

# *systemPipeR*: NGS workflow and report generation environment

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## 1 Introduction

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*systemPipeR* provides utilities for building *end-to-end* analysis workflows with automated report generation for next generation sequence (NGS) applications such as RNA-Seq, ChIP-Seq, VAR-Seq and many others ([Girke, 2014](#)). An important feature is support for running command-line software, such as NGS aligners, on both single machines or compute clusters. This includes both interactive job submissions or batch submissions to queuing systems of clusters (tested only with Torque). For instance, *systemPipeR* can be used with most command-line aligners such as BWA ([Li, 2013](#); [Li and Durbin, 2009](#)), TopHat 2 ([Kim et al., 2013](#)) and Bowtie 2 ([Langmead and Salzberg, 2012](#)), as well as the R-based NGS aligner *Rsubread* ([Liao et al., 2013](#)). Efficient handling of complex sample sets and experimental designs is facilitated by a well-defined sample annotation infrastructure which improves reproducibility and user-friendliness of many typical analysis workflows in the NGS area ([Lawrence et al., 2013](#)).

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## 2 Getting Started

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### 2.1 Installation

The R software can be downloaded from CRAN (<http://cran.at.r-project.org/>) and the *systemPipeR* package from GitHub (<https://github.com/tgirke/systemPipeR>). The *systemPipeR* package can be installed from R using the `install.packages` command after downloading and uncompressing the package directory.

```
> system("R CMD build systemPipeR") # Builds package
> install.packages("systemPipeR.X.X.X.tar.gz", repos=NULL, type="source") # Installs the package
```

### 2.2 Loading the Package and Documentation

```
> library("systemPipeR") # Loads the package
> library(help="systemPipeR") # Lists all functions and classes
> vignette("systemPipeR") # Opens this PDF manual from R
```

### 2.3 Sample FASTQ Files

The mini sample FASTQ files used by this overview vignette as well as the associated workflow reporting vignettes can be downloaded from [here](#). The chosen data set [SRP010938](#) contains 18 paired-end (PE) read sets from *Arabidopsis thaliana* Howard et al. (2013). To minimize processing time during testing, each FASTQ file has been subsetting to 90,000-100,000 random sampled PE reads that map to the first 100,000 nucleotides of each chromosome of the *A. thaliana* genome. The corresponding reference genome sequence (FASTA) and its GFF annotation files (provided in the same download) have been truncated accordingly. This way the entire test sample data set is less than 200MB in storage space. A PE read set has been chosen for this test data set for flexibility, because it can be used for testing both types of analysis routines requiring either SE (single end) reads or PE reads.

## 3 Structure of targets file

---

The targets file defines all FASTQ files and sample comparisons of an analysis workflow. The following shows the format of a sample targets file provided by this package.

```
> library(systemPipeR)
> targetspath <- paste0(system.file("extdata", package="systemPipeR"), "/targets.txt")
> read.delim(targetspath, comment.char = "#")
```

	FileName	SampleName	Factor	SampleLong	Experiment	Date
1	./data/SRR446027_1.fastq	M1A	M1	Mock.1h.A	1	23-Mar-2012
2	./data/SRR446028_1.fastq	M1B	M1	Mock.1h.B	1	23-Mar-2012
3	./data/SRR446029_1.fastq	A1A	A1	Avr.1h.A	1	23-Mar-2012
4	./data/SRR446030_1.fastq	A1B	A1	Avr.1h.B	1	23-Mar-2012
5	./data/SRR446031_1.fastq	V1A	V1	Vir.1h.A	1	23-Mar-2012
6	./data/SRR446032_1.fastq	V1B	V1	Vir.1h.B	1	23-Mar-2012
7	./data/SRR446033_1.fastq	M6A	M6	Mock.6h.A	1	23-Mar-2012
8	./data/SRR446034_1.fastq	M6B	M6	Mock.6h.B	1	23-Mar-2012
9	./data/SRR446035_1.fastq	A6A	A6	Avr.6h.A	1	23-Mar-2012
10	./data/SRR446036_1.fastq	A6B	A6	Avr.6h.B	1	23-Mar-2012

```

11 ./data/SRR446037_1.fastq      V6A      V6      Vir.6h.A      1 23-Mar-2012
12 ./data/SRR446038_1.fastq      V6B      V6      Vir.6h.B      1 23-Mar-2012
13 ./data/SRR446039_1.fastq      M12A     M12     Mock.12h.A    1 23-Mar-2012
14 ./data/SRR446040_1.fastq      M12B     M12     Mock.12h.B    1 23-Mar-2012
15 ./data/SRR446041_1.fastq      A12A     A12     Avr.12h.A     1 23-Mar-2012
16 ./data/SRR446042_1.fastq      A12B     A12     Avr.12h.B     1 23-Mar-2012
17 ./data/SRR446043_1.fastq      V12A     V12     Vir.12h.A     1 23-Mar-2012
18 ./data/SRR446044_1.fastq      V12B     V12     Vir.12h.B     1 23-Mar-2012

```

Structure of targets file for paired end (PE) samples.

```

> library(systemPipeR)
> targetspath <- paste0(system.file("extdata", package="systemPipeR"), "/targetsPE.txt")
> read.delim(targetspath, comment.char = "#")[1:2,1:6]

```

	FileName1	FileName2	SampleName	Factor	SampleLong	Experiment
1	./data/SRR446027_1.fastq	./data/SRR446027_2.fastq	M1A	M1	Mock.1h.A	1
2	./data/SRR446028_1.fastq	./data/SRR446028_2.fastq	M1B	M1	Mock.1h.B	1

Comparisons are defined in the header lines of the targets starting with '# <CMP>'. The function readComp imports the comparison and stores them in a list.

```

> readComp(file=targetspath, format="vector", delim="-")

$CMPset1
[1] "M1-A1" "M1-V1" "A1-V1" "M6-A6" "M6-V6" "A6-V6" "M12-A12" "M12-V12" "A12-V12"

$CMPset2
[1] "M1-A1" "M1-V1" "M1-M6" "M1-A6" "M1-V6" "M1-M12" "M1-A12" "M1-V12" "A1-V1"
[10] "A1-M6" "A1-A6" "A1-V6" "A1-M12" "A1-A12" "A1-V12" "V1-M6" "V1-A6" "V1-V6"
[19] "V1-M12" "V1-A12" "V1-V12" "M6-A6" "M6-V6" "M6-M12" "M6-A12" "M6-V12" "A6-V6"
[28] "A6-M12" "A6-A12" "A6-V12" "V6-M12" "V6-A12" "V6-V12" "M12-A12" "M12-V12" "A12-V12"

```

## 4 Structure of param file and SYSargs container

The param file defines the parameters of the command-line software. The following shows the format of a sample param file provided by this package.

```

> parampath <- paste0(system.file("extdata", package="systemPipeR"), "/tophat.param")
> read.delim(parampath, comment.char = "#")

```

	PairSet	Name	Value
1	modules	<NA>	bowtie2/2.1.0
2	modules	<NA>	tophat/2.0.8b
3	software	<NA>	tophat
4	cores	-p	4
5	other	<NA>	-g 1 --segment-length 25 -i 30 -I 3000
6	outfile1	-o	<FileName1>
7	outfile1	path	./results/
8	outfile1	remove	<NA>
9	outfile1	append	.tophat
10	outfile1	outextension	.tophat/accepted_hits.bam
11	reference	<NA>	./data/tair10.fasta
12	infile1	<NA>	<FileName1>
13	infile1	path	<NA>

```

14  infile2          <NA>                                <FileName2>
15  infile2          path                                <NA>

```

The `systemArgs` function imports the definitions of both the `param` file and the `targets` file, and stores all relevant information as `SYSargs` object.

```

> args <- systemArgs(sysma=parampath, mytargets=targetspath)
> args

```

An instance of 'SYSargs' for running 'tophat' on 18 samples

Several accessor functions are available that are named after the slot names of the `SYSargs` object class.

```

> names(args)

[1] "modules"  "software" "cores"    "other"    "reference" "results"  "infile1"
[8] "infile2"  "outfile1" "sysargs"  "outpaths"

> modules(args)

[1] "bowtie2/2.1.0" "tophat/2.0.8b"

> cores(args)

[1] 4

> outpaths(args)[1]

```

M1A

```
"/rhome/tgirke/Projects/github/systemPipeR/vignettes/results/SRR446027_1.fastq.tophat/accepted_hits.bam"
```

```
> sysargs(args)[1]
```

```
"tophat -p 4 -g 1 --segment-length 25 -i 30 -I 3000 -o /rhome/tgirke/Projects/github/systemPipeR/vignettes/
```

The content of the `param` file can be returned as JSON object as follows (requires `rjson` package).

```

> systemArgs(sysma=parampath, mytargets=targetspath, type="json")

[1] "{\"modules\":{\"n1\":\"\", \"v2\":\"bowtie2/2.1.0\", \"n1\":\"\", \"v2\":\"tophat/2.0.8b\"}, \"software\"

```

## 5 Workflow

---

### 5.1 Define environment settings and samples

Load package:

```
> library(systemPipeR)
```

Construct `SYSargs` object from `param` and `targets` files.

```
> args <- systemArgs(sysma="tophat.param", mytargets="targetsPE.txt")
```

### 5.2 FASTQ quality report

The following `seeFastq` and `seeFastqPlot` functions generate and plot a series of useful quality statistics for a set of FASTQ files including per cycle quality box plots, base proportions, base-level quality trends, relative k-mer diversity, length and occurrence distribution of reads, number of reads above quality cutoffs and mean quality distribution.

```
> fqlist <- seeFastq(fastq=infile1(args), batchsize=10000, klength=8)
> pdf("./results/fastqReport.pdf", height=18, width=4*length(fqlist))
> seeFastqPlot(fqlist)
> dev.off()
```

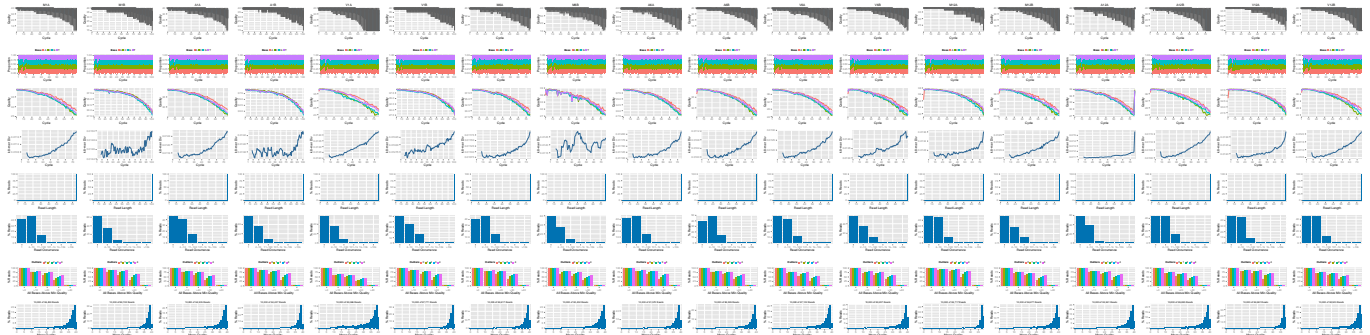


Figure 1: QC report for 18 FASTQ files.

### 5.3 Alignment with Tophat 2

Build Bowtie 2 index.

```
> moduleload(modules(args)) # Skip if module system is not available
> system("bowtie2-build ./data/tair10.fasta ./data/tair10.fasta")
```

Execute SYSargs on a single machine without submitting to a queuing system of a compute cluster.

```
> bampaths <- runCommandline(args=args)
```

Submit to compute nodes.

```
> qsubargs <- getQsubargs(queue="batch", cores=cores(args), memory="mem=10gb", time="walltime=20:00:00")
> (joblist <- qsubRun(args=args, qsubargs=qsubargs, Nqsubs=18, package="systemPipeR"))
```

Alignment Stats

```
> read_statsDF <- alignStats(args, fqgz=TRUE)
> write.table(read_statsDF, "results/alignStats.xls", row.names=FALSE, quote=FALSE, sep="\t")
```

### 5.4 Create symbolic links for viewing BAM files in IGV

```
> symLink2bam(sysargs=args, htmlDir=c("~/html/", "somedir/"),
+             urlbase="http://myserver.edu/~username/",
+             urlfile="IGVurl.txt")
```

### 5.5 Alignment with Bowtie 2 (here for miRNA profiling experiment)

Run as single process without submitting to cluster, e.g. via qsub -l.

```
> args <- systemArgs(sysma="bowtieSE.param", mytargets="targets.txt")
> bampaths <- runCommandline(args=args)
```

Submit to compute nodes

```
> qsubargs <- getQsubargs(queue="batch", cores=cores(args), memory="mem=10gb", time="walltime=20:00:00")
> (joblist <- qsubRun(args=args, qsubargs=qsubargs, Nqsubs=18, package="systemPipeR"))
```

## 5.6 Read counting for mRNA profiling experiments

Create txdb (do only once)

```
> library(GenomicFeatures)
> txdb <- makeTranscriptDbFromGFF(file="data/tair10.gff", format="gff", dataSource="TAIR", species="A. thaliana")
> saveDb(txdb, file="./data/tair10.sqlite")
```

Read counting with summarizeOverlaps in parallel mode with multiple cores

```
> library(BiocParallel)
> txdb <- loadDb("./data/tair10.sqlite")
> eByg <- exonsBy(txdb, by="gene")
> bfl <- BamFileList(outpaths(args), yieldSize=50000, index=character())
> multicoreParam <- MulticoreParam(workers=4); register(multicoreParam); registered()
> counteByg <- bplapply(bfl, function(x) summarizeOverlaps(eByg, x, mode="Union", ignore.strand=TRUE, inter.feature=FALSE))
> countDFeByg <- sapply(seq(along=counteByg), function(x) assays(counteByg[[x]])$counts)
> rownames(countDFeByg) <- names(rowData(counteByg[[1]])); colnames(countDFeByg) <- names(bfl)
> rpkmDFeByg <- apply(countDFeByg, 2, function(x) returnRPKM(counts=x, ranges=eByg))
> write.table(countDFeByg, "results/countDFeByg.xls", col.names=NA, quote=FALSE, sep="\t")
> write.table(rpkmDFeByg, "results/rpkmDFeByg.xls", col.names=NA, quote=FALSE, sep="\t")
```

## 5.7 Read counting for miRNA profiling experiments

Download miRNA genes from miRBase

```
> system("wget ftp://mirbase.org/pub/mirbase/19/genomes/My_species.gff3 -P ./data/")
> gff <- import.gff("./data/My_species.gff3", asRangedData=FALSE)
> gff <- split(gff, elementMetadata(gff)$ID)
> bams <- names(bampaths); names(bams) <- targets$SampleName
> bfl <- BamFileList(bams, yieldSize=50000, index=character())
> countDFmiR <- summarizeOverlaps(gff, bfl, mode="Union", ignore.strand=FALSE, inter.feature=FALSE) # Note
> rpkmDFmiR <- apply(countDFmiR, 2, function(x) returnRPKM(counts=x, gffsub=gff))
> write.table(assays(countDFmiR)$counts, "results/countDFmiR.xls", col.names=NA, quote=FALSE, sep="\t")
> write.table(rpkmDFmiR, "results/rpkmDFmiR.xls", col.names=NA, quote=FALSE, sep="\t")
```

## 5.8 Correlation analysis of samples

```
> library(ape)
> rpkmDFeByg <- read.table("./results/rpkmDFeByg.xls", check.names=FALSE)
> rpkmDFeByg <- rpkmDFeByg[rowMeans(rpkmDFeByg) > 50,]
> d <- cor(rpkmDFeByg, method="spearman")
> hc <- hclust(as.dist(1-d))
> plot.phylo(as.phylo(hc), type="p", edge.col="blue", edge.width=2, show.node.label=TRUE, no.margin=TRUE)
```

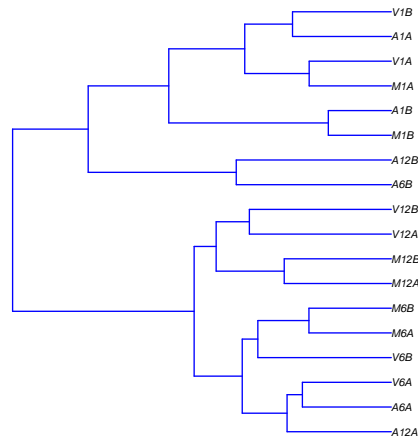


Figure 2: Correlation dendrogram of samples.

## 5.9 DEG analysis with edgeR

```
> library(edgeR)
> targets <- read.delim(targetspath, comment="#")
> cmp <- readComp(file=targetspath, format="matrix", delim="-")
> cmp[[1]]
```

```
      [,1] [,2]
[1,] "M1"  "A1"
[2,] "M1"  "V1"
[3,] "A1"  "V1"
[4,] "M6"  "A6"
[5,] "M6"  "V6"
[6,] "A6"  "V6"
[7,] "M12" "A12"
[8,] "M12" "V12"
[9,] "A12" "V12"
```

Run *edgeR*

```
> edgeDF <- run_edgeR(countDF=countDFeByg, targets=targets, cmp=cmp[[1]], independent=FALSE, mdsplot="")
```

Filter and plot DEG results for up and down regulated genes. Because of the toy sample set used in this vignette, the FDR value has been set to a relatively high threshold (here 10%). More commonly used FDR cutoffs are 1% or 5%.

```
> DEG_list <- filterDEGs(degDF=edgeDF, filter=c(Fold=2, FDR=10))
```

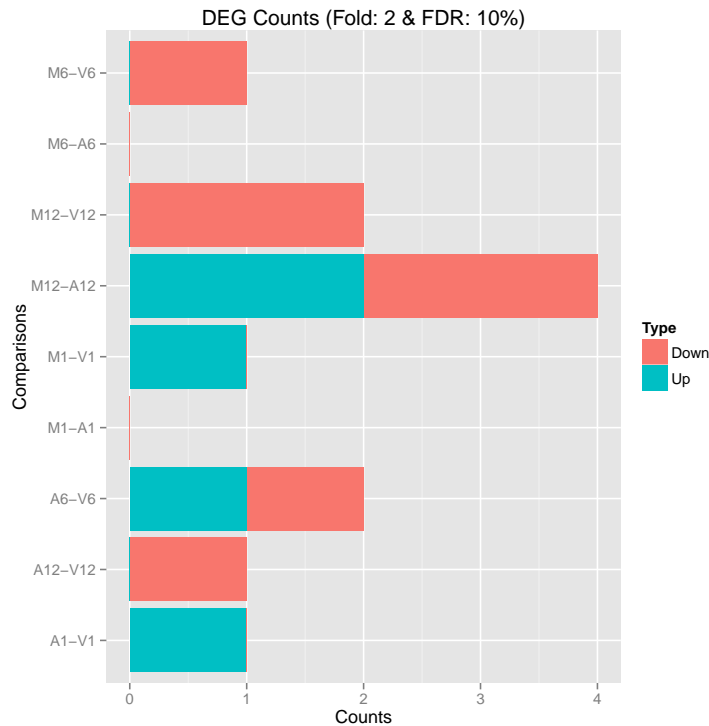


Figure 3: Up and down regulated DEGs.

```
> names(DEG_list)
> DEG_list$Summary
```

## 5.10 GO term enrichment analysis of DEGs

The following shows how to obtain gene-to-GO mappings from *biomaRt* (here for *A. thaliana*) and how to organize them for the downstream GO term enrichment analysis. Alternatively, the gene-to-GO mappings can be obtained for many organisms from Bioconductor's \*.db genome annotation packages or GO annotation files provided by various genome databases. For each annotation this relatively slow preprocessing step needs to be performed only once. Subsequently, the preprocessed data can be loaded with the `loadData` function as shown in the next subsection.

```
> library("biomaRt")
> listMarts() # Choose BioMart databases, here vb_mart_22 (VectorBase)
> m <- useMart("ENSEMBL_MART_PLANT"); listDatasets(m) # Choose genome from VectorBase, here aaegypti_eg_gene
> m <- useMart("ENSEMBL_MART_PLANT", dataset="athaliana_eg_gene")
> listAttributes(m) # Choose data types you want to download
> go <- getBM(attributes=c("go_accession", "tair_locus", "go_namespace_1003"), mart=m)
> go <- go[go[,3]!="",]; go[,3] <- as.character(go[,3])
> go[go[,3]=="molecular_function", 3] <- "F"; go[go[,3]=="biological_process", 3] <- "P"; go[go[,3]=="cellular_component", 3] <- "C"
> go[1:4,]
> dir.create("./data/GO")
> write.table(go, "data/GO/GOannotationsBiomart_mod.txt", quote=FALSE, row.names=FALSE, col.names=FALSE, sep="\t")
> readGOorg(myfile = "data/GO/GOannotationsBiomart_mod.txt", outdir="data/GO", org="", colno = c(1,2,3))
> gene2GOlist(outdir="data/GO", rootUK=FALSE)
```

Apply the enrichment analysis to the DEG sets obtained the above differential expression analysis. Note in the following example the FDR filter is set here to an unreasonably high value, simply because of the small size of the toy data set



used in this vignette. Batch enrichment analysis of many gene sets is performed with the `GOCluster_Report` function. When `method="all"`, it returns all GO terms passing the p-value cutoff specified under the `cutoff` arguments. When `method="slim"`, it returns only the GO terms specified under the `myslimv` argument. The given example shows how a GO slim vector for a specific organism can be obtain from BioMart.

```
> loadData("data/GO")
> DEG_list <- filterDEGs(degDF=edgeDF, filter=c(Fold=2, FDR=50), plot=FALSE)
> up_down <- DEG_list$UporDown; names(up_down) <- paste(names(up_down), "_up_down", sep="")
> up <- DEG_list$Up; names(up) <- paste(names(up), "_up", sep="")
> down <- DEG_list$Down; names(down) <- paste(names(down), "_down", sep="")
> DEGlist <- c(up_down, up, down)
> DEGlist <- DEGlist[sapply(DEGlist, length) > 0]
> BatchResult <- GOCluster_Report(setlist=DEGlist, method="all", id_type="gene", CLSZ=2, cutoff=0.9, gocat="MF")
> library("biomaRt"); m <- useMart("ENSEMBL_MART_PLANT", dataset="athaliana_eg_gene")
> goslimvec <- as.character(getBM(attributes=c("goslim_goa_accession"), mart=m)[,1])
> BatchResultslim <- GOCluster_Report(setlist=DEGlist, method="slim", id_type="gene", myslimv=goslimvec, CLSZ=2, cutoff=0.9, gocat="MF")
```

Sample plots of GO slim term analysis results. The data.frame generated by `GOCluster_Report` can be plotted with the `goBarplot` function. Because of the variable size of the sample sets, it may not be always desirable to show the results from different DEG sets in the same bar plot. Plotting single sample sets is achieved by subsetting the input data frame as shown in the first line of the following example.

```
> gos <- BatchResultslim[grep("M6-V6_up_down", BatchResultslim$CLID), ]
> gos <- BatchResultslim
> pdf("GOslimbarplotMF.pdf", height=8, width=10); goBarplot(gos, gocat="MF"); dev.off()
> goBarplot(gos, gocat="BP")
> goBarplot(gos, gocat="CC")
```



Figure 4: GO Slim Barplot for MF Ontology.

## 6 Version Information

```
> toLatex(sessionInfo())
```

- R version 3.1.0 (2014-04-10), x86\_64-unknown-linux-gnu
- Locale: C
- Base packages: base, datasets, grDevices, graphics, grid, methods, parallel, stats, utils
- Other packages: AnnotationDbi 1.26.0, BSgenome 1.32.0, Biobase 2.24.0, BiocGenerics 0.10.0, BiocParallel 0.6.1, Biostrings 2.32.0, Category 2.30.0, DBI 0.2-7, GO.db 2.14.0, GOstats 2.30.0, GenomInfoDb 1.0.2, GenomicAlignments 1.0.1, GenomicRanges 1.16.3, IRanges 1.22.9, Matrix 1.1-4, RSQLite 0.11.4, Rsamtools 1.16.1, ShortRead 1.22.0, XVector 0.4.0, annotate 1.42.0, edgeR 3.6.2, ggplot2 1.0.0,

- graph 1.42.0, limma 3.20.6, rjson 0.2.14, systemPipeR 1.0.11
- Loaded via a namespace (and not attached): AnnotationForge 1.6.1, BBmisc 1.7, BatchJobs 1.2, BiocStyle 1.2.0, GSEABase 1.26.0, MASS 7.3-33, RBGL 1.40.0, RColorBrewer 1.0-5, Rcpp 0.11.2, XML 3.98-1.1, bitops 1.0-6, brew 1.0-6, checkmate 1.0, codetools 0.2-8, colorspace 1.2-4, digest 0.6.4, fail 1.2, foreach 1.4.2, genefilter 1.46.1, gtable 0.1.2, hwriter 1.3, iterators 1.0.7, lattice 0.20-29, latticeExtra 0.6-26, munsell 0.4.2, plyr 1.8.1, proto 0.3-10, reshape2 1.4, scales 0.2.4, sendmailR 1.1-2, splines 3.1.0, stats4 3.1.0, stringr 0.6.2, survival 2.37-7, tools 3.1.0, xtable 1.7-3, zlibbioc 1.10.0

## 7 Funding

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## 8 References

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- Thomas Girke. systemPipeR: NGS workflow and report generation environment, 28 June 2014. URL <https://github.com/tgirke/systemPipeR>.
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