



Analysis of Bisulfite Sequencing PCR

User guide

Version 1.0.0

Marie Denoulet and Chann Lagadec

Univ. Lille, CNRS, Inserm, CHU Lille, UMR9020-U1277 - CANTHER - Cancer Heterogeneity Plasticity and Resistance to Therapies, F-59000 Lille, France

June 13, 2022

Contents

List of Figures	3
List of Code Listings	3
1 General information	4
1.1 What ABSP does do?	4
1.2 What are the advantages of using ABSP?	4
1.3 Bisulfite Sequencing PCR	5
1.4 How does ABSP work?	7
1.5 License	9
2 How to proceed with analysis using ABSP?	12
2.1 Open ABSP for the first time	12
2.1.1 Download and installations	12
2.1.2 Content of the ABSP main directory	12
2.1.3 Launch the app	13
2.2 Open ABSP (not for the first time)	14
2.2.1 Launch the app	14
2.3 Update ABSP	14
2.3.1 Launch the app	14
2.4 Individual analysis	14
2.4.1 Input files requirements	14
2.4.2 Procedure	16
2.4.3 Output report	17
2.4.4 Output files	22
2.5 Grouped analysis	24
2.5.1 Procedure	24
2.5.2 Output report	25
2.5.3 Output files	27
2.6 Multiple analyses	33
2.6.1 Input files requirements	33
2.6.2 Procedure	34
2.6.3 Output files	35
3 Complementary information	35
3.1 Some recommendations for the BSP experiment	35
3.2 Detailed workflow of ABSP individual analysis	35
3.3 Code modifications	37
3.3.1 List of reference genomes	37
3.3.2 Modify the default thresholds	38
3.3.3 Modify the plots colors and point shapes	39

4	Troubleshooting guide	41
4.1	General	41
4.2	Individual analysis	43
4.3	Grouped analysis	46
5	Acknowledgments	47

List of Figures

1	Bisulfite Sequencing PCR experimental principle	5
2	Bisulfite Sequencing PCR analysis strategies for both direct-BSP and cloning-BSP	6
3	Workflow of the ABSP analytic process	8
4	Detailed workflow of the ABSP analytic process	10
5	Diagram of the possible ways to launch ABSP analyses	11
6	Example of <i>.fasta</i> file for the reference DNA input required for ABSP individual analysis	15
7	IGV (Integrative Genomics Viewer) software window	16
8	Example of trimming plot from the output report	20
9	Diagram of output directories to locate output files from individual analysis	23
10	Visualization plots of all replicates (direct-BSP)	28
11	Visualization plots of all clones from one sample (cloning-BSP) . .	29
12	Visualization plots of groups (means of replicates/clones per sample)	30
13	Boxplots and methylation profile plots	32
14	Diagram of output directories to locate output files from individual analysis	32
15	Detailed workflow of the individual analysis	36

List of Code Listings

1	List of reference genomes displayed in the drop-down lists	37
2	Genome name selected by default in the individual analysis tab. . .	37
3	Genome name selected by default in the grouped analysis tab. . .	38
4	Default thresholds in the individual analysis script	38
5	Default thresholds in the grouped analysis script	39
6	Colors and shapes setting for plots in the grouped analysis script .	40

1 General information

1.1 What ABSP does do?

ABSP, standing for "[Analysis of Bisulfite Sequencing PCR](#)", is an R-based tool to analyze CpG methylation profiles using data from Bisulfite Sequencing PCR (BSP) experiment results. It was developed to help researchers to estimate and compare methylation percentages of a DNA region studied using BSP experiments. It provides a complete automated workflow, from trace file sequencing results to data visualization and statistics.

1.2 What are the advantages of using ABSP?

- **A complete workflow.** ABSP uses as input the chromatogram trace files as the sequencing results, and through a two-steps analysis, it (1) computes the methylation percentages of individual samples after validating the sequencing quality and (2) gathers the methylation levels from all samples to summarize methylation data, generate publication-ready figures and perform comparative statistics to answer to the experiment hypothesis on the DNA methylation differences between conditions.
- **A fully automated process.** ABSP uses a shiny app on R to provide a user-friendly interface. To launch the analytic process the user is guided to provide the required inputs and can launch the desired analysis with one click. For each analysis, an HTML report file is generated to visualize the results and keep a record of them. Additionally, output files, such as tables and figures, are automatically saved in the corresponding result folders. For an even more automated use, several analyses can be launched with the help of pre-filled input tables (spreadsheet document to fill) in the special tab "Multiple analyses", which is useful for large amounts of samples.
- **Analyses of both direct-BSP and cloning-BSP sequencing data.** ABSP can analyze results from both BSP methods. No existing tool is currently able to analyze both. It allows continuity in the experiment analytic process, as the direct-BSP approach can be performed before cloning the PCR products to have preliminary insights on DNA methylation, and then further confirmed/validated using cloning-BSP.
- **Accessible and flexible.** ABSP is coded using R, a cross-platform tool language increasingly used in biology research, making it very accessible to any researchers. Additionally, for researchers accustomed to R coding, as the entire scripts are provided, ABSP is fully upgradeable. Also, we provide specific guidelines to easily modify some features to adapt ABSP to experiment needs, such as adjusting quality thresholds or changing graphical parameters (see section 3.3 Code modifications at page 37).

1.3 Bisulfite Sequencing PCR

The Bisulfite Sequencing PCR (BSP) is an experimental technique aiming to estimate methylation levels of CpG sites on a specific DNA region of interest, among a population of DNA molecules. The method was originally developed by Frommer *et al.* in 1992¹ and Clark *et al.* in 1994² and was named BSP in opposition to the methylation-specific PCR (MSP) method by Li *et al.* in 2002.³

This method is composed of three steps, described in figure 1:

1. A DNA bisulfite conversion
2. A PCR amplification and an optional cloning
3. A sequencing of either PCR products or individual subclones

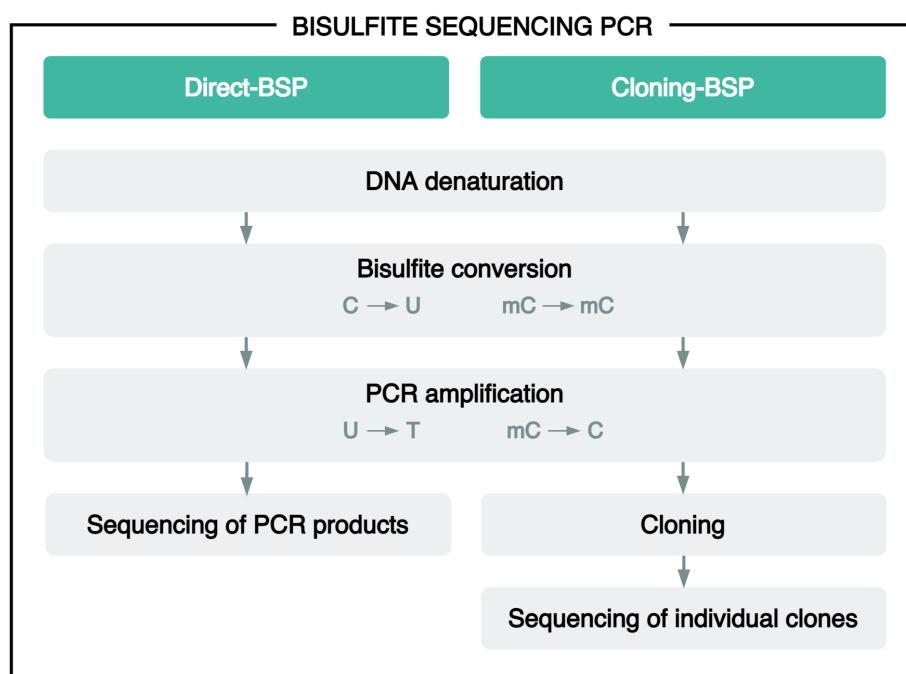


Figure 1. Bisulfite Sequencing PCR experimental principle.

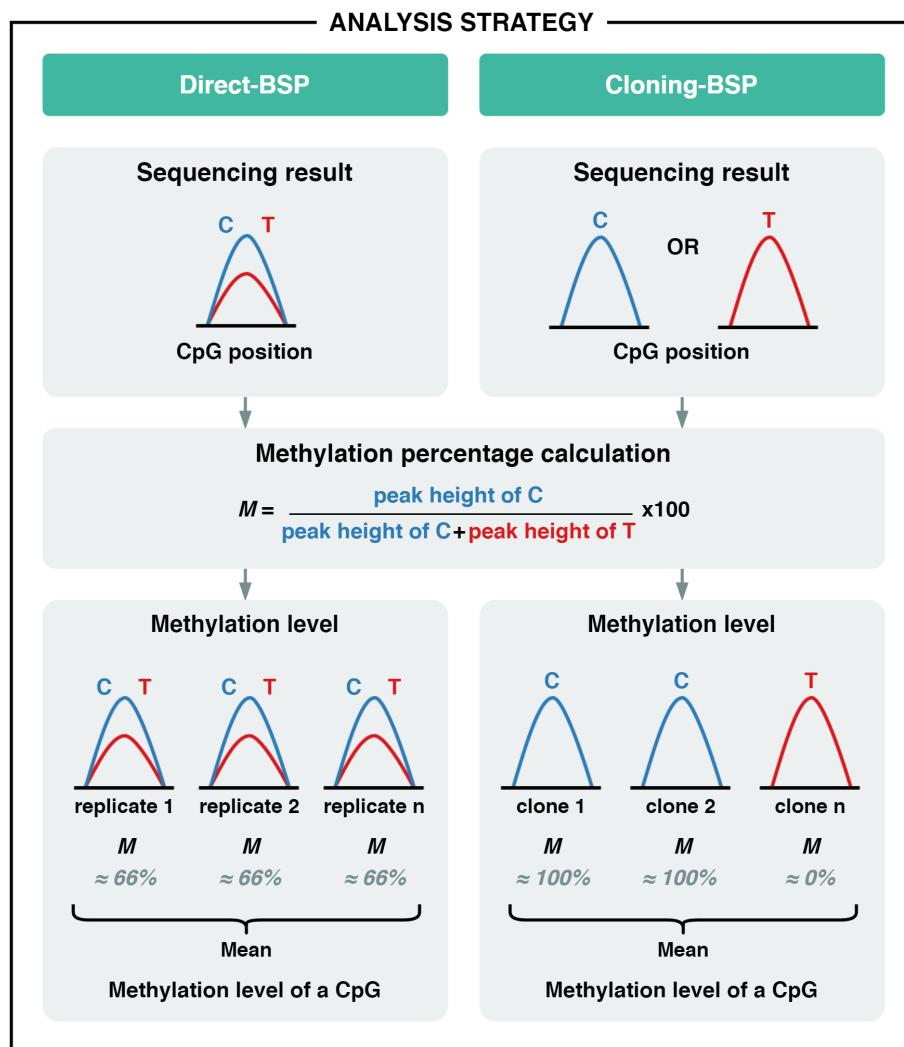
Two approaches of BSP could be used (figure 1). The *direct-BSP* method is characterized by the direct sequencing of PCR products, whereas the *cloning-BSP* consists of cloning PCR products within a specific vector and sequencing several individual clones.

¹M Frommer et al. "A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands." In: *Proceedings of the National Academy of Sciences* 89.5 (1992), pp. 1827–1831. ISSN: 0027-8424. doi: [10.1073/pnas.89.5.1827](https://doi.org/10.1073/pnas.89.5.1827).

²S J Clark et al. "High sensitivity mapping of methylated cytosines." In: *Nucleic acids research* 22.15 (1994), pp. 2990–7. ISSN: 0305-1048. doi: [10.1093/nar/22.15.2990](https://doi.org/10.1093/nar/22.15.2990).

³Long-Cheng Li and Rajvir Dahiya. "MethPrimer: designing primers for methylation PCRs". In: *Bioinformatics* 18.11 (2002), pp. 1427–1431. ISSN: 1367-4803. doi: [10.1093/bioinformatics/18.11.1427](https://doi.org/10.1093/bioinformatics/18.11.1427).

As described in [figure 2](#), the analysis strategies of these two sub-methods are different on some points.



[Figure 2](#). Bisulfite Sequencing PCR analysis strategies for both direct-BSP and cloning-BSP.

First, in **direct-BSP**, a mix of DNA molecules with different unknown methylation statuses are sequenced, thereby, at each CpG site, two base signals can co-exist: the methylated signal (C) and the unmethylated signal (T). By calculating the signal ratio, the methylation level of a CpG in the DNA population can directly be estimated, but for reproducibility and statistical significance purposes, it still needs to be repeated in several biological replicates to obtain the final methylation level of a CpG (direct-BSP results are considered less quantitative than cloning-BSP ones).

Secondly, in **cloning-BSP**, as each clone represents only one PCR product, the methylation status of a CpG can be either methylated (C) or unmethylated (T),

thereby only one of these two signals can exist. Accordingly, the signal ratio can only give either 0% or 100% of methylation (partial methylation is considered biased results), revealing the methylation status. For each CpG, the proportion of clones with a methylated status reveals the methylation level of the CpG in the original DNA population, in general, a minimum of 10 clones is recommended to have a 10% accuracy of the methylation level.

1.4 How does ABSP work?

As a first step, each BSP sequencing result is defined by a combination of experiment information ([figure 3](#)):

- **Sequence.** The sequence identifier refers to a unique amplicon sequence produced by the BSP experiment, using a unique set of primers. For example, if several regions of a gene are analyzed by BSP, as each region corresponds to a unique amplicon they must have distinct sequence names (e.g. *CDH1promoter* and *CDH1exon1*; *CDH1-1*, *CDH1-2*, and *CDH1-3*). Make sure the sequence name is strictly identical for all samples of the same sequence.
- **Collection.** The collection corresponds to a separation of samples above groups. Samples from different collections can not be compared, even if they belong to the same group. For example, collections can be different cell lines, organs, or patients, in which the same groups are compared but not between the different collections. To compare these types of samples, consider them as groups. Make sure the collection name is strictly identical for all samples of the same collection.
- **Group.** The group corresponds to the condition that will be compared with other groups/conditions in the grouped analysis. For example, groups can be the "control" and "treated" conditions. Make sure the group name is strictly identical for all samples of the same group.
- **Replicate.** Information has to be provided only when using the direct-BSP approach. The replicate number refers to the repetition identifier number of the sequencing. To have robust and reproducible data and to perform comparative statistics, each sample needs to be sequenced at least three times (in both directions).
- **Clones.** Information has to be provided only when using the cloning-BSP approach. To estimate the methylation levels among the DNA population, the methylation statuses of several individual clones needs to be sequenced. The ratio of methylated and unmethylated clones for a CpG position will give the methylation level estimation.
- **Sequencing files.** The sequencing read of each sample is performed in both directions, using a forward primer and a reverse primer.
- **Experiment.** The term "experiment (data)" refers to the unique combination

of collection, group, and replicate or clone, for a specific sequence, corresponding to the sample information for the sequencing read.

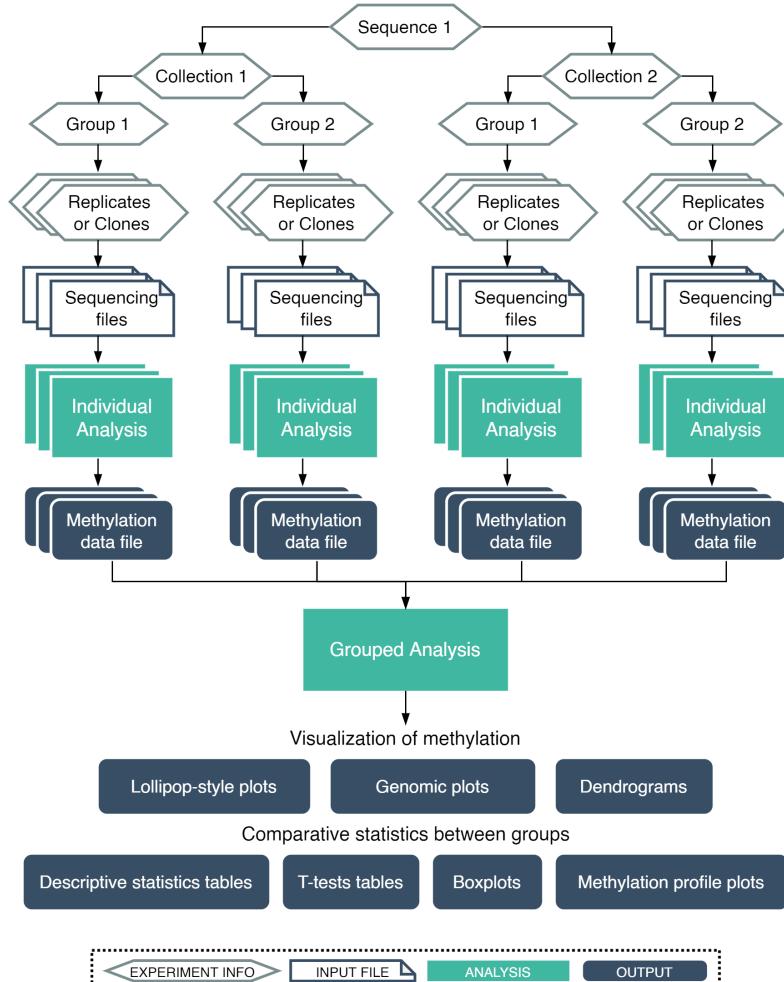


Figure 3. Workflow of the ABSP analytic process.

Then, the ABSP analysis is divided into two steps, corresponding to two scripts (R markdown scripts), as illustrated in the workflow in figure 3.

- **Individual analysis.** For each individual experiment point, two sequencing .ab1 files (one from forward direction, one from the reverse direction) are used as input for the individual analysis. First, the sequencing reads are trimmed based on quality to get the correct sequence for alignments with the reference DNA input. Then, results from the alignments go through a quality control step to check for mismatches, gaps, length of aligned sequences, and bisulfite conversion rates (calculated on cytosines outside CpG that should be thymines). If the results are defined as correct, the methylation levels of CpG can be calculated and visualized on a genomic plot. Several output files (e.g. chromatograms, sequences, tables) are saved in folders, especially the methylation data file as a result of the individual sequencing experiment

analyzed.

- **Grouped analysis.** All methylation data files from the same sequence are gathered by the grouped analysis. First, a preprocessing step is performed to organize data. Then, visualization plots, lollipop-style plots, and genomic plots (with associated clustering dendograms) are generated to view methylation data differences. Finally, a statistical analysis is performed, descriptive statistics tables and Student's t-test p-values tables are generated, as well as boxplots with t-test p-values and methylation profile plots with Kruskal–Wallis test p-values, to display the significant methylation differences.

For more details, the inputs, processes, and outputs of these two steps, individual analysis, and grouped analysis, are displayed in the [figure 4](#), where the 3 tabs of ABSP are represented: "**Individual analysis**", "**Grouped analysis**", and "**Multiple analyses**".

The two steps, individual analysis and grouped analysis, require the manual entry of input data in the corresponding tab, and therefore only one analysis can be launched at the same time ([figure 5](#)). An additional tab has been implemented to launch multiple analyses all at once. This multiple analyses tab can be used to launch either several individual analyses and/or several grouped analyses at the same time, by using tables (.x/sx files or .csv files) as input instead of the manual entry of input for unique analysis.

1.5 License

ABSP, Analysis of Bisulfite Sequencing PCR

Copyright © 2022 by the CANTHER laboratory, France (absp@univ-lille.fr)

Released under the GPL-3 license.

This program is free software: you can redistribute it and/or modify it under the terms of the GNU General Public License as published by the Free Software Foundation, either version 3 of the License, or (at your option) any later version. This program is distributed in the hope that it will be useful, but WITHOUT ANY WARRANTY; without even the implied warranty of MERCHANTABILITY or FITNESS FOR A PARTICULAR PURPOSE. See the GNU General Public License for more details.

You should have received a copy of the GNU General Public License along with this program. If not, see <https://www.gnu.org/licenses/>.

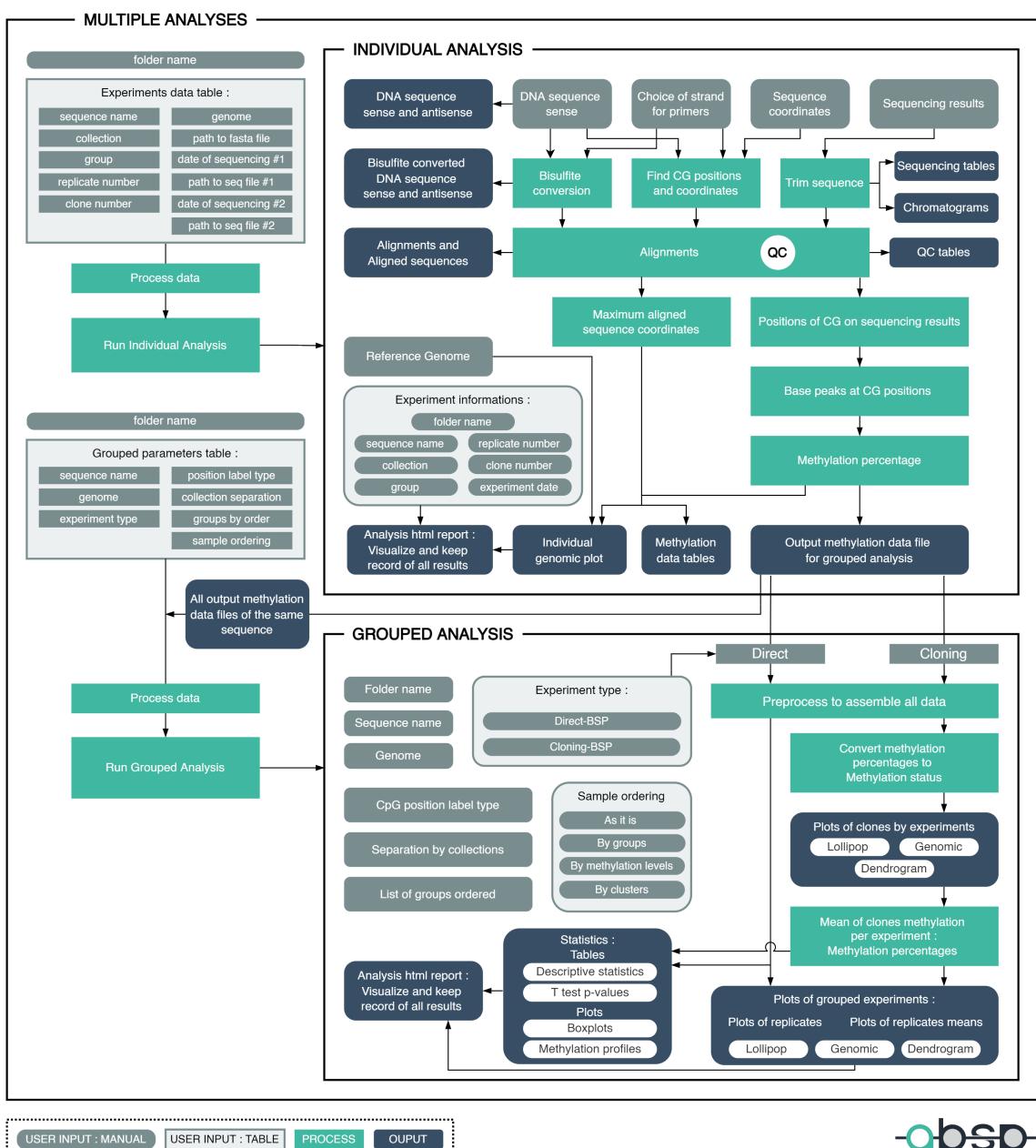


Figure 4. Detailed workflow of the ABSP analytic process.

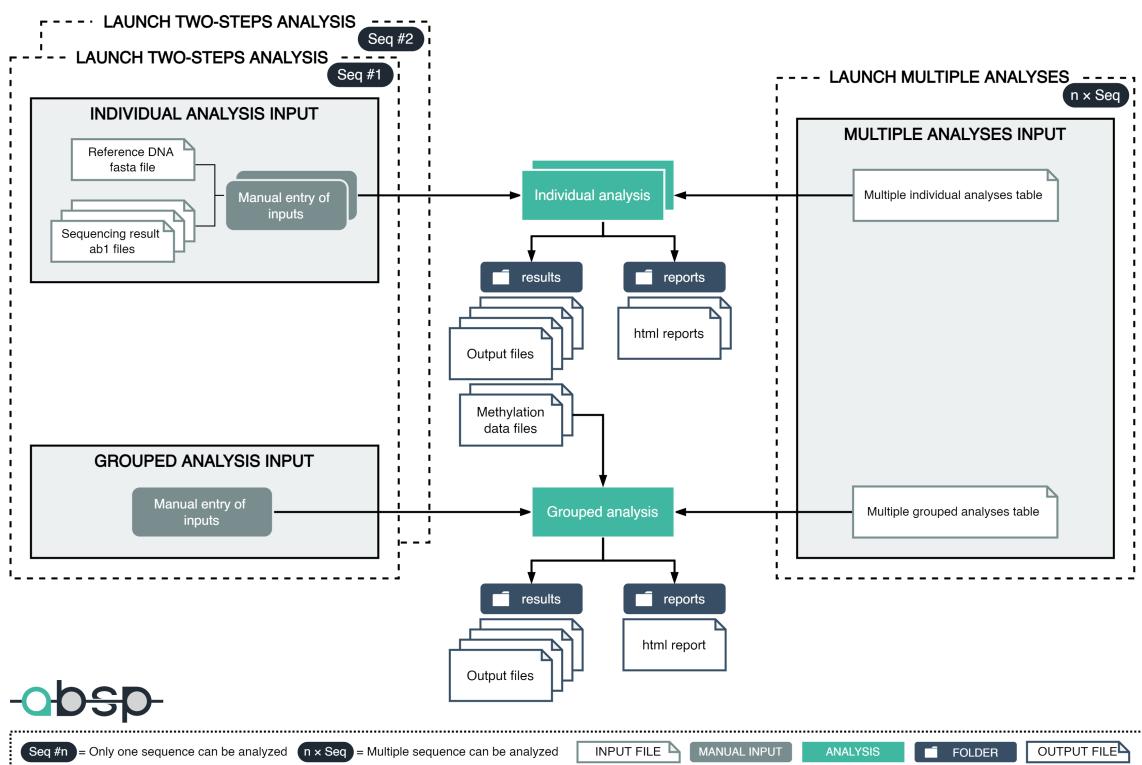


Figure 5. Diagram of the possible ways to launch ABSP analyses.

2 How to proceed with analysis using ABSP?

2.1 Open ABSP for the first time

2.1.1 Download and installations

- Install R: <https://www.r-project.org/>
- Install RStudio: <https://www.rstudio.com/>
- Download the ABSP files on github: <https://github.com/ABSP-methylation-tool/ABSP>

2.1.2 Content of the ABSP main directory

The main ABSP folder is organized as follows:

- **documents** *folder for documents available to the user*
 - List of BSgenomes.xlsx *file listing the available genomes*
 - multiple_grouped_parameters_table.xlsx *table of inputs to launch multiple grouped analyses*
 - multiple_grouped_parameters_table.ods *table of inputs to launch multiple grouped analyses*
 - multiple_individual_analyses_table.xlsx *table of inputs to launch multiple individual analyses*
 - multiple_individual_analyses_table.ods *table of inputs to launch multiple individual analyses*
- **renv** *folder for R environment and packages*
 - **library** *folder for the private project library*
 - activate.R *activation script run by the project .Rprofile*
- **reports** *folder for analysis reports*
- **results** *folder for analysis results (data, tables, graphics...)*
- **scripts** *folder for scripts and associated files required to run analysis*
 - ABSP_functions.R *R script providing the necessary functions for ABSP*
 - ABSP_grouped_analysis.RMD *R markdown script of grouped analysis*
 - ABSP_individual_analysis.RMD *R markdown script of individual analysis*
 - custom.css *CSS script for custom theme settings of the .html report files*
 - logo.svg *ABSP logo vector image*

- **WWW** *folder for files necessary for the shiny app*
 - ABSP - Analysis.svg *diagram of ABSP analysis strategy*
 - ABSP - BSP.svg *diagram of BSP experiment principle*
 - ABSP - fasta file.png *image of a reference sequence .fasta file example*
 - ABSP - Launch analysis.svg *diagram of the different ways to launch ABSP analyses*
 - ABSP - Workflow simple.svg *diagram of the BSP workflow*
 - custom_app.css *CSS script for custom theme settings of the app interface*
 - logo.svg *ABSP logo vector image*
- .Rprofile *file used to activate renv for new R sessions launched in the project*
- ABSP RProject.Rproj *R project file*
- ABSP User Guide.pdf *reference manual to use ABSP*
- app.R *shiny app file*
- renv.lock *lockfile describing the state of the project's library at some point in time*

For ABSP to function properly, all the aforementioned files must be downloaded and present in the ABSP main folder with the same structure.

Make sure not to rename, move or delete the provided folders and files. If you want to reorganize files or folders it is better to copy to other directories than to modify the files. However, new folders can be added to the ABSP main folder without causing issues.

2.1.3 Launch the app

- Open the ABSP Rproject.Rproj file with RStudio (It might take a few minutes to open).
- Open the app.R file with RStudio.
- Find the "Run App" button in the upper right corner, click on the arrow right next to it and select "Run external" (for a better display).
- Click on the "Run App" button to launch the app.
- A pop-up window should appear if the Shiny package was not already installed on your device, click on "Yes" to accept the Shiny installation.

Once this procedure is done, the package installation can start and it might take a few minutes until the app can be opened in the default web browser.

2.2 Open ABSP (not for the first time)

2.2.1 Launch the app

- Open the ABSP Rproject.Rproj file with RStudio.
- Open the app.R file with RStudio.
- Click on the "Run App" button to launch the app.

To launch the different analyses refer to the following sections below which describes the individual analysis, grouped analysis, and multiples analyses ([figure 5](#)).

2.3 Update ABSP

Download the latest version of ABSP files on GitHub: <https://github.com/ABSP-methylation-tool/ABSP>.

2.3.1 Launch the app

Use the new ABSP main directory (latest version) to open ABSP.

- Open the ABSP Rproject.Rproj file with RStudio (It might take a few minutes to open).
- Open the app.R file with RStudio.
- Click on the "Run App" button to launch the app.

You can use your previous input files, but make sure they are compatible with the new version (e.g. the *multiple_individual_analyses_table* and *multiple_grouped_analyses_table* files templates for multiple launches of analyses might have been changed).

As the R project and app are from the new ABSP main directory (latest version), the results and reports will be saved in this directory.

2.4 Individual analysis

2.4.1 Input files requirements

Sequencing result .ab1 files As input, ABSP requires the chromatogram trace file (.ab1) from the sequencing run (Sanger) using the bisulfite converted DNA PCR products as templates. It is highly recommended to have both directions sequenced, by a primer on each side of the PCR product: a forward primer and a reverse primer. Although, the analysis can be run using only one trace file instead of both. The directions do not need to be specified as the analysis will determine it automatically.

Reference DNA sequence and information .fasta file ABSP also requires a *.fasta* file containing information about the reference DNA. A *.fasta* file is composed of a **header** and a **body**. The header must contain both the **genomic coordinates** of this sequence and the **choice of strand**, the one used for primer design, the one that will be amplified by PCR. Indeed, as both strands are no longer complementary after bisulfite conversion, the primers have to be designed on only one bisulfite converted strand as template DNA. The body must contain the **nucleotide sequence** of the reference DNA from the **plus strand** (upper/sense strand) of the genome.

Formats for the *.fasta* file header:

- The **genomic coordinates** must be written in the format *chr#:# #####-#####* (e.g. *chr16:68771087-68771462*).
- The **choice of strand amplified** must be either "*primers=plus*" or "*primers=minus*". If none of these character strings are present in the *.fasta* file, by default the plus strand is chosen.

An example of a *.fasta* file content is depicted in [figure 6](#). Note that any other information in the header, such as the sequence name, for example, can be added without consequences if they do not interfere with the previously described formats.

```
> CDH1 chr16:68770900-68771299 primers=plus
CCAAGTGTAAAAGCCCTTTCTGATCCCAGGTCTTAGTGAGCCACCGCGGGGCTGGGATTGAAACCCAGTGGAAATCAGAAC
CGTGCAGGTCCCATAACCCACCTAGACCTAGCAACTCCAGGCTAGAGGGTCACCGCGTCTATGCGAGGCCGGTGGCGG
GCCGTCAGCTCCGCCCTGGGGAGGGGTCGGCGCTGCTGATTGGCTGTGGCCGGCAGGTGAACCCCTAGCCAATCAGCGTA
CGGGGGGGCGGTGCCCTCCGGGGCTCACCTGGCTGCAGCCACCGCACCCCTCTCAGTGGCGTCGGAACTGCAAAGCACCTGTG
AGCTTGCAGAAGTCAGTTCAGACTCCAGCCCCGCTCCAGCCCCGGCCGACCCGACCGCACCCGGCGCCTGCCCTCGC
```

Figure 6. Example of *.fasta* file for the reference DNA input required for ABSP individual analysis.

As a recommendation, to help with the creation of the *.fasta* file, the [IGV \(Integrative Genomics Viewer\)⁴](#) software can be easily used to navigate the genome and to get the nucleotide sequence of a specific region, alongside with its genomic coordinates. After navigating on the genome, the current viewed region can be added as a region of interest by selecting the "*Regions*" > "*Region Navigator*" > "*Add*" ([figure 7](#)). In this "*Regions of Interest*" panel, the added region will appear in the list of regions, its coordinates can be adjusted and it can be annotated with a description. By right-clicking on a region from this list, a context menu appears and two options can be selected, "*Copy Sequence*" or "*Copy Details*". The first one copies to the clipboard the nucleotide sequence of the region, and the second one copies the genomic coordinates (in the correct format for the *.fasta* file) as well as the description associated with the region. Make sure to properly verify the first and last nucleotides, as there can be a one nucleotide difference between the coordinates and the actual sequence.

⁴<https://software.broadinstitute.org/software/igv/>

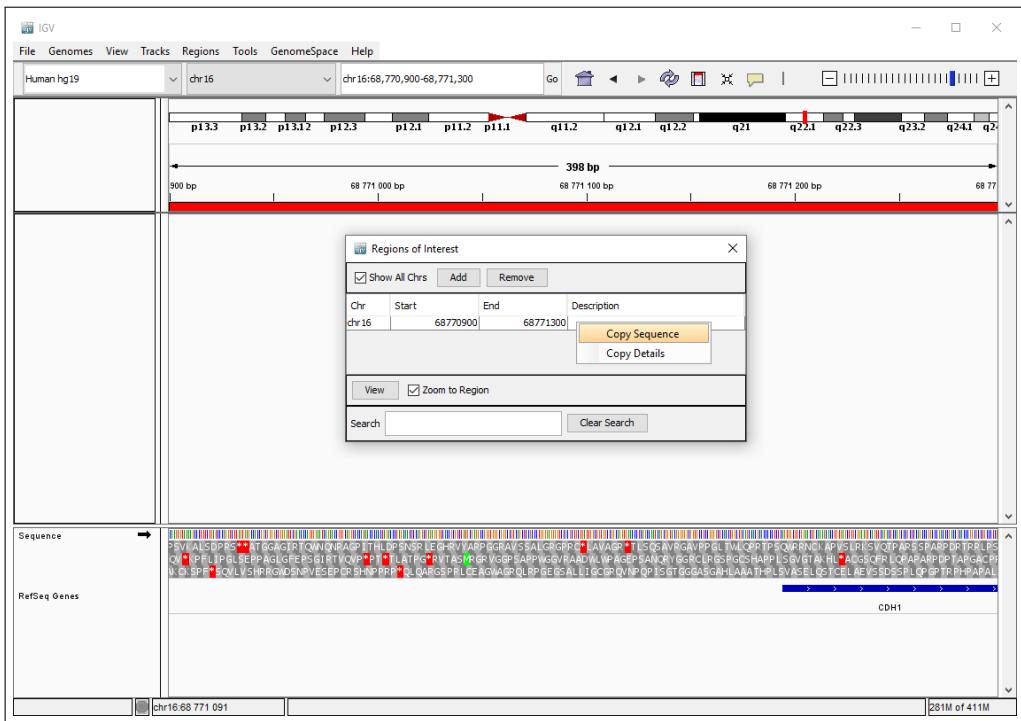


Figure 7. IGV (Integrative Genomics Viewer) software window.

2.4.2 Procedure

In the **individual analysis tab**, the left panel is to launch analysis, and the right panel provides entry information.

Experiment information

- **Select an existing folder or enter a new folder name.** Located in the *AB-SP/results* folder, all of the analysis results will be saved in this folder. Having different folders of results can be used to separate the different analyses by projects, experiments, or users. Note that the six first letters of the folder name will appear in the report file name.
- **Select an existing sequence folder or enter a new sequence name.** The sequence folder is located in the previously selected folder. All of the analysis results will be saved in this folder corresponding to the same sequence. Note that this sequence name will be used in output files (tables, plots...) to refer to the sequence.
- **Enter the collection name.** The collection corresponds to a separation of samples above groups (*details in section 1.4 How does ABSP work? at page 7*). Make sure the collection name is strictly identical for all samples of the same collection.
- **Enter the group name.** The group corresponds to the condition to compare (*details in section 1.4 How does ABSP work? at page 7*). Make sure the group

name is strictly identical for all samples of the same group.

- **For direct-BSP only: Enter replicate number.** In the case of direct sequencing of PCR products only, the replicate number corresponds to the repetition identifier of the sample (*details in section 1.4 How does ABSP work? at page 7*).
- **For cloning-BSP only: Enter clone number.** In the case of clone sequencing only, the clone number corresponds to the identifier number of each clone from the same condition (*details in section 1.4 How does ABSP work? at page 7*).

Reference DNA sequence

- **Select the reference genome.** It will only be used to display the genomic sequence in the genomic plot. Make sure to click on the "Pre-install genome" button if the selected genome is used for the first time. Only a short list of genomes is displayed in the drop-down list but more genome assemblies are available. Go to section 3.3 *Code modifications at page 37* to get information on how to add another genome in the drop-down list. The complete list of available genomes can be found in the "List of BSgenomes.xlsx" file in the "ABSP/documents" folder.
- **Select .fasta file of reference DNA sequence.** As described above in the Input files requirements section, the .fasta file of the reference DNA sequence needs to be selected from your folders.

2.4.3 Output report

The HTML report file of the analysis is automatically saved in the *reports* folder in the ABSP directory.

Header First, in the top panel, the information about the sample experimental conditions is displayed in a table.

- Folder name
- Sequence name
- Collection
- Group
- Replicate number (for direct-BSP only)
- Cloning number (for cloning-BSP only)
- Date of sequencing #1
- Date of sequencing #2
- Date of analysis
- Prefix of output files

Reference DNA This tab summarizes all the data computed from the reference DNA sequence .fasta file.

- **Reference DNA sequence** General information on the sequence (name, strand used for primer design, length, and genomic coordinates) and genomic sequence from plus strand (given by the reference DNA .fasta file) and minus strand (reverse complement).
- **Localization of CG dinucleotides** Detection of CpG sites on the reference DNA (on plus and minus strand) with attribution of the CG number, from 1 to n on the plus strand.
- **Bisulfite converted sequences** Sequences of reference DNA after theoretical bisulfite conversion (CpG sites considered as methylated). The bisulfite conversion is performed on the strand used for PCR primer design, as only this strand is amplified during PCR. The PCR regenerates the opposite strand, corresponding to the reverse complement of the bisulfite converted DNA template.

Sequencing trimming This tab summarizes the trimming of sequencing reads based on quality. Two parameters are used: the Phred quality score of each base retrieved from the sequencing file, and the mixed base peak ratio.

- **Summary** In the first tab, the default thresholds used to trim the sequencing results are displayed.
 - Minimum length of the trimmed sequence (default is 30 bp)
 - Minimum Phred quality score (default is 30, corresponding to a base-calling error probability of 0.001%)
 - Minimum ratio of primary peak (default is 0.75)
 - Minimum percentage of non-mixed positions (default is 75%)
- Below the threshold table, the trimming summary for both sequencing reads is displayed and indicates whether or not the trimming was successful (correct trimmed sequence quality) or failed (incorrect trimmed sequence quality).
- **Details per sequencing**
 - **Raw sequence** The sequence, chromatogram, and data table of the sequencing results are displayed.
 - **Quality report** The first trimming is based on the base-calling quality as it uses the Phred quality scores of each base to find the best sequence to trim. This step is provided by the [SangeranalyseR package](#)^{5,6}. The thresholds and the results of this quality trimming are displayed.
 - **Mixed base peak report** The second trimming is based on the primary peak ratio over the other peaks for each position. At each position, the signal ratio of the primary peak is computed using the peak height

⁵<https://sangeranalyser.readthedocs.io/en/latest/index.html>

⁶Kuan-Hao Chao et al. "sangeranalyseR: simple and interactive analysis of Sanger sequencing data in R". in: *bioRxiv* (2020), p. 2020.05.18.102459. doi: [10.1101/2020.05.18.102459](https://doi.org/10.1101/2020.05.18.102459).

values for each base, with formula: if $peak_C > \{peak_A, peak_T, peak_G\}$

$$\text{Primary peak ratio} = \frac{peak_C}{peak_A + peak_T + peak_G + peak_C}$$

If the ratio is above the threshold (default is 0.75), the position is considered non-mixed; if the ratio is below the threshold, the position is considered mixed. All the possible trimmed sequences are obtained by selecting the sequence between n (from 3 to 15) consecutive non-mixed positions. For each one of the possible trimmed sequences, the percentage of non-mixed positions is calculated. Among those, the trimmed sequence which is selected corresponds to the one with a percentage of non-mixed positions above the threshold (default is 75%) with the minimum of consecutive non-mixed positions at extremities (this number is displayed).

- **Trimming plot** The two previous report steps give two different trimmed sequences that can be viewed on the trimming plot ([figure 8](#)). The top panel represents a dot plot of the Phred quality score per position, values in green are above the threshold and values in red below, in which the start and end positions of the trimmed sequence are represented by orange vertical lines. The second panel is also a dot plot but it represents the primary peak ratio per position, values in green are above the threshold (considered as non-mixed) and values in red below (considered as mixed), in which the start and end positions of the trimmed sequence are represented by cyan vertical lines. In the last panel, the two trimmed sequences are represented in the same color, orange, and cyan. The raw sequence is displayed in red. The overlapping of the two previous trimmed sequences gives the final trimmed sequence, in green, which is the one kept for the rest of the analysis and corresponds to the information given in the summary tab and the final trimmed sequence tab.
- **Final trimmed sequence** The sequence, chromatogram, and data table of the final trimmed sequencing results are displayed.

Quality score and primary base peak ratio plots

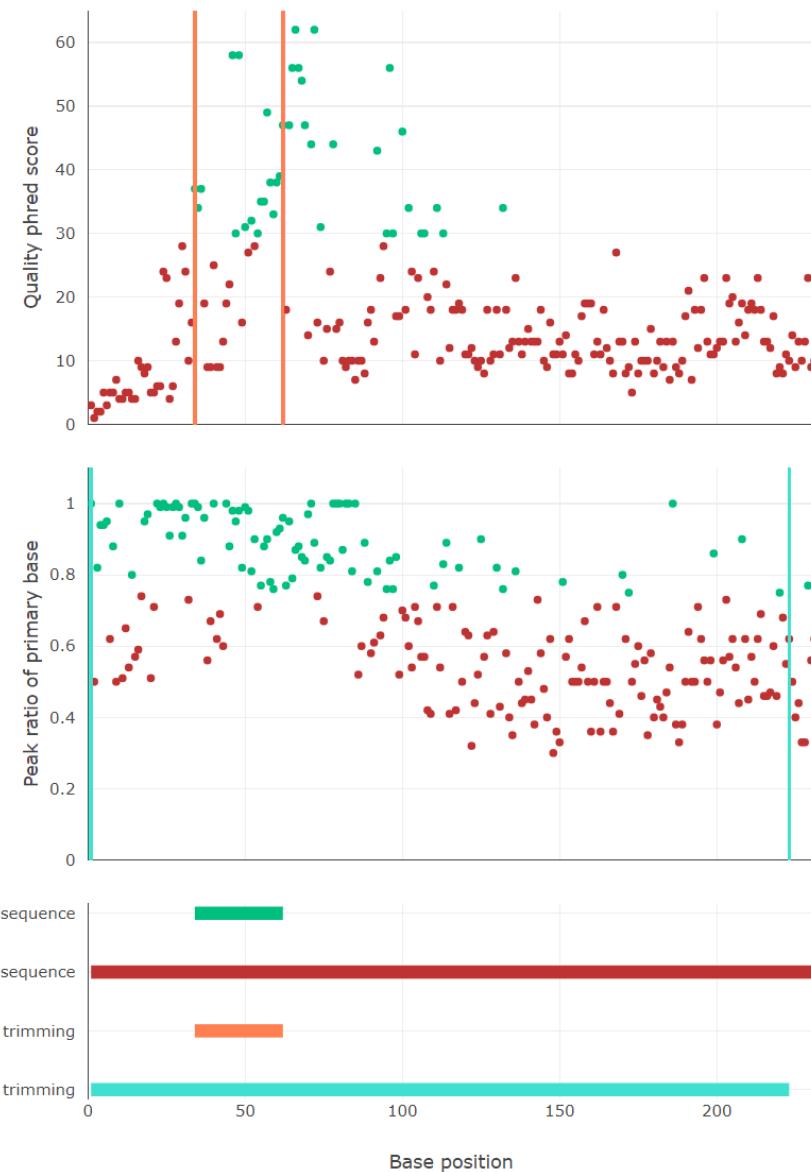


Figure 8. Example of trimming plot from the output report.

Alignments In the first two tabs, each sequencing read is aligned with either the sense sequence (bisulfite converted sequence from the template strand) as if it is a forward sequencing, or the antisense sequence (reverse complement of bisulfite converted sequence from the template strand) as if it is a reverse sequencing. The direction of each sequencing result is determined based on the aligned sequence length: the alignment which gives the longest aligned sequence is considered the correct one. If for one sequencing read the aligned sequences are equal between alignment as forward and as reverse, the direction determination depends on the other one. The two last tabs display the correct alignments and aligned sequences.

Quality control This tab summarizes the quality of the trimmed sequencing result aligned with the reference DNA.

- **Summary** In the first tab, the default thresholds are used to control the quality of the aligned sequencing result.

- Minimum length of the aligned sequence (default is 30 bp)
- Minimum identity percentage of alignment (default is 75%)
- Minimum of bisulfite conversion rate mean (default is 0.90, corresponding to 90% conversion efficacy)

Below the threshold table, the quality summaries for both sequencing results are displayed and indicate whether or not the aligned sequencing results have a sufficient quality (correct) or insufficient (incorrect) relative to thresholds.

- **Mismatch positions** For both sequencing reads a table indicates the mismatched positions and nucleotides, on both the sequencing read and reference DNA sequence.
- **Insertions/deletions** For both sequencing reads a table indicates the insertions/deletions (gaps) found in either the sequencing read or the reference DNA sequence.
- **Conversion rates** For both sequencing reads a table indicates the bisulfite conversion rate for each cytosine outside a CpG in the aligned sequence. The first column corresponds to the identifier number of the cytosine on the reference DNA sequence, in the second one is displayed its position on the reference DNA sequence, in the third one its position on the trimmed sequencing result, and in the fourth its position on the raw sequencing result. The position matching is obtained thanks to the alignment of sequences. For each position on the raw sequencing result, the peak height values of signals are extracted and used to compute the conversion rate, with the following formulas (for forward and reverse sequencing respectively):

$$\text{Bisulfite conversion rate} = \frac{\text{peak}_T}{\text{peak}_C + \text{peak}_T}$$

$$\text{Bisulfite conversion rate} = \frac{\text{peak}_A}{\text{peak}_G + \text{peak}_A}$$

For each position a bisulfite conversion is obtained. The mean of rates from

all positions is indicated in the summary table in the first tab, as well as standard deviation.

- **Maximum aligned sequence** The maximum aligned sequence corresponds to the sequence covered by at least one of the two sequencing results. Its information such as its length, its coordinates, and its nucleotide sequence are displayed.

Methylation

- For both sequencing reads, the tables of computed **methylation percentages** are displayed. The first column corresponds to the CpG site identifier number on the reference DNA sequence (list of all CpG sites in the *Reference DNA* tab, *Localization of CG dinucleotides* tab). In the next three columns, its coordinates are specified. Then, the position of the methylated cytosine (the C in forward, the G in reverse) is displayed, as well as its position in the raw and trimmed sequencing results. The position matching is obtained thanks to the alignment of sequences. For each position on the raw sequencing result, the peak height values of signals are extracted and used to compute the methylation percentage, with the following formulas, for forward and reverse sequencing respectively:

$$\text{Methylation percentage} = \frac{\text{peak}_C}{\text{peak}_C + \text{peak}_T} \times 100$$

$$\text{Methylation percentage} = \frac{\text{peak}_G}{\text{peak}_G + \text{peak}_A} \times 100$$

- **Combined** Methylation results from both sequencing results are then combined in a unique table with the calculation of the average methylation and standard deviation per position.
- **Individual methylation plot** Finally, a plot is generated to visualize results relative to the genomic sequence.

Output data

- **Directories** A diagram of output files directories is displayed ([figure 9](#)).
- **Files** A list of all the output files with links to local folders is displayed.
- **Methylation data file preview** The output methylation data file which will be used as input for the grouped analysis is displayed as a table (The "alg_coord_start" and "alg_coord_end" columns contain a unique value corresponding to the start and end coordinates of the maximum aligned sequence for the individual analysis).

2.4.4 Output files

All the output files are located in the *results* directory, as depicted by the diagram in [figure 9](#).

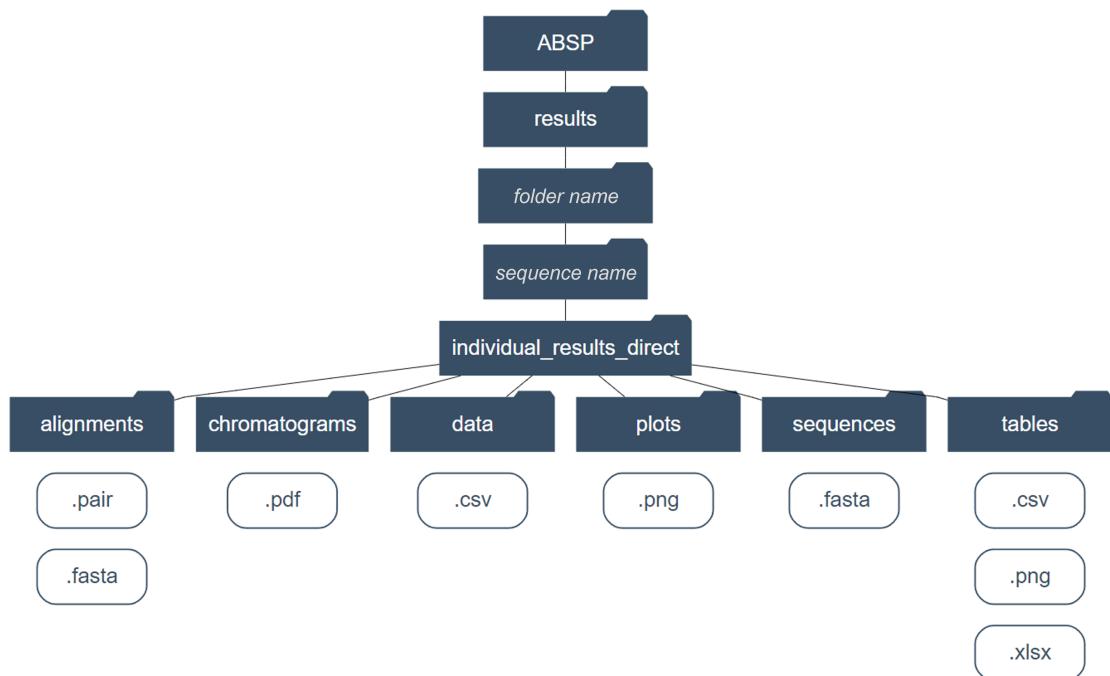


Figure 9. Diagram of output directories to locate output files from the individual analysis. The “*folder name*” and “*sequence name*” depend on the input entries when launching the analysis.

As for the same sequence (same primer set) results from both direct-BSP and cloning-BSP can be generated, the two types of outputs are separated into two subfolders: “*individual_results_direct*” and “*individual_results_cloning*”.

- **alignments** In subfolders specific to each individual analysis, the .pair files of the alignments and the .fasta files of the aligned sequence are saved.
- **chromatograms** In subfolders specific to each individual analysis, the chromatograms of raw sequencing reads and the chromatograms of the trimmed sequencing are saved as .pdf files.
- **data** It contains all the methylation data .csv files for the grouped analysis.
- **plots** It contains all the individual methylation plots as well as legends for plots as .png image files.
- **sequences** The reference DNA sequences from plus and minus strands and the bisulfite converted sequence of the template strand are saved as .fasta files.
- **tables** In subfolders specific to each individual analysis, the data tables of raw sequencing results, the data tables of trimmed sequencing results, the summary table of sequencing trimming, the summary table of quality control, the bisulfite conversion rates table and the methylation percentages tables

are saved as .csv .png and .xlsx files.

2.5 Grouped analysis

2.5.1 Procedure

In the **grouped analysis tab**, the left panel is to launch analysis, and the right panel provides entry information.

Experiment information

- **Select an existing folder.** Located in the "ABSP/results" folder, all of the analysis results will be saved in this folder. Having different folders of results can be used to separate the different analyses by projects, experiments, or users. Note that the six first letters of the folder name will appear in the report file name.
- **Select an existing sequence folder.** Located in the "ABSP/results/previous folder" folder, all of the analysis results will be saved in this folder for this sequence. This sequence name will be used in output files (tables, plots...) to refer to the sequence.
- **Select the reference genome.** It will only be used to display the genomic sequence in the genomic plot. See section 3.3 Code modifications at page 37 to add another genome in the drop-down list. The complete list of available genomes can be found in the "List of BSgenomes.xlsx" file in the "ABSP/documents" folder.
- **Select the experiment type.** The choice of the experiment can be either Direct-BSP or Cloning-BSP. The correct experiment type entry is essential to retrieve the methylation data files either in the "individual_results_direct" or "individual_results_cloning" folders.

Plot parameters

- **Select position labels for plots.** The CpG positions on plots can be referred to by different label types:
 - The **CpG coordinates** label type displays the genomic coordinates of the CpG site in the format *chr#:#####-#####* (e.g. "chr16:68771230-68771231").
 - The **CpG numbers** label type displays the CpG site identifier number on the represented sequence, from 1 to n.
 - The **None** label type displays blank labels, which can be a suitable alternative in case of extremely close CpG positions as labels may overlap.
- **Choose to separate plots by collections.** For lollipop plots, genomic plots, and boxplots and only for display purposes (it does not affect data). If this parameter is not ticked (default), all samples from different collections will be displayed on the same plot. If this parameter is ticked, a plot is generated

by collection, displaying samples from one collection only.

- **Indicate the order of groups for display.** For this input to work, the folder, sequence, and experiment type have to be selected and correct. In this case, the group names are extracted from methylation data files corresponding to the previously selected entries. The groups must all be selected, in the desired order for display.
- **Select the types of sample ordering for plots.** Four different sample ordering (ordinate axis ordering) are available for visualization plots, each provides a specific way of ordering samples on the ordinate axis based on different parameters. At least one must be selected, up to the four of them.
 - **As it is** arranges samples by alphabetic order of collections. If none or one collection is present, this order is equivalent to the *By groups* one.
 - **By groups** arranges samples by the provided group order above.
 - **By methylation levels** arranges samples depending on their methylation mean.
 - **By clusters** arranges samples depending on the hierarchical clustering calculated and represented by an associated dendrogram.

2.5.2 Output report

The HTML report *.html* file of the analysis is automatically saved in the "reports" folder in the ABSP directory.

Header First, in the top panel, the information about the sample experimental conditions is displayed in a table.

- Folder name
- Sequence name
- Reference genome
- Type of experiment
- Group order
- Date of analysis

Files content This tab summarizes all the data that have been used for this analysis.

- **Data files content** General information about the data is listed: sequence name, collections, groups, and replicates or clones.
- **Data files paths** Paths of the methylation files that were found and used for the analysis are listed.

Methylation data This tab regroups the methylation data in tables from the retrieved files.

- **Methylation data of replicates/clones** For each replicate or clone, depending on the experiment type, the methylation percentages of each CpG site

are displayed in a table, with the mean of all positions and the associated standard deviation by replicate/clone.

For clones: Methylation percentages calculated from sequencing results are converted to 0% or 100% methylation status.

- A CpG site is considered unmethylated (0%) when the methylation percentage is between 0% and the defined threshold (default is 20%).
- A CpG site is considered methylated (100%) when the methylation percentage is between the defined threshold (default is 80%) and 100%.
- A CpG site partially methylated, with methylation percentage between 20% and 80%, is removed and annotated as NA (Not Available).
- For one clone, if more than a threshold percentage (default is 20%) of CpG sites are partially methylated, the clone is considered as defective and all of its CpG sites are annotated as NA (Not Available).

The thresholds can be modified in the script, please refer to section 3.3 Code modifications at page 37.

- **Methylation data of groups** For each group, the mean of methylation percentages of each CpG site are displayed in a table, with the mean of all positions and the associated standard deviation by groups.

Plots of replicates Only for direct-BSP. This tab provides plots to visualize the methylation data of replicates.

The Lollipop plots (condensed and proportional), the genomic plot, and the cluster dendrogram plot are as illustrated in [figure 10](#).

Plots of clones Only for cloning-BSP. This tab provides plots to visualize the methylation data of clones from each sample in separated plots.

Lollipop plots (condensed and proportional), genomic plot and cluster dendrogram plot are illustrated in [figure 11](#).

Plots of groups This tab provides plots to visualize the methylation data of groups per collection. For each sample, the mean of replicates or clones is calculated per CpG, and the results are displayed in this tab.

Lollipop plots (condensed and proportional), genomic plot and cluster dendrogram plot are illustrated in [figure 12](#).

Statistics This tab aims to compare methylation between groups, to find statistically significant differences, either by comparing CpG site per CpG site or by comparing the entire region.

- **Descriptive statistics of groups** Two tables of methylation data descriptive statistics are displayed: one for methylation data by CpG positions, and one for methylation data of all CpG positions for each sample.
- **Student's T test** Two tables display T-tests p-values between groups 2 by 2, one table for methylation data by CpG positions, and one for methylation data of all CpG positions for each sample.

- **Boxplots** Boxplots of methylation data with T tests p-values as numbers or symbols are generated, one plot details data of each CpG position ([figure 13 A](#) for direct-BSP data and [figure 13 D](#) for cloning-BSP), and another one data from methylation mean of the region ([figure 13 B](#) for direct-BSP data and [figure 13 E](#) for cloning-BSP).
- **Methylation profile plots** Methylation profile plots with Kruskal-Wallis tests p-values are generated for each collection ([figure 13 C and F](#)).

The [Methylation plotter](#)^{7,8} tool served as a base to develop the different types of plots with different sample ordering.

Output data

- **Directories** A diagram of output files directories is displayed ([figure 14](#)).
- **Files** A list of all the output files with links to local folders is displayed.

2.5.3 Output files

All the output files are located in the *results* directory, as depicted by the diagram in [figure 14](#).

As results for the same sequence from both direct-BSP and cloning-BSP can be generated, the two types of outputs are separated into two subfolders: "*grouped_results_direct*" and "*grouped_results_cloning*".

- **boxplots** Boxplots are saved as *.png* files.
- **dendro_plots** Cluster dendograms are saved as *.png* files.
- **genomic_plots** Genomic plots are saved as *.png* files, in subfolders for plots of replicates/clones and plots of groups.
- **lollipop_plots** Lollipop plots are saved as *.png* files, in subfolders for plots of replicates/clones and plots of groups.
- **meth_profile_plots** Methylation profile plots are saved as *.png* files
- **tables** The methylation data tables, the descriptive statistics tables of positions or means (means of all positions), and the Student's T test tables are saved as *.csv* and *.xlsx* files.

⁷http://maplab.imppc.org/methylation_plotter/index.html

⁸Izaskun Mallona, Anna Díez-Villanueva, and Miguel A Peinado. "Methylation plotter: a web tool for dynamic visualization of DNA methylation data". In: *Source Code for Biology and Medicine* 9.1 (2014). Methylation plotter, p. 11. doi: [10.1186/1751-0473-9-11](https://doi.org/10.1186/1751-0473-9-11).

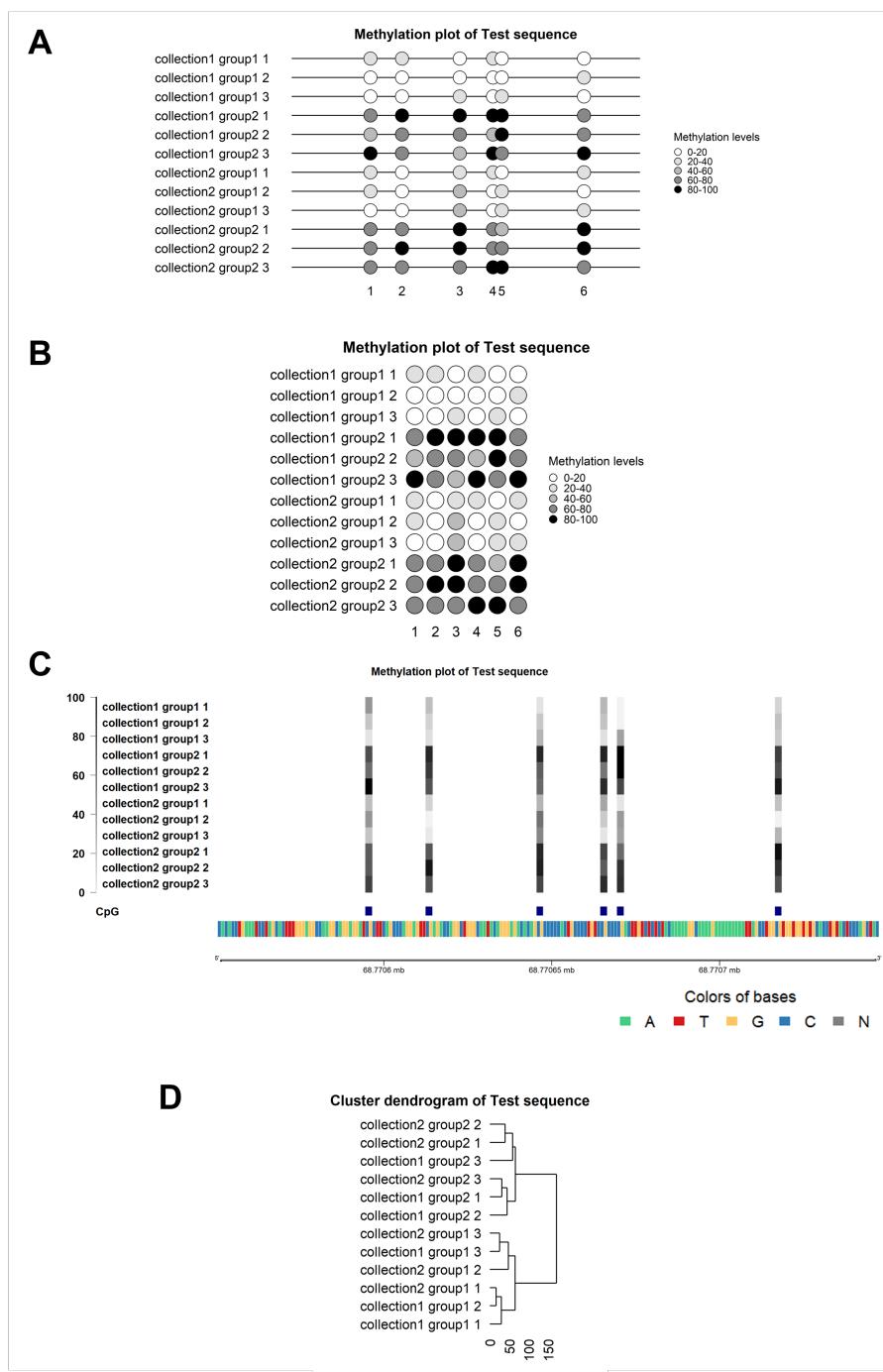


Figure 10. Visualization plots of all replicates (direct-BSP). The plots were generated based on mock methylation data for a test sequence. Three replicates (1, 2, and 3) per sample are represented on the plots, from two groups (group1 and group2) and two collections (collection1 and collection2) (sample ordering *as it is*). The methylation levels are given as percentages and correspond directly to the methylation output data from the individual analyses. **A.** Proportional lollipop plot. **B.** Condensed lollipop plot. **C.** Genomic plot. **D.** Cluster dendrogram (for sample ordering *by clusters*).

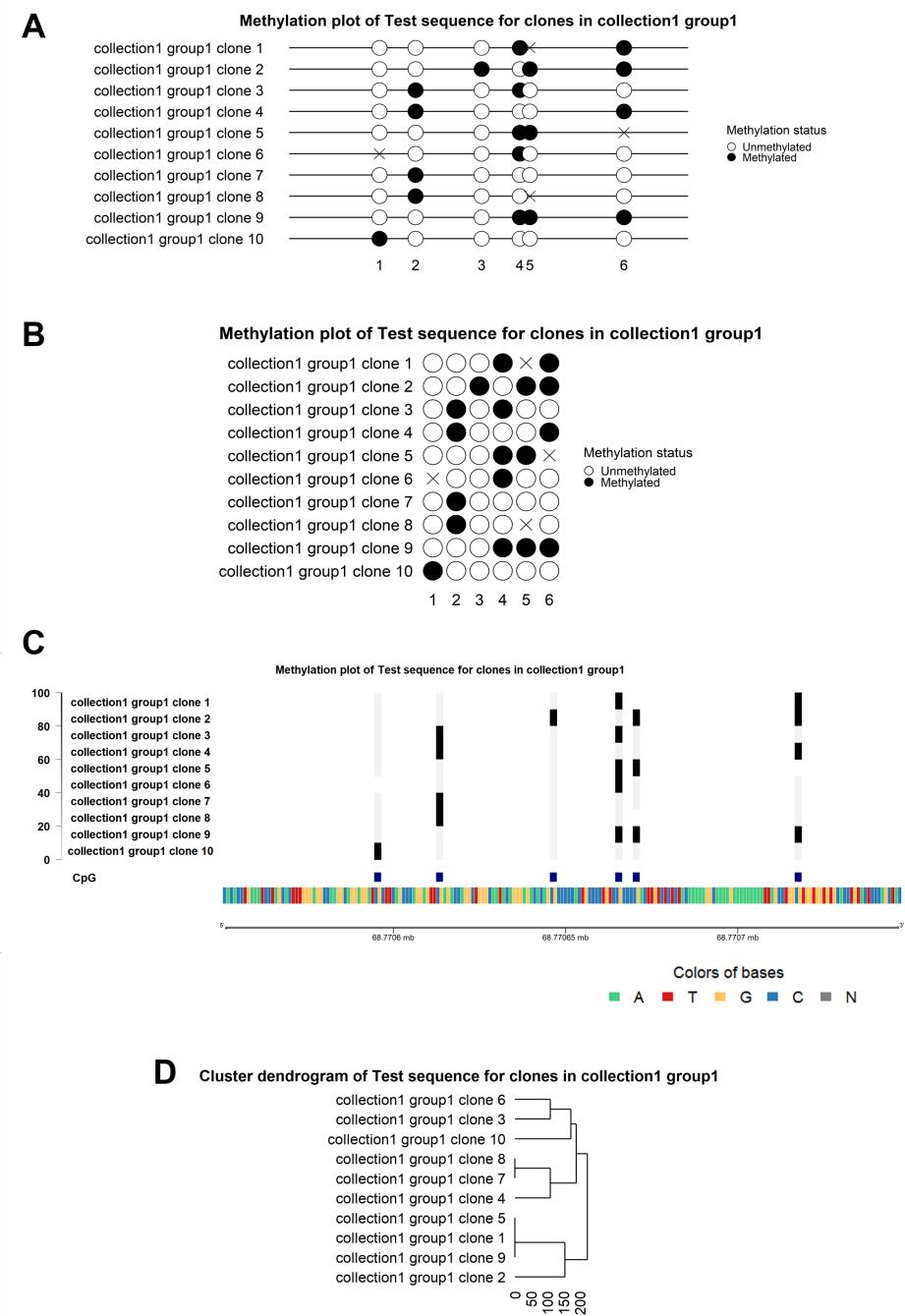


Figure 11. Visualization plots of all clones from one sample (cloning-BSP). The plots were generated based on mock methylation data for a test sequence. Ten clones (from 1 to 10) for the sample "*collection1 group1*" are represented on the plots (sample ordering *as it is*). The methylation status corresponds to the conversion of methylation percentages from the individual analyses into unmethylated (0%) or methylated (100%) (or not available) methylation statuses. **A.** Proportional lollipop plot. **B.** Condensed lollipop plot. **C.** Genomic plot. **D.** Cluster dendrogram (for sample ordering *by clusters*).

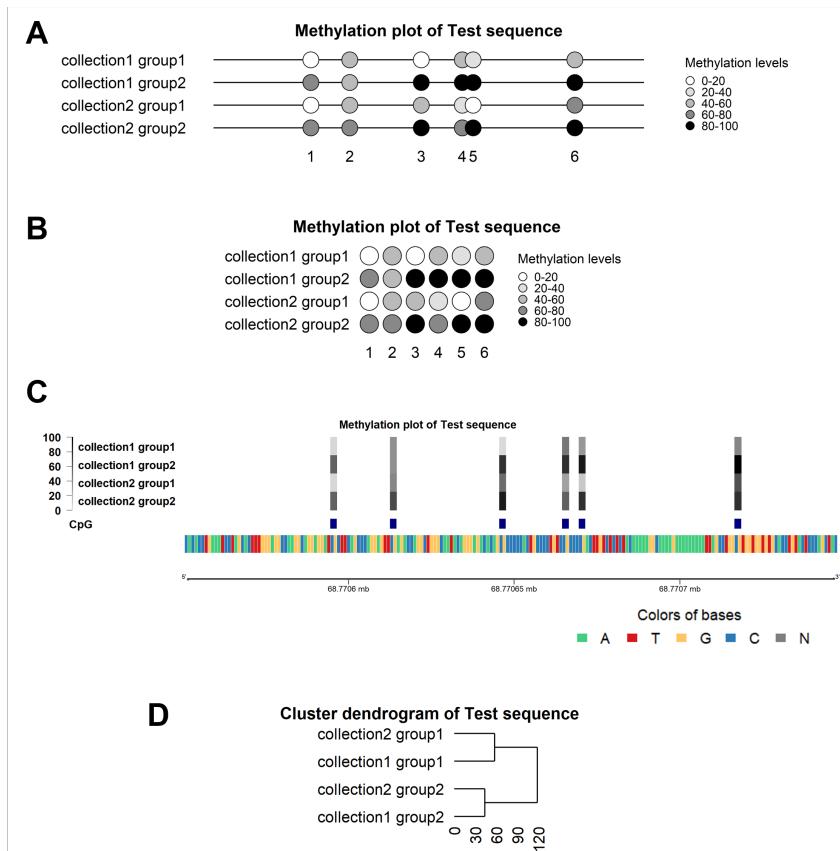


Figure 12. Visualization plots of groups (means of replicates/ clones per sample). The plots were generated based on mock methylation data for a test sequence. Plots represent the means of methylation percentages of ten clones per sample and per CpG position (sample ordering as *it is*). **A.** Proportional lollipop plot. **B.** Condensed lollipop plot. **C.** Genomic plot. **D.** Cluster dendrogram (for sample ordering by clusters).

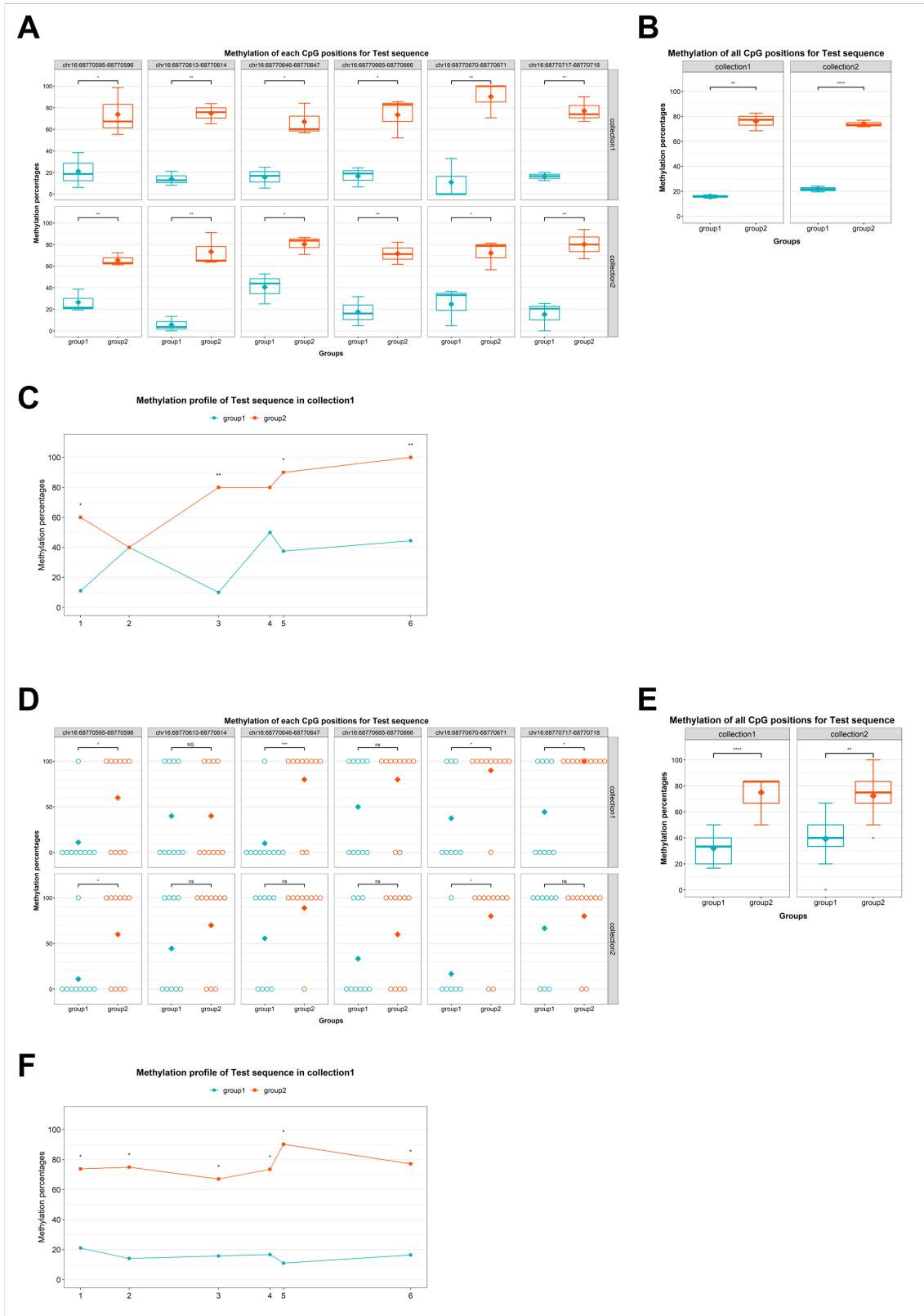


Figure 13. Boxplots and methylation profile plots. The plots were generated based on mock methylation data for a test sequence. **A.** Boxplots of direct-BSP methylation results for each CpG site. **B.** Boxplots of direct-BSP methylation results for means of all CpG sites. **C.** Methylation profile plot of direct-BSP methylation results for one of the two collections. **D.** Boxplots of cloning-BSP methylation results for each CpG site. As methylation levels of CpG from clones can only be either 0% or 100% thereby boxes can't be drawn, instead, each clone is represented in the plot by a circle. **E.** Boxplots of cloning-BSP methylation results for means of all CpG sites. **F.** Methylation profile plot of cloning-BSP methylation results for one of the two collections. In boxplots, symbols represent significance levels of Student's T-test p-values (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). In methylation profile plots, symbols represent significance levels of Kruskal-Wallis test p-values (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

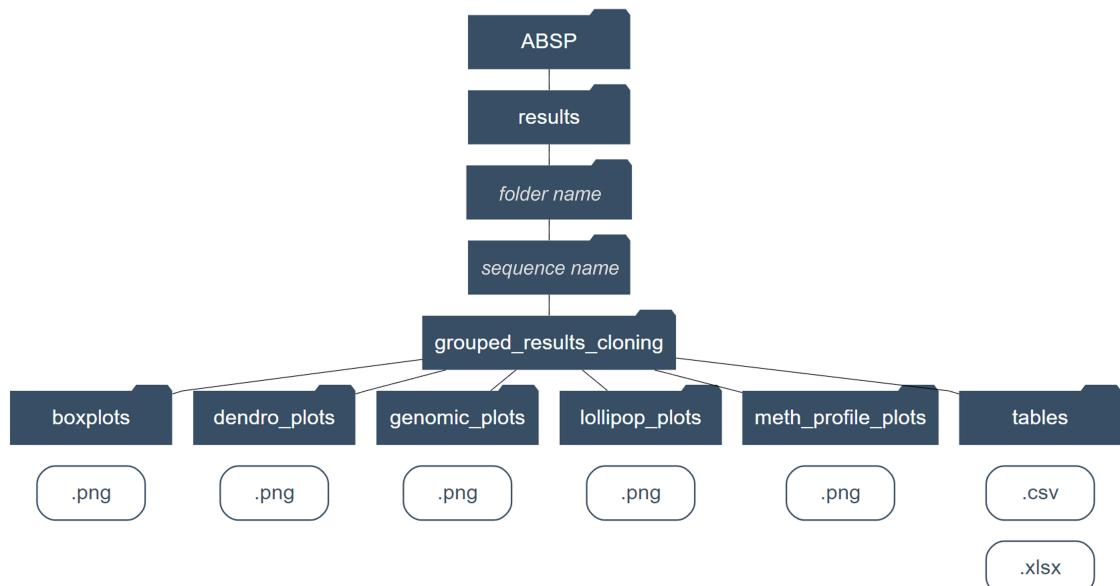


Figure 14. Diagram of output directories to locate output files from the individual analysis. The "folder name" and "sequence name" depend on the input entries when launching the analysis.

2.6 Multiple analyses

As described in the [figure 5](#), multiple analyses can be launched at the same time using data tables as input with all the required information. Both individual and grouped analyses can be launched, at the same time or separately.

2.6.1 Input files requirements

Two files are provided in the "documents" folder. They must be filled with the desired input entries, all information about how to fill the documents is also indicated within the documents as notes.

The documents are provided in the .xlsx (Microsoft Excel Open XML Format Spreadsheet) and .ods (OpenDocument Spreadsheet) formats, but the input file format must be either .xlsx or .csv (Comma-Separated Values) (.ods files must be converted to one of those formats).

- **Experiments data table for individual analyses:**

[*"multiple_individual_analyses_table.xlsx"*](#)

- SEQUENCE NAME. The sequence name should be unique and consistent for each amplicon. It must not contain any special character.
- COLLECTION. The collection refers to a separation of samples above the groups/conditions. Leave empty if you do not want to indicate a collection.
- GROUP. The group refers to the condition that is to be compared. It must not contain any special character.
- REPLICATE NUMBER. The replicate number refers to the experiment repetition identifier. It must be an integer ≥ 1 . Only for direct-BSP experiments, leave empty otherwise.
- CLONE NUMBER. The clone number refers to the individual clone identifier. It must be an integer ≥ 1 . Only for cloning-BSP experiments, leave empty otherwise.
- GENOME. The genome refers to the reference genome assembly used for coordinates and plots displaying the genomic sequence. Only a short list of genomes is displayed in the cells, but you can use another available genome. Please refer to the provided document: "*List of BSgenomes*" to get the list of available genomes assemblies and the correct spelling.
- PATH TO FASTA FILE OF REFERENCE DNA. Path to the .fasta file of the reference DNA sequence on your computer. On Windows, you can copy the file path by holding down shift then right-clicking on the file, and selecting "Copy as path" in the menu. On macOS, you can copy the file path by right-clicking on the file to display the menu then holding down the option key and selecting "Copy ... as Pathname".
- DATE SEQUENCING #1. Date of the sequencing result #1 for traceability. The date format must be YYYY-MM-DD and the cell format must be set to "date".
- PATH TO SEQUENCING FILE #1. Path to sequencing result #1 .ab1 file.
- DATE OF SEQUENCING #2. Date of the sequencing result #2 for traceability.

The date format must be YYYY-MM-DD and the cell format must be set to "date".

- PATH TO SEQUENCING FILE #2. Path to sequencing result #2 .ab1 file.

- **Parameters table for grouped analyses:**

"multiple_grouped_analyses_table.xlsx".

- SEQUENCE NAME. The sequence name should be unique and consistent for each amplicon. It must not contain any special character.
- GENOME. The genome refers to the reference genome assembly used for coordinates and plots displaying the genomic sequence. Only a short list of genomes is displayed in the cells, but you can use another available genome. Please refer to the provided document: "List of BSgenomes" to get the list of available genomes assemblies and the correct spelling.
- EXPERIMENT TYPE. The experiment type can be either Direct-BSP or Cloning-BSP.
- CpG POSITION LABEL TYPE. The CpG position label type refers to the displayed element corresponding to the CpG position on plots.
- SEPARATION OF COLLECTIONS. This parameter refers to the generation of plots: if FALSE, all samples from all collections will be displayed on the same plot, and if TRUE, samples will be split into different plots, one plot per collection.
- LIST OF GROUPS ORDERED. The cell must contain all of the groups within the experiment in the order you want them to be displayed. Type your groups separated by commas. It must not contain any special character.
- TYPE OF SAMPLE ORDERING. The type of sample ordering refers to the parameter used for ordering samples on plots. Four types of ordering are available: As it is, By groups, By methylation levels, and By clusters. Multiple ordering can be chosen by typing the ordering names separated by commas.

2.6.2 Procedure

- **Select an existing folder** within the ABSP results folder to locate all of the analysis results. To create a new folder, select the "Create new folder" entry and enter the name of the new folder in the text input. Note that the six first letters will appear in the report file name.
- **Select the filled table as input.** Both the experiments data table and the grouped parameters table can be provided at the same time to launch individual analyses followed by grouped analyses, or only one of the two tables can be provided and will launch the corresponding analyses, either individual analyses or grouped analyses.
- **Launch the analyses** by clicking on the bottom button "Run analyses".

2.6.3 Output files

The reports and output files of analyses are saved in the *reports* and *results* folders, in the same way as for the manual launch of individual and grouped analyses, see sections 2.4 Individual analysis at page 14 and 2.5 Grouped analysis at page 24.

3 Complementary information

3.1 Some recommendations for the BSP experiment

The length of the PCR products should not exceed 350-400 bp, as the bisulfite treatment causes DNA strand breakages long amplicon can not be properly amplified, and ABSP plot display for sequences above 400 bp with numerous CpG is not optimal.

Several tools can be used to design BSP primers, several are listed in the [Methtools⁹](#) list of tools, such as [Methprimer¹⁰](#) to design primers and [BiSearch¹¹](#) to check for unintended PCR products, on bisulfite-treated DNA.

3.2 Detailed workflow of ABSP individual analysis

In [Figure 15](#), the individual analysis input, steps and outputs are described in more detail.

⁹<http://bigd.big.ac.cn/methbank/methTool/list/>

¹⁰<https://www.urogene.org/methprimer/>

¹¹<http://bisearch.enzim.hu/>

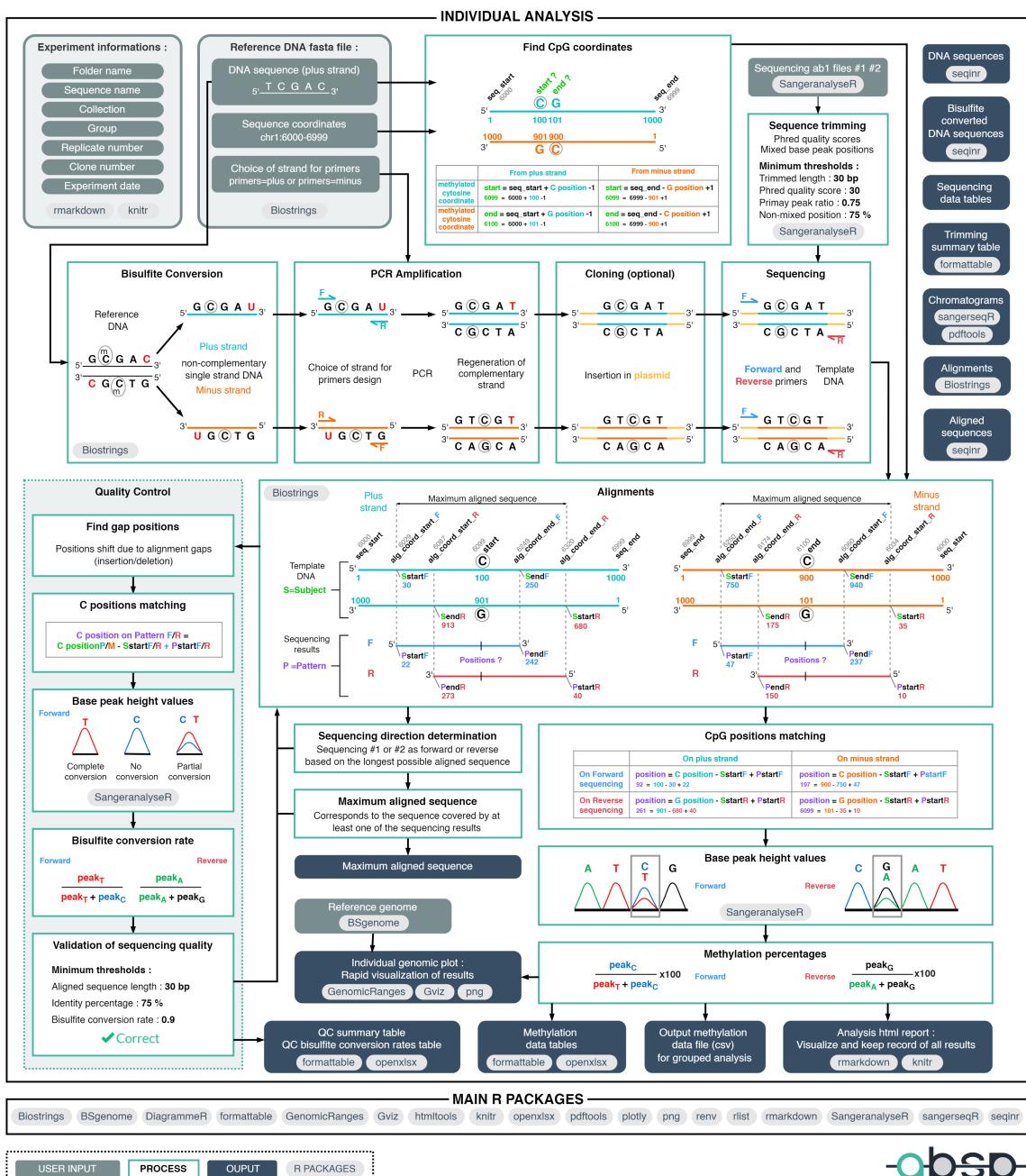


Figure 15. Detailed workflow of the individual analysis.

3.3 Code modifications

3.3.1 List of reference genomes

In the individual analysis and grouped analysis tabs of the app, the drop-down list to select the reference genome is limited. If your reference genome does not appear it doesn't mean that it is not available, and can be manually added. The complete list of [genomes assemblies](#)¹² with the correct spelling can be found in the "List of BSgenomes.xlsx" file in the "ABSP/documents" folder.

To modify the displayed drop-down list items, the *app.R* script has to be modified. In the [Code listing 1](#), the "*list_genomes*" object corresponds to the vector of character strings listing the displayed reference genome. Any of the other reference genomes can be added to the list.

[Code listing 1.](#) List of reference genomes displayed in the drop-down lists as selectable inputs for analyses. From the "*app.R*" script.

```
14 # Here, to simplify, a short list of genomes is displayed but all BSgenome  
15 # can be used  
16 # To get the list of all genomes of BSgenome package run : 'BSgenome::  
17 # available.genomes()', more information on genomes at https://genome.ucsc.edu/cgi-bin/hgGateway  
18 # A new genome can be added to the list displayed just below :  
19 list_genomes <- c(  
20   "BSgenome.Hsapiens.UCSC.hg19", "BSgenome.Hsapiens.UCSC.hg38", "  
   BSgenome.Mmusculus.UCSC.mm10", "BSgenome.Mmusculus.UCSC.mm39",  
   "BSgenome.Rnorvegicus.UCSC.rn6", "BSgenome.Rnorvegicus.UCSC.rn7", "  
   BSgenome.Cfamiliaris.UCSC.canFam3", "BSgenome.Mmulatta.UCSC.rheMac8",  
   "BSgenome.Ggallus.UCSC.galGal6", "BSgenome.Drerio.UCSC.danRer11", "  
   BSgenome.Celegans.UCSC.ce11", "BSgenome.Dmelanogaster.UCSC.dm6")
```

To modify the genome name selected by default in the drop-down "Select genome" list of either the individual or grouped analysis tabs, change the name of the genome in the following lines ([Code listing 2](#) and [Code listing 3](#)):

[Code listing 2.](#) Genome name selected by default in the drop-down list of the individual analysis tab. From the "*app.R*" script.

```
221 selectInput("genomeI",label ="Select genome",choices=list_genomes,selected  
              ="BSgenome.Hsapiens.UCSC.hg19")
```

¹²<https://genome.ucsc.edu/cgi-bin/hgGateway>

Code listing 3. Genome name selected by default in the drop-down-list of the grouped analysis tab. From the "app.R" script.

```
496 selectInput("genomeG", label = "Select genome", choices=list_genomes, selected  
= "BSgenome.Hsapiens.UCSC.hg19"),
```

3.3.2 Modify the default thresholds

Individual analysis

The default thresholds used for the individual analysis can be modified ([Code listing 4](#)).

Code listing 4. Default thresholds in the individual analysis script. From the "ABSP_individual_analysis.Rmd" script.

```
607 '``{r Thresholds, include=F}  
608  
609 # Thresholds  
610  
611 # Maximum base-calling error probability (value per position):  
612 th_quality_error <- 0.001  
613 # Minimum phred quality score, logarithmically linked to error probability  
# (value per position):  
614 th_quality_phred <- (-10*log(th_quality_error,10))  
615  
616 # Minimum ratio of primary peak, corresponding to the primary peak value  
# over the total of peak value, to consider a position as non-mixed (value per position):  
617 th_mixed_position <- 0.75  
618 # Minimum percentage of non-mixed positions in the trimmed sequence to be  
# considered as non-mixed (value for the total trimmed sequence):  
619 th_mixed_perc <- 75 # %  
620  
621 # Minimum length of trimmed sequences  
622 th_min_trim <- 30 # bp  
623  
624 # Minimum length of aligned sequences  
625 th_min_alg <- 30 # bp  
626 '``
```

Grouped analysis

The default thresholds used for the grouped analysis, for clones methylation percentage conversion into methylation status (0% or 100%), can be modified ([Code listing 5](#)).

Code listing 5. Default thresholds in the grouped analysis script. From the "*ABSP_grouped_analysis.Rmd*" script.

```
529  '''{r Thresholds, include=F}
530
531 # Thresholds (used for cloning only) :
532
533 # unmethylated clones : methylation between 0% and 20%
534 th_unmethylated_max <- 20
535
536 # methylated clones : methylation between 80% and 100%
537 th_methylated_min <- 80
538
539 # maximum proportion of partial positions allowed : 20% of CpG positions
540 th_partialpos_ratio <- 0.2
541
542 clone_thresholds <- c(th_unmethylated_max, th_methylated_min, th_
543   partialpos_ratio)
544'''
```

3.3.3 Modify the plots colors and point shapes

For the grouped analysis, the plot colors and point shape parameters can be modified. As depicted in [Code listing 6](#), the color of bases can be changed in the *bases_colors* object, the plot colors for each group in the *plot_colors* object, and the point shapes for each group in the *plot_shapes* object.

In the *ABSP_grouped_analysis.Rmd* script, the code chunk below the one depicted in the [Code listing 6](#) gives examples of different palette colors and points shapes, with functions to visualize them.

Code listing 6. Colors and shapes setting for plots in the grouped analysis script. From the *ABSP_grouped_analysis.Rmd* script.

```
364 '``{r colors and shapes settings, include=F}
365 # Colors of sequence track for genomic plots
366 bases_colors <- c(A="#43CD80", T="#D7191C", G="#FDC661", C="#2C7BB6", N="#
367   7F7F7F")
368
369 # Colors of groups for plots
370 plot_colors <- c("aqua"="#00AFBB",
371   "tangerine"="#FC4E07",
372   "sun"="#E7B800",
373   "berry"="#d30446",
374   "lime"="#90c613",
375   "grape"="#7839de",
376   "flamingo"="#d12a97",
377   "jade"="#00b673",
378   "ink"="#1221ed",
379   "terracotta"="#a93b2c")
380
381 # Shapes of groups for methylation profile plots
382 plot_shapes <- c("round"=19,
383   "square"=15,
384   "triangle"=17,
385   "round_border"=21,
386   "square_border"=22,
387   "triangle_border"=24,
388   "diamond"=18,
389   "small_round"=20,
390   "reverse_triangle_border"=25,
391   "diamond_border"=23)
392
393 ''
```

4 Troubleshooting guide

4.1 General

Error	Cause	Solution
After following the opening procedure (for the first time) and clicking on the "Run App" button, the app interface is not opening.	Package installation failed or some packages are not fully installed. Plus, to finalize some package installation, R needs to be restarted.	In the RStudio console, type "renv::restore(prompt=F)" and press enter to run it. The download and installation of packages should start. Then, in the top bar, click on "Session" and "Restart R". Then try to open the app by clicking on the "Run app" button. If the problem is not solved, please contact us with a description of the issue.
Warning message about RStudio version in the RStudio console. <i>"R graphics engine version X is not supported by this version of RStudio. The Plots tab will be disabled until a newer version of RStudio is installed."</i>	The RStudio installed on the device is not up-to-date.	Download and install the newest version of RStudio at https://www.rstudio.com/products/rstudio/download/ .
Prompt message displayed in the RStudio console during the selected BSgenome installation. <i>"Do you want to install from sources the package which needs compilation? (Yes/no/cancel)"</i>	The installation of the BSgenome requires other packages to be installed.	Type "Yes" in the console panel and press enter to run it.

<p>Warning message when opening the ABSP about project configuration and R version (and the app does not function).</p> <p><i>"This project is configured to use R version 'X.X.X', but 'X.X.X' is currently being used."</i></p>	<p>The R version does not match the R project configuration, the R version is either older or newer than the one used to generate the ABSP project.</p>	<p>Either download the latest version of R to match the one of the project configuration (https://www.r-project.org/) or download the latest version of ABSP to match the R version (https://github.com/ABSP-methylation-tool/ABSP). If the currently available ABSP version does not match the currently available R version, download the corresponding version of R and contact us, for us to update ABSP as soon as possible to match the current R version.</p>
--	---	---

4.2 Individual analysis

Error	Cause	Solution
<p>Analysis report aborted after the reference DNA step. <i>Error: Length of reference DNA sequence does not match with the provided genomic coordinates. Please verify concordance between the reference DNA sequence and genomic coordinates.</i></p>	The provided reference sequence in the fasta file has not had the same length as the one calculated based on genomic coordinates provided in the fasta file header. So the sequence and coordinates might not match.	Check the reference and its coordinates. If the IGV (Integrative Genomics Viewer) software is used to get them, make sure to properly verify the first and last nucleotides, as there can be a one nucleotide difference between the coordinates and the actual sequence.
<p>Analysis report aborted after the sequencing trimming step. <i>Error: Analysis has been stopped as none of the sequencing results are of sufficient quality to be used.</i></p>	The provided sequencing results are not of good quality, the trimming step was not able to find a trimmed sequence long enough (below length threshold) and/or with poor quality (below the quality score and/or non-mixed positions thresholds).	Lowering a bit the trimming thresholds might solve the issue, but if not, no solution can be provided. The best recommendation is to perform the sequencing run one more time. If the sequencing is not of good quality again, then there must be an experimental issue with BSP samples or sequencing runs.
<p>Analysis report aborted after the alignment step. <i>Error: Analysis has been stopped as none of the possible alignments are of sufficient length (< N bp) to be used.</i></p>	Even if the sequencing results were of good quality and successfully trimmed, their alignments with the reference DNA sequence give too short aligned sequences to pursue the analysis.	Check if the reference DNA sequence is the correct one. Check if the sequencing results passed the trimming step with values just above thresholds that might explain the alignment results. In this case, lowering some thresholds may solve the issue. If not, the best option is to provide new results from a new sequencing run.

<p>Analysis report aborted after the alignment step.</p> <p><i>Error: Analysis has been stopped as sequencing result direction (forward or reverse) could not be found.</i></p>	<p>This error occurs when the determined direction for both sequencing results happened to be the same, or when only one sequencing has been successfully trimmed and aligned, but alignments as forward or reverse have the same length.</p>	<p>For the first case, the results might have passed the alignment step with values just below thresholds, explaining the identical length of the aligned sequence in both directions, still, the best option should be to provide new sequencing results to have better trimmed and aligned results. For the second case, lowering a bit some thresholds might help the other sequencing to pass the trimming and alignment steps, still, the best option should be to provide new sequencing results to have better trimmed and aligned results.</p>
<p>Analysis report aborted after the alignment step.</p> <p><i>Error: Analysis has been stopped as none of the provided sequencing reads are of sufficient quality to be used.</i></p>	<p>The provided sequencing results are not of good quality, they passed the trimming step but not the alignment step. The aligned sequences were not long enough (below length threshold) or direction determination failed.</p>	<p>Lowering a bit the trimming and alignment thresholds might solve the issue, but if not, no solution can be provided. The best recommendation is to perform the sequencing run one more time. If the sequencing is not of good quality again, then there must be an experimental issue with BSP samples or sequencing runs.</p>
<p>Analysis report aborted after the Quality Control (QC) step.</p> <p><i>Error: Analysis has been stopped as sequencing results are defined as incorrect by Quality Control. The analysis can not be performed.</i></p>	<p>The provided sequencing results are not of good quality when compared to the reference DNA; they passed the trimming and alignment steps but the quality control found the sequencing results incorrect: either the identity percentage or the mean bisulfite conversion rates are below the threshold.</p>	<p>Lowering a bit the thresholds might solve the issue, but if not, no solution can be provided. The best recommendation is to perform the sequencing run one more time. If the sequencing is not of good quality again, then there must be an experimental issue with BSP samples or sequencing runs.</p>

Analysis report aborted after the Quality Control (QC) step. <i>Error: Analysis has been stopped as no CpG sites were found covered by sequencing results.</i>	Even if the sequencing results passed the trimming, alignment, and quality control steps, the aligned results are not long enough and do not cover any CpG sites on the sequence, thereby methylation levels can not be computed.	Lowering a bit the thresholds might solve the issue to get longer aligned sequences, but if not, no solution can be provided. The best recommendation is to perform the sequencing run one more time. If the sequencing is not of good quality again, then there must be an experimental issue with BSP samples or sequencing runs.
Analysis failed and no report has been generated	Unexpected error.	Please contact us and send us the error message appearing in the RStudio console.

4.3 Grouped analysis

Error	Cause	Solution
Analysis report aborted after the header. <i>Error: No file methylation data files found. Check inputs: folder name and sequence name, and check the 'data' directory in the individual results folder.</i>	No methylation data files from the individual analysis, corresponding to the input information, were able to be retrieved.	Check if the individual analyses have already been run, if the files were not moved to another folder, they should be in the 'data' directory in the individual results folder, or if the input information, folder name, sequence name, and experiment type are correct. If the issue is not solved by these recommendations, please contact us.
Analysis failed and no report has been generated.	Unexpected error.	Please contact us and send us the error message appearing in the RStudio console.

5 Acknowledgments

An important part of the plot generation work for the grouped analysis was based on the R code provided by the [Methylation plotter](#)^{13,14} tool.

Main R packages used:

- arrangements
- BiocManager
- Biostrings
- BSgenome
- compareGroups
- DiagrammeR
- dplyr
- formattable
- GenomeInfoDb
- GenomicRanges
- ggdendro
- ggplot2
- ggpubr
- Gviz
- htmltools
- htmlwidgets
- knitr
- openxlsx
- pdfTools
- plotly
- png
- purrr
- RColorBrewer
- readr
- renv
- rlist
- rmarkdown
- Rmisc
- rstatix
- sangeranalyseR
- sangerseqR
- seqinr
- shiny
- shinythemes
- webshot

¹³http://maplab.imppc.org/methylation_plotter/index.html

¹⁴Mallona, Díez-Villanueva, and Peinado, “Methylation plotter: a web tool for dynamic visualization of DNA methylation data”.

List of all R packages used:

- **abind 1.4.5** - Plate T, Heiberger R (2016). *_abind: Combine Multidimensional Arrays_*. R package version 1.4-5, <https://CRAN.R-project.org/package=abind>.
- **ade4 1.7.19** - Dray S, Dufour A (2007). "The ade4 Package: Implementing the Duality Diagram for Ecologists." *_Journal of Statistical Software_*, *22*(4), 1-20. doi:10.18637/jss.v022.i04 <https://doi.org/10.18637/jss.v022.i04>.
- **AnnotationDbi 1.58.0** - Pagès H, Carlson M, Falcon S, Li N (2022). *_AnnotationDbi: Manipulation of SQLite-based annotations in Bioconductor_*. R package version 1.58.0, <https://bioconductor.org/packages/AnnotationDbi>.
- **AnnotationFilter 1.20.0** - Morgan M, Rainer J (2022). *_AnnotationFilter: Facilities for Filtering Bioconductor Annotation Resources_*. R package version 1.20.0, <https://github.com/Bioconductor/AnnotationFilter>.
- **arrangements 1.1.9** - Lai R (2020). *_arrangements: Fast Generators and Iterators for Permutations, Combinations, Integer Partitions and Compositions_*. R package version 1.1.9, <https://CRAN.R-project.org/package=arrangements>.
- **askpass 1.1** - Ooms J (2019). *_askpass: Safe Password Entry for R, Git, and SSH_*. R package version 1.1, <https://CRAN.R-project.org/package=askpass>.
- **assertthat 0.2.1** - Wickham H (2019). *_assertthat: Easy Pre and Post Assertions_*. R package version 0.2.1, <https://CRAN.R-project.org/package=assertthat>.
- **backports 1.4.1** - Lang M, R Core Team (2021). *_backports: Reimplementations of Functions Introduced Since R-3.0.0_*. R package version 1.4.1, <https://CRAN.R-project.org/package=backports>.
- **base64enc 0.1.3** - Urbanek S (2015). *_base64enc: Tools for base64 encoding_*. R package version 0.1-3, <https://CRAN.R-project.org/package=base64enc>.
- **BH 1.78.0.0** - Eddelbuettel D, Emerson JW, Kane MJ (2021). *_BH: Boost C++ Header Files_*. R package version 1.78.0-0, <https://CRAN.R-project.org/package=BH>.
- **Biobase 2.56.0** - Huber W, Carey VJ, Gentleman R, Anders S, Carlson M, Carvalho BS, Bravo HC, Davis S, Gatto L, Girke T, Gottardo R, Hahne F, Hansen KD, Irizarry RA, Lawrence M, Love MI, MacDonald J, Obenchain V, Ole's AK, Pag'es H, Reyes A, Shannon P, Smyth GK, Tenenbaum D, Waldron L, Morgan M (2015). "Orchestrating high-throughput genomic analysis with Bioconductor." *_Nature Methods_*, *12*(2), 115-121. <http://www.nature.com/nmeth/journal/v12/n2/full/nmeth.3252.html>.
- **BiocGenerics 0.42.0** - Huber, W., Carey, J. V, Gentleman, R., Anders, S., Carlson, M., Carvalho, S. B, Bravo, C. H, Davis, S., Gatto, L., Girke, T., Gottardo, R., Hahne, F., Hansen, D. K, Irizarry, A. R, Lawrence, M., Love, I. M, MacDonald, J., Obenchain, V., Ole's, K. A, Pag'es, H., Reyes, A., Shannon, P., Smyth, K. G, Tenenbaum, D., Waldron, L., Morgan, M. (2015). "Orchestrating high-throughput genomic analysis with Bioconductor." *_Nature Methods_*, *12*(2), 115-121. <http://www.nature.com/nmeth/journal/v12/n2/full/nmeth.3252.html>.
- **BiocManager 1.30.17** - Morgan M (2022). *_BiocManager: Access the Bioconductor Project Package Repository_*. R package version 1.30.17, <https://CRAN.R-project.org/package=BiocManager>.
- **BiocParallel 1.30.0** - Morgan M, Wang J, Obenchain V, Lang M, Thompson R, Turaga N (2022). *_BiocParallel: Bioconductor facilities for parallel evaluation_*. R package version 1.30.0, <https://github.com/Bioconductor/BiocParallel>.
- **BiocStyle 2.24.0** - Oleś A (2022). *_BiocStyle: Standard styles for vignettes and other Bioconductor documents_*. R package version 2.24.0, <https://github.com/Bioconductor/BiocStyle>

[BiocStyle](#).

- **Biostrings 2.64.0** - Pagès H, Aboyoun P, Gentleman R, DebRoy S (2022). *_Biostrings: Efficient manipulation of biological strings_*. R package version 2.64.0, <https://bioconductor.org/packages/Biostrings>.
- **bit 4.0.4** - Oehlschlägel J, Ripley B (2020). *_bit: Classes and Methods for Fast Memory-Efficient Boolean Selections_*. R package version 4.0.4, <https://CRAN.R-project.org/package=bit>.
- **bit64 4.0.5** - Oehlschlägel J, Silvestri L (2020). *_bit64: A S3 Class for Vectors of 64bit Integers_*. R package version 4.0.5, <https://CRAN.R-project.org/package=bit64>.
- **bitops 1.0.7** - port SobSDiR, revised ebMM, Dutky mbS (2021). *_bitops: Bitwise Operations_*. R package version 1.0-7, <https://CRAN.R-project.org/package=bitops>.
- **blob 1.2.3** - Wickham H (2022). *_blob: A Simple S3 Class for Representing Vectors of Binary Data ('BLOBS')_*. R package version 1.2.3, <https://CRAN.R-project.org/package=blob>.
- **bookdown 0.26** - Xie Y (2022). *_bookdown: Authoring Books and Technical Documents with R Markdown_*. R package version 0.26, <https://github.com/rstudio/bookdown>.
- **brio 1.1.3** - Hester J, Csárdi G (2021). *_brio: Basic R Input Output_*. R package version 1.1.3, <https://CRAN.R-project.org/package=brio>.
- **broom 0.8.0** - Robinson D, Hayes A, Couch S (2022). *_broom: Convert Statistical Objects into Tidy Tibbles_*. R package version 0.8.0, <https://CRAN.R-project.org/package=broom>.
- **BSgenome 1.64.0** - Pagès H (2022). *_BSgenome: Software infrastructure for efficient representation of full genomes and their SNPs_*. R package version 1.64.0, <https://bioconductor.org/packages/BSgenome>.
- **bslib 0.3.1** - Sievert C, Cheng J (2021). *_bslib: Custom 'Bootstrap' 'Sass' Themes for 'shiny' and 'rmarkdown'_*. R package version 0.3.1, <https://CRAN.R-project.org/package=bslib>.
- **cachem 1.0.6** - Chang W (2021). *_cachem: Cache R Objects with Automatic Pruning_*. R package version 1.0.6, <https://CRAN.R-project.org/package=cachem>.
- **callr 3.7.0** - Csárdi G, Chang W (2021). *_callr: Call R from R_*. R package version 3.7.0, <https://CRAN.R-project.org/package=callr>.
- **car 3.0.13** - Fox J, Weisberg S (2019). *_An R Companion to Applied Regression_*, Third edition. Sage, Thousand Oaks CA. <https://socialsciences.mcmaster.ca/jfox/Books/Companion/>.
- **carData 3.0.5** - Fox J, Weisberg S, Price B (2022). *_carData: Companion to Applied Regression Data Sets_*. R package version 3.0-5, <https://CRAN.R-project.org/package=carData>.
- **checkmate 2.1.0** - Lang M (2017). "checkmate: Fast Argument Checks for Defensive R Programming." *_The R Journal_*, *9*(1), 437-445. doi:10.32614/RJ-2017-028 <https://doi.org/10.32614/RJ-2017-028>.
- **chron 2.3.56** - James D, Hornik K (2020). *_chron: Chronological Objects which Can Handle Dates and Times_*. R package version 2.3-56. S original by David James, R port by Kurt Hornik., <https://CRAN.R-project.org/package=chron>.
- **cli 3.3.0** - Csárdi G (2022). *_cli: Helpers for Developing Command Line Interfaces_*. R package version 3.3.0, <https://CRAN.R-project.org/package=cli>.

- **clipr 0.8.0** - Lincoln M (2022). *_clipr: Read and Write from the System Clipboard_*. R package version 0.8.0, <https://CRAN.R-project.org/package=clipr>.
- **colorspace 2.0.3** - Zeileis A, Fisher JC, Hornik K, Ihaka R, McWhite CD, Murrell P, Stauffer R, Wilke CO (2020). "colorspace: A Toolbox for Manipulating and Assessing Colors and Palettes." *_Journal of Statistical Software_*, *96*(1), 1-49. doi:10.18637/jss.v096.i01 <https://doi.org/10.18637/jss.v096.i01>.
- **commonmark 1.8.0** - Ooms J (2022). *_commonmark: High Performance CommonMark and Github Markdown Rendering in R_*. R package version 1.8.0, <https://CRAN.R-project.org/package=commonmark>.
- **compareGroups 4.5.1** - Subirana I, Sanz H, Vila J (2014). "Building Bivariate Tables: The compareGroups Package for R." *_Journal of Statistical Software_*, *57*(12), 1-16. <https://www.jstatsoft.org/v57/i12/>.
- **corrplot 0.92** - Wei T, Simko V (2021). *_R package 'corrplot': Visualization of a Correlation Matrix_*. (Version 0.92), <https://github.com/taiyun/corrplot>.
- **cowplot 1.1.1** - Wilke C (2020). *_cowplot: Streamlined Plot Theme and Plot Annotations for 'ggplot2'_*. R package version 1.1.1, <https://CRAN.R-project.org/package=cowplot>.
- **cpp11 0.4.2** - Hester J, François R (2021). *_cpp11: A C++11 Interface for R's C Interface_*. R package version 0.4.2, <https://CRAN.R-project.org/package=cpp11>.
- **crayon 1.5.1** - Csárdi G (2022). *_crayon: Colored Terminal Output_*. R package version 1.5.1, <https://CRAN.R-project.org/package=crayon>.
- **crosstalk 1.2.0** - Cheng J, Sievert C (2021). *_crosstalk: Inter-Widget Interactivity for HTML Widgets_*. R package version 1.2.0, <https://CRAN.R-project.org/package=crosstalk>.
- **curl 4.3.2** - Ooms J (2021). *_curl: A Modern and Flexible Web Client for R_*. R package version 4.3.2, <https://CRAN.R-project.org/package=curl>.
- **data.table 1.14.2** - Dowle M, Srinivasan A (2021). *_data.table: Extension of 'data.frame'_*. R package version 1.14.2, <https://CRAN.R-project.org/package=data.table>.
- **DBI 1.1.2** - R Special Interest Group on Databases (R-SIG-DB), Wickham H, Müller K (2021). *_DBI: R Database Interface_*. R package version 1.1.2, <https://CRAN.R-project.org/package=DBI>.
- **dbplyr 2.1.1** - Wickham H, Girlich M, Ruiz E (2021). *_dbplyr: A 'dplyr' Back End for Databases_*. R package version 2.1.1, <https://CRAN.R-project.org/package=dbplyr>.
- **DelayedArray 0.22.0** - Pagès H, Hickey wcfP, Lun A (2022). *_DelayedArray: A unified framework for working transparently with on-disk and in-memory array-like datasets_*. R package version 0.22.0, <https://bioconductor.org/packages/DelayedArray>.
- **desc 1.4.1** - Csárdi G, Müller K, Hester J (2022). *_desc: Manipulate DESCRIPTION Files_*. R package version 1.4.1, <https://CRAN.R-project.org/package=desc>.
- **DiagrammeR 1.0.9** - Iannone R (2022). *_DiagrammeR: Graph/Network Visualization_*. R package version 1.0.9, <https://CRAN.R-project.org/package=DiagrammeR>.
- **dichromat 2.0.0.1** - Lumley T (2022). *_dichromat: Color Schemes for Dichromats_*. R package version 2.0-0.1, <https://CRAN.R-project.org/package=dichromat>.
- **diffobj 0.3.5** - Gaslam B (2021). *_diffobj: Diffs for R Objects_*. R package version 0.3.5, <https://CRAN.R-project.org/package=diffobj>.
- **digest 0.6.29** - Lucas DEwcbA, Tuszynski J, Bengtsson H, Urbanek S, Frasca M, Lewis B, Stokely M, Muehlenleisen H, Murdoch D, Hester J, Wu W, Kou Q, Onkelinx T, Lang M, Simko V, Hornik K, Neal R, Bell K, de Queljoe M, Suruceanu I, Denney B, Schumacher D, Chang. aW

(2021). *_digest: Create Compact Hash Digests of R Objects_*. R package version 0.6.29, <https://CRAN.R-project.org/package=digest>.

- **downloader 0.4** - Chang W (2015). *_downloader: Download Files over HTTP and HTTPS_*. R package version 0.4, <https://CRAN.R-project.org/package=downloader>.
- **dplyr 1.0.9** - Wickham H, François R, Henry L, Müller K (2022). *_dplyr: A Grammar of Data Manipulation_*. R package version 1.0.9, <https://CRAN.R-project.org/package=dplyr>.
- **DT 0.23** - Xie Y, Cheng J, Tan X (2022). *_DT: A Wrapper of the JavaScript Library 'DataTables'_*. R package version 0.23, <https://CRAN.R-project.org/package=DT>.
- **ellipsis 0.3.2** - Wickham H (2021). *_ellipsis: Tools for Working with_*. R package version 0.3.2, <https://CRAN.R-project.org/package=ellipsis>.
- **ensemblDb 2.20.1** - Rainer J, Gatto L, Weichenberger CX (2019). "ensemblDb: an R package to create and use Ensembl-based annotation resources." *_Bioinformatics_*. doi:10.1093/bioinformatics/btz031 <https://doi.org/10.1093/bioinformatics/btz031>, <https://academic.oup.com/bioinformatics/advance-article/doi/10.1093/bioinformatics/btz031/5301311>.
- **evaluate 0.15** - Wickham H, Xie Y (2022). *_evaluate: Parsing and Evaluation Tools that Provide More Details than the Default_*. R package version 0.15, <https://CRAN.R-project.org/package=evaluate>.
- **excelR 0.4.0** - Bista S (2020). *_excelR: A Wrapper of the 'JavaScript' Library 'jExcel'_*. R package version 0.4.0, <https://CRAN.R-project.org/package=excelR>.
- **fansi 1.0.3** - Gaslam B (2022). *_fansi: ANSI Control Sequence Aware String Functions_*. R package version 1.0.3, <https://CRAN.R-project.org/package=fansi>.
- **farver 2.1.0** - Pedersen T, Nicolae B, François R (2021). *_farver: High Performance Colour Space Manipulation_*. R package version 2.1.0, <https://CRAN.R-project.org/package=farver>.
- **fastmap 1.1.0** - Chang W (2021). *_fastmap: Fast Data Structures_*. R package version 1.1.0, <https://CRAN.R-project.org/package=fastmap>.
- **fastmatch 1.1.3** - Urbanek S (2021). *_fastmatch: Fast 'match()' Function_*. R package version 1.1-3, <https://CRAN.R-project.org/package=fastmatch>.
- **filelock 1.0.2** - Csárdi G (2018). *_filelock: Portable File Locking_*. R package version 1.0.2, <https://CRAN.R-project.org/package=filelock>.
- **flextable 0.7.0** - Gohel D (2022). *_flextable: Functions for Tabular Reporting_*. R package version 0.7.0, <https://CRAN.R-project.org/package=flextable>.
- **fontawesome 0.2.2** - Iannone R (2021). *_fontawesome: Easily Work with 'Font Awesome' Icons_*. R package version 0.2.2, <https://CRAN.R-project.org/package=fontawesome>.
- **formatR 1.12** - Xie Y (2022). *_formatR: Format R Code Automatically_*. R package version 1.12, <https://CRAN.R-project.org/package=formatR>.
- **formattable 0.2.1** - Ren K, Russell K (2021). *_formattable: Create 'Formattable' Data Structures_*. R package version 0.2.1, <https://CRAN.R-project.org/package=formattable>.
- **Formula 1.2.4** - Zeileis A, Croissant Y (2010). "Extended Model Formulas in R: Multiple Parts and Multiple Responses." *_Journal of Statistical Software_*, *34*(1), 1-13. doi:10.18637/jss.v034.i01 <https://doi.org/10.18637/jss.v034.i01>.
- **fs 1.5.2** - Hester J, Wickham H, Csárdi G (2021). *_fs: Cross-Platform File System Operations Based on 'libuv'_*. R package version 1.5.2, <https://CRAN.R-project.org/package=fs>.
- **futile.logger 1.4.3** - Rowe BLY (2016). *_futile.logger: A Logging Utility for R_*. R package version 1.4.3, <https://CRAN.R-project.org/package=futile.logger>.

- **futile.options 1.0.1** - Rowe BLY (2018). *_futile.options: Futile Options Management_*. R package version 1.0.1, <https://CRAN.R-project.org/package=futile.options>.
- **gdtools 0.2.4** - Gohel D, Wickham H, Henry L, Ooms J (2022). *_gdtools: Utilities for Graphical Rendering_*. R package version 0.2.4, <https://CRAN.R-project.org/package=gdtools>.
- **generics 0.1.2** - Wickham H, Kuhn M, Vaughan D (2022). *_generics: Common S3 Generics not Provided by Base R Methods Related to Model Fitting_*. R package version 0.1.2, <https://CRAN.R-project.org/package=generics>.
- **GenomeInfoDb 1.32.1** - Arora S, Morgan M, Carlson M, Pagès H (2022). *_GenomeInfoDb: Utilities for manipulating chromosome names, including modifying them to follow a particular naming style_*. R package version 1.32.1, <https://bioconductor.org/packages/GenomeInfoDb>.
- **GenomicAlignments 1.32.0** - Lawrence M, Huber W, Pagès H, Aboyoun P, Carlson M, Gentleman R, Morgan M, Carey V (2013). "Software for Computing and Annotating Genomic Ranges." *_PLoS Computational Biology_*, *9*. doi:10.1371/journal.pcbi.1003118 <https://doi.org/10.1371/journal.pcbi.1003118>, <http://www.ploscompbiol.org/article/info%3Adoi%2F10.1371%2Fjournal.pcbi.1003118>.
- **GenomicFeatures 1.48.1** - Lawrence M, Huber W, Pagès H, Aboyoun P, Carlson M, Gentleman R, Morgan M, Carey V (2013). "Software for Computing and Annotating Genomic Ranges." *_PLoS Computational Biology_*, *9*. doi:10.1371/journal.pcbi.1003118 <https://doi.org/10.1371/journal.pcbi.1003118>, <http://www.ploscompbiol.org/article/info%3Adoi%2F10.1371%2Fjournal.pcbi.1003118>.
- **GenomicRanges 1.48.0** - Lawrence M, Huber W, Pagès H, Aboyoun P, Carlson M, Gentleman R, Morgan M, Carey V (2013). "Software for Computing and Annotating Genomic Ranges." *_PLoS Computational Biology_*, *9*. doi:10.1371/journal.pcbi.1003118 <https://doi.org/10.1371/journal.pcbi.1003118>, <http://www.ploscompbiol.org/article/info%3Adoi%2F10.1371%2Fjournal.pcbi.1003118>.
- **ggdendro 0.1.23** - de Vries A, Ripley BD (2022). *_ggdendro: Create Dendograms and Tree Diagrams Using 'ggplot2'_*. R package version 0.1.23, <https://CRAN.R-project.org/package=ggdendro>.
- **ggplot2 3.3.6** - Wickham H (2016). *_ggplot2: Elegant Graphics for Data Analysis_*. Springer-Verlag New York. ISBN 978-3-319-24277-4, <https://ggplot2.tidyverse.org>.
- **ggpubr 0.4.0** - Kassambara A (2020). *_ggpubr: 'ggplot2' Based Publication Ready Plots_*. R package version 0.4.0, <https://CRAN.R-project.org/package=ggpubr>.
- **ggrepel 0.9.1** - Slowikowski K (2021). *_ggrepel: Automatically Position Non-Overlapping Text Labels with 'ggplot2'_*. R package version 0.9.1, <https://CRAN.R-project.org/package=ggrepel>.
- **ggsci 2.9** - Xiao N (2018). *_ggsci: Scientific Journal and Sci-Fi Themed Color Palettes for 'ggplot2'_*. R package version 2.9, <https://CRAN.R-project.org/package=ggsci>.
- **ggsignif 0.6.3** - Constantin A, Patil I (2021). "ggsignif: R Package for Displaying Significance Brackets for 'ggplot2'." *_PsyArxiv_*. doi:10.31234/osf.io/7awm6 <https://doi.org/10.31234/osf.io/7awm6>, <https://psyarxiv.com/7awm6>.
- **glue 1.6.2** - Hester J, Bryan J (2022). *_glue: Interpreted String Literals_*. R package version 1.6.2, <https://CRAN.R-project.org/package=glue>.
- **gmp 0.6.5** - Lucas A, Scholz I, Boehme R, Jasson S, Maechler M (2022). *_gmp: Multiple Precision Arithmetic_*. R package version 0.6-5, <https://CRAN.R-project.org/package=gmp>.

- **gridExtra 2.3** - Auguie B (2017). *_gridExtra: Miscellaneous Functions for "Grid" Graphics_*. R package version 2.3, <https://CRAN.R-project.org/package=gridExtra>.
- **gttable 0.3.0** - Wickham H, Pedersen T (2019). *_gttable: Arrange 'Grobs' in Tables_*. R package version 0.3.0, <https://CRAN.R-project.org/package=gttable>.
- **Gviz 1.40.1** - Hahne F, Ivanek R (2016). "Statistical Genomics: Methods and Protocols." In Mathé E, Davis S (eds.), chapter Visualizing Genomic Data Using Gviz and Bioconductor, 335-351. Springer New York, New York, NY. ISBN 978-1-4939-3578-9, doi:10.1007/978-1-4939-3578-9_16 https://doi.org/10.1007/978-1-4939-3578-9_16, http://dx.doi.org/10.1007/978-1-4939-3578-9_16.
- **HardyWeinberg 1.7.5** - Graffelman J (2015). "Exploring Diallelic Genetic Markers: The HardyWeinberg Package." *_Journal of Statistical Software_*, *64*(3), 1-23. <https://www.jstatsoft.org/v64/i03/>.
- **highr 0.9** - Xie Y, Qiu Y (2021). *_highr: Syntax Highlighting for R Source Code_*. R package version 0.9, <https://CRAN.R-project.org/package=highr>.
- **Hmisc 4.7.0** - Harrell Jr F (2022). *_Hmisc: Harrell Miscellaneous_*. R package version 4.7-0, <https://CRAN.R-project.org/package=Hmisc>.
- **hms 1.1.1** - Müller K (2021). *_hms: Pretty Time of Day_*. R package version 1.1.1, <https://CRAN.R-project.org/package=hms>.
- **htmlTable 2.4.0** - Gordon M, Gragg S, Konings P (2022). *_htmlTable: Advanced Tables for Markdown/HTML_*. R package version 2.4.0, <https://CRAN.R-project.org/package=htmlTable>.
- **htmltools 0.5.2** - Cheng J, Sievert C, Schloerke B, Chang W, Xie Y, Allen J (2021). *_htmltools: Tools for HTML_*. R package version 0.5.2, <https://CRAN.R-project.org/package=htmltools>.
- **htmlwidgets 1.5.4** - Vaidyanathan R, Xie Y, Allaire J, Cheng J, Sievert C, Russell K (2021). *_htmlwidgets: HTML Widgets for R_*. R package version 1.5.4, <https://CRAN.R-project.org/package=htmlwidgets>.
- **httpuv 1.6.5** - Cheng J, Chang W (2022). *_httpuv: HTTP and WebSocket Server Library_*. R package version 1.6.5, <https://CRAN.R-project.org/package=httpuv>.
- **httr 1.4.3** - Wickham H (2022). *_httr: Tools for Working with URLs and HTTP_*. R package version 1.4.3, <https://CRAN.R-project.org/package=httr>.
- **igraph 1.3.1** - Csardi G, Nepusz T (2006). "The igraph software package for complex network research." *_InterJournal_*, *Complex Systems*, 1695. <https://igraph.org>.
- **influenceR 0.1.0.1** - Simon J, Aditya K (2021). *_influenceR: Software Tools to Quantify Structural Importance of Nodes in a Network_*. R package version 0.1.0.1, <https://CRAN.R-project.org/package=influenceR>.
- **IRanges 2.30.0** - Lawrence M, Huber W, Pagès H, Aboyoun P, Carlson M, Gentleman R, Morgan M, Carey V (2013). "Software for Computing and Annotating Genomic Ranges." *_PLoS Computational Biology_*, *9*. doi:10.1371/journal.pcbi.1003118 <https://doi.org/10.1371/journal.pcbi.1003118>, <http://www.ploscompbiol.org/article/info%3Adoi%2F10.1371%2Fjournal.pcbi.1003118>.
- **isoband 0.2.5** - Wilke C, Pedersen T (2021). *_isoband: Generate Isolines and Isobands from Regularly Spaced Elevation Grids_*. R package version 0.2.5, <https://CRAN.R-project.org/package=isoband>.
- **jpeg 0.1.9** - Urbanek S (2021). *_jpeg: Read and write JPEG images_*. R package version 0.1-9, <https://CRAN.R-project.org/package=jpeg>.

- **jquerylib 0.1.4** - Sievert C, Cheng J (2021). `_jquerylib`: Obtain 'jQuery' as an HTML Dependency Object_. R package version 0.1.4, <https://CRAN.R-project.org/package=jquerylib>.
- **jsonlite 1.8.0** - Ooms J (2014). "The jsonlite Package: A Practical and Consistent Mapping Between JSON Data and R Objects." `_arXiv:1403.2805 [stat.CO]`_. <https://arxiv.org/abs/1403.2805>.
- **kableExtra 1.3.4** - Zhu H (2021). `_kableExtra`: Construct Complex Table with 'kable' and Pipe Syntax_. R package version 1.3.4, <https://CRAN.R-project.org/package=kableExtra>.
- **knitr 1.39** - Xie Y (2022). `_knitr`: A General-Purpose Package for Dynamic Report Generation in R_. R package version 1.39, <https://yihui.org/knitr/>.
- **labeling 0.4.2** - Talbot, J (2020). `_labeling`: Axis Labeling_. R package version 0.4.2, <https://CRAN.R-project.org/package=labeling>.
- **lambda.r 1.2.4** - Rowe BLY (2019). `_lambda.r`: Modeling Data with Functional Programming_. R package version 1.2.4, <https://CRAN.R-project.org/package=lambda.r>.
- **later 1.3.0** - Chang W, Cheng J (2021). `_later`: Utilities for Scheduling Functions to Execute Later with Event Loops_. R package version 1.3.0, <https://CRAN.R-project.org/package=later>.
- **latticeExtra 0.6.29** - Sarkar D, Andrews F (2019). `_latticeExtra`: Extra Graphical Utilities Based on Lattice_. R package version 0.6-29, <https://CRAN.R-project.org/package=latticeExtra>.
- **lazyeval 0.2.2** - Wickham H (2019). `_lazyeval`: Lazy (Non-Standard) Evaluation_. R package version 0.2.2, <https://CRAN.R-project.org/package=lazyeval>.
- **lifecycle 1.0.1** - Henry L, Wickham H (2021). `_lifecycle`: Manage the Life Cycle of your Package Functions_. R package version 1.0.1, <https://CRAN.R-project.org/package=lifecycle>.
- **lme4 1.1.29** - Bates D, Mächler M, Bolker B, Walker S (2015). "Fitting Linear Mixed-Effects Models Using lme4." `_Journal of Statistical Software`, *67*(1), 1-48. doi:10.18637/jss.v067.i01 <https://doi.org/10.18637/jss.v067.i01>.
- **logger 0.2.2** - Daróczi G (2021). `_logger`: A Lightweight, Modern and Flexible Logging Utility_. R package version 0.2.2, <https://CRAN.R-project.org/package=logger>.
- **magrittr 2.0.3** - Bache S, Wickham H (2022). `_magrittr`: A Forward-Pipe Operator for R_. R package version 2.0.3, <https://CRAN.R-project.org/package=magrittr>.
- **maptools 1.1.4** - Bivand R, Lewin-Koh N (2022). `_maptools`: Tools for Handling Spatial Objects_. R package version 1.1-4, <https://CRAN.R-project.org/package=maptools>.
- **MatrixGenerics 1.8.0** - Ahlmann-Eltze C, Hickey P, Pagès H (2022). `_MatrixGenerics`: S4 Generic Summary Statistic Functions that Operate on Matrix-Like Objects_. R package version 1.8.0, <https://bioconductor.org/packages/MatrixGenerics>.
- **MatrixModels 0.5.0** - Bates D, Maechler M (2021). `_MatrixModels`: Modelling with Sparse and Dense Matrices_. R package version 0.5-0, <https://CRAN.R-project.org/package=MatrixModels>.
- **matrixStats 0.62.0** - Bengtsson H (2022). `_matrixStats`: Functions that Apply to Rows and Columns of Matrices (and to Vectors)_. R package version 0.62.0, <https://CRAN.R-project.org/package=matrixStats>.
- **memoise 2.0.1** - Wickham H, Hester J, Chang W, Müller K, Cook D (2021). `_memoise`: 'Memoisation' of Functions_. R package version 2.0.1, <https://CRAN.R-project.org/package=memoise>.

- **mice 3.14.0** - van Buuren S, Groothuis-Oudshoorn K (2011). "mice: Multivariate Imputation by Chained Equations in R." *_Journal of Statistical Software_*, *45*(3), 1-67. doi:10.18637/jss.v045.i03 <https://doi.org/10.18637/jss.v045.i03>.
- **mime 0.12** - Xie Y (2021). *_mime: Map Filenames to MIME Types_*. R package version 0.12, <https://CRAN.R-project.org/package=mime>.
- **minqa 1.2.4** - Bates D, Mullen KM, Nash JC, Varadhan R (2014). *_minqa: Derivative-free optimization algorithms by quadratic approximation_*. R package version 1.2.4, <https://CRAN.R-project.org/package=minqa>.
- **munsell 0.5.0** - Wickham C (2018). *_munsell: Utilities for Using Munsell Colours_*. R package version 0.5.0, <https://CRAN.R-project.org/package=munsell>.
- **numDeriv 2016.8.1.1** - Gilbert P, Varadhan R (2019). *_numDeriv: Accurate Numerical Derivatives_*. R package version 2016.8-1.1, <https://CRAN.R-project.org/package=numDeriv>.
- **officer 0.4.2** - Gohel D (2022). *_officer: Manipulation of Microsoft Word and PowerPoint Documents_*. R package version 0.4.2, <https://CRAN.R-project.org/package=officer>.
- **openssl 2.0.1** - Ooms J (2022). *_openssl: Toolkit for Encryption, Signatures and Certificates Based on OpenSSL_*. R package version 2.0.1, <https://CRAN.R-project.org/package=openssl>.
- **openxlsx 4.2.5** - Schäuberger P, Walker A (2021). *_openxlsx: Read, Write and Edit xlsx Files_*. R package version 4.2.5, <https://CRAN.R-project.org/package=openxlsx>.
- **pbkrtest 0.5.1** - Halekoh U, Højsgaard S (2014). "A Kenward-Roger Approximation and Parametric Bootstrap Methods for Tests in Linear Mixed Models - The R Package pbkrtest." *_Journal of Statistical Software_*, *59*(9), 1-30. <https://www.jstatsoft.org/v59/i09/>.
- **pdftools 3.2.0** - Ooms J (2022). *_pdftools: Text Extraction, Rendering and Converting of PDF Documents_*. R package version 3.2.0, <https://CRAN.R-project.org/package=pdftools>.
- **phangorn 2.8.1** - Schliep K (2011). "phangorn: phylogenetic analysis in R." *_Bioinformatics_*, *27*(4), 592-593. doi:10.1093/bioinformatics/btq706 <https://doi.org/10.1093/bioinformatics/btq706>.
- **pillar 1.7.0** - Müller K, Wickham H (2022). *_pillar: Coloured Formatting for Columns_*. R package version 1.7.0, <https://CRAN.R-project.org/package=pillar>.
- **pixmap 0.4.12** - Bivand R, Leisch F, Maechler M (2021). *_pixmap: Bitmap Images / Pixel Maps_*. R package version 0.4-12, <https://CRAN.R-project.org/package=pixmap>.
- **pkgconfig 2.0.3** - Csárdi G (2019). *_pkgconfig: Private Configuration for 'R' Packages_*. R package version 2.0.3, <https://CRAN.R-project.org/package=pkgconfig>.
- **pkgload 1.2.4** - Wickham H, Chang W, Hester J, Henry L (2021). *_pkgload: Simulate Package Installation and Attach_*. R package version 1.2.4, <https://CRAN.R-project.org/package=pkgload>.
- **plogr 0.2.0** - Müller K (2018). *_plogr: The 'plog' C++ Logging Library_*. R package version 0.2.0, <https://CRAN.R-project.org/package=plogr>.
- **plotly 4.10.0** - Sievert C (2020). *_Interactive Web-Based Data Visualization with R, plotly, and shiny_*. Chapman and Hall/CRC. ISBN 9781138331457, <https://plotly-r.com>.
- **plyr 1.8.7** - Wickham H (2011). "The Split-Apply-Combine Strategy for Data Analysis." *_Journal of Statistical Software_*, *40*(1), 1-29. <https://www.jstatsoft.org/v40/i01/>.
- **png 0.1.7** - Urbanek S (2013). *_png: Read and write PNG images_*. R package version 0.1-7, <https://CRAN.R-project.org/package=png>.

- **polynom 1.4.1** - Venables B, Hornik K, Maechler M (2022). *_polynom: A Collection of Functions to Implement a Class for Univariate Polynomial Manipulations_*. R package version 1.4-1. S original by Bill Venables, packages for R by Kurt Hornik and Martin Maechler., <https://CRAN.R-project.org/package=polynom>.
- **praise 1.0.0** - Csardi G, Sorhus S (2015). *_praise: Praise Users_*. R package version 1.0.0, <https://CRAN.R-project.org/package=praise>.
- **prettyunits 1.1.1** - Csardi G (2020). *_prettyunits: Pretty, Human Readable Formatting of Quantities_*. R package version 1.1.1, <https://CRAN.R-project.org/package=prettyunits>.
- **processx 3.5.3** - Csárdi G, Chang W (2022). *_processx: Execute and Control System Processes_*. R package version 3.5.3, <https://CRAN.R-project.org/package=processx>.
- **progress 1.2.2** - Csárdi G, FitzJohn R (2019). *_progress: Terminal Progress Bars_*. R package version 1.2.2, <https://CRAN.R-project.org/package=progress>.
- **promises 1.2.0.1** - Cheng J (2021). *_promises: Abstractions for Promise-Based Asynchronous Programming_*. R package version 1.2.0.1, <https://CRAN.R-project.org/package=promises>.
- **ProtGenerics 1.28.0** - Gatto L, Rainer J (2022). *_ProtGenerics: Generic infrastructure for Bioconductor mass spectrometry packages_*. R package version 1.28.0, <https://github.com/RforMassSpectrometry/ProtGenerics>.
- **ps 1.7.0** - Loden J, Daeschler D, Rodola' G, Csárdi G (2022). *_ps: List, Query, Manipulate System Processes_*. R package version 1.7.0, <https://CRAN.R-project.org/package=ps>.
- **purrr 0.3.4** - Henry L, Wickham H (2020). *_purrr: Functional Programming Tools_*. R package version 0.3.4, <https://CRAN.R-project.org/package=purrr>.
- **qpdf 1.1** - Ooms J (2019). *_qpdf: Split, Combine and Compress PDF Files_*. R package version 1.1, <https://CRAN.R-project.org/package=qpdf>.
- **quadprog 1.5.8** - dpodi/LINPACK) SobBATRpbAW<FcfcCM (2019). *_quadprog: Functions to Solve Quadratic Programming Problems_*. R package version 1.5-8, <<https://CRAN.R-project.org/package=quadprog>>
- **quantreg 5.93** - Koenker R (2022). *_quantreg: Quantile Regression_*. R package version 5.93, <https://CRAN.R-project.org/package=quantreg>.
- **R6 2.5.1** - Chang W (2021). *_R6: Encapsulated Classes with Reference Semantics_*. R package version 2.5.1, <https://CRAN.R-project.org/package=R6>.
- **rappdirs 0.3.3** - Ratnakumar S, Mick T, Davis T (2021). *_rappdirs: Application Directories: Determine Where to Save Data, Caches, and Logs_*. R package version 0.3.3, <https://CRAN.R-project.org/package=rappdirs>.
- **RColorBrewer 1.1.3** - Neuwirth E (2022). *_RColorBrewer: ColorBrewer Palettes_*. R package version 1.1-3, <https://CRAN.R-project.org/package=RColorBrewer>.
- **Rcpp 1.0.8.3** - Eddelbuettel D, François R (2011). "Rcpp: Seamless R and C++ Integration." *_Journal of Statistical Software_*, *40*(8), 1-18. doi:10.18637/jss.v040.i08 <https://doi.org/10.18637/jss.v040.i08>.
- **RcppEigen 0.3.3.9.2** - Bates D, Eddelbuettel D (2013). "Fast and Elegant Numerical Linear Algebra Using the RcppEigen Package." *_Journal of Statistical Software_*, *52*(5), 1-24. doi:10.18637/jss.v052.i05 <https://doi.org/10.18637/jss.v052.i05>.
- **RCurl 1.98.1.6** - Temple Lang D (2022). *_RCurl: General Network (HTTP/FTP/...) Client Interface for R_*. R package version 1.98-1.6, <https://CRAN.R-project.org/package=RCurl>.

- **readr 2.1.2** - Wickham H, Hester J, Bryan J (2022). *_readr: Read Rectangular Text Data_*. R package version 2.1.2, <https://CRAN.R-project.org/package=readr>.
- **rematch2 2.1.2** - Csárdi G (2020). *_rematch2: Tidy Output from Regular Expression Matching_*. R package version 2.1.2, <https://CRAN.R-project.org/package=rematch2>.
- **renv 0.15.4** - Ushey K (2022). *_renv: Project Environments_*. R package version 0.15.4, <https://CRAN.R-project.org/package=renv>.
- **reshape2 1.4.4** - Wickham H (2007). "Reshaping Data with the reshape Package." *_Journal of Statistical Software_*, *21*(12), 1-20. <http://www.jstatsoft.org/v21/i12/>.
- **restfulr 0.0.13** - Lawrence M (2017). *_restfulr: R Interface to RESTful Web Services_*. R package version 0.0.13, <https://CRAN.R-project.org/package=restfulr>.
- **rjson 0.2.21** - Couture-Bell A (2022). *_rjson: JSON for R_*. R package version 0.2.21, <https://CRAN.R-project.org/package=rjson>.
- **rlang 1.0.2** - Henry L, Wickham H (2022). *_rlang: Functions for Base Types and Core R and 'Tidyverse' Features_*. R package version 1.0.2, <https://CRAN.R-project.org/package=rlang>.
- **rlist 0.4.6.2** - Ren K (2021). *_rlist: A Toolbox for Non-Tabular Data Manipulation_*. R package version 0.4.6.2, <https://CRAN.R-project.org/package=rlist>.
- **rmarkdown 2.14** - Allaire J, Xie Y, McPherson J, Luraschi J, Ushey K, Atkins A, Wickham H, Cheng J, Chang W, Iannone R (2022). *_rmarkdown: Dynamic Documents for R_*. R package version 2.14, <https://github.com/rstudio/rmarkdown>.
- **Rmisc 1.5.1** - Hope RM (2022). *_Rmisc: Ryan Miscellaneous_*. R package version 1.5.1, <https://CRAN.R-project.org/package=Rmisc>.
- **rprojroot 2.0.3** - Müller K (2022). *_rprojroot: Finding Files in Project Subdirectories_*. R package version 2.0.3, <https://CRAN.R-project.org/package=rprojroot>.
- **Rsamtools 2.12.0** - Morgan M, Pagès H, Obenchain V, Hayden N (2022). *_Rsamtools: Binary alignment (BAM), FASTA, variant call (BCF), and tabix file import_*. R package version 2.12.0, <https://bioconductor.org/packages/Rsamtools>.
- **RSQlite 2.2.14** - Müller K, Wickham H, James DA, Falcon S (2022). *_RSQlite: SQLite Interface for R_*. R package version 2.2.14, <https://CRAN.R-project.org/package=RSQlite>.
- **rstatix 0.7.0** - Kassambara A (2021). *_rstatix: Pipe-Friendly Framework for Basic Statistical Tests_*. R package version 0.7.0, <https://CRAN.R-project.org/package=rstatix>.
- **rstudioapi 0.13** - Ushey K, Allaire J, Wickham H, Ritchie G (2020). *_rstudioapi: Safely Access the RStudio API_*. R package version 0.13, <https://CRAN.R-project.org/package=rstudioapi>.
- **rtracklayer 1.56.0** - Lawrence M, Gentleman R, Carey V (2009). "rtracklayer: an R package for interfacing with genome browsers." *_Bioinformatics_*, *25*, 1841-1842. doi:10.1093/bioinformatics/btp328 <https://doi.org/10.1093/bioinformatics/btp328>, <http://bioinformatics.oxfordjournals.org/content/25/14/1841.abstract>.
- **rvest 1.0.2** - Wickham H (2021). *_rvest: Easily Harvest (Scrape) Web Pages_*. R package version 1.0.2, <https://CRAN.R-project.org/package=rvest>.
- **S4Vectors 0.34.0** - Pagès H, Lawrence M, Aboym P (2022). *_S4Vectors: Foundation of vector-like and list-like containers in Bioconductor_*. R package version 0.34.0, <https://bioconductor.org/packages/S4Vectors>.
- **sangeranalyseR 1.6.1** - Chao K, Barton K, Palmer S, Lanfear R (2021). "sangeranalyseR: simple and interactive analysis of Sanger sequencing data in R." *_Genome Biology and*

Evolution_. doi:10.1093/gbe/evab028 <https://doi.org/10.1093/gbe/evab028>.

- **sangerseqR 1.32.0** - Hill JT, Demarest BL, Bisgrove BW, Su Y, Smith M, Yost HJ (2014). "Poly peak parser: Method and software for identification of unknown indels using sanger sequencing of polymerase chain reaction products." _Developmental Dynamics_. doi:10.1002/dvdy.24183. <https://doi.org/10.1002/dvdy.24183..>
- **sass 0.4.1** - Cheng J, Mastny T, Iannone R, Schloerke B, Sievert C (2022). _sass: Syntactically Awesome Style Sheets ('Sass')_. R package version 0.4.1, <https://CRAN.R-project.org/package=sass>.
- **scales 1.2.0** - Wickham H, Seidel D (2022). _scales: Scale Functions for Visualization_. R package version 1.2.0, <https://CRAN.R-project.org/package=scales>.
- **selectr 0.4.2** - Potter S (2012). "Introducing the selectr Package." The University of Auckland, Auckland, New Zealand. <http://stattech.wordpress.fos.auckland.ac.nz/2012-10-introducing-the-selectr-package/>.
- **sessioninfo 1.2.2** - Wickham H, Chang W, Flight R, Müller K, Hester J (2021). _sessioninfo: R Session Information_. R package version 1.2.2, <https://CRAN.R-project.org/package=sessioninfo>.
- **shiny 1.7.1** - Chang W, Cheng J, Allaire J, Sievert C, Schloerke B, Xie Y, Allen J, McPherson J, Dipert A, Borges B (2021). _shiny: Web Application Framework for R_. R package version 1.7.1, <https://CRAN.R-project.org/package=shiny>.
- **shinycssloaders 1.0.0** - Sali A, Attali D (2020). _shinycssloaders: Add Loading Animations to a 'shiny' Output While It's Recalculating_. R package version 1.0.0, <https://CRAN.R-project.org/package=shinycssloaders>.
- **shinydashboard 0.7.2** - Chang W, Borges Ribeiro B (2021). _shinydashboard: Create Dashboards with 'Shiny'_. R package version 0.7.2, <https://CRAN.R-project.org/package=shinydashboard>.
- **shinyjs 2.1.0** - Attali D (2021). _shinyjs: Easily Improve the User Experience of Your Shiny Apps in Seconds_. R package version 2.1.0, <https://CRAN.R-project.org/package=shinyjs>.
- **shinythemes 1.2.0** - Chang W (2021). _shinythemes: Themes for Shiny_. R package version 1.2.0, <https://CRAN.R-project.org/package=shinythemes>.
- **shinyWidgets 0.7.0** - Perrier V, Meyer F, Granjon D (2022). _shinyWidgets: Custom Inputs Widgets for Shiny_. R package version 0.7.0, <https://CRAN.R-project.org/package=shinyWidgets>.
- **snow 0.4.4** - Tierney L, Rossini AJ, Li N, Sevcikova H (2021). _snow: Simple Network of Workstations_. R package version 0.4-4, <https://CRAN.R-project.org/package=snow>.
- **sourcetools 0.1.7** - Ushey K (2018). _sourcetools: Tools for Reading, Tokenizing and Parsing R Code_. R package version 0.1.7, <https://CRAN.R-project.org/package=sourcetools>.
- **sp 1.4.7** - Pebesma EJ, Bivand RS (2005). "Classes and methods for spatial data in R." _R News_, *5*(2), 9-13. <https://CRAN.R-project.org/doc/Rnews/>.
- **SparseM 1.81** - Koenker R (2021). _SparseM: Sparse Linear Algebra_. R package version 1.81, <https://CRAN.R-project.org/package=SparseM>.
- **stringr 1.4.0** - Wickham H (2019). _stringr: Simple, Consistent Wrappers for Common String Operations_. R package version 1.4.0, <https://CRAN.R-project.org/package=stringr>.

- **SummarizedExperiment 1.26.1** - Morgan M, Obenchain V, Hester J, Pagès H (2022). *_SummarizedExperiment: SummarizedExperiment container_*. R package version 1.26.1, <https://bioconductor.org/packages/SummarizedExperiment>.
- **svglite 2.1.0** - Wickham H, Henry L, Pedersen T, Luciani T, Decorde M, Lise V (2022). *_svglite: An 'SVG' Graphics Device_*. R package version 2.1.0, <https://CRAN.R-project.org/package=svglite>.
- **sys 3.4** - Ooms J (2020). *_sys: Powerful and Reliable Tools for Running System Commands in R_*. R package version 3.4, <https://CRAN.R-project.org/package=sys>.
- **systemfonts 1.0.4** - Pedersen T, Ooms J, Govett D (2022). *_systemfonts: System Native Font Finding_*. R package version 1.0.4, <https://CRAN.R-project.org/package=systemfonts>.
- **testthat 3.1.4** - Wickham H (2011). "testthat: Get Started with Testing." *_The R Journal_, *3*, 5-10.* https://journal.r-project.org/archive/2011-1/RJournal_2011-1_Wickham.pdf.
- **tibble 3.1.7** - Müller K, Wickham H (2022). *_tibble: Simple Data Frames_*. R package version 3.1.7, <https://CRAN.R-project.org/package=tibble>.
- **tidy 1.2.0** - Wickham H, Girlich M (2022). *_tidy: Tidy Messy Data_*. R package version 1.2.0, <https://CRAN.R-project.org/package=tidy>.
- **tidyselect 1.1.2** - Henry L, Wickham H (2022). *_tidyselect: Select from a Set of Strings_*. R package version 1.1.2, <https://CRAN.R-project.org/package=tidyselect>.
- **tinytex 0.39** - Xie Y (2022). *_tinytex: Helper Functions to Install and Maintain TeX Live, and Compile LaTeX Documents_*. R package version 0.39, <https://github.com/rstudio/tinytex>.
- **truncnorm 1.0.8** - Mersmann O, Trautmann H, Steuer D, Bornkamp B (2018). *_truncnorm: Truncated Normal Distribution_*. R package version 1.0-8, <https://CRAN.R-project.org/package=truncnorm>.
- **tzdb 0.3.0** - Vaughan D (2022). *_tzdb: Time Zone Database Information_*. R package version 0.3.0, <https://CRAN.R-project.org/package=tzdb>.
- **utf8 1.2.2** - Perry PO (2021). *_utf8: Unicode Text Processing_*. R package version 1.2.2, <https://CRAN.R-project.org/package=utf8>.
- **uuid 1.1.0** - Urbanek S, Ts'o T (2022). *_uuid: Tools for Generating and Handling of UUIDs_*. R package version 1.1-0, <https://CRAN.R-project.org/package=uuid>.
- **VariantAnnotation 1.42.0** - Obenchain V, Lawrence M, Carey V, Gogarten S, Shannon P, Morgan M (2014). "VariantAnnotation: a Bioconductor package for exploration and annotation of genetic variants." *_Bioinformatics_, *30*(14), 2076-2078. doi:10.1093/bioinformatics/btu168* <https://doi.org/10.1093/bioinformatics/btu168>.
- **vctrs 0.4.1** - Wickham H, Henry L, Vaughan D (2022). *_vctrs: Vector Helpers_*. R package version 0.4.1, <https://CRAN.R-project.org/package=vctrs>.
- **viridis 0.6.2** - Garnier, Simon, Ross, Noam, Rudis, Robert, Camargo, Pedro A, Sciaini, Marco, Scherer, Cédric (2021). *_viridis - Colorblind-Friendly Color Maps for R_*. doi:10.5281/zenodo.4679424 <https://doi.org/10.5281/zenodo.4679424>, R package version 0.6.2, <https://sjmgarnier.github.io/viridis/>.
- **viridisLite 0.4.0** - Garnier, Simon, Ross, Noam, Rudis, Robert, Camargo, Pedro A, Sciaini, Marco, Scherer, Cédric (2021). *_viridis - Colorblind-Friendly Color Maps for R_*. doi:10.5281/zenodo.4679424 <https://doi.org/10.5281/zenodo.4679424>, R package version 0.4.0, <https://sjmgarnier.github.io/viridis/>.

- **visNetwork 2.1.0** - Almende B.V. and Contributors, Thieurmel B, Robert T (2021). `_visNetwork: Network Visualization using 'vis.js' Library_`. R package version 2.1.0, <https://CRAN.R-project.org/package=visNetwork>.
- **vroom 1.5.7** - Hester J, Wickham H, Bryan J (2021). `_vroom: Read and Write Rectangular Text Data Quickly_`. R package version 1.5.7, <https://CRAN.R-project.org/package=vroom>.
- **waldo 0.4.0** - Wickham H (2022). `_waldo: Find Differences Between R Objects_`. R package version 0.4.0, <https://CRAN.R-project.org/package=waldo>.
- **webshot 0.5.3** - Chang W (2022). `_webshot: Take Screenshots of Web Pages_`. R package version 0.5.3, <https://CRAN.R-project.org/package=webshot>.
- **withr 2.5.0** - Hester J, Henry L, Müller K, Ushey K, Wickham H, Chang W (2022). `_withr: Run Code 'With' Temporarily Modified Global State_`. R package version 2.5.0, <https://CRAN.R-project.org/package=withr>.
- **writexl 1.4.0** - Ooms J (2021). `_writexl: Export Data Frames to Excel 'xlsx' Format_`. R package version 1.4.0, <https://CRAN.R-project.org/package=writexl>.
- **xfun 0.31** - Xie Y (2022). `_xfun: Supporting Functions for Packages Maintained by 'Yihui Xie'_`. R package version 0.31, <https://CRAN.R-project.org/package=xfun>.
- **XML 3.99.0.9** - Temple Lang D (2022). `_XML: Tools for Parsing and Generating XML Within R and S-Plus_`. R package version 3.99-0.9, <https://CRAN.R-project.org/package=XML>.
- **xml2 1.3.3** - Wickham H, Hester J, Ooms J (2021). `_xml2: Parse XML_`. R package version 1.3.3, <https://CRAN.R-project.org/package=xml2>.
- **xtable 1.8.4** - Dahl D, Scott D, Roosen C, Magnusson A, Swinton J (2019). `_xtable: Export Tables to LaTeX or HTML_`. R package version 1.8-4, <https://CRAN.R-project.org/package=xtable>.
- **XVector 0.36.0** - Pagès H, Aboyoun P (2022). `_XVector: Foundation of external vector representation and manipulation in Bioconductor_`. R package version 0.36.0, <https://bioconductor.org/packages/XVector>.
- **yaml 2.3.5** - Garbett SP, Stephens J, Simonov K, Xie Y, Dong Z, Wickham H, Horner J, reikoch, Beasley W, O'Connor B, Warnes GR, Quinn M, Kamvar ZN (2022). `_yaml: Methods to Convert R Data to YAML and Back_`. R package version 2.3.5, <https://CRAN.R-project.org/package=yaml>.
- **zeallot 0.1.0** - Teetor N (2018). `_zeallot: Multiple, Unpacking, and Destructuring Assignment_`. R package version 0.1.0, <https://CRAN.R-project.org/package=zeallot>.
- **zip 2.2.0** - Csárdi G, Podgórski K, Geldreich R (2021). `_zip: Cross-Platform 'zip' Compression_`. R package version 2.2.0, <https://CRAN.R-project.org/package=zip>.
- **zlibbioc 1.42.0** - Morgan M (2022). `_zlibbioc: An R packaged zlib-1.2.5_`. R package version 1.42.0, <https://bioconductor.org/packages/zlibbioc>.
- **base 4.2.0** - R Core Team (2022). `_R: A Language and Environment for Statistical Computing_`. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.
- **class 7.3.20** - Venables WN, Ripley BD (2002). `_Modern Applied Statistics with S_`, Fourth edition. Springer, New York. ISBN 0-387-95457-0, <https://www.stats.ox.ac.uk/pub/MASS4/>.
- **cluster 2.1.3** - Maechler M, Rousseeuw P, Struyf A, Hubert M, Hornik K (2022). `_cluster: Cluster Analysis Basics and Extensions_`. R package version 2.1.3 - For new features, see the 'Changelog' file (in the package source), <https://CRAN.R-project.org/package=cluster>.

- **codetools 0.2.18** - Tierney L (2020). *_codetools: Code Analysis Tools for R_*. R package version 0.2-18, <https://CRAN.R-project.org/package=codetools>.
- **compiler 4.2.0** - R Core Team (2022). *_R: A Language and Environment for Statistical Computing_*. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.
- **datasets 4.2.0** - R Core Team (2022). *_R: A Language and Environment for Statistical Computing_*. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.
- **foreign 0.8.82** - R Core Team (2022). *_foreign: Read Data Stored by 'Minitab', 'S', 'SAS', 'SPSS', 'Stata', 'Systat', 'Weka', 'dBase',_* R package version 0.8-82, <https://CRAN.R-project.org/package=foreign>.
- **graphics 4.2.0** - R Core Team (2022). *_R: A Language and Environment for Statistical Computing_*. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.
- **grDevices 4.2.0** - R Core Team (2022). *_R: A Language and Environment for Statistical Computing_*. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.
- **grid 4.2.0** - R Core Team (2022). *_R: A Language and Environment for Statistical Computing_*. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.
- **KernSmooth 2.23.20** - Wand M (2021). *_KernSmooth: Functions for Kernel Smoothing Supporting Wand & Jones (1995)_*. R package version 2.23-20, <https://CRAN.R-project.org/package=KernSmooth>.
- **lattice 0.20.45** - Sarkar D (2008). *_Lattice: Multivariate Data Visualization with R_*. Springer, New York. ISBN 978-0-387-75968-5, <http://lmdvr.r-forge.r-project.org>.
- **MASS 7.3.56** - Venables WN, Ripley BD (2002). *_Modern Applied Statistics with S_*, Fourth edition. Springer, New York. ISBN 0-387-95457-0, <https://www.stats.ox.ac.uk/pub/MASS4/>.
- **Matrix 1.4.1** - Bates D, Maechler M, Jagan M (2022). *_Matrix: Sparse and Dense Matrix Classes and Methods_*. R package version 1.4-1, <https://CRAN.R-project.org/package=Matrix>.
- **methods 4.2.0** - R Core Team (2022). *_R: A Language and Environment for Statistical Computing_*. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.
- **nlme 3.1.157** - Pinheiro J, Bates D, R Core Team (2022). *_nlme: Linear and Nonlinear Mixed Effects Models_*. R package version 3.1-157, <https://CRAN.R-project.org/package=nlme>.
- **nnet 7.3.17** - Venables WN, Ripley BD (2002). *_Modern Applied Statistics with S_*, Fourth edition. Springer, New York. ISBN 0-387-95457-0, <https://www.stats.ox.ac.uk/pub/MASS4/>.
- **parallel 4.2.0** - R Core Team (2022). *_R: A Language and Environment for Statistical Computing_*. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.
- **rpart 4.1.16** - Therneau T, Atkinson B (2022). *_rpart: Recursive Partitioning and Regression Trees_*. R package version 4.1.16, <https://CRAN.R-project.org/package=rpart>.
- **spatial 7.3.15** - Venables WN, Ripley BD (2002). *_Modern Applied Statistics with S_*, Fourth edition. Springer, New York. ISBN 0-387-95457-0, <https://www.stats.ox.ac.uk/pub/>

[MASS4/](#).

- **splines 4.2.0** - R Core Team (2022). *_R: A Language and Environment for Statistical Computing_*. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.
- **stats 4.2.0** - R Core Team (2022). *_R: A Language and Environment for Statistical Computing_*. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.
- **stats4 4.2.0** - R Core Team (2022). *_R: A Language and Environment for Statistical Computing_*. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.
- **survival 3.3.1** - Therneau T (2022). *_A Package for Survival Analysis in R_*. R package version 3.3-1, <https://CRAN.R-project.org/package=survival>.
- **tcltk 4.2.0** - R Core Team (2022). *_R: A Language and Environment for Statistical Computing_*. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.
- **tools 4.2.0** - R Core Team (2022). *_R: A Language and Environment for Statistical Computing_*. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.
- **utils 4.2.0** - R Core Team (2022). *_R: A Language and Environment for Statistical Computing_*. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.