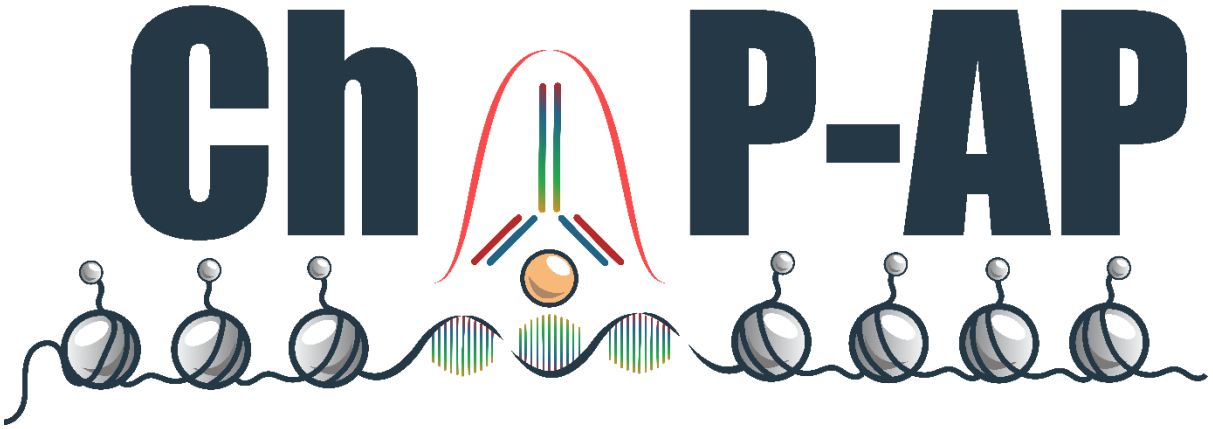


Chromatin **I**mmuno**P**recipitation Sequencing **A**nalysis **P**ipeline

Inherently Simple ChIP-Seq Analysis

Developed by:

Jeremiah Suryatenggara

Mahmoud Bassal

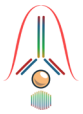
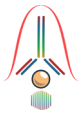


Table of Contents



Introduction

ChIP-Seq is a technique used to analyse protein-DNA interactions and identify the binding sites of said protein. Briefly, protein is fixed/attached to the DNA, then, samples are fragmented and a pull-down is performed with an anti-body targeted to the protein of interest to extract the protein-DNA complexes where binding to DNA has occured. The samples are then sequenced and analysed. A key step in the bioinformatics analysis is the calling of "peaks," or regions of enrichment, corresponding to binding sites of the protein to the DNA.

It has been widely reported that of all the bioinformatics analysis steps, the step most likely to affect study outcomes is peak calling (Chen et al., 2012; Johnson et al., 2007). Numerous reviews (Koohy et al., 2014; Laajala et al., 2009; Wilbanks and Facciotti, 2010) have therefore been published benchmarking available peak callers to ascertain superiority of one caller over others. Consistently however, these benchmarking papers will report mixed, potentially conflicting, findings with no single peak caller out-performing others. Rather, each peak-caller excels in specific types of datasets and one cannot know the overall best performance of a single peak-caller in advance. Using a sub-optimal peak caller will result in few peaks called, or worse, a large number of false-positive peaks which, short of validating every peak manually, will be indescriminable from true-positive peaks. Compounding issues further, benchmarking reviews have consistently shown little overlap between called peak sets of different peak callers. Therefore, we can conclude that each peak caller has distinct selectivity and specificity characteristics which are often not additive and seldom completely overlap in many scenarios. As such, results obtained from individual peak callers, even sub-optimal callers, will contain sub-sets of true and false-positive peaks that are exclusive to that caller.

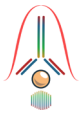
This leads the user to the peak-caller dilemma, if there is no universal peak caller to use, which peak caller *does* one use for their dataset? We therefore rationalized that without a perfect peak caller (which is near impossible owing to the significant variability introduced from wet-lab experiments), the only option is to leverage the best performing, currently available peak callers and identify unique and/or overlapping peaks to better discern the confidence of peaks without manual validation, and identify overlapping, high-confidence peak sub-sets.

To that end, our **ChIP-Seq Analysis Pipeline (ChIP-AP)** utilizes multiple peak callers, which enables filtering in/out results from every peak caller used in this pipeline (MACS2, GEM/SICER2, HOMER, and Genrich) to:

1. Selectively obtain high confidence peaks based on overlaps by different peak callers
2. Avoid the caveats of depending on a single peak caller in datasets with different peak characteristics and signal-to-noise ratios
3. Freely choose the "sweet spot" between the consensus peaks set (intersection of detected peaks from all peak callers) and the total peaks set (the union of all detected peaks), that answers one's biological questions which may be supported by additional evidence from other experiments. This can be done from the output without re-processing data.

ChIP-AP is a fully automated ChIP-seq data processing and analysis pipeline.

1. For input, it takes unaligned sequencing reads in fastq format (extension: .fastq / .fq / .fastq.gz / .fq.gz) or previously aligned reads in bam format (extension: .bam) from any aligner
2. ChIP-AP is capable of processing and analyze multiple sample replicates
3. ChIP-AP is a complete, integrated workflow which performs all analysis steps (QC, cleanup, alignment, peak-calling, pathway analysis) and outputs a final integrated peak set
4. The output of ChIP-AP is a detected peaks list, annotated with information on associated gene names, IDs, functions, gene ontology, and related pathways



System requirements

OS – Linux (Ubuntu-variants tested), MacOS (10.15 or later), Windows 10 (v1903 or later)

CPU – (minimum) Quad-Core Intel/AMD CPU, (recommended) Octa-Core Intel/AMD CPU. ChIP-AP will NOT run on newer Apple Silicone (AS) M1 or later CPU's – not yet anways…

RAM – (minimum) 8Gb, (recommended) 16Gb+

Storage (SSD/HDD) – Installation alone takes ~60Gb. Roughly, an additional 30-100Gb space is required for processed and analysing samples

Screen Resolution – A minimum resolution of 1920*1080 is required for the dashboard interface. If your screen resolution is less than this, you will be limited to only using the wizard.

Quick start – For Command line User Only

ChIP-AP is capable of handling multiple sample replicates in a single run. It is also capable of hanlding an un-balance number of sample replicates (ie 3 ChIP, 2 Controls). It does so by merging each corresponding sample type (ie merge all ChIP samples together and controls together) following alignment and these merged files are used for all down-stream processing.

For controls, input (not IgG) is recommended and is consensually considered best practice.

For peak calling, peaks are called as ChIP over control.

Example : To process *single-end unaligned reads* with default settings:

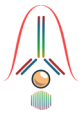
```
chipap  --mode single \
        --chipR1 [chip fastq replicate1] [chip fastq replicate2] … \
        --ctrlR1 [control fastq replicate1] [control fastq replicate2] … \
        --genome [path to genome folder] \
        --output [path to output save folder] \
        --setname [dataset name]
```

Example : To process *paired-end unaligned reads* with default settings:

```
chipap  --mode paired \
        --chipR1 [chip fastq replicate1, first read] [chip fastq replicate2, first read] … \
        --chipR2 [chip fastq replicate1, second read] [chip fastq replicate2, second read] … \
        --ctrlR1 [control fastq replicate1, first read] [control fastq replicate2, first read] … \
        --ctrlR2 [control fastq replicate1, second read] [control fastq replicate2, second read] … \
        --genome [path to output save folder] \
        --output [path to output save folder] \
        --setname [dataset name]
```

Example : To process *single/paired-end aligned reads* with default settings:

```
chipap  --mode single / paired \
        --chipR1 [chip bam replicate1] [chip bam replicate2],… \
        --ctrlR1 [control bam replicate1] [control bam replicate2],… \
        --genome [path to genome folder] \
        --output [path to output save folder] --setname [dataset name]
```



Software Installation

ChIP-AP has been designed to be as simple as possible to run for end-users be they bioinformaticians or wet-lab biologists with no coding experience. For non-experienced users however, there is a little command-line dabbling required in order to get everything set up just right. Thankfully, we have prepared full installation guides located on our github wiki page for your operating system (OS) of choice. These guides are complete, step-by-step guides with accompanying screenshots to walk users through every step of the installation processes. Below are brief installation notes for reference only. Installation files can be downloaded from our github (https://github.com/JSuryatenggara/ChIP-AP)

Pre-Configured Images and Available Online Portal

For users who do not wish to try to configure things on their own, we provide 2 usage options

1 – Users can download a pre-configured virtual-machine image to run in VirtualBox (https://www.virtualbox.org/). Setup guides for VirtualBox can be found online. The pre-configured image can be downloaded from (https://www.dropbox.com/s/4d3adb6ckof5rti/ChIP-AP_Ubuntu_LTS2004.vhd).

2 – Users can use the Cancer Science Institute of Singapore's Next-Generation Sequencing Portal (CSI NGS Portal, (An et al., 2020), https://csibioinfo.nus.edu.sg/csingsportal/login/home.php).

Using the NGS Portal, users can upload their data to a secure processing server, setup a ChIP-AP run, and wait for the results. This option does not require users to set anything up on their local machines, simply upload your data and use the service. Data uploaded is accessible only to your user account and not shared publically. Data and analyses are deleted from the server after 30 days. This is an ideal option if you will only do 1/2 analyses or do not have the hardware to run ChIP-AP yourself – and can be done without a bioinformaticians input. The pipeline will run with default parameters and so no configuration is required.

Installing ChIP-AP on local machine or laptop

Before attempting to install ChIP-AP on your local machine, make sure your hardware is capable of running ChIP-AP. ChIP-AP will run on Linux (Ubuntu-variants tested), MacOS (excluding Apple Silicone (M1) macs) and Windows 10. For running, ensure your computer/laptop has at least a quad-core cpu and a minimum of 8Gb of RAM. Storage on your SSD/HDD will vary depending on the size of your sequencing files but at least 30Gb of free storage space is a good start.

1 - Before setting up ChIP-AP, Anaconda 3 ***MUST*** be installed first. Simply search "anaconda 3" in your search-engine of choice and download and install the correct setup for your operating system (OS). We'll wait here till you've done this…

2 - ChIP-AP can be downloaded from our GitHub (https://github.com/JSuryatenggara/ChIP-AP)

3 - After downloading ChIP-AP, move the downloaded ChIP-AP package folder to a directory of your choosing. This will be the installation directory for ChIP-AP

4 - To install, run: **chipap_install.py** from the command line inside the unpacked folder

Running ChIP-AP with Graphical Interfaces

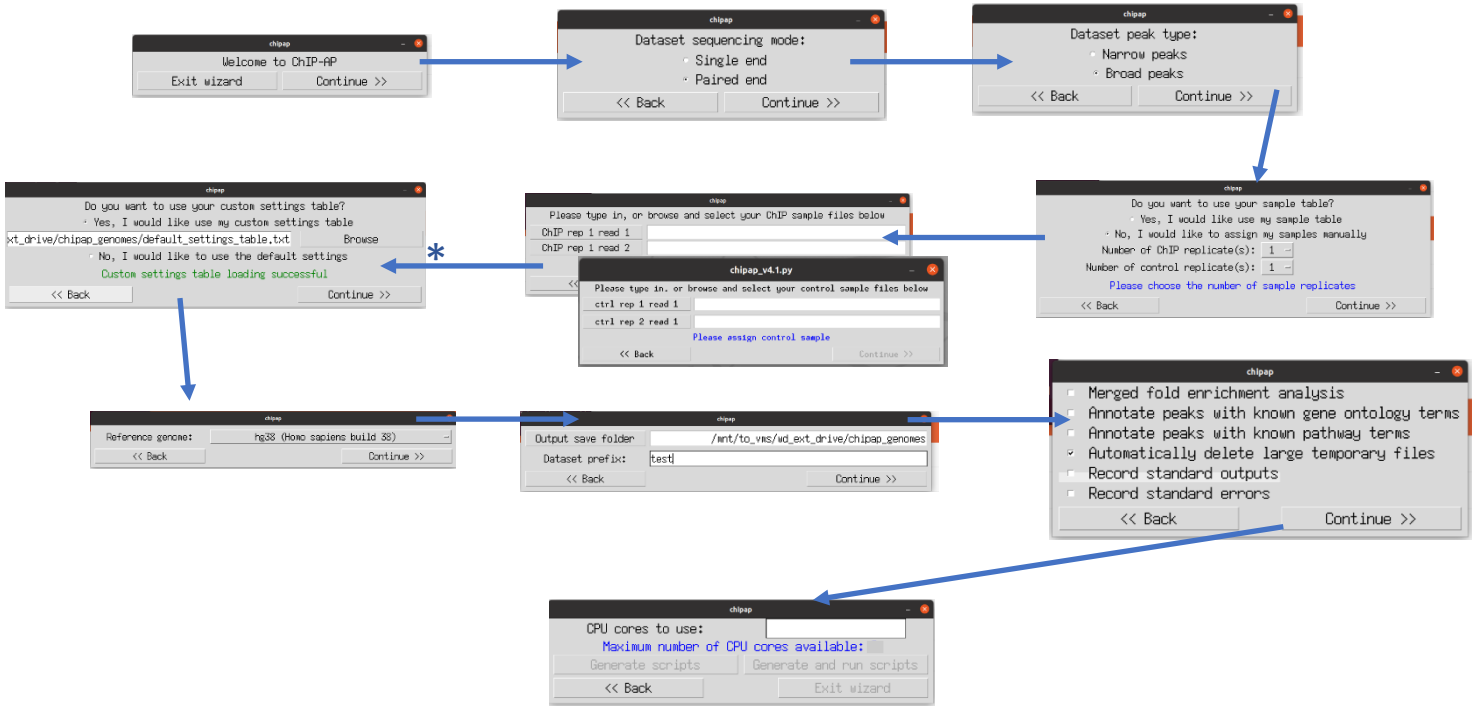
ChIP-AP offers 2 graphical interfaces for running – depending on a users proficiency with ChIP-AP. They are the **chipap_dashboard** and the **chipap_wizard**.

We recommend new users to ChIP-AP first use the **wizard**. Proficient users of ChIP-AP should use the **dashboard** as it enables inputting the data more quickly.

Running the Wizard

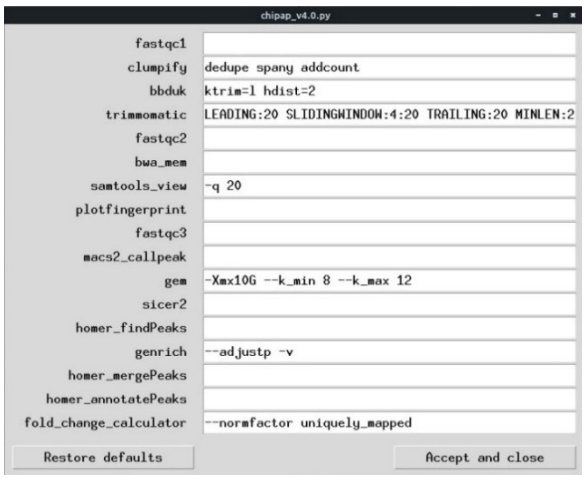
Once ChIP-AP is setup, at the command line type: **chipap_wizard.py**

to use the wizard graphical user interface (GUI). The multiple windows that appear ask users questions sequentially regarding information required for a run. This ensure data is provided in the correct order without overwhelming the user with all questions and options simultaneously. This option is therefore recommended for inexperienced ChIP-AP users. Tooltip boxes appear over entry fields providing brief explanations of input requirements.



For advanced users

A customized pipeline run is possible by providing the required flag arguments for each individual program in the pipeline at the * stage above. By selecting the right option, the secondary window to the right will appear containing multiple entry fields for such flag arguments. *This option is for advanced users only.* For valid flags, please refer to the documentaiton of each program specifically. If you really made a mess of things, theres a

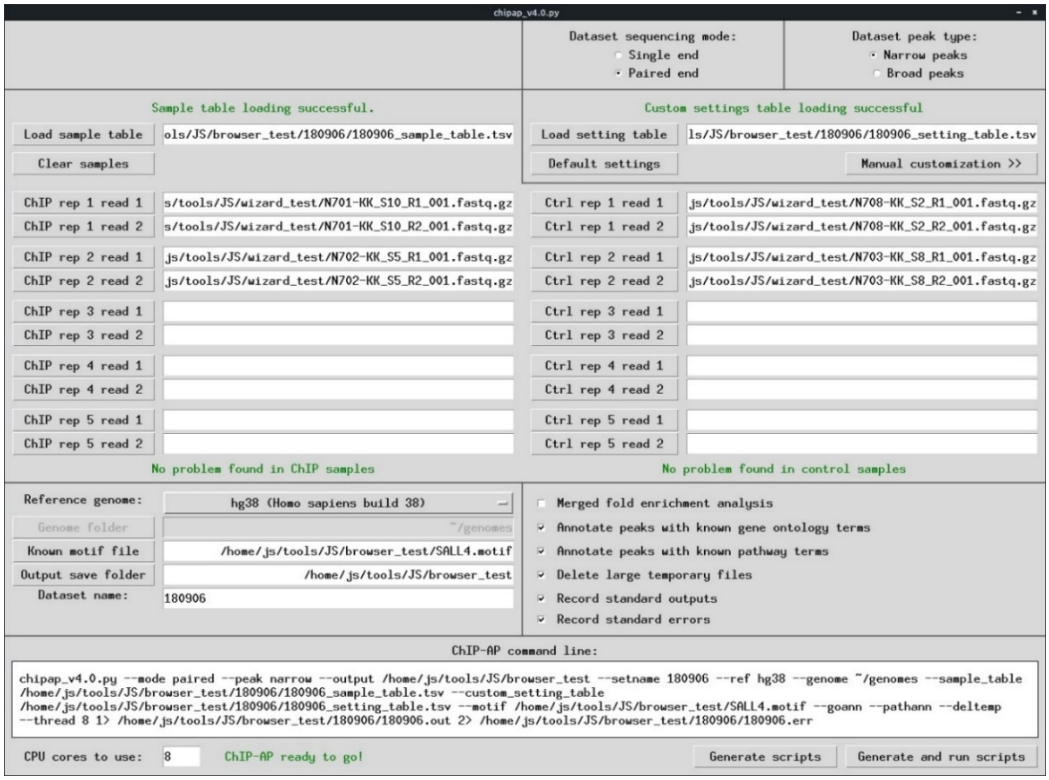


"Restore defaults" option to restore everything to default parameters again.

Running the Dashboard

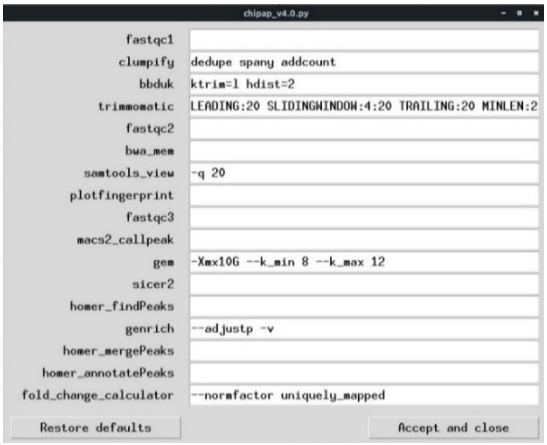
Once ChIP-AP is setup, at the command line type: **chipap_dashboard.py**

The GUI below will appear enabling users to input everything required for a run. Tooltip boxes will appear over each field providing brief explanations of whats required in said field. ***NOTE***: For you to use the dashboard you need to have a minimum screen resolution of at least 1920*1080. If less than this, then all the dashboard elements will not appear on the. So either use an external monitor with a resolution of 1920*180 or larger, or the wizard.



For advanced users

A customized pipeline run is possible by providing the required flag arguments for each individual program in the pipeline. For the settings table option, clicking the "manual customization" button will bring out a secondary window (shown right) containing multiple entry fields for such flag arguments. *This option is for advanced users only.* For valid flags, please refer to the documentation of each program specifically.



In each of these entry fields, user may key in the flag arguments as they are written for each corresponding program (e.g., "-q 20" for samtools view which will filter reads with a MAPQ > 20). This requires user to read dedicated official manuals to understand what and how these arguments can be given to the corresponding program in the pipeline. This, accompanied with the fact that bad/wrong flag arguments may break the pipeline run, shows that this manual customization of pipeline programs settings is *recommended for experienced users only*, and that inexperienced users should stick with the default settings or get a bioinformaticians input.

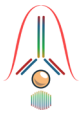
Usage notes and Command Line Flags / Parameters

Required Arguments

--mode	*single / paired*	Single-end or paired-end sequencing analysis. If a paired-end run, files will typically be labelled ending in *_R1 and *_R2 before the file extension. If these labels aren't present then ***likely***, you have single-ended sequencing data and select the "single" option.
--genome	*[directory]*	Your genome folder directory. Required full path, ***not*** relative path. This is the folder where the pre-computed genome alignment and processing files are saved. These genomes can be downloaded from (https://www.dropbox.com/s/ioqd3hwdahh9xon/genomes.zip) or instead you can compute your own (a guide for this is coming soon actually… Keep an eye on the wiki…)
--output	*[directory]*	Your desired output folder. Required full path, ***not*** relative path.
--setname	*[text]*	The prefix to label output and intermediate files (no space allowed). ChIP-AP will rename all processed data files to have this "setname" prefix.

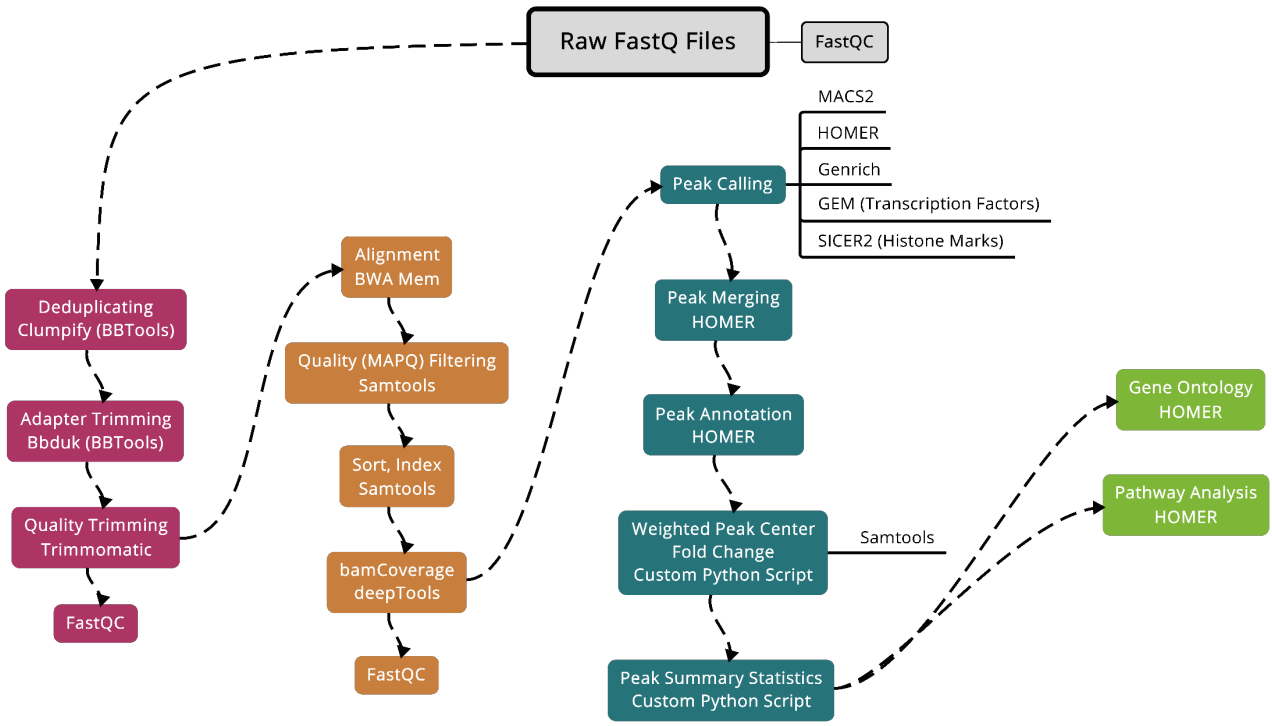
Optional Arguments

--peak	*narrow / broad*	Narrow peaks for transcription factors (default). Broad peaks for histone modifiers. If unsure what will work best for your dataset, you may need to run ChIP-AP once in each mode and inspect the output to ensure you get what you want from the results. Pay close attention to the width of the peaks called and regions of enrichment.
--chipR1	*[repl1 repl2 …]*	Your ChIP datasets: ordered by replicate, separated by space. Best to include full path rather than relative paths.
--chipR2	*[repl1 repl2 …]*	**[Paired-end Only]** Your ChIP datasets second read: ordered by replicate, separated by space. Best to include full path rather than relative paths.
--ctrlR1	*[repl1 repl2 …]*	Your control datasets: ordered by replicate, separated by space. Best to include full path rather than relative paths.
--ctrlR2	*[repl1 repl2 …]*	**[Paired-end Only]** Your control datasets second read: ordered by replicate, separated by space. Best to include full path rather than relative paths.
--sample	*[file]*	Rather than including the input sample file names and paths on the commandline, one can make a sample table containing the same information and input this instead. The sample-table is a 4*n-sized table (with n = number of replicates) containing the absolute paths to each of your ChIP and control replicates (See *pg 19* for more information regarding this file and its layout). When this option is used, this table will disregard any assigned values to **--chipR1, --chipR2, --ctrlR1,** and **--ctrlR2**.



--setting	*[file]*	**[For Advanced Users <u>ONLY</u>]** The settings-table allows you to fine-tune the behaviour of every program as part of ChIP-AP. *Hence very disasterous if you get wrong!* If you are unsure even a little about what you're doing, then stick to default settings please – this goes even for bioinformaticians. This txt file is a 2*n-sized table (with n = number of replicates) containing custom arguments for every program as part of ChIP-AP. The default settings table is provided in the genome folder. You can <u>*COPY*</u> this file and make changes as necessary. To use your customs settings table, provide full path to updated txt file. See *pg 19* for more information regarding this file and its layout.
--ref	*hg19 / hg38 / mm9 / mm10 / dm6 / sacCer3*	Your sample genome reference build. Default is hg38 (human). The genomes listed to the left are provided, pre-calculated by us and are the only genomes used and tested for now. We will provide added functionality soon to add your own custom genome to ChIP-AP. Watch this space (actually the github…)!
--motif	*[file]*	Your predicted/known motif file, in HOMER matrix format (.motif). If provided, once peaks are called, HOMER motif discovery will be run on the total called peak set for this motif. Many users prefer MEME-ChIP instead… we know… WIP :)
--fcmerge		This flag will force fold change analysis to be computed based on merged replicates instead of on each replicate seperately.
--goann		This flag will instruct to annotate peaks with all relevant GO terms as provided by HOMER.
--pathann		This flag will instruct to annotate peaks with all relevant pathway and interaction enrichment terms as provided by HOMER.
--deltemp		This flag will instruct to delete large intermediary files right after they are not going to be used for further processes (eg intermediary fq files). This option will save a significant amount of space by the end of your analysis, so recommended.
--thread	*[integer]*	Maximum number of processes to use. Default is half the maximum available on your system so as to not choke it during the run. If running on a laptop or low-thread count cpu, best to push this up to maximum number threads available -1 – but this **<u>will</u>** significantly slow your laptop if attempting other tasks while ChIP-AP is running.
--run		Use to immediately run the suite by running the master script. This is the big red button ok... Use at your own risk! When not used, the generated master script (MASTER_script.sh) in the output folder can be run manually by user. We have made sure that when you tell ChIP-AP "do not press the big red button" that it will behave and do as you say.

ChIP-AP Graphical Overview



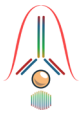
Detailed Explanation of Steps and Methodology Used

1. **Acquisition of raw sequencing files**. ChIP-AP can directly process the output files of sequencing instruments. Files may be in FASTQ, or compressed FASTQ (.gz) format. ChIP-AP can also process aligned reads in BAM format. Reads may be single or paired ends. Background control is compulsory, no unmatched samples allowed here.

2. **Sample recognition and registration**. Performed by the main script. Each input sample is registered into the system and given a new name according to their sample category (ChIP or background control), replicate number, and whether it's the first or second read file (in case of paired end sequencing data). Afterwards, their formats and compression status is recognized and processed into gun-zipped FASTQ as necessary.

3. **Generation of multiple modular scripts**. Each process in ChIP-AP is executed from individual scripts generated. This was an intentional design decision as it allows for easy access for modifications of any step within the pipeline without hunting through the master ChIP-AP scripts. You can thank us later if you have to modify and tailor something later and don't have to drudge through the trenches of someone else's code. Choclate treats always welcome!

4. **Copying, compressing, and renaming of the raw sequencing reads**. In the very beginning, ChIP-AP makes (in the user-designated output folder) a copy of each unaligned sequence reads file (e.g., fastq), compresses them into a gunzipped file (if not already), and renames them with the prepared new name from step "**2. Sample recognition and registration"**. If the given inputs are aligned reads (bam files), the pipeline starts at step "**12. Sorting and indexing of aligned reads files"** (see below)



and the copying and renaming are taken over by "**08_results_script.sh"** where the original bam files are directly sorted and the pipeline proceeds normally from there.

Modular script used: **00_raw_data_script.sh**

- Operation : cp, gzip, mv (Bash)
- Input : [origin directory] / [original ChIP/control filename]
- Process : Copy, compress, and rename raw reads files
- Output : [output directory] / 00_raw_data / [setname]_[chip/ctrl]_rep[#]_R[1/2].fq.gz

5. **Raw sequencing reads quality assessment**. Performed by FastQC. Reads quality assessment is performed to check for duplicates, adapter sequences, base call scores, etc. Assessment results are saved as reports for user viewing. If the final results are not as expected, its worthwhile to go through the multiple QC steps and track the quality of the data as its processed. If the default QC steps aren't cleaning up the data adequately, you may need to modify some parameters to be more/less stringent with cleanup. From our testing, our default values seem to do a faily adequate job though for most datsets.

   Modular script used: **01_raw_reads_quality_control_script.sh**

   - Calls : fastqc
   - Input : 00_raw_data / [setname]_[chip/ctrl]_rep[#]_R[1/2].fq.gz
   - Process : Generate raw reads quality assessment reports
   - Output : 01_raw_reads_quality_control / [setname]_[chip/ctrl]_rep[#]_R[1/2]_fastqc.html

6. **Deduplication of reads**. Performed by clumpify from BBMap package. Necessary command line argument is given to clumpify in order to remove optical duplicates and tile-edge duplicates from the reads file in addition to PCR duplicates. Optimization of file compression is also performed by clumpify during deduplication process, in order to minimize storage space and speed up reads file processing.

   Modular script used: **02_deduplicating_script.sh**

   - Calls : clumpify.sh
   - Input : 00_raw_data / [setname]_[chip/ctrl]_rep[#]_R[1/2].fq.gz
   - Process : Remove PCR duplicates, optical duplicates, and tile-edge duplicates
   - Output : 02_deduplicating / [setname]_[chip/ctrl]_rep[#]_R[1/2].deduped.fq.gz

7. **Adapter trimming of reads**. Performed by BBDuk from BBMap package. BBDuk scans every read for adapter sequence, based on the reference list adapters given in the command line argument. The standard BBDuk adapter sequence reference list 'adapter.fa' is be used as a default in the pipeline. Any sequencing adapter present in the reads is removed. Custom adapter sequence can be used whenever necessary or by modifying the adapter.fa file with your new sequences.

   Modular script used: **03_adapter_trimming_script.sh**

   - Calls : bbduk.sh
   - Input : 02_deduplicating / [setname]_[chip/ctrl]_rep[#]_R[1/2].deduped.fq.gz
     [path to genome folder] / bbmap / adapters.fa *(file provided by ChIP-AP)*
   - Process : Trim away adapter sequences based on given sequences in file 'adapters.fa'
   - Output : 03_adapter_trimming / [setname]_[chip/ctrl]_rep[#]_R[1/2].adaptertrimmed.fq.gz

8. **Quality trimming of reads**. Performed by trimmomatic. Trimmomatic scans every read trims low quality base calls from reads. Additionally, it scans with a moving window along the read and cuts the remainder of the read when the average quality of base calls within the scanning window drops below the set threshold. Finally, it discards the entirety of a read of it gets too short post-trimming for alignment to reference genome, minimizing the chance of reads being multi-mapped to multiple genomic locations.

Modular script used: **04_quality_trimming_script.sh**

- Calls : trimmomatic
- Input : 03_adapter_trimming / [setname]_[chip/ctrl]_rep[#]_R[1/2].adaptertrimmed.fq.gz
- Process : Remove reads with low PHRED (base calling) score
- Output : 04_quality_trimming / [setname]_[chip/ctrl]_rep[#]_R[1/2].qualitytrimmed.fq.gz

9. **Pre-processed reads quality assessment**. Performed by FastQC. Quality assessment is performed to check for the efficiency of cleanup. Results are saved as reports.

   Modular script used: **05_preprocessed_reads_quality_control_script.sh**

   - Calls : fastqc
   - Input : 04_quality_trimming / [setname]_[chip/ctrl]_rep[#]_R[1/2].qualitytrimmed.fq.gz
   - Process : Generate preprocessed reads quality assessment reports
   - Output : 05_preprocessed_reads_quality_control / [setname]_[chip/ctrl]_rep[#]_R[1/2]_fastqc.html

10. **Reads alignment to reference genome**. Performed by the mem algorithm in BWA aligner. Appropriate genome reference for the sample organism is given as a command line argument. The default genome reference is hg38. Precomputed genome references hg38, hg19, mm9, mm10, dm6, and sacCer3 are downloaded as part of the ChIP-AP installation process. Tutorials for custom/different genome references can be found on the ChIP-AP github (coming soon!). BWA is used in preference to other alignes such as Bowtie2 as in benchmarking papers (Thankaswamy-Kosalai et al., 2017), we were more statisfied with the results of BWA, hence its inclusion in this pipeline.

    Modular script used: **06_bwa_mem_aligning_script.sh**

    - Calls : bwa mem
    - Input : 04_quality_trimming / [setname]_[chip/ctrl]_rep[#]_R[1/2].qualitytrimmed.fq.gz
      [path to genome folder] / bwa / *(reference genome provided by ChIP-AP)*
    - Process : Align preprocessed reads to the designated reference genome
    - Output : 06_bwa_mem_aligning / [setname]_[chip/ctrl]_rep[#].aligned.bam

11. **Alignment score quality filtering**. Performed by samtools view. This filter (if set) will remove all reads with alignment score (MAPQ) below a user defined threshold. Reads with suboptimal fit into the genome and/or reads with multiple ambiguous mapped locations can easily be excluded from the reads file using this filter step also. To disable MAPQ filtering, simply remove all flags from the settings table for this step.

    Modular script used: **06_bwa_mem_aligning_script.sh**

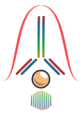
    - Calls : samtools view
    - Input : 06_bwa_mem_aligning / [setname]_[chip/ctrl]_rep[#].aligned.bam
    - Process : Remove reads with low MAPQ (alignment) score
    - Output : 07_MAPQ_filtering / [setname]_[chip/ctrl]_rep[#].mapqfiltered.bam

12. **Sorting and indexing of aligned reads files**. Performed by samtools sort and samtools index, which do nothing to the aligned reads files other than sorting and indexing, priming the aligned reads files for further processing.

    Modular script used: **08_results_script.sh**

    - Calls : samtools sort
    - Input : 07_MAPQ_filtering / [setname]_[chip/ctrl]_rep[#].mapqfiltered.bam
    - Process : Sort all bam files based on coordinate
    - Output : 08_results / [setname]_[chip/ctrl]_rep[#].bam

    - Calls : samtools merge
    - Input : 08_results / [setname]_chip_rep[#].bam
      08_results / [setname]_ctrl_rep[#].bam
    - Process : Merge all sorted ChIP bam files and all sorted control bam files.



- Output : 08_results / [setname]_chip_merged.bam
  08_results / [setname]_ctrl_merged.bam
- *Condition: --fcmerge flag is used OR unequal number of ChIP and control samples OR peak type is broad*

- Calls : samtools index
- Input : 08_results / [setname]_[chip/ctrl]_rep[#].bam
  08_results / [setname]_[chip/ctrl]_merged.bam
- Process : Make indices for all coordinate-sorted bam files
- Output : 08_results / [setname]_[chip/ctrl]_rep[#].bam.bai
  08_results / [setname]_[chip/ctrl]_merged.bam.bai

- Calls : samtools sort -n
- Input : 08_results / [setname]_[chip/ctrl]_rep[#].bam
- Process : Sort all bam files based on read name
- Output : 08_results / [setname]_[chip/ctrl]_rep[#]_namesorted.bam

13. **ChIP pulldown efficiency assessment**. Performed by plotFingerprint from the deeptools package, which generates fingerprint plots. These serve as a quality control figure that shows DNA pulldown efficiency of the ChIP experiment. Refer to the appropriate documentation for full details but in short – the input should be as close to the 1:1 diagonal as possible and the better enrichment seen in your sample, the more its curve will bend towards the bottom right. You want (ideally) a large gap between the chip and the control samples. PNG files are provided for easy viewing, SVG files provided if you want to make HQ verions later for publication.

    Modular script used: **08_results_script.sh**
    - Calls : plotFingerprint
    - Input : 08_results / [setname]_[chip/ctrl]_rep[#].bam
    - Process : Generate fingerprint plots for all bam files
    - Output : 08_results / fingerprint_plots/[setname].[png/svg]
      08_results / fingerprint_plots/[setname]_merged.[png/svg]

14. **Visualization track generation of aligned reads files**. Performed by bamCoverage from the deeptools package. Generates bigwig files for quick and simple visualization of reads distribution along the referenced genome using local tools such as IGV. The Coverage tracks can be uploaded to genome browsers such as UCSC, however a track hub needs to be generated – something ChIP-AP does not do at this stage.

    Modular script used: **08_results_script.sh**
    - Calls : bamCoverage
    - Input : 08_results / [setname]_[chip/ctrl]_rep[#].bam
      08_results / [setname]_[chip/ctrl]_merged.bam
    - Process : Generate BigWig coverage file for each individual bam file
    - Output : 08_results / [setname]_[chip/ctrl]_rep[#].bw
      08_results / [setname]_[chip/ctrl]_merged.bw

15. **Aligned reads quality assessment**. Processed by FastQC. Quality assessment is performed to check for the alignment efficiency, such as how many reads failed to be mapped. Assessment results are saved as reports.

    Modular script used: **09_aligned_reads_quality_control_script.sh**
    - Calls : fastqc
    - Input : 08_results / [setname]_[chip/ctrl]_rep[#].bam

08_results / [setname]_[chip/ctrl]_merged.bam
- Process : Generate raw reads quality assessment reports
- Output : 08_results / [setname]_[chip/ctrl]_rep[#]_fastqc.html
  08_results / [setname]_[chip/ctrl]_merged_fastqc.html

16. **Peak calling**.

    For Transcription Factors - Performed by MACS2 (default setting), GEM, HOMER (factor setting), and Genrich for transcription factor proteins of interest.

    For Broad Peaks (Histone Marks) - Performed by MACS2 (broad setting), SICER2, HOMER (broad setting), and Genrich for histone modifier protein of interest.

    The same track of aligned reads is scanned for potential protein-DNA binding sites. The process returns a list of enriched regions in various formats.

    Modular script used: **11_macs2_peak_calling_script.sh**
    - Calls : macs2 callpeak
    - Input : 08_results / [setname]_[chip/ctrl]_rep[#].bam
    - Process : Generate a list of called peaks
    - Output : 11_macs2_peak_calling / [setname]_MACS2_peaks.narrowPeak

    Modular script used: **12_gem_peak_calling_script.sh**
    - Calls : gem
    - Input : 08_results / [setname]_[chip/ctrl]_rep[#].bam
    - Process : Generate a list of called peaks
    - Output : 12_gem_peak_calling / [setname]_GEM_GEM_events.txt
    - *Condition: Peak type is narrow. GEM is replaced by SICER2 for broad peak type.*

    Modular script used: **12_sicer2_peak_calling_script.sh**
    - Calls : sicer
    - Input : 08_results / [setname]_[chip/ctrl]_merged.bam
    - Process : Generate a list of called peaks
    - Output : 12_sicer2_peak_calling / [setname]-W*-G*-islands-summary
      (* depends on -w and -g flag arguments. Defaults are 200 and 600, respectively)
    - *Condition: Peak type is broad. SICER2 is replaced by GEM for narrow peak type.*

    Modular script used: **13_homer_peak_calling_script.sh**
    - Calls : findPeaks
    - Input : 08_results / [setname]_[chip/ctrl]_rep[#].bam
    - Process : Generate a list of called peaks
    - Output : 13_homer_peak_calling / [setname]_HOMER.peaks

    Modular script used: **14_genrich_peak_calling_script.sh**
    - Calls : Genrich
    - Input : 08_results / [setname]_[chip/ctrl]_rep[#]_namesorted.bam
    - Process : Generate a list of called peaks
    - Output : 14_genrich_peak_calling / [setname]_Genrich.narrowPeak

17. **Peaks merging**. Performed by a custom script and HOMER's mergePeaks. The custom script reformats necessary peak caller outputs into HOMER region list format. mergePeaks looks for overlaps between the regions in the four peak caller outputs and lists the merged regions in multiple files based on the peak caller(s) that calls them. These multiple files are then concatenated together into a single regions list file.

    Modular script used: **21_peaks_merging_script.sh**

- Calls : mergePeaks
- Input : 11_macs2_peak_calling / [setname]_MACS2_peaks.narrowPeak
  12_gem_peak_calling / [setname]_GEM_GEM_events.txt (narrow peak only)
  12_sicer2_peak_calling / [setname]-W*-G*-islands-summary (broad peak only)
  13_homer_peak_calling / [setname]_HOMER.peaks
  14_genrich_peak_calling / [setname]_Genrich.narrowPeak
- Process : Generate multiple lists of merged peak coordinates based on peak callers
- Output : 21_peaks_merging / [setname]_merged_peaks*
  * is the combination of peak callers where the listed peaks in are found in
  (e.g., [setname] _merged_peaks_MACS2_Genrich)

Modular script used: **22_peaks_processing_script.sh**

- Operation : cat (Bash)
- Input : 21_peaks_merging / [setname]_merged_peaks*
- Process : Generate concatenated list of peak coordinates
- Output : 22_peaks_processing / [setname]_all_peaks_concatenated.tsv

18. **Peaks annotation**. Performed by annotatePeaks from HOMER package. Each region in the concatenated list is annotated based on its genomic location for the genome specified. The process returns the same list of regions, with each entry row appended with various information pertaining to the gene name, database IDs, category, and instances of motif (if HOMER known motif matrix file is provided to ChIP-AP), etc.

    Modular script used: **22_peaks_processing_script.sh**

    - Calls : annotatePeaks
    - Input : 22_peaks_processing/[setname]_all_peaks_concatenated.tsv
    - Process : Append gene annotations to the list of peak coordinates
    - Output : 22_peaks_processing/[setname]_all_peaks_annotated.tsv

19. **Fold enrichment calculations**. Performed by a custom script (full details pg 26), with the help of samtools depth and view modules. For weighted peak center fold enrichment calculation in cases of narrow peak type datasets, the custom script sends out the reformatted genomic regions as command line arguments for multi-threaded samtools depth runs. Samtools depth returns a list of read depths at each base within the region and saves them in a temporary file. The script then reads the temporary files and determine the weighted peak centers and returns the read depth values along with the base locations. The custom script sends out the weighted peak center base locations as command line arguments for multi-threaded samtools view runs. Samtools view returns the read depth values at the given base locations. The custom script then calculates the fold enrichment values, corrected based on ChIP-to-control normalization factor.

    For average fold enrichment calculation in cases of broad peak type datasets, samtools view simply sums up the number of reads in the whole peak region, then calculates the fold enrichment values, corrected based on ChIP-to-control normalization factor.

    In addition, the custom-made script also makes some reformatting and provides additional information necessary for downstream analysis.

    Modular script used: **22_peaks_processing_script.sh**

    - Calls : fold_change_calculator_suite_xx.py
    - Input : 22_peaks_processing / [setname]_all_peaks_annotated.tsv
    - Process : Calculate ChIP tag counts (read depth)
      Calculate weighted center fold change (narrow peak only)
      Calculate average fold change (broad peak only)

Calculate number of peak callers overlaps
Calculate number of user-provided (via --motif flag) motif instances found

- Output : 22_peaks_processing / [setname]_all_peaks_calculated.tsv
  (Ready to view, if user does not wish for gene ontology or pathway annotations)

20. **Peak statistics summary**. Performed by a custom script designed for quality assessment of called peaks. Returns a summary text file containing information pertaining to the peak read depth, peak fold enrichment, known motif hits, and positive peak hits (based on known motif presence), in each peak set along the continuum between single peak callers and the absolute consensus of all four peak callers.

    Modular script used: **22_peaks_processing_script.sh**
    - Calls : peak_caller_stats_suite.py
    - Input : 22_peaks_processing / [setname]_all_peaks_calculated.tsv
    - Process : Generate a separate summary table of key statistics in peak callers performance
    - Output : 22_peaks_processing / [setname]_peak_caller_combinations_statistics.tsv

21. **(Optional) Downstream analysis: Gene ontology enrichment**. Each peak in the concatenated list is appended with all the gene ontology terms associated with its gene annotation. The gene ontology terms are derived from biological processes, molecular functions, and cellular compartments databases. This enables list filtering based on the gene ontology terms of the study's interest

    Modular script used: **23_go_annotation_script.sh**
    - Calls : go_annotate_suite_xx.py
    - Input : 22_peaks_processing / [setname]_all_peaks_calculated.tsv
    - Process : Append related gene ontology terms to the list of peaks
    - Output : 23_supplementary_annotations / [setname]_all_peaks_go_annotated.tsv

22. **(Optional) Downstream analysis: Pathway enrichment**. Each peak in the concatenated list is appended with all the related biological pathways associated with its gene annotation. The biological pathway terms are derived from KEGG, SMPDB, Biocyc, Reactome, Wikipathways, and pathwayInteractionDB databases. This enables list filtering based on the biological pathways of the study's interest. Additionally, this analysis also adds other terms pertaining to known interactions with common proteins and known gene mutations found in malignant cases, derived from common protein interaction and COSMIC databases, respectively.

    Modular script used: **23_pathway_annotation_script.sh**
    - Calls : pathway_annotate_suite_xx.py
    - Input : 22_peaks_processing / [setname]_all_peaks_calculated.tsv
    - Process : Append known pathways and interactions to the list of peaks
    - Output : 23_supplementary_annotations / [setname]_all_peaks_pathway_annotated.tsv

Main Pipeline Output

Final Analysis Table (including supplementary annotations)

The table below shows the contents of *[filename]_all_peaks_go_pathway_annotated.tsv*. Smaller sized and less verbose variants of this table are saved in the output folder with suffixes: concatenated, annotated, and calculated (see Source in the table below)

Column #	Peak Attribute	Source
Column 1 (A)	Peak ID (unique peak ID)	Pipeline script: 22_peaks_ processing _script.sh Called program: cat (Bash) Output file: [filename]_all_peaks_concatenated.tsv Output folder: 22_peaks_processing
Column 2 (B)	Chr (chromosome)	
Column 3 (C)	Start (peak start coordinate)	
Column 4 (D)	End (peak end coordinate)	
Column 5 (E)	Strand (strand on which peak is found)	
Column 6 (F)	Peak Caller Combination	
Column 7 (G)	Peak Caller Overlaps	Pipeline script: 22_peaks_processing_script.sh Called script: fold_change_calculator_suite_xx.py Output file: [filename]_all_peaks_calculated.tsv Output folder: 22_peaks_processing
Column 8 (H)	ChIP Tag Count	
Column 9 (I)	Control Tag Count	
Column 10 (J)	Fold Change	
Column 11 (K)	Number of Motifs	
Column 12 (L)	Annotation	Pipeline script: 22_peaks_processing_script.sh Called program: HOMER annotatePeaks Output file: [filename]_all_peaks_annotated.tsv Output folder: 22_peaks_processing
Column 13 (M)	Detailed Annotation	
Column 14 (N)	Distance to TSS	
Column 15 (O)	Nearest PromoterID	
Column 16 (P)	Entrez ID	
Column 17 (Q)	Nearest Unigene	
Column 18 (R)	Nearest Refseq	
Column 19 (S)	Nearest Ensembl	
Column 20 (T)	Gene Name	
Column 21 (U)	Gene Alias	
Column 22 (V)	Gene Description	
Column 23 (W)	Gene Type	
Column 24 (X)	CpG%	
Column 25 (Y)	GC%	
Column 26 (Z)	Biological Process	Pipeline script: 23_go_annotation_script.sh Called script: GO_annotate_suite_xx.py Output file: [filename]_all_peaks_go_annotated.tsv Output folder: 23_supplementary_annotations
Column 27 (AA)	Molecular Function	
Column 28 (AB)	Cellular Component	
Column 29 (AC)	Interaction with Common Protein	Pipeline script: 23_pathway_annotation_script.sh Called script: pathway_annotate_suite_xx.py Output file: [filename]_all_peaks_pathway_annotated.tsv Output folder: 23_supplementary_annotations
Column 30 (AD)	Somatic Mutations (COSMIC)	
Column 31 (AE)	Pathway (KEGG)	
Column 32 (AF)	Pathway (BIOCYC)	
Column 33 (AG)	Pathway (pathwayInteractionDB)	
Column 34 (AH)	Pathway (REACTOME)	
Column 35 (AI)	Pathway (SMPDB)	
Column 36 (AJ)	Pathway (Wikipathways)	

Miscellaneous Pipeline Outputs

Multiple peak callers statistics summary

The table below shows the contents of *[filename]_peak_caller_combinations_statistics.tsv*.

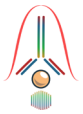
Column 1 (A)	**Peak Callers Combination**
Column 2 (B)	Exclusive Peak Count
Column 3 (C)	Exclusive Positive Peak Count
Column 4 (D)	Exclusive Motif Count
Column 5 (E)	Exclusive Positive Peak Hit Rate
Column 6 (F)	Exclusive Motif Hit Rate
Column 7 (G)	Exclusive ChIP Peak Read Depth
Column 8 (H)	Exclusive ChIP Peak Fold Change
Column 9 (I)	Inclusive Peak Count
Column 10 (J)	Inclusive Positive Peak Count
Column 11 (K)	Inclusive Motif Count
Column 12 (L)	Inclusive Positive Peak Hit Rate
Column 13 (M)	Inclusive Motif Hit Rate
Column 14 (N)	Inclusive ChIP Peak Read Depth
Column 15 (O)	Inclusive ChIP Peak Fold Change

- Exclusive: Only counts for a specific peak caller combination (e.g., Exclusive peak count of MACS2 only counts for peaks that is exclusively called by MACS2 alone).
- Inclusive: Counts for other peak caller combinations containing the same peak callers (e.g., Inclusive peak count of MACS2|GEM also counts for all other peaks in MACS2|GEM|HOMER, MACS2|GEM|Genrich, and MACS2|GEM|HOMER|Genrich).

Pipeline Run Info

This file summarizes the assignment of the files (IP sample or control, read 1 or 2; replicate number) and the file name conversion for every unaligned or aligned sequencing reads to be processed. Each line tells the user what the original files have been renamed into. Check this file if you suspect the order of samples were incorrectly entered (ie swapped chip with control)

```
Chromatin IP dataset replicate 1, 1st read : Original filename = a.fastq --> New filename = setname_chip_rep1_R1.fq.gz
Chromatin IP dataset replicate 2, 1st read : Original filename = b.fastq --> New filename = setname _chip_rep2_R1.fq.gz
Chromatin IP dataset replicate 1, 2nd read : Original filename = c.fastq --> New filename = setname _chip_rep1_R2.fq.gz
Chromatin IP dataset replicate 2, 2nd read : Original filename = d.fastq --> New filename = setname _chip_rep2_R2.fq.gz
Control dataset replicate 1, 1st read : Original filename = e.fastq --> New filename = setname _ctrl_rep1_R1.fq.gz
Control dataset replicate 2, 1st read : Original filename = f.fastq --> New filename = setname _ctrl_rep2_R1.fq.gz
Control dataset replicate 1, 2nd read : Original filename = g.fastq --> New filename = setname _ctrl_rep1_R2.fq.gz
Control dataset replicate 2, 2nd read : Original filename = h.fastq --> New filename = setname _ctrl_rep2_R2.fq.gz
```



Pipeline Run Command

Contains the input command line that was used to call the pipeline in a text file: *[filename]_command_line.txt* in the output save folder. This is useful for documentation of the run, and for re-running of the pipeline after a run failure or some tweaking if need be.

```
[chipap directory]/chipap_xx.py --mode paired --ref [genome_build] --genome [path_to_computed_genome_folders]
--output [full_path_to_output_save_folder] --setname [dataset name] --sample_table [path_to_sample_table_file]
--custom_setting_table [path_to_setting_table_file].tsv --motif [path_to_known_motif_file]
--fcmerge --goann --pathann --deltemp --thread [#_of_threads_to_use] --run
```

Sample Table

Contains the full path of each input ChIP and control sample in the pipeline run in a tab-separated value file: *[filename]_sample_table.tsv* in the output save folder in ChIP-AP sample table format. This is useful for documentation of the run, and for re-running of the pipeline after a run failure or some tweaking if need be. Below is an example of sample table file content (header included), given paired-end samples with two ChIP replicates and two control replicates.

chip_read_1	**chip_read_2**	**ctrl_read_1**	**ctrl_read_2**
… /a.fastq	… /c.fastq	… /e.fastq	… /g.fastq
… /b.fastq	… /d.fastq	… /f.fastq	… /h.fastq

If your sample is single-ended, then the sample table can simply be formatted as follows.

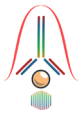
chip_read_1	**ctrl_read_1**
… /a.fastq	… /e.fastq
… /b.fastq	… /f.fastq

Setting Table & Default Parameters

A *cornerstone* of ChIP-AP's functionality is the settings table. ChIP-AP, with the raw fq files and the settings table, is able to reproduce (near) identically any analysis that was performed (provided the same program version numbers are used). The 'near identically' statements is owing to the fact that reported alignments of multi-mappers may, in some cases, give every so slightly different results. This ambiguity can be alleviated however by filtering out alignemnts with low MAPQ scores in the corresponding alignment filter step, post-alignment to ensure consistent results from every analysis run. The provision of the settings table therefore ensures reproducibility of any analysis with minimal effort and bypasses the usually sparse and significantly under-detailed methods sections of publications. Science is supposed to be reproducible, yet bioinformatics analysis are typically black-boxes which are irrepdoducible. This 1 file, changes that!

The structure of the settings table is simple. It is a 2 column tab-separated value file with the names of the programs on the 1st column, and the necessary flags required or changed in the 2nd column. If making your own custom table, then the 1st column below must be copied as-is and not changed. These 2 columns together, list the flags and argument values for each program used in the pipeline.

When ChIP-AP is run, a copy of the used settings table is saved as a tab-separated value file: *[filename]_ setting_table.tsv* in the output save folder. If you have a custom settings table



made and provided it as input, then ChIP-AP will make a 2nd copy of this table in the same output save folder. This decision is made as it is useful documentation of the run performed. This file is also useful for re-running of the pipeline after run failure or some tweaking if necessary. If submitting an issue request on Github, you ***must*** provide us your settings table used as well as all other requested information. See Github for details regarding this.

We consider the discemination of the information of this file as vital and essential along with results obtained. The table can be included as a supplemental table in a manuscript or can be included as a processed data file when submitting data to GEO – either way, the information of this file must be presented when publishing data.

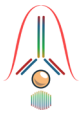
Below is an example of setting table file in its default-setting state.

fastqc1	
clumpify	dedupe spany addcount
bbduk	ktrim=l hdist=2
trimmomatic	LEADING:20 SLIDINGWINDOW:4:20 TRAILING:20 MINLEN:20
fastqc2	
bwa_mem	
samtools_view	-q 20
plotfingerprint	
fastqc3	
macs2_callpeak	
gem	-Xmx10G --k_min 8 --k_max 12
sicer2	
homer_findPeaks	
genrich	--adjustp -v
homer_mergePeaks	
homer_annotatePeaks	
fold_change_calculator	--normfactor uniquely_mapped

As can see, certain flags and values for some programs have been preset as per our testing and opinions. A point to note however, some flags for programs, such as -BAMPE in MACS2, are not listed since they are “hard-coded” into the pipeline and cannot be modified. For this example of -BAMPE in MACS2, this is “hard-coded” because this flag is essential for running peak calling in paired-end datsets. Parameters and flags like this that must be set are “hard-coded” and hidden and cannot be changed unless by choosing the appropriate narrow/broad run modes. A listing of all these “hard-coded” parameters can be found on pg 21.

Below however is a listing of the values listed above in the default-settings with an explanation as to why we set these values as they are.

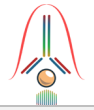
Program	Flag / Argument	Explanation
clumpify	dedupe spany addcount	These parameters are added since they instruct clumpify to remove optical duplicates (dedupe spany) and count the reads input. Clumpify will automatically optimize compression of output gz files with no additional parameters.
bbduk	ktrim=l hdist=2	The flag ktrim=l will instruct bbduk to operate in lef-trimming mode for matched adaptor kmers, ie, trim from the left/5' end of the read. The flag hdist=2 flag instructs bbduk to use a hamming distance of 2 when processing kmers.
Trimmomatic	LEADING:20 SLIDINGWINDOW:4:20 TRAILING:20 MINLEN:20	These 4 flags and parameters instruct Trimmomatic (T) on how to trim reads. The LEADING:20 flag directs T to remove bases at the 5' start of the read with PHRED scores <20. Likewise, TRAILING:20 instructs T to do the same but for the 3' end of the read. The SLIDINGWINDOW:4:20 paramater instructs T to use a sliding window of 4bp, and to scan the entire read and when the average PHRED score drops below 20, then T will trim the remainder of the read at that point. Finally, the MINLEN:20 flag instructs T to drop all trimmed reads with a length <20bp as we believe shorter reads will likely map more ambiguously to the genome and not be informative.
samtools view	-q 20	This option instructs samtools to remove all aligned reads with a MAPQ score < 20. This is seen as an optional but recommended step to remove reads with less favourable mapping scores. For more information on MAPQ scores, search online as that discussion is beyond the scope of this explanation.
GEM	-Xmx10G --k_min 8 --k_max 12	As GEM is java jar package, the amount of ram required to run needs to be specified. For this, the -Xmx10G flag is used which allocates 10Gb of ram for GEM to run. This can (and probably) should be increased if your system has the ram to accommodate it, to avoid any potential issues with out of memory errors and crashes. The –k_min and –k_max flags instruct GEM to search for kmers between 8 and 12bp. Depending on your datset you may need to change this, but for defaults these values will suffice.
Genrich	--adjustp	This flag is ***NOT*** part of Genrich's default behaviour. We noted aberrant peak calling with low coverage datasets in our dataset and confirmed this behaviour with the developer in private communications. Through our testing, we derived equations that allow us to curtail Genrich's aberrant behaviour in such scenarios. This



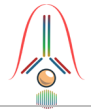
		adjustment is performed and derived by us and is not attirubuted to Genrich and/or its developer(s).
FC Calculator	--normfactor uniquely_mapped	This flag instructs the fold-change calculator script to only consier uniquely-mapped reads in the fold-change calculation. While multi-mapped reads might be important to consider in some scenario's, we deemed it more accurate to calculate FC based on uniquely-mapped reads.

The next pages are a listing of all the "hard-coded" settings hidden in ChIP-AP's code with a brief explanation of why they are used. Full details can be found in the official documentation for each program.

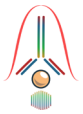
Program Sub-cmd	Flag	Definition	Explanation
fastqc1	-t	Number of threads	Determined by ChIP-AP **--thread**
	-o	Output directory	Locked to *[output directory] / 01_raw_data*
clumpify	in=	Sequencing data input	Locked by ChIP-AP file naming and pathing
	out=	Sequencing data output	Locked by ChIP-AP file naming and pathing
	in2=	Sequencing data input	Locked by ChIP-AP file naming and pathing
	out2=	Sequencing data output	Locked by ChIP-AP file naming and pathing
bbduk	in=	Sequencing data input	Locked by ChIP-AP file naming and pathing
	out=	Sequencing data output	Locked by ChIP-AP file naming and pathing
	in2=	Sequencing data input	Locked by ChIP-AP file naming and pathing
	out2=	Sequencing data output	Locked by ChIP-AP file naming and pathing
	ref=	Path to adapter sequences file	Locked to *[path to genome folder] / bbmap / adapters.fa*
trimmomatic	SE	Flag to use single-end reads mode	Determined by ChIP-AP --mode
	PE	Flag to use paired-end reads mode	Determined by ChIP-AP --mode
	-threads	Number of threads	Determined by ChIP-AP --thread
fastqc2	-t	Number of threads	Determined by ChIP-AP --thread
	-o	Output directory	Locked by ChIP-AP file naming and pathing
bwa_mem	-t	Number of threads	Determined by ChIP-AP --thread
Samtools view	-@	Number of threads	Determined by ChIP-AP --thread
	-h	Flag to include SAM file header	Locked to use -h
	-b	Flag to produce result in BAM format	Locked to use -b
plotfingerprint	-p	Number of threads	Determined by ChIP-AP --thread
	-b	Sequencing data input/output	Locked by ChIP-AP file naming and pathing
	-l	Sample input/output labels	Locked by ChIP-AP file naming and pathing



	-o	Output directory	Locked by ChIP-AP file naming and pathing
fastqc3	-t	Number of threads	Determined by ChIP-AP --thread
	-o	Output directory	Locked by ChIP-AP file naming and pathing
macs2 callpeak	-f	Input format	Locked to SAM format
	-t	Sequencing data input/output	Locked by ChIP-AP file naming and pathing
	-c	Sequencing data input/output	Locked by ChIP-AP file naming and pathing
	-g	Sample genome size	Determined by ChIP-AP internal genome size data
	--name	Output prefix	Locked by ChIP-AP file naming and pathing
	--outdir	Output directory	Locked by ChIP-AP file naming and pathing
	--broad	Flag to use broad peak calling mode	Determined by ChIP-AP --peak
gem	--expt	Sequencing data input/output	Locked by ChIP-AP file naming and pathing
	--ctrl	Sequencing data input/output	Locked by ChIP-AP file naming and pathing
	--t	Number of threads	Determined by ChIP-AP --thread
	--d	Path to file containing sample reads distribution	Locked to *[path to genome folder] / GEM / Read_Distribution_default.txt*
	--g	Path to file containing sample genome chromosome sizes	Locked to *[path to genome folder] / GEM / [sample genome reference build].chrom.sizes*
	--genome	Path to file containing sample whole genome FASTA sequence	Locked to *[path to genome folder] / GEM / [sample genome reference build]_Chr_FASTA*
	--s	Sample genome size	Determined by ChIP-AP internal genome size data
	--f	Input format	Locked to BAM format
	--out	Output directory	Locked by ChIP-AP file naming and pathing
sicer2	-cpu	Number of threads	Determined by ChIP-AP --thread
	-t	Sequencing data input/output	Locked by ChIP-AP file naming and pathing
	-c	Sequencing data input/output	Locked by ChIP-AP file naming and pathing
	-s	Sample genome reference build	Determined by ChIP-AP --ref
Homer findPeaks	-style	Sample peak type	Determined by ChIP-AP --peak



	-gsize	Sample genome size	Determined by ChIP-AP internal genome size data
	-o	Output file name	Locked by ChIP-AP file naming and pathing
	-i	Input directory	Locked by ChIP-AP file naming and pathing
genrich	--mode	Sample read mode	Determined by ChIP-AP --mode
	-y	Flag to keep unpaired reads	Determined by ChIP-AP --mode
	-t	Sequencing data input/output	Locked by ChIP-AP file naming and pathing
	-o	Output file name	Locked by ChIP-AP file naming and pathing
	-c	Sequencing data input/output	Locked by ChIP-AP file naming and pathing
Homer mergePeaks	-prefix	Output merged peaks files prefix	Locked to [setname]_merged_peaks
	-matrix	Output data matrix files prefix	Locked to matrix; useful but not mandatory for subsequent processes for now
	-venn	Output venn diagram file name	Locked to venn.txt; useful but not mandatory for subsequent processes for now
Homer annotatePeaks	-m	Path to file containing known motifs (.motif)	Determined by ChIP-AP --motif
	-nmotifs	Flag to report the number of motifs per peak	Mandatory for subsequent processes
	-matrix	Output data matrix files prefix	Locked to *[output directory] / 22_peaks_processing*
	-go	Output directory for HOMER genome ontology analyses	Mandatory for subsequent processes
fold_change_ calculator	--thread	Number of threads that will be used to run this	Determined by ChIP-AP –thread
	--input_tsv	Path to input peak list	Locked to *[output directory] / 22_peaks_processing / [setname]_all_peaks_annotated.tsv*
	--output_tsv	Path to output peak list	Locked to *[output directory] / 22_peaks_processing / [setname]_all_peaks_calculated.tsv*
	--chip_bam	Sequencing data input/output	Locked by ChIP-AP file naming and pathing
	--ctrl_bam	Sequencing data input/output	Locked by ChIP-AP file naming and pathing
	--peak	Sample peak type	Determined by ChIP-AP --peak



Effective Genome Sizes

hg19, hg38, mm9, mm10, and dm6 effective genome size are derived from:

https://deeptools.readthedocs.io/en/develop/content/feature/effectiveGenomeSize.html

hg19_effective_genome_size = '2864785220'

hg38_effective_genome_size = '2913022398'

mm9_effective_genome_size = '2620345972'

mm10_effective_genome_size = '2652783500'

dm6_effective_genome_size = '142573017'

The sacCer3 effective genome size was manually calculated based on the number of non-N characters in the genome's FASTA file.

sacCer3_effective_genome_size = '12071326'

ChIP vs Control fold change calculation

For transcription factor samples (narrow peaks), ChIP weighted peak center coordinate is determined by the median coordinate of all reads in the peak region. Fold change was then calculated as read depth in ChIP sample divided by non-zero read depth of control sample at the weighted peak center coordinate.

For histone modifier samples (broad peak type), fold change was simply calculated based on average read depth in ChIP sample, divided by non-zero average read depth of control sample, along the same peak region.

To correct for read depth discrepancies due to imbalanced number of reads between ChIP and control samples, ChIP-AP uses one of three available normalization factors depending on the commandline flag specified:

- (Default) Based on only uniquely mapped reads in ChIP vs control bam files (filtered using samtools view -F256 and counted using samtools view -c)
- Based on only successfully mapped reads in ChIP vs control bam files (filtered using samtools view -F4 and counted using samtools view -c)
- Based on user-determined value.

Advanced users may choose to change this by changing the argument field for fold_change_calculator in the settings table (details on pg 22) as follows:

- --normfactor uniquely_mapped
    - Default - based on uniquely mapped reads; default setting
- --normfactor mapped
    - change to all mapped reads
- --normfactor user_value --chip_norm [x] --ctrl_norm [y]
    - change to user-determined normalization factor, where x / y is the user-intended ratio of the number of ChIP reads to the number of control reads

Genrich p-value threshold adjustment formula

Based on our testing, Genrich tends to misbehave when processing datasets with low read depth. It starts to call low enrichment regions as peaks, leaving users with an impossibly large number of called peaks. We contacted the developer of Genrich and confirmed such behaviour with them. Through our testing, we derived equations that allow us to curtail Genrich's aberrant behaviour in such scenarios. This adjustment is performed and derived by us and is not attirubuted to Genrich and/or its developer(s).

To avoid the afore mentioned peak calling depth issue, we curtail such behaviour by setting up an auto-adjusting p value threshold that responsively raises the default limit of peak's minimum p value as the read depth gets lower using the following equations Q1, Q2, Q3.

Q1 - genrich_negative_log_p_threshold =

$$2^{[((-0.016 * mapped\_read\_count\_average) + 0.5) * \log_2(mapped\_read\_count\_average)\ ]}$$

Q2 - adjusted_genrich_negative_log_p_threshold =

$$0.5 * genrich\_negative\_\log\_p\_threshold$$

Q3 - genrich_p_threshold =

$$10^{-adjusted\_genrich\_neg\_\log\ \_p\_threshold}$$

Interpreting ChIP-AP Output

Ok so ChIP-AP does report a fair amount of stuff. If you ran it locally you have a swath of folders and you have nooooo clue what to look for and its all confusing. We get that. The reality though its very simple to know what to look for to know your experimental run worked and in this section were going to walk you through that!

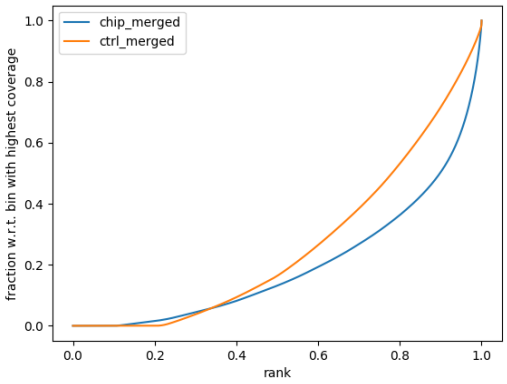
Did my analysis work?

There are a couple of things to look for to answer this question. 1, the fingerprint plot and 2, the venn diagram of the merged peaks. Lets begin…

1 – The fingerprint plot

The fingerprint plot tells us how well the enrichment of your samples worked. It is generated by the function from the deeptools package and is generated after the alignment files. As such, the plots are found in the *"08_results"* folder and are labelled "fingerpring_xxxxx.png/svg." The PNG files allow you to view them in any image viewer, the SVG files are for opening in Adobe Illustrator or Inkscape to make HQ publication figures later if you need.

To interpret the fingerprint plot, (more information can be found on the deeptools documentation site), but the put simply (image to the right), the input control should be a diagonal line as close as possible toward the 1:1 diagonal. Your ChIP sample should have a bend/kink towards the bottom right corner. The greater the separation between the input and the chip sample, the greater the enrichment you will see in the final result (ie lots of peaks). If the lines are overlapping, then you will see little enrichment and your experiment didn't work that well. If you're sample lines are switched – then you proabably switched the sample names and we recommend doing the right thing and repeating the experiment and not simply switch sample names for the sake of a publication.



In this example, there is reasonable enrichment in our chip samples. And so we are confident we can see enrichment.

2 – The Venn Digaram (well Venn Text)

In the folder *"21_peaks_merging"* folder, you will find the *"venn.txt"* file. This will show you a textual venn diagram of the overlap between the called peaks across all peak callers. To know your experiment worked well, then you should see a full list with combinations of all peak callers and relatively large numbers for the consensus peak sets (ie peaks called by multiple peak callers) – this is the ideal case. However, from our experiment, there will almost always be 1 maybe 2 peak callers that don't like a dataset for some reason and so you may find a peak caller performed poorly but the others performed admirably. This is still a good and valid result. If you look at this file and only see small number of peaks and little overlap, and only 1 peak caller seems to have dominated peak

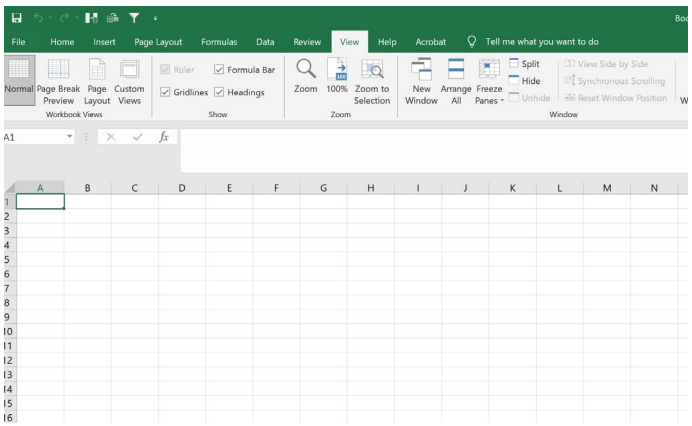
MACS2	SICER2	HOMER	Genrich	Total	Name
			X	103	Genrich
		X		2151	HOMER
		X	X	12	HOMER|Genrich
	X			14499	SICER2
	X		X	328	SICER2|Genrich
	X	X		10346	SICER2|HOMER
	X	X	X	687	SICER2|HOMER|Genrich
X				522	MACS2
X			X	606	MACS2|Genrich
X		X		78	MACS2|HOMER
X		X	X	44	MACS2|HOMER|Genrich
X	X			1115	MACS2|SICER2
X	X		X	714	MACS2|SICER2|Genrich
X	X	X		12833	MACS2|SICER2|HOMER
X	X	X	X	28549	MACS2|SICER2|HOMER|Genrich

calling, then likely your experiment didn't work that great. Just because only 1 peak caller performed well though, doesn't mean the experiment is a write-off and a failure. It can still be valid and so doings some manual validations on the top FC differential peaks by chip-PCR might give you an indication whether there is salvageable data or not. Also if you have other confirmatory experimental evidence then even 1 peak calling getting results is fine. This is why we implemented multiple peak callers, because there are many instances where the signal:noise just creates a mess for most peak callers but generally 1 will be the super-hero of the day in such a situation.

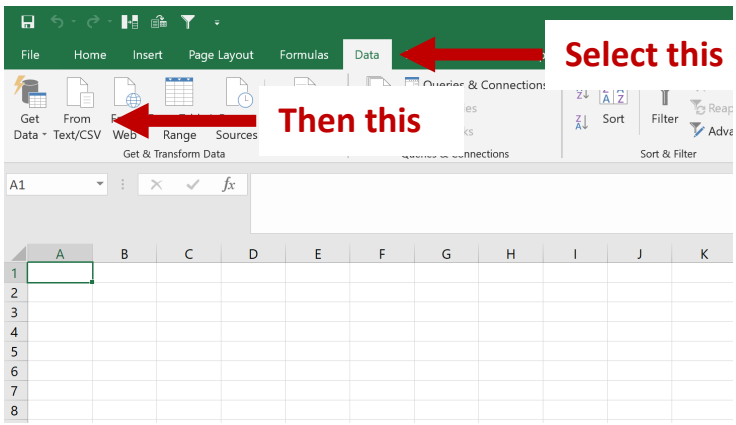
3 – What results files do I look at exactlty?

Valid question. In the folder "22_peak_processing," open the "xxxx_all_peaks_calculated.tsv" file in excel and you're good to go. Now to open it there is a little step to do…

Open a new blank workbook in excel

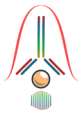


In the ribbon at the top, go to "Data", then select "From Text/CSV"



In the dialog box that opens up, find and open the peaks files "xxxx_all_peaks_calculated.tsv." Follow all the prompts and keep pressing "Next" / "Proceed" till the end and the file opens. Opening the peak file this way circumvents an issue that Excel constantly makes which is it will interpret some gene names such as OCT1 as a date, when its not. So by following the afore mentined steps, excel will not do this stupid conversion and instead, when you save the file as an xlsx, it will ensure that this issue doesn't happen (seen it in sooooo many publications its not funny – just import data this way please people?)

From this file, you can view all the results and data for you analysis. Refer to Interpreting ChIP-AP Output for the definition of what each column means.



4 – How do I view my alignments and data?

People typically want to view their results on UCSC or other genome browsers. As we don't have a web-server to host such coverage files (and making accessible ucsc hub is a real pain and we don't want to implement that), the onus is on you to view them locally on your machine. All laptops, whether then can run ChIP-AP or not can run IGV Downloads | Integrative Genomics Viewer (broadinstitute.org) and view the coverage and bam files. The coverage and bam failes can be located in the *"08_results"* folder.

Download IGV, install it (super easy) and then load the coverage and bam files needed. Make sure you load the right genome build however! That's critical. From section Main Pipeline Output, you can copy columns B,C,D straight into IGV and it will take you to the peak section.

5 – In Short, whats relevant?

Easy answers

1 – check fingerprint plot and make sure it looks good

2 – check venn.txt file and make sure you get good spread of peaks

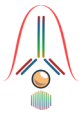
Together points 1 and 2 tell you your experiment worked!

3 – Your final peak file is in *"22_peak_processing"* open the *"xxxx_all_peaks_calculated.tsv"* – This is the file you need to upload to GEO as your processed data file for your analysis and the only file you need to work with when looking through your data.

4 – Also as part of your submission to GEO or as a supplemental table in your manuscript, you MUST include the settings table named *"default_settings_table.txt"* located in the root analysis directory. This provided with the raw fq files, which must be uploaded to GEO, will ensure complete reproducibility of the analysis performed.

5 – Manuscript details for M&M. A statement such as the following should suffice.

For processing our ChIP-Seq analysis, we utilized ChIP-AP (REF). Raw fq files are uploaded to GEO with accession number XXXXX, and the custom settings table utilized for analysis can be found on GEO as a processed settings file and also in supplemental table XX in our manuscript. Full details of ChIP-AP and its function can be found in its corresponding manscruipt (REF).



Q&A

1 – What does ChIP-AP stand for?

A – ChIP-Seq Analysis Pipeline

2 – What should I use as my control? Input or IgG?

A – The consensually accepted control to be used for ChIP-Seq experiments is input control and not IgG. You're free to do so if you wish but that's not whats commonly done in the literature. Take a look at consortia such as ENCODE and you won't find IgG controls, only input.

3 – How are peaks called? In what order?

A – Peaks are called as your ChIP-IP over Input control.

4 – Can ChIP-AP handle an un-balanced number of replicates? (ie 3 ChIP and 1 input?)

A – Yes it can. The initial QC and cleanup steps are done on a per replicate basis, but after the alignment step, samples are merged and the remaining peak-calling, annotation, FC calculations etc… are done on the merged sample sets.

5 – I've only input 1 sample ChIP and 1 Input control, why are there "_merged" files in the output directories?

A – Yeah… ideally ChIP-AP shouldn't do this and we're aware of this behaviour. The contents of the single replicate file and the merged are the same though so theres not really an issue which file you refer to as long as you're consistent. We will address this small issue in future updates.

6 – Why are you using BWA instead of other aligners such as Bowtie2?

A - BWA is used in preference to other aligners such as Bowtie2 as in benchmarking papers such as (Thankaswamy-Kosalai et al., 2017) and others, we were more statisfied with the results and performance of BWA over others, hence its inclusion in this pipeline. Personal preference ppl.

7 – Can ChIP-AP handle bam files from other aligners as input, or only BWA?

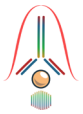
A – Technically it should be able to handle alignment bam files from any aligner provided the files are formatted correctly. We have not tested it thoroughly though, but should still be fine. If there are issues let us know through the githib and we can work together to resolve such matters.

8 – I have developed this incredibly awesome new peak caller called Peak2DaMax, can it be incorporated into ChIP-AP?

A – Owing to the modular nature of ChIP-AP, it is realtively easy for additional programs to be added/removed if need be. You can attempt to modify the source files to get this working and reach out if you have any issues. As for officially including it into ChIP-AP… Reach out to us and we can zoom.

9 – Can ChIP-AP be setup on a shared computing cluster?

A – Technically yes it can. Have we done it? Yes (and no, we have access to 2 clusters, we've set it up on 1 and not the other yet). Was the installation more involved? Yes. Every shared computing cluster has its own configuration in terms of available software and personal profile configuration requirements. Its too difficult for us to do every possible combination in advance. Refer to the References and Citations section to see what programs need to be installed/available to for



ChIP-AP to run and setup your environment accordingly to access those programs. ChIP-AP will also run a pre-flight check before each run to ensure everything is available and accessible. So check the logs to make sure ChIP-AP is happy and then you will be ok. If unfamiliar with working in said environments, contact the required IT support for the shared cluster to help you.

10 – The installer script downloads genomes for which I'm never going to use (like fly), what gives? Why cant I chose what to install?

A – We have tried to streamline the installation as much as possible so we just install everything by default. Maybe in the future we will go through and make a more thorough installer with more fine-grained options but for now this is how it is. You can modify the installer script if you are savy enough to remove those commands – that's up to you. This is why we provide all the code used for everything, so you can tinker with the pipeline how you want. If you break it though… The other option is wait till everything installs and then delete what you don't want – the HOMER install directories are quite large for sure.

For now its an all or nothing installer. Future updates should address being able to add/remove aspects of the installation without having to wipe the slate clean and start over. For a 1st release though, we are happy with this behaviour for now.

11 – If I get ChIP-AP working on my institutes computing cluster SuperUltraMegaVoltron, can you include the guide on the wiki?

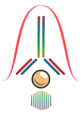
A – If you're computing cluster is called SuperUltraMegaVoltron then most certainly YES we can work together to get your installation guide up on the wiki simply to say we have it working on SuperUltraMegaVoltron (but does it have a V-MAX option??). Even if It doesn't have an exciting name as that and is simply called BigPuddle, we will still work with you to get the installation guide up on the wiki. We want ChIP-AP to become the go-to tool for analyses. So we will work towards that goal with anyone who is willing to help and collaborate.

12 – Is there a difference in the output between the GUI and command line versions of ChIP-AP?

*A – Nope! We have designed ChIP-AP to be just as functional from the GUI or command line. Any feature accessible on the command line is also accessible from the GUI. However, with great power comes great responsibility! If you modify the settings table without knowing what you're doing you **will** break the pipeline run and get wrong results. This option, while available for modification in the GUI, really is intended for the most advanced of users. Even most "bioinformaticians" wont know how to take full advantage of this functionality and will break the pipeline more times than improving its efficiency. So, the ability is there, but don't use it unless you **REALLLLLYYYYY** know what you're doing. This really goes for everyone, biologist or bioinformatician.*

13 – What versions of python are compatible?

A – From our testing, python3 is required, ChIP-AP is not compatible with python2. However, we have also noted that on macOS in particular, python 3.7.x is required and ChIP-AP will not work with python 3.8.x. This is because of how multi-processing is handled in 3.8 on macOS in particular. This doesn't appear to be an issue in our usage with Linux based installations though.



References and Citations

If you use ChIP-AP in your analysis, please cite the us and all the following programs

Programs	References
ChIP-AP v4.1	Guide: https://github.com/JSuryatenggara/ChIP-AP/wiki/ChIP-AP-Guide Github: https://github.com/JSuryatenggara/ChIP-AP Citation: (coming soon…)
Python3 Linux 3.7.x/3.8.x macOS 3.7.x	We have noted in our testing that there is a change in python 3.8 on macOS in how multi-threading is handled which breaks ChIP-AP. As such, for macOS installs you must ensure that ptyhon3.7.x is installed. If using our installation guides, the provided yml files will ensure all the correct dependencies and requirements are met automatically.
FastQC v0.11.9	Guide: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ GitHub: https://github.com/s-andrews/FastQC
Clumpify v38.18 (BBmap)	Introduction: https://www.biostars.org/p/225338/ Guide: https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/clumpify-guide/ GitHub: https://github.com/BioInfoTools/BBMap/blob/master/sh/clumpify.sh Citation: https://www.osti.gov/biblio/1241166-bbmap-fast-accurate-splice-aware-aligner
BBDuk v38.18 (BBmap)	Introduction: http://seqanswers.com/forums/showthread.php?t=42776 Guide: https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/ GitHub: https://github.com/BioInfoTools/BBMap/blob/master/sh/bbduk.sh Citation: https://www.osti.gov/biblio/1241166-bbmap-fast-accurate-splice-aware-aligner
Trimmomatic v0.39	Guide: http://www.usadellab.org/cms/?page=trimmomatic Downloadable manual page: http://www.usadellab.org/cms/uploads/supplementary/Trimmomatic/TrimmomaticManual_V0.32.pdf GitHub: https://github.com/timflutre/trimmomatic Citation: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4103590/
bwa v0.7.17	Guide: http://bio-bwa.sourceforge.net/bwa.shtml GitHub: https://github.com/lh3/bwa Citation: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2705234/
samtools view v1.9 (samtools)	Guide: http://www.htslib.org/doc/samtools-view.html GitHub: https://github.com/samtools/samtools Citation: https://pubmed.ncbi.nlm.nih.gov/19505943/
deeptools plotFingerprint v3.5.0 (deepTools)	Guide: https://deeptools.readthedocs.io/en/develop/content/tools/plotFingerprint.html Citation: https://academic.oup.com/nar/article/44/W1/W160/2499308?login=true
MACS2 v2.2.6	Guide: https://hbctraining.github.io/Intro-to-ChIPseq/lessons/05_peak_calling_macs.html Citation: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2732366/ GitHub: https://github.com/macs3-project/MACS/wiki
GEM v2.7	Guide: https://groups.csail.mit.edu/cgs/gem/ GitHub: https://github.com/gifford-lab/GEM Citation: https://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1002638
SICER2 v1.0.2	Guide: https://zanglab.github.io/SICER2/ GitHub: https://github.com/bioinf/SICER2 Citation: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2732366/

HOMER findPeaks v4.11 (HOMER)	Guide: http://homer.ucsd.edu/homer/ngs/peaks.html Citation: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2898526/
Genrich v0.6	Guide: https://informatics.fas.harvard.edu/atac-seq-guidelines.html GitHub: https://github.com/jsh58/Genrich
Homer mergePeaks v4.11 (HOMER)	Guide: http://homer.ucsd.edu/homer/ngs/mergePeaks.html Citation: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2898526/
HOMER annotatePeaks v4.11 (HOMER)	Guide: http://homer.ucsd.edu/homer/ngs/annotation.html Citation: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2898526/

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