

GEO2RNAseq: An easy-to-use R pipeline for complete pre-processing of RNA-seq data

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1 ABSTRACT

2 In transcriptomics, the study of the total set of RNAs transcribed by the cell, RNA sequencing
3 (RNA-seq) has become the standard tool for analysing gene expression. The primary goal is
4 the detection of genes whose expression changes significantly between two or more conditions,
5 either for a single species or for two or more interacting species at the same time (dual RNA-seq,
6 triple RNA-seq and so forth). The analysis of RNA-seq can be simplified as many steps of the
7 data pre-processing can be standardised in a pipeline.

8 In this publication we present the “GEO2RNAseq” pipeline for complete, quick and concurrent
9 pre-processing of single, dual, and triple RNA-seq data. It covers all pre-processing steps
10 starting from raw sequencing data to the analysis of differentially expressed genes, including
11 various tables and figures to report intermediate and final results. Raw data may be provided
12 in FASTQ format or can be downloaded automatically from the Gene Expression Omnibus
13 repository. GEO2RNAseq strongly incorporates experimental as well as computational metadata.
14 GEO2RNAseq is implemented in R, lightweight, easy to install via Conda and easy to use, but still
15 very flexible through using modular programming and offering many extensions and alternative
16 workflows.

17 GEO2RNAseq is publicly available at <https://anaconda.org/xentrics/r-geo2rnaseq>
18 and https://bitbucket.org/thomas_wolf/geo2rnaseq/overview, including source
19 code, installation instruction, and comprehensive package documentation.

20 **Keywords:** next-generation sequencing, RNA-seq, dual RNA-seq, differentially expressed genes, comparative transcriptomics,
21 bioinformatics

1 INTRODUCTION

22 Organisms constantly change the expression of their genes to adapt to changes in the environment. With
23 the help of transcriptomics scientists are able to study the gene expression of a given organism allowing
24 to get insights in the interplay of genes dependent on environmental alterations. The primary goal is

25 the detection of genes whose expression changes significantly between two or more conditions, and the
26 function relationship of these genes. RNA sequencing (RNA-seq) offers a complete, fast, and cheap way
27 to perform transcriptomics of single organisms using next-generation-sequencing technologies (Mardis,
28 2008). However, species do not exist in isolation. In fact, interspecies interactions are a major part of
29 environmental adaptation. The special variant dual RNA-seq can be applied to analyse the transcriptome
30 of two interacting species at the same time by separating their RNA *in silico* (Schulze et al., 2016; Wolf
31 et al., 2018). The concept of dual RNA-seq can be further extended: triple RNA-seq allows investigating
32 the interaction of three organisms, *e. g.* a host and two competing pathogens.

33 Both, the number of scientists applying RNA-seq and the number of published datasets have been growing
34 exponentially ¹ (Deelen et al., 2014). However, the bottlenecks in transcriptomics are the small number
35 of experts able to pre-process and analyse RNA-seq data, and the small number of easy-to-use tools. A
36 number of pipelines were published in R to handle this issue, but none of them includes the complete set of
37 pre-processing steps and none exploits the available metadata fully.

38 Extensive utilisation of metadata is highly important. Wet-lab metadata, *e. g.* temperature or pH, and
39 dry-lab metadata, *e. g.* p-value cut-offs or reference genome version, both influence the outcome of RNA
40 -seq analysis and are often indispensable for the reproducibility of results (Rayner et al., 2006). Metadata
41 help to decide if and how different datasets are comparable. Especially when many samples were used
42 or different datasets were combined, metadata help to understand to keep track of the structure of a
43 dataset. Knowledge of wet-lab metadata is essential for the interpretation computational results. To allow
44 comparability of different analyses of the same datasets it is important to provide as much metadata as
45 possible. Providing metadata for a possible subsequent analysis of the current data is as important as
46 incorporating available metadata into a new analysis.

2 THE GEO2RNASEQ PIPELINE

47 GEO2RNAseq offers a complete analysis of RNASeq data starting at FASTQ files or with a download
48 from Gene Expression Omnibus (GEO).

49 At each step of the pipeline, the user has the possibility to change all parameters of the applied tools,
50 which gives complete control over the processing steps. GEO2RNAseq is completely modular, allowing
51 users to skip processing steps they do not need, integrate other R packages, or start the analysis at a
52 later step. A restart-upon-error mechanism is implemented which detects incomplete and unprocessed
53 samples. The mechanisms also allows the user to add new samples easily to the analysis without the need
54 to re-run the most time consuming steps, *i.e.* quality control, mapping and gene abundance estimation.
55 Every function is documented with a complete describing of input parameters, default values and output.
56 Usually, only the paths to input files or The pipeline is highly scalable. Runnig it with one thread/core
57 on a laptop or on a server with multiple processors and threads/cores is a matter of changing one variable
58 [MAX_CPUS] in the R pipeline script and results in heavy parallelisation of almost all processing steps.
59 For cluster computing, the `Rmpi` package is installed with GEO2RNAseq and can be used to pre-process
60 batches of read data on different machines. Figure 1 illustrates the workflow of GEO2RNAseq.

61 GEO2RNAseq automatically extracts metadata from GEO according to the MINSEQE standard hereby
62 extending the IDF and SDRF formats (Rayner et al., 2006). If available from GEO, the following IDF
63 features are retrieved automatically: investigation title, experiment class (*e. g.* RNA-seq, microarray),

¹ <https://www.ncbi.nlm.nih.gov/sra/docs/sragrowth/>

64 experimental description, experimental design according to the MGED ontology (*e. g.* “in_vitro_design”),
65 experimental factors (*e. g.* strain or line), quality control information (*e. g.* “biological_replicate”), data
66 release information, personal information of data submitter(s) and investigator(s) (*e. g.* Name, e-mail
67 address, affiliation), and information about publications based on the dataset (*e. g.* PubMed ID). Again,
68 if available from GEO, the following SDRF features are retrieved automatically per each sample: source
69 information (*e.g.* organism part, sampling time point, culture conditions), applied protocols or standard
70 operation procedures (SOP) for RNA extraction (*e. g.* extraction method), sequencing (*e. g.* technology
71 type, read length, library source), and data processing (*e. g.* software parameters). Most importantly, the
72 fold change assignment (“design matrix”), is extracted giving information on which comparisons of what
73 samples have been or should be used for differentially expression analysis. The user can change or extend
74 the metadata as necessary. During processing, the pipeline automatically extends the already available
75 metadata by detailed information about each applied processing step and their parameters.

76 The pipeline starts with downloading raw sequencing data and corresponding metadata from GEO for
77 a given GSE accession number. Next, it converts Sequence Read Archive (SRA) files into FASTQ files.
78 Users can start directly with a set of FASTQ files as well. Quality check of FASTQ files is checked
79 using FastQC². Next, quality trimming of low-quality regions and adapter trimming is performed with
80 Trimmomatic (Bolger et al., 2014). rRNA reads can be removed by using SortMeRNA (Kopylova et al.,
81 2012) if required. SortMeRNA searches for rRNA sequences on a domain-of-life-basis, *i.e.* it distinguishes
82 rRNA from bacterial, eukaryotic and archaeal origin. We implemented convenient wrappers to either (i)
83 filter for rRNA from only one of the domains, (ii) filter for all three at the same time but keep a separate
84 report, and (iii), to speed up SortMeRNA runtime, filter for all three as a whole and only report a total
85 sum of rRNA content. At this point, users can decide to run FastQC again to check how trimming and
86 rRNA removal improved the overall quality of the dataset. For mapping against a reference genome, the
87 user can choose between TopHat2 (Kim et al., 2013) and HISAT2 (Kim et al., 2015). Other mappers, like
88 STAR (Dobin et al., 2013) or BWA (Li and Durbin, 2009) can be easily integrated into the GEO2RNAseq
89 workflow. In case of prokaryotic data, we suggest to use HISAT2 with the `--no-spliced-alignment`
90 argument. To count the number of reads per gene, the pipeline uses featureCounts (Liao et al., 2013). As an
91 indicator for rRNA contamination or high levels of globin-derived RNA in whole-blood datasets (Zhao
92 et al., 2018), the pipeline automatically detects genes with particularly high read coverage. If such genes
93 were detected, the user may consider to remove these genes from the count data, but this is not mandatory.

94 MultiQC (Ewels et al., 2016) is used to summarise quality control, read mapping and counting by creating
95 interactive tables and various quality plots. GEO2RNAseq includes a MultiQC configuration file, which is
96 optimised to work best with the pipeline’s output files and default directory structure. Next, comprehensive
97 mapping statistics, including genome and transcriptome coverage, are calculated. Clustering of samples
98 using different clustering methods (single-, complete-, average-linkage, median, centroid and all other
99 methods as available in the `hclust` function) and visualizations (Figure 2 and Figure 3), and a principal
100 component analysis (PCA, Figure 4) is performed and plotted.

101 Multiple different normalisation methods for count matrices (raw read counts), clustering, and PCA are
102 implemented. These include counts per million (CPM), transcripts per million (TPM), read per kilobase
103 million (RPKM), median by-ratio normalisation (MRN) (Anders and Huber, 2010), log-ratio, and variance
104 stabilisation transformation (VST).

² <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>

105 Finally, GEO2RNAseq calculates significantly differentially expressed genes (DEGs) according to a
106 given q-value cut-off and, optionally, \log_2 fold-change cut-off, for all comparisons defined manually by
107 the user or automatically by using metadata. By default, the tools DESeq (Anders and Huber, 2012),
108 DESeq2 (Love et al., 2014), edgeR (Robinson et al., 2010), and limma (McCarthy and Smyth, 2009) are
109 executed concurrently and used to calculate adjusted p-values (q values) for each gene and comparison. In
110 addition, baySeq (Hardcastle and Kelly, 2010), NOIseq (Tarazona et al., 2012), SAMseq (Tibshirani et al.,
111 2011), and PoissonSeq (Li et al., 2012) are available. See Gierliński et al. (2015), Schurch et al. (2016), and
112 Froussios et al. (2017) for in-depth analyses of strengths and weaknesses of various DEG tools. Moulos
113 *et al.* Moulos and Hatzis (2015) demonstrate how the specificity, *i.e.* reducing type 2 errors for detected
114 DEGs, is improved by using the maximum p-values across different statistical tools. We implemented the
115 same strategy in GEO2RNAseq. Sensitivity can be increased by either using less stringent tools (e.g. only
116 DESeq2 and edgeR) or increasing the q value threshold. Fold-change filtering can be applied on MRN,
117 TPM or RPKM transformed abundance values as well.

118 The GEO2RNAseq vignette (see supplement file 1 `Vignette.html`) provides a comprehensive
119 introduction to the basic concepts of an RNA-seq analysis and a step-by-step guide for using GEO2RNAseq.
120 It explains the common steps of the RNA-seq pre-processing workflow and the different types of GEO
121 datasets. Most importantly, the vignette describes each step of the GEO2RNAseq pipeline and the data
122 structures introduced by this package. It includes the rationale behind each pre-processing step, a short
123 description of each applied tool, example R codes, and the results produced by each code snippet.

124 Supplement file 2 `example_pipeline.R` may be used as a “minimal RNA-seq pipeline”. We also
125 provide more comprehensive pipeline scripts for complete pre-processing of single (regular), dual, triple,
126 and mixed single-end-paired-end RNA-seq datasets.

127 GEO2RNAseq combines 14 mandatory functions (see next section) that can be complemented with a
128 collection of 132 functions for complete RNA-seq data pre-processing. GEO2RNAseq is implemented in
129 R version 3.4.1 and publicly available at <https://anaconda.org/xentrics/r-geo2rnaseq>.
130 The Conda³ package supports Linux and Mac systems. It includes a basic R installation as well as all R,
131 external tool, and system dependencies required to run the pipeline. Therefore, it is very easy to install and
132 use the pipeline within different computer environments. A tar ball for Conda-independent installation,
133 source code, installation instructions, manual, and vignette are provided alongside the Conda package.

134 To ensure fast processing of RNA-seq data, GEO2RNAseq uses parallelisation, *i.e.* multi-threading
135 and concurrent execution, wherever possible. The most time consuming and critical steps of RNA-seq
136 data processing are handled by third-party software, *e.g.*, Trimmomatic (Bolger et al., 2014), Sort-
137 MeRNA (Kopylova et al., 2012), TopHat2 (Kim et al., 2013) or HISAT2 (Kim et al., 2015). Thus, run-time,
138 accuracy and so on mainly depend on these third-party software products and are intensively discussed in
139 their respective references. By parallelising the execution of third-party tools in addition to any intermediate
140 R code, we are able to reduce the total runtime of the pipeline drastically. Therefore, the total execution
141 time almost only depends on the size of the dataset, quality control and mapping of reads, the speed of the
142 data drives, and the number of CPUs available to run GEO2RNAseq. The overall accuracy depends on the
143 specifications of the third party software applied.

³ (Ana-) Conda is a package, dependency, and environment management system for many programming languages and operating systems (<https://conda.io/docs/>)

3 APPLICATION AND EXAMPLE RESULTS

144 To exemplarily present the capabilities of GEO2RNAseq, we re-analysed the RNA-seq dataset GSE55663
145 Valiante et al. (2015), which is freely available at GEO⁴, using only metadata provided within GSE55663.
146 This dataset is based on RNA-seq results from of *Aspergillus fumigatus* treated with Caspofungin and
147 Siamycin. It consists of three replicates for each condition, including a control condition. The R script
148 for this re-analysis is provided in supplement `example_pipeline.R`. Furthermore, GEO2RNAseq has
149 been successfully applied for the analysis of other single, dual, and also triple RNA-seq datasets.

150 For the complete pre-processing of GSE55663, we downloaded raw sequencing files and meta-
151 data using the function `getGEOdata()`. The automatically downloaded metadata can be found in
152 supplement files 3 `GSE55663_SDRF.tsv` and 4 `GSE55663_IDF.tsv`. Next, we checked the se-
153 quencing quality of raw read data using the function `runFastQC()`, performed adapter and quality
154 trimming of the same read data using the function `runTrimmomatic()`, and checked the qual-
155 ity of these trimmed reads. Trimmed reads were mapped to the reference genome of *A. fumigatus*
156 strain A1163 using the function `runHisat2()`. The number of reads per gene were counted us-
157 ing `runfeatureCounts()`. Reference genome and annotation files (in FASTA and GTF format)
158 were downloaded from <http://www.aspergillusgenome.org/>. Only the download location
159 of these files must be supplied to the functions. The index of the reference genome was created us-
160 ing `makeHisat2index()`. The latter required about 1 Gb of RAM. The results of all processing
161 steps mentioned so far were summarised by the function `runMultiQC()`. They can be found in
162 supplement file `multiqc_report.zip`. GEO2RNAseq calculated detailed mapping statistics us-
163 ing the function `calc_mapping_stats()`, the result of which can be found in supplement file
164 `mapping_stats.xls`. We then parsed the treatment information from the column 'characteristics'. For
165 normalisation, we used the function `getmrn()`. After normalisation, we used `makehclustplot()`,
166 `makeheatclusteringplot()`, and `makePCAplot()` to generate the clustering (Figure 2, Fig-
167 ure 3) and PCA (Figure 4) plots. Based on the treatment conditions, we generated all pairwise tests
168 using `create_design_matrix()`. Finally, we calculated differentially expressed genes for each test
169 using `calculate_DEGs()` (Figure 5, Figure 6, Figure 7, Figure 8). For each comparison, this function
170 creates Venn diagrams, intersection bar, volcano and MA plots as well. In parallel, the pipeline collected
171 metadata for each processing step, like tools, their respective versions and parameters. The resulting
172 enriched metadata table is provided as supplement file `meta_data_final.csv`.

173 Intermediate and final results are saved in tabular form (Excel and CSV) and are supported by various
174 plots (Table 1). The DEG analysis results include (i) normalised count values for each condition and for
175 several normalisation methods, (ii) log fold-change values, and (iii) adjusted p-values from each applied
176 DEG tool. More results are available as R objects and can be saved in the RData format (R workspace file)
177 or can be further examined.

178 GEO2RNAseq can be applied to analyse huge datasets, *e. g.* with more than 1000 samples, in a server
179 environment or small datasets on a personal computer with limited capacities. We ran the example
180 workflow (supplement `example_pipeline.R`), which consists of about 200 million raw reads in total,
181 on a desktop computer. Intentionally we ran the computation on a 1 TB hard disk, which is much slower
182 than a modern solid state drive. We allowed the pipeline to utilise up to six CPU cores. The entire system
183 never allocated more than 8 GB of memory. The total execution time was roughly 45 minutes, excluding
184 the GEO download. Quality check with FastQC took 5 minutes, trimming with Trimmomatic 17 minutes,

⁴ <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55663>

185 Mapping with HISAT2 about 20 minutes, featureCounts 2 minutes, PCA, clustering and statistical testing
186 roughly 1 minute. This shows that the usage of GEO2RNAseq can be scaled up and applied to analyse
187 vast datasets, *e. g.* with more than 1000 samples, in a server environment or small datasets on a personal
188 computer with limited capacities.

189 Furthermore, GEO2RNAseq has been successfully applied for the analysis of other single, dual, and also
190 triple RNA-seq datasets, for example by Schulze et al. (2015) and Sieber et al. (2018) (data not shown). For
191 dual (and triple) RNA-seq, the pipeline requires one fitting reference genome in FASTA format and one
192 annotation file in GTF format for each species. Annotation files must have the same attribute for gene IDs
193 (usually the tag "gene_id") or should be converted beforehand. Reference genomes are simply concatenated
194 and then indexed together (using `make_HiSat2_index()`). Annotation files are concatenated together
195 as well. The pipeline is executed the same as it was for single RNA-seq until gene abundance estimation
196 was performed. The gene expression matrix can be separated into separate matrices for the two (or three)
197 species. Hence, gene expression can then be analysed further per species or together. The latter, for
198 example, is done when inferring inter-species gene expression correlations. In this case, normalization (*e.g.*
199 MRN) is applied based using all genes from all species. This method removes the kind of bias that single
200 RNA-seq of multiple species introduces by having different sequencing depths and hence normalization
201 factors *between species*. In addition, the proportion of expression per species can be determined in dual
202 RNA-seq which is not possible in single RNA-seq. Mapping statistics for dual RNA-seq are performed
203 using `calc_dual_mapping_stats()` (or `calc_triple_mapping_stats()` for triple RNA-seq)
204 which considers each species separately. Among other things, it calculates percentage contribution of
205 RNA, exon and genome coverage per species. Example pipelines for performing dual and triple RNA-seq
206 analyzes are supplied with the Geo2RNAseq R package.

4 COMPARISON TO OTHER RNA-SEQ PIPELINES

207 The very popular Galaxy workflow engine (Afgan et al., 2016) offers a scientific workflow, data integration,
208 and analysis platform. It primarily aims to make computational biology accessible to scientists without
209 programming skills. Today, Galaxy is a well-established platform, but installation, adaptation, and aug-
210 mentation is still challenging. To achieve simplicity in usage, flexibility and output options of tools are
211 limited when compared to their full capabilities. Using non-implemented tools and performing other follow
212 up analyses requires to manually integrate and import such tools into Galaxy and to manually export the
213 results from it. This difficulty potentially causes a barrier for efficient and error-free data processing when
214 using novel tools.

215 The R software environment⁵ offers an easy framework for performing simple and complex statistics.
216 Users have access to a huge number of packages for transcriptome data analysis, data interpretation, plotting
217 and more. In this sense, R is much more flexible than Galaxy, but requires at least some programming skills
218 of the user. In fact, many important steps of RNA-seq data processing, like the detection of differentially
219 expresses genes (DEGs), are natively implemented in R.

220 Two pipelines for processing of RNAseq data exist and were widely used. The Total RNA-Seq
221 Analysis Package for R (TRAPR) (Lim et al., 2017) is a partial RNA-seq pipeline implemented in R.
222 However, does not include mapping and counting, and uses counts per genes as input. Given counts,
223 TRAPR performs all following steps necessary for the detection of DEGs, but the statistical analysis is
224 limited to DESeq2 and edgeR.

⁵ <https://www.r-project.org>

225 EasyRNASeq (Delhomme et al., 2012) is a package available through Bioconductor⁶. Again, it can only
226 be used after initial mapping was performed. As well the statistical analysis is limited to DESeq2 and
227 edgeR.

228 In computer science, the programming language Python is often used in conjunction or as an alternative
229 to R. READemption (Förstner et al., 2014) is a Python pipeline for processing differential RNA-seq data.
230 However, quality trimming of reads is incomplete, it uses only DESeq2 for statistical analysis, and the
231 processing steps are fixed. READemption utilised Segemehl (?) for mapping, which is rarely used as of
232 today.

233 None of the R or Python pipelines mentioned before are able to use raw sequencing data available in the
234 Gene Expression Omnibus (GEO) repository. None of them handles metadata according to the MINSEQE⁷
235 standard, and none of them can handle dual or triple RNA-seq datasets. See Table 1 for a detailed feature
236 comparison.

237 In conclusion, GEO2RNAseq is complete and modular RNAseq pre-processing pipeline. In contrast to
238 the other pipelines mentioned, users can process data in standardised ways or fine-tune the processing steps
239 using all capabilities that R offers. Between each step, some of which are optional, the user may choose to
240 use other R code or packages to fit their particular needs. GEO2RNAseq ensures this by keeping input and
241 output of processed data comprehensive, but simple. Finally, GEO2RNAseq is highly scalable because it
242 makes heavy use of parallelisation in form of concurrent execution and multi-threading.

CONFLICT OF INTEREST STATEMENT

243 The authors declare that the research was conducted in the absence of any commercial or financial
244 relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

245 JL designed the project, workflow, and supported acquisition and analysis of data. BS, TW, JL, SP, SM,
246 and SG programmed and shipped the tool. BS and TW wrote the manuscript. JL and RG contributed on
247 writing the manuscript. All authors read, revised and approved the final manuscript.

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FIGURES

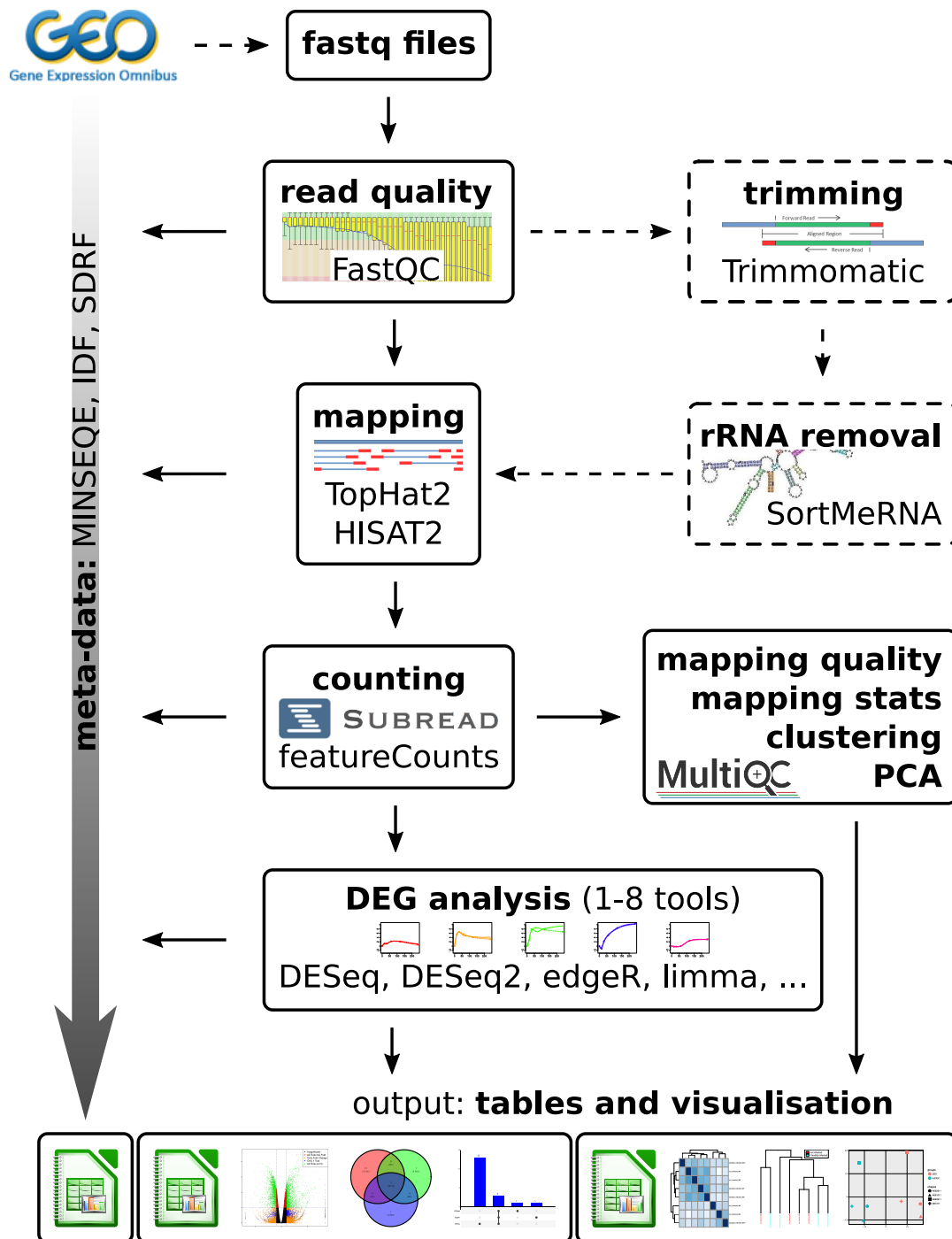


Figure 1. GEO2RNAseq workflow overview. The pipeline can download raw sequencing and metadata from the Gene Expression Omnibus (GEO) repository with a given GSE accession number. It automatically parses metadata according to the MINSEQE standard. It pre-processes raw sequencing data using many different tools. Some steps are optional, others offer alternative tools to apply. Differentially expressed genes are identified using up to eight different tools. By default, intermediate and final results are reported using tables in CSV and Excel format, various quality plots, hierarchical clustering and PCA, Venn diagrams, intersection bar plots, and volcano plots; more are available. Please note that this figure illustrates the *logical* order of processing steps as suggested by the authors. The arrows connecting each tool do not necessarily represent direct input/output interactions.

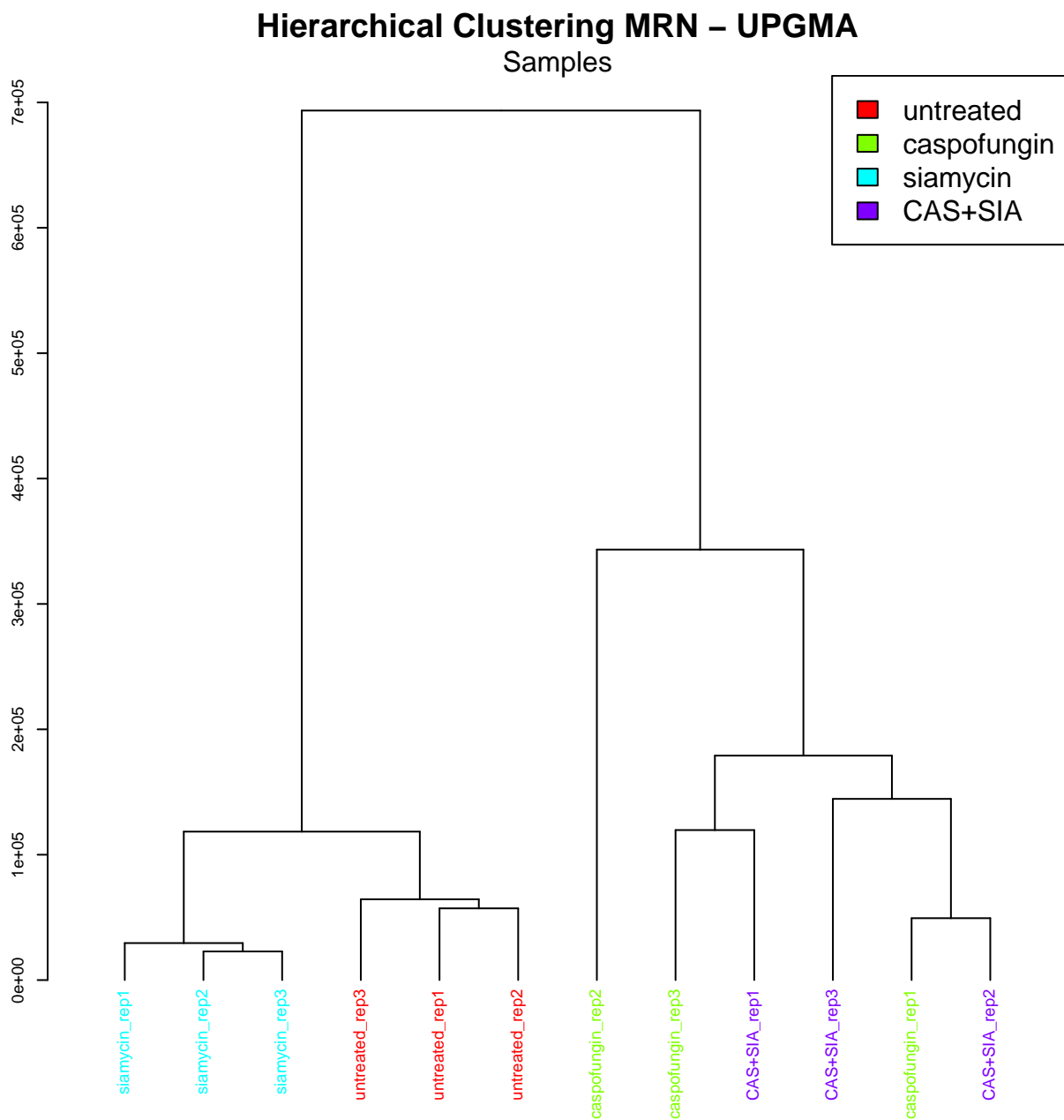


Figure 2. Hierarchical clustering of raw (not normalised) read count values. Samples can be coloured by user specified conditions or by conditions parsed from metadata. Here: Clear separation of samples treated with Caspofungin (green) or Caspofungin and Siamycin (CAS+SIA; purple) in the right subtree and samples treated with Siamycin only (blue) and the control sample (untreated; red) in the left subtree.

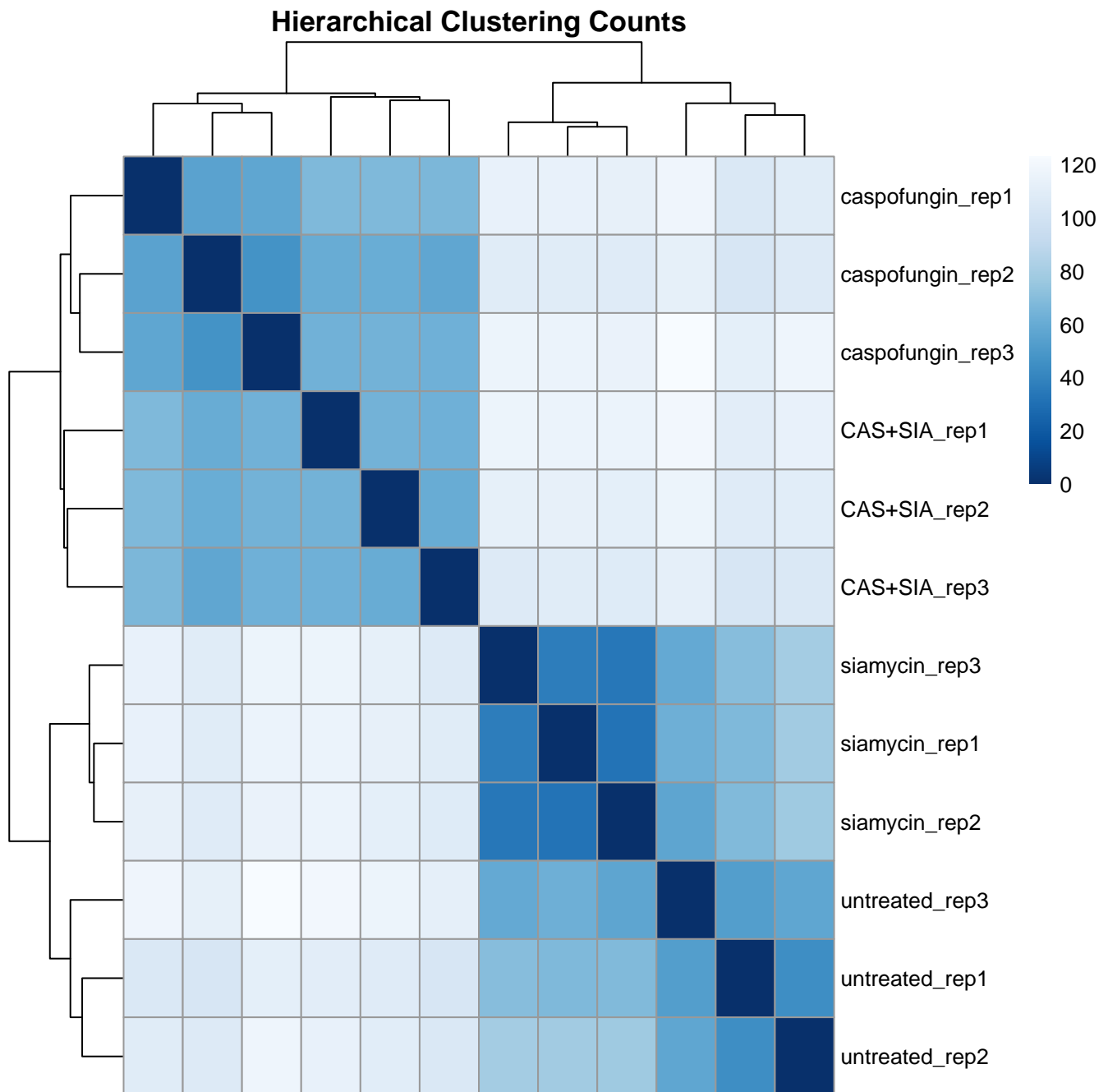


Figure 3. Similarity heatmap of MRN (Anders and Huber, 2010) normalised read count values with hierarchical clustering. The samples/replicates treated with Caspofungin only as well as Siamycin and Caspofungin (CAS+SIA) are in the upper left corner. The samples treated with Siamycin only and the control (untreated) are in the bottom right corner.

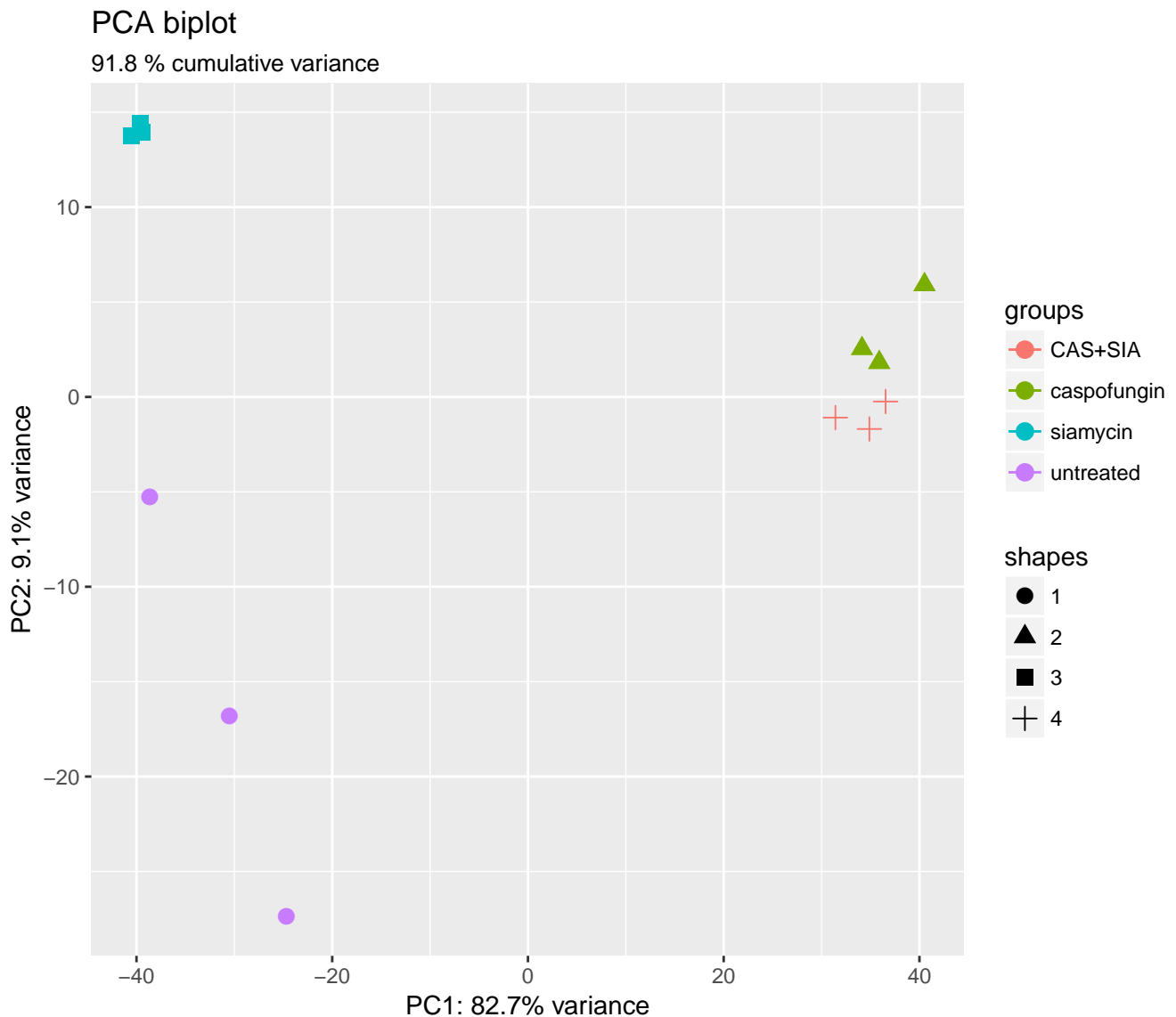


Figure 4. Principal component analysis (PCA) of MRN (Anders and Huber, 2010) normalised read count values showing a clear separation of samples treated with Caspofungin (green) or Caspofungin and Siamycin (CAS+SIA; red) on the right – and samples treated with Siamycin only (blue) and the control sample (untreated; purple) on the left. The three replicates of the control sample show a much higher variance, compared to the other samples. The plot can be further extended using ggplot2 in R.

Venn Diagram

total number of DEGs: 1728

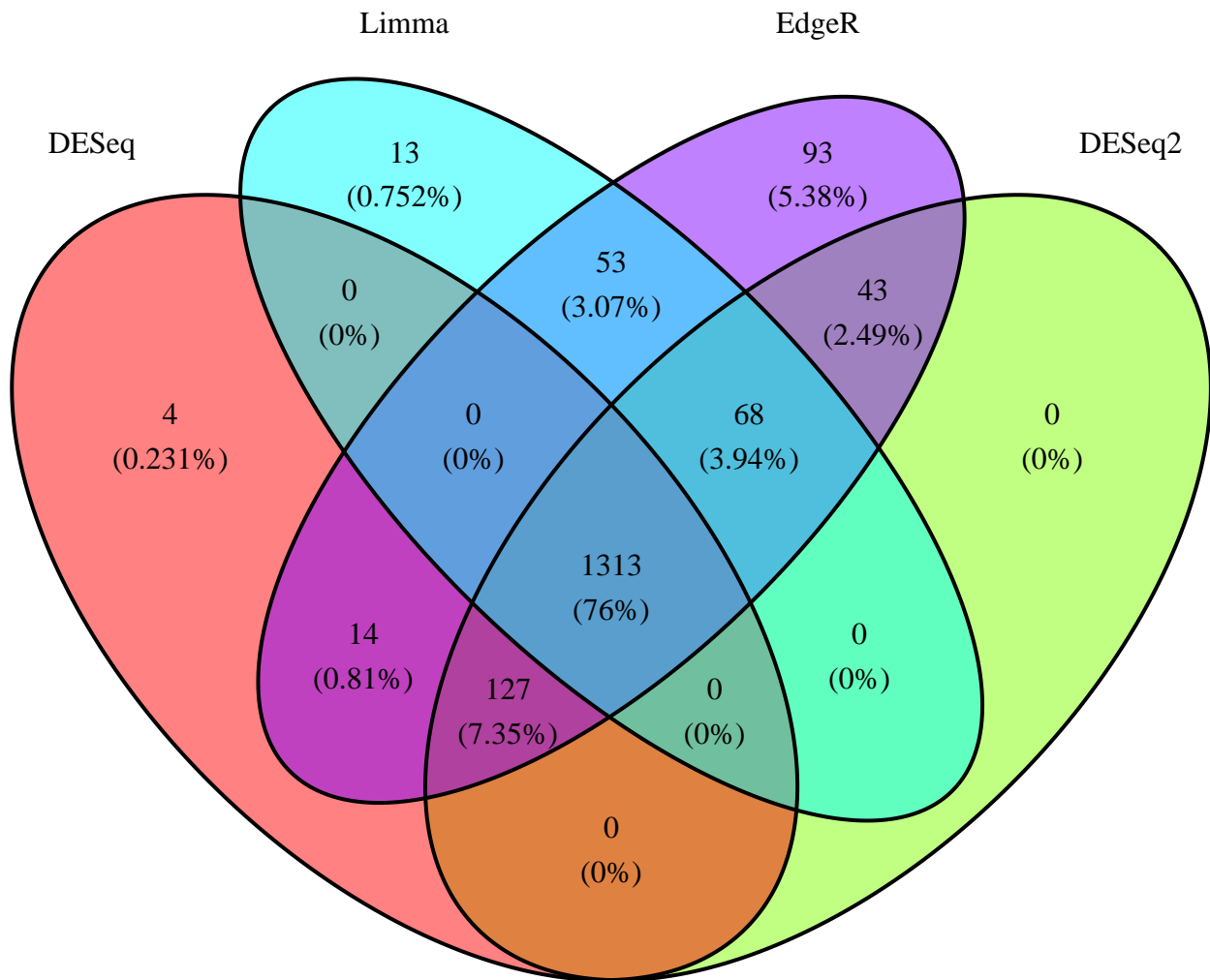


Figure 5. Venn diagram showing the overlap and difference of four sets of differentially expressed genes (DEGs) from the four DEG analysis tools DESeq (Anders and Huber, 2012), DESeq2 (Love et al., 2014), edgeR (Robinson et al., 2010), and limma (McCarthy and Smyth, 2009), which are used by default in GEO2RNAseq. Up to eight different DEG tools are supported. More than four are visualised using intersection bar plots (see Figure 6).

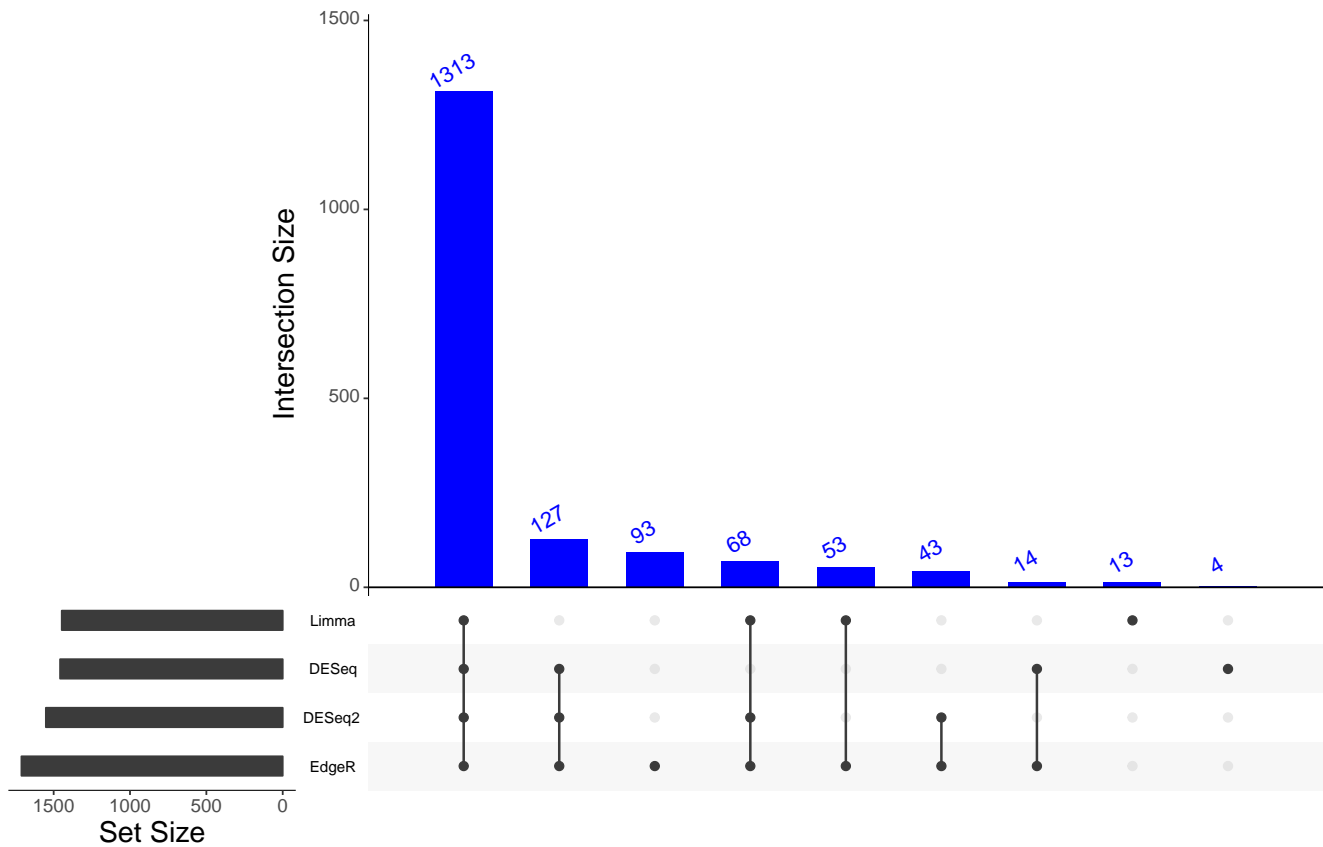


Figure 6. The intersection bar plot is an alternative for Venn diagrams and especially helpful if comparing more than four sets. Here: Four sets of differentially expressed genes (DEGs) from the four DEG analysis tools DESeq (Anders and Huber, 2012), DESeq2 (Love et al., 2014), edgeR (Robinson et al., 2010), and limma (McCarthy and Smyth, 2009), which are used by default in GEO2RNAseq. Up to eight different DEG tools are supported by GEO2RNAseq.

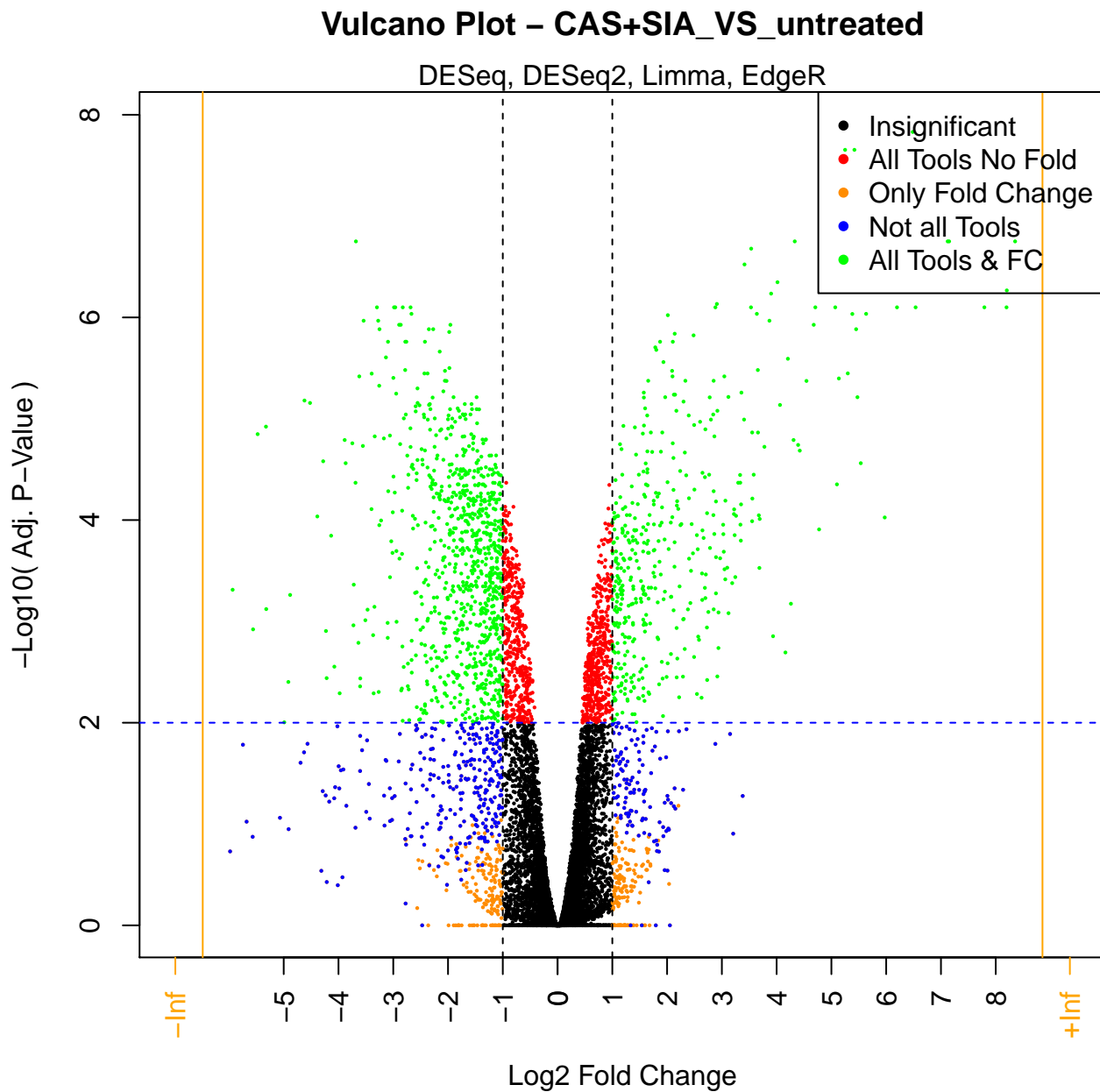


Figure 7. Volcano plot showing the distribution of \log_2 fold-changes and adjusted p-values from the DEG analysis of the comparison “control (untreated) against treatment with Caspofungin and Siamycin (CAS+SIA)”. When multiple DEG analysis tools are used, the maximum (“worst”) p-value per gene over these tools is used. Therefore, precision for DEGs is maximised and sensitivity minimised in the volcano plot. Genes with an adjusted p-value worse than the p-value cut-off (insignificant) are coloured black. If a fold-change filter was used during DEG analysis, vertical lines are added to indicate this threshold. Genes not achieving the fold-change threshold are coloured red. Insignificant genes achieving the fold-change cut-off are coloured orange. Blue coloured genes did not satisfy the adjusted p-value cut-off for all used tools, but may satisfy it for a subset of tools. Finally, genes satisfying both cut-offs for all tools are coloured green.

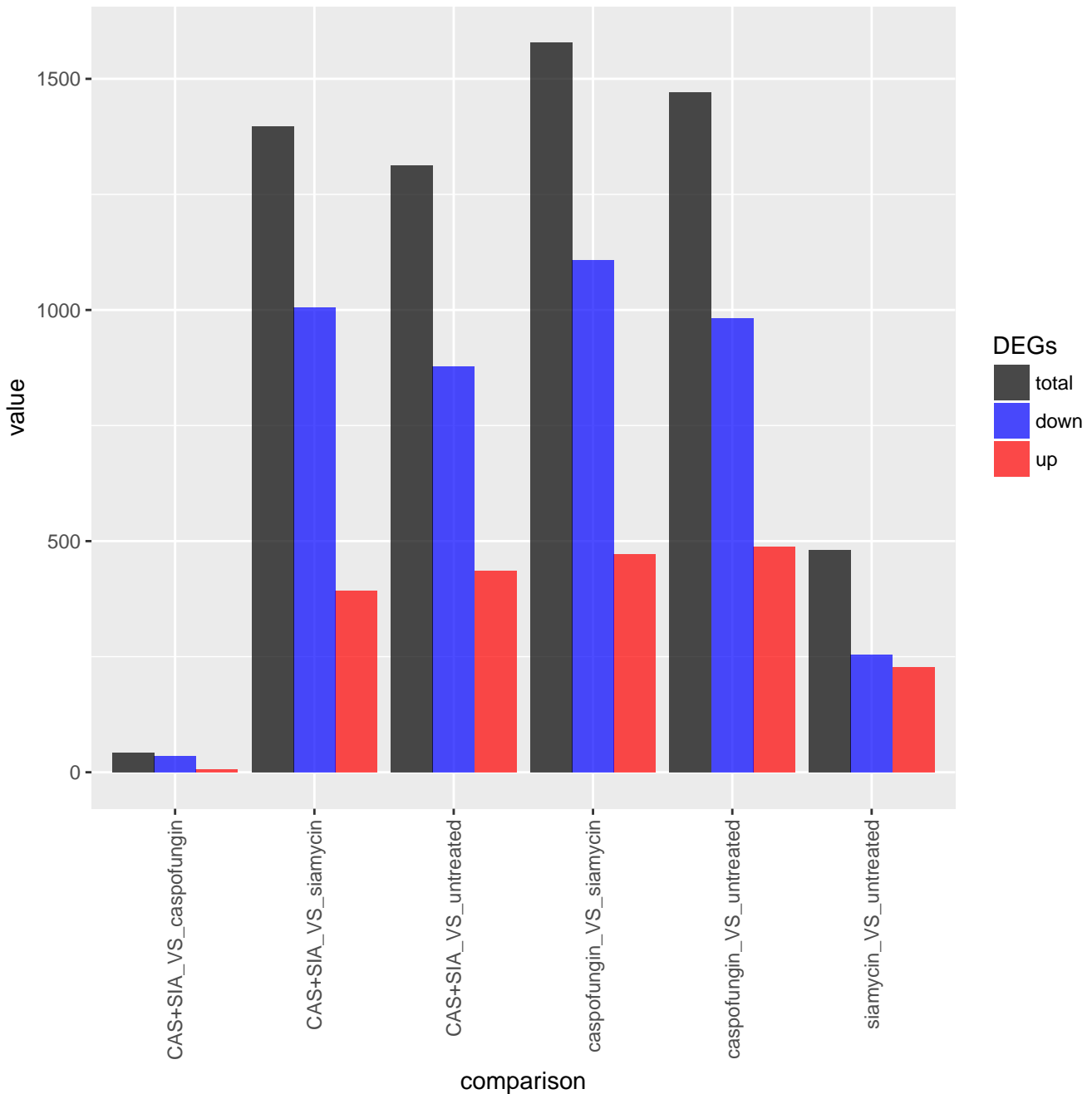


Figure 8. Bar plot showing the number of differentially expressed genes (DEGs) per comparison. This plot helps to quickly identify the subset of comparisons with a stronger treatment reaction according to the number of DEGs. It also shows whether a treatment induces more up- or downregulation. For the chosen comparisons, we see that a treatment with siamycin has a much weaker effect with respect to the number of DEGs when compared to caspofungin treatment. In addition, we see that the combination of caspofungin and siamycin treatment does not lead to substantially more DEGs. All treatments seem to induce an overall downregulation.

TABLES

Table 1. Feature comparison of RNA-seq processing pipelines.

	GEO2RNAseq ^a	TRAPR ^b	EasyRNASeq ^c	READemption ^d
wrapper language	R	R	R	Python
complete	yes	no	no	no ^e
input type	GEO ^f , FASTQ	counts	counts	FASTQ
quality control	FastQC	no	no	in-house
trimming	Trimmomatic	no	no	in-house
rRNA removal	SortMeRNA	no	no	no
mapping	TopHat2, HISAT2	no	no	Segemehl
counting	featureCounts	no	bam2hits	in-house
counting result	raw, RPKM, TPM, MRN, rlog, vst	quantile, TMM, MRN	raw, RPKM, vst	raw, TNOAR ^g
mapping statistics	MultiQC, CSV	no	no	CSV
sample correlation	yes	yes	yes	yes
sample clustering	yes (Figure 2)	no	yes	no
sample heatmap	yes (Figure 3)	yes	yes	no
sample PCA	yes (Figure 4)	no	yes	no
DEG tools	8 ^h	3	2	1
DEG results				
Venn diagram	yes (Figure 5)	no	no	no
UpSetR plot	yes (Figure 6)	no	no	no
Volcano plot	yes (Figure 7)	yes	no	yes
MA plot	yes	yes	no	yes
overview bar plot	yes (Figure 8)	no	no	no
modular	yes	yes	yes	no
dual/triple RNA-seq	yes	no	no	no
read/write metadata	yes	no	no	no
parallelisation	wrapper, external tools	no	yes	yes
Conda package	yes	no	yes	yes

^a <https://anaconda.org/xentrics/r-geo2rnaseq>

^b Lim et al. (2017)

^c Delhomme et al. (2012)

^d Förstner et al. (2014)

^e quality trimming of reads is incomplete

^f GSE accession

^g normalised by the total number of aligned reads (TNOR) and then multiplied by the lowest number of aligned reads of all considered libraries

^h four concurrent by default