Enter the window size (e.g., 200).
3. Upload up to 5 BED files containing enhancer/histone data.
4. Select an output file name.

5. Click 'Run Overlap Analysis'

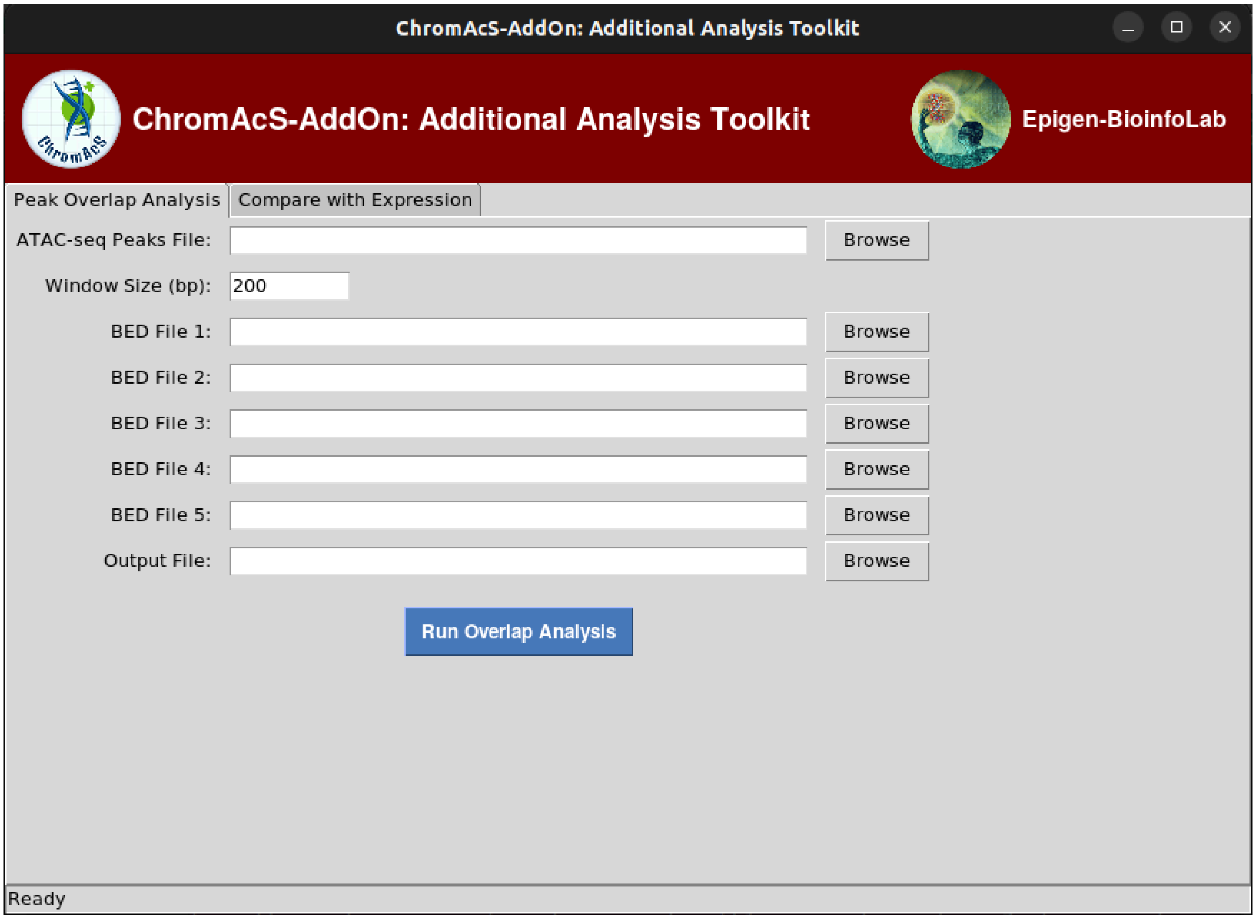
**What it does:**

- Cleans and formats the ATAC-seq file
- Extends the peaks by the specified window
- Normalizes chromosome naming (chr prefix)
- Uses bedtools intersect to find overlapping regions
- Outputs a combined overlap file

# Output Files

- Overlap.tsv: Contains ATAC-seq peaks with overlaps against BED files

## Screenshot: Peak Overlap Analysis Tab



ChromAcS-AddOn: Additional Analysis Toolkit

ATAC-seq Peaks File:

Window Size (bp): 200

BED File 1:

BED File 2:

BED File 3:

BED File 4:

BED File 5:

Output File:

Ready

# Tab 2: ATAC-seq Compare with Expression

This module merges annotated ATAC-seq peaks with gene expression data.

**Inputs:**
- Annotated ATAC-seq file (from ChromAcS). Only tab-separated text file WITH header.
- Gene expression matrix (tab-separated text file with header line)
- Gene ID column number in each file.

Example (RNA_expression.tsv):

| ID | logFC | AveExpr | t | P.Value | adj.P.Val | B |
|---|---|---|---|---|---|---|
| PEG3 | 13.14 | 0.94 | 96.70 | 0.001 | 0.002 | 19.02 |
| RP11-572P18.1 | 11.83 | 0.29 | 66.56 | 0.001 | 0.002 | 17.19 |
| DCT | 8.12 | 2.67 | 66.14 | 0.001 | 0.002 | 18.80 |
| NEFM | 6.47 | 5.43 | 63.65 | 0.001 | 0.002 | 19.41 |

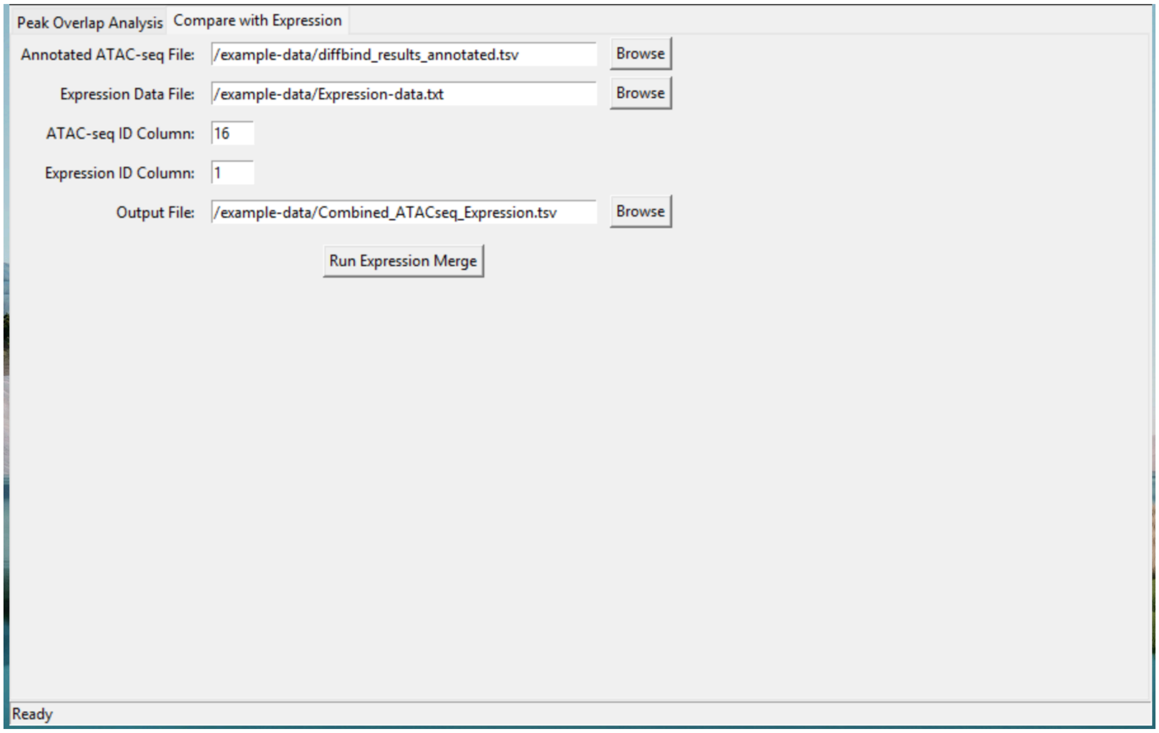
**Steps:**
1. Upload the annotated ATAC-seq file (or annotated differential ATAC-seq file)
2. Upload the expression file.
3. Enter the matching gene ID (or gene name/symbol) column number for both files.
4. Select an output file.
5. Click 'Run Expression Merge'.

**What it does:**
- Matches gene IDs across both files
- Appends expression values to the peak annotation
- Handles missing or unmatched values with 'NA'

# Output Files

- Combined_ATACseq_Expression.tsv: Merged table of peaks + gene expression

## Screenshot: Compare with Expression Tab



Annotated ATAC-seq File: /example-data/diffbind_results_annotated.tsv

Expression Data File: /example-data/Expression-data.txt

ATAC-seq ID Column: 16

Expression ID Column: 1

Output File: /example-data/Combined_ATACseq_Expression.tsv

Ready

# Troubleshooting

- Ensure all files are tab-delimited.
- Use a similar format for chromosome name.
- Ensure bedtools is installed and in the system PATH.
- For large files, allow a few minutes to complete.

# Support

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**Website:** www.epigen-bioinfolab.com