Transcriptome Data Analysis

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@FedeBioinfo

Let's do a quick introduction round!

I'd like to know

- Your name
- The link between you and RNA-seq data
- A fun fact about you

I'll start :)

Hi, I am Federico, I am the head of the Bioinformatics group at the IMBEI in Mainz.
People told me I am somehow well versed with RNA-seq data.
I like to cook and talk about food.

Questions

What are the steps to process RNA-Seq data?

- How to convert RNA-seq reads into counts?
- How to perform quality control (QC) of RNA-seq reads?

How to identify differentially expressed genes across multiple experimental conditions?

- How to properly analyze RNA count data using DESeq2?
- How to perform quality control (QC) and exploratory data analysis (EDA) of RNA-seq count data?

What are the biological functions impacted by the differential expression of genes?

- How can I perform a gene ontology enrichment analysis?

How can I create neat visualizations of the data?

- How can I visualize the results for my enrichment analysis?

How can I generate interactive reports to summarise my analyses?

What you will learn

- the basics of RNA-seq data
- the basics of RNA-seq data analysis
- to get familiar with the concepts of gene expression, high-dimensional data, expression quantification, differential expression analysis
- the importance to pose the right question, in order to get the right answer :)

This material has been developed together with Charlotte Soneson in the scope of the GTIPI Summer School (https://imbeimainz.github.io/GTIPI2022)

Setup for practical sessions

Got R/RStudio?

Latest versions highly recommended!

See https://imbeimainz.github.io/GTIPI2022/material.html for details!

+

This repo should contain it all: https://github.com/imbeimainz/MSE_GenEpi_2024

Decomposing the title

RNA

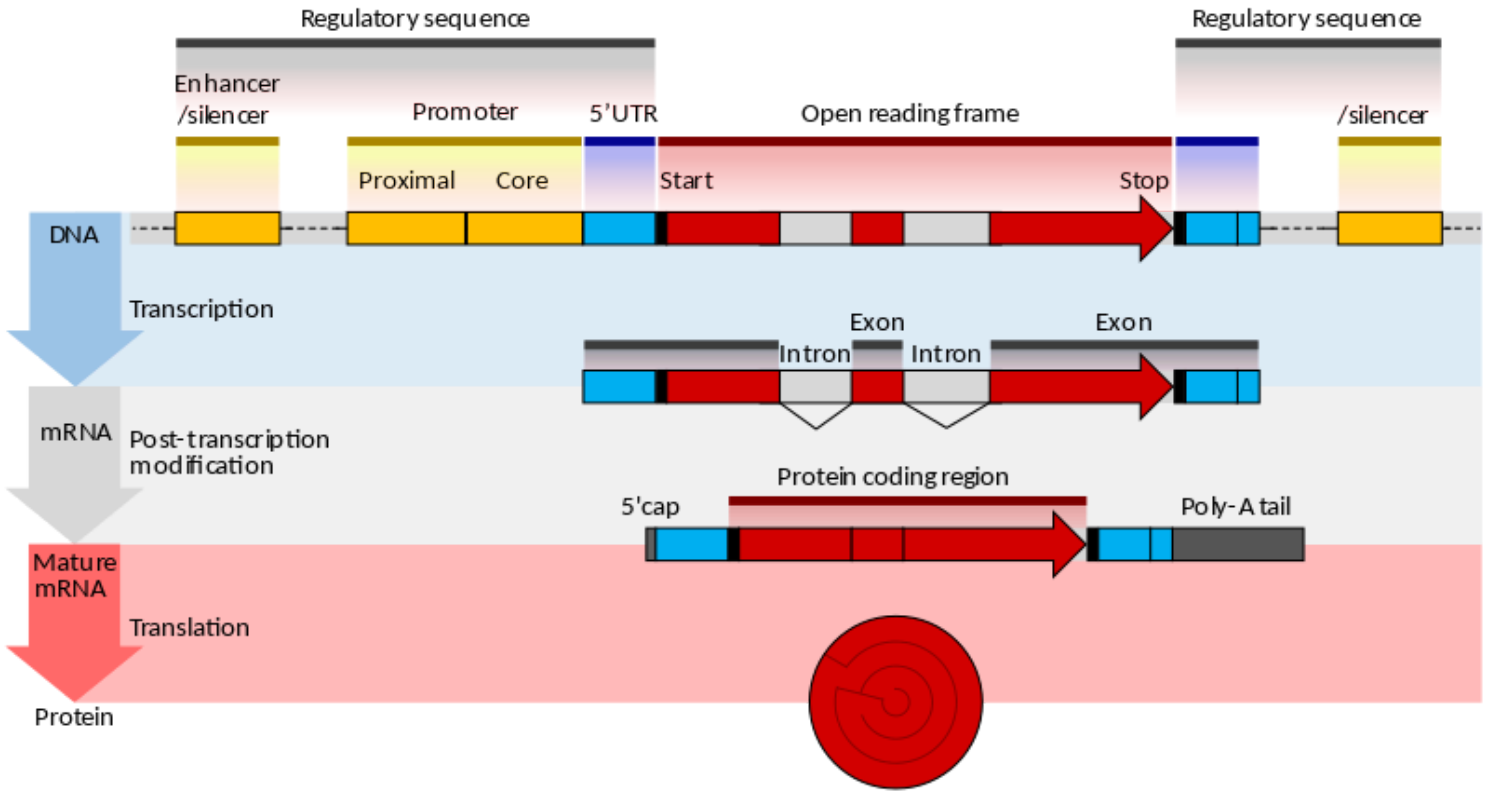
Sequencing

Bioinformatics

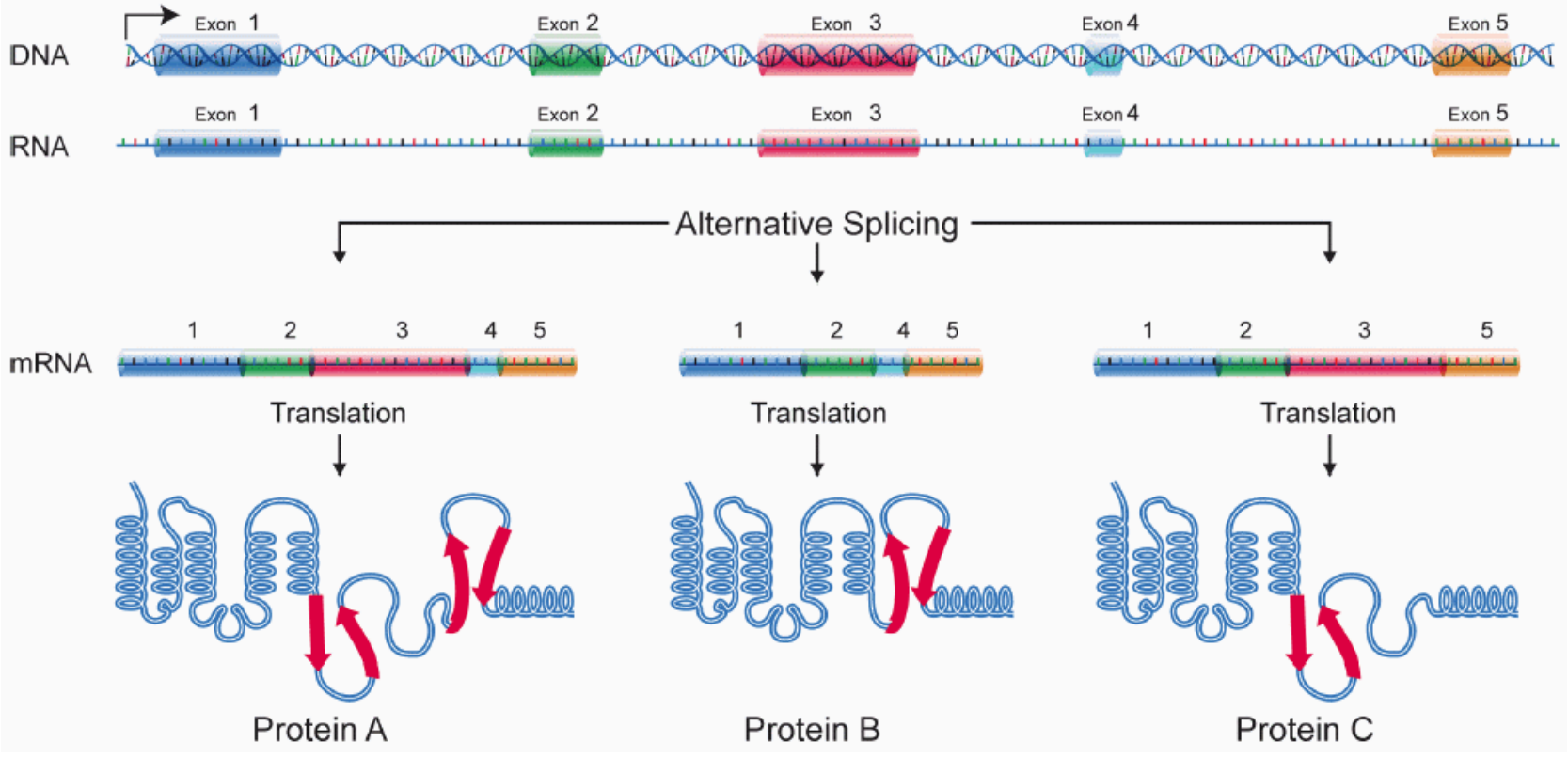
Transcriptome

Analysis

(messenger) RNA

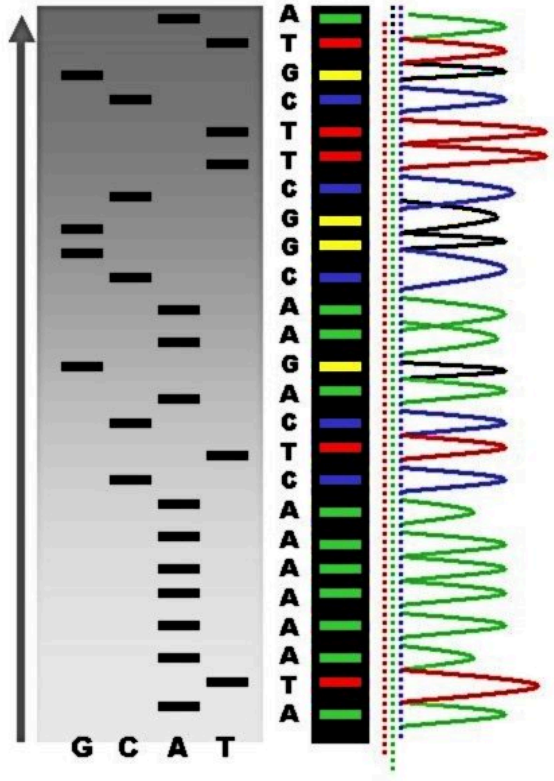


(messenger) RNA



Exons, introns, transcripts, isoforms

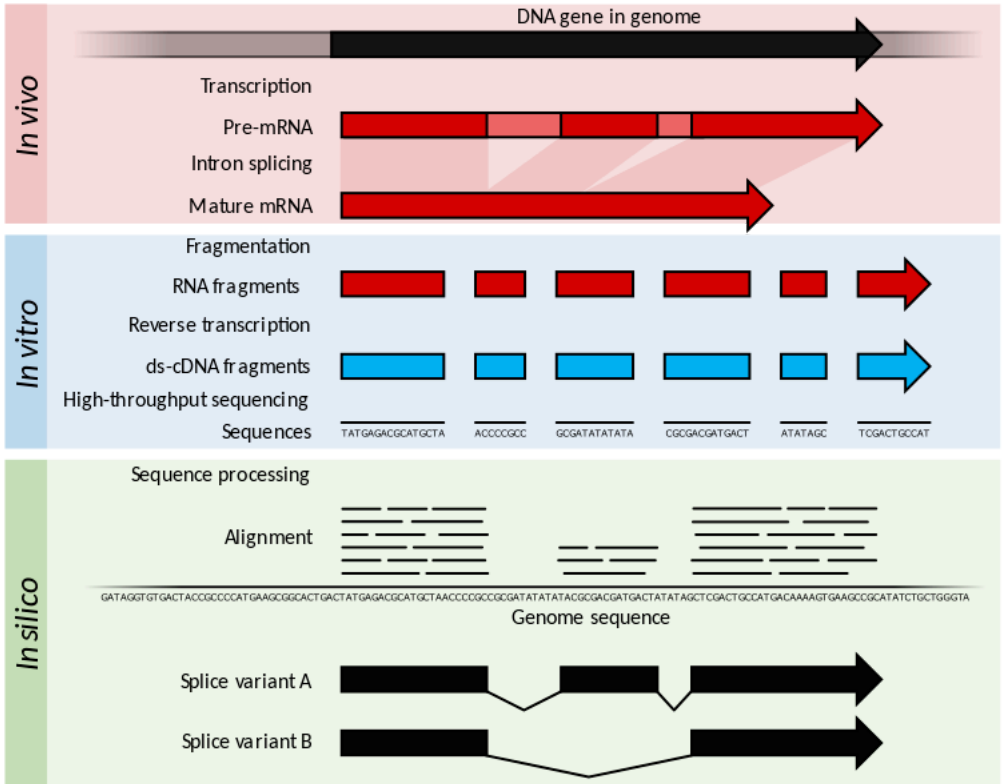
Sequencing



4 base pairs, 21 aminoacids

Excellent review on next-generation sequencing: https://www.nature.com/articles/nrg.2016.49

RNA-sequencing



- RNA quantification at single base resolution
- Cost efficient analysis of the whole transcriptome in a high-throughput manner

Challenges in RNA-seq

- Different origin for the sample RNA and the reference genome
- Presence of incompletely processed RNAs or transcriptional background noise
- Sequencing biases (e.g. PCR library preparation)

Benefits

- sensitive
- specific
- high-throughput
- cost-efficient
- basepair resolution

You can see: transcripts, splicing, lncRNA, circRNA, gene fusions

Bioinformatics

> "an interdisciplinary field that develops methods and software tools for understanding biological data, in particular when the data sets are large and complex."

A combination of

- biology
- computer science
- information engineering
- mathematics
- statistics

... to analyze and interpret the biological data

Transcriptome

Gene expression is a fundamental level at which the results of various genetic and regulatory programs are observable.

RNA sequencing (RNA-seq) provides a quantitative and open system for profiling transcriptional outcomes on a large scale

Much has been learned about the characteristics of the RNA-seq data sets, as well as the performance of the myriad of methods developed

"RNA Sequencing Data: Hitchhiker's Guide to Expression Analysis" ->
https://www.annualreviews.org/doi/abs/10.1146/annurev-biodatasci-072018-021255

Analysis

There's data involved!

and these datasets have particular properties (how they are generated, ...)

How to make sense out of it?

There is a large diversity of applications to deal with

Among the most widely adopted workflows:

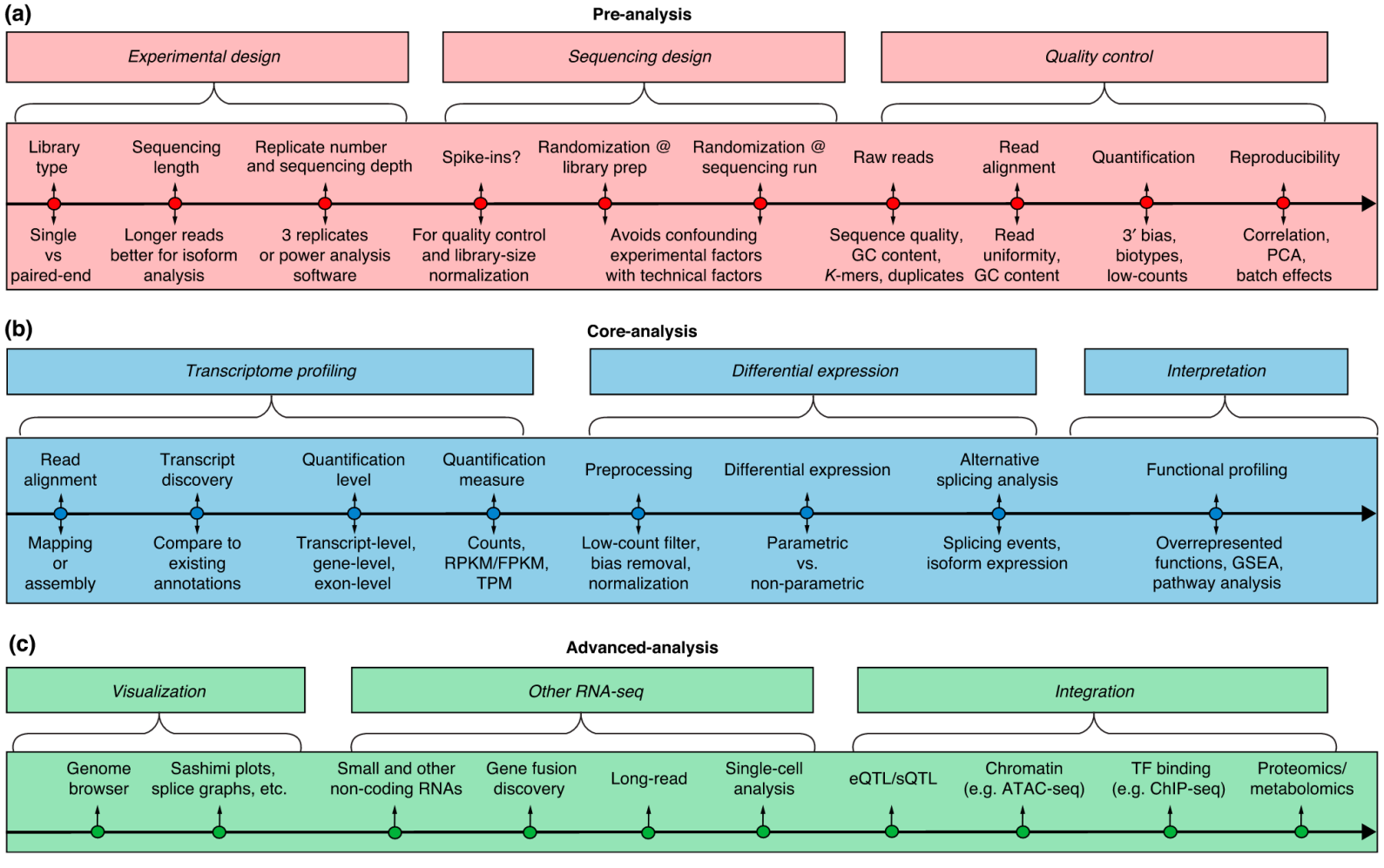
- Transcript discovery

  > *Which RNA molecules are in my sample?*
  >
  > Novel isoforms and alternative splicing, Non-coding RNAs, Single nucleotide variations, Fusion genes

- RNA quantification

  > *What is the concentration of RNAs?*
  >
  > Absolute gene expression (within sample), Differential expression (between biological samples)

Analysis - a bird's eye view



The essential

- Expression quantification
- Data exploration: principal components analysis, gene plots
- Differential analysis: DE modeling, design, effect size, variability, significance
- Functional interpretation: gene sets, pathways, biological themes

Differential analysis types for RNA-seq

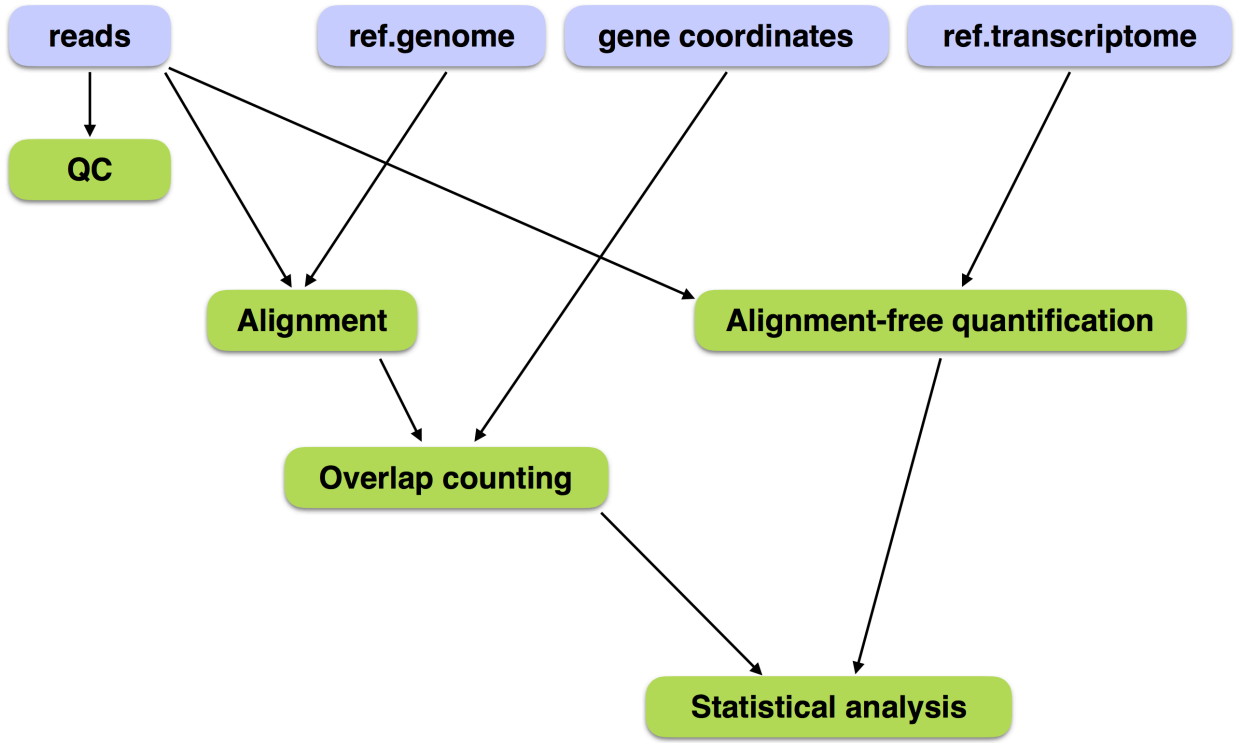
No single available standardized workflow
Multiple possible best practices for every dataset

To get the right answer, you have to pose the right question.

- Does the total output of a gene change between conditions? Differential Gene Expression (DGE)
- Does the expression of individual transcripts change? Differential Transcript Expression (DTE)
- Does any isoform of a given gene change? DTE+G
- Does the isoform composition for a given gene change? Differential Transcript Usage/Differential Exon Usage (DTU/DEU)

Each needs different computational approaches (quantifications + tests)

Overview of the processing workflow



Ingredients + operations

The raw data: sequencing reads

FASTQ files: sequence + base quality (phred score)

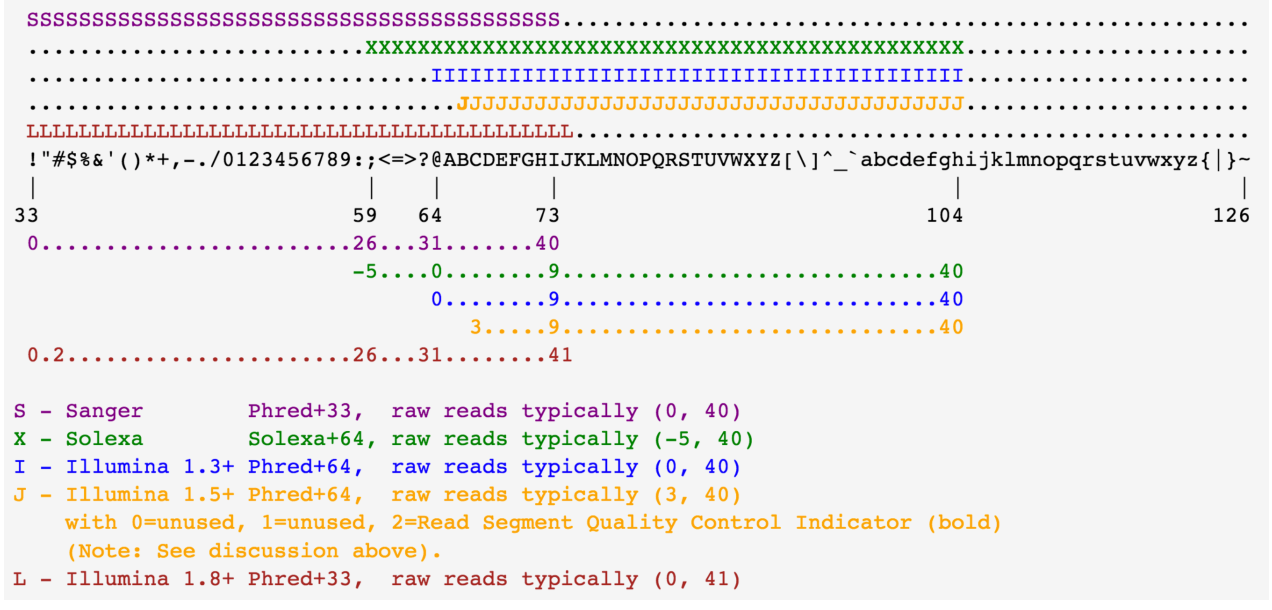
First lines of a FASTQ file

```
@SRR1055095.1 HWI-ST156:397:D09NJACXX:5:1101:1222:1915/1
NGCTGCTGGACTCCGAAGATGGGCGGTATATCATCCCACTGCTGACTCTN
+
#1=DDFFFHFHHHJJHIIGHIJJGIJAFDDHGGGIJJGJIJJJIJJIIH#
@SRR1055095.2 HWI-ST156:397:D09NJACXX:5:1101:1245:1920/1
NCTTTTCTTTGTTCTCATCATCTTCAGGAGGAGGAGGGTCATCCTTGTGN
+
#1=BDB:?FFDFFDF?EF<FFF>B>?C@CF<1??CFB:09;09BFE9DB#
```

Repeat this tens of millions of times, and you'll have *one* sample

The raw data: sequencing reads

Different quality encodings exist



The raw data

Demo: quality control report, from FastQC

http://www.bioinformatics.babraham.ac.uk/projects/fastqc/good_sequence_short_fastqc.html

Your next best friend: MultiQC

Common sequence artefacts in NGS data:

- read errors
- base calling errors
- small insertions and deletions
- poor quality reads
- primer/adapter contamination

Solutions: Quality trimming & filtering (wide range of QC tools available)

Reference files

Reference genome sequences (in `fasta` format), required for genome alignment.

Think of the alignment as the address of each read in a 3-billion houses street, where the elements along the street can also end up repeating themselves.

What's out there?

Ensembl: http://www.ensembl.org/info/data/ftp/index.html

Gencode (human & mouse): https://www.gencodegenes.org/

UCSC: http://hgdownload.cse.ucsc.edu/downloads.html

iGenome: http://support.illumina.com/sequencing/sequencing_software/igenome.html

Reference files

Some critical points:

Be consistent!

Different chromosome identifiers!

Reference genomes and annotations are continuously refined, extended and improved

Keep track of version and be consistent!

Naming of genes can vary across versions, in some databases!

Reference files

An example:

http://ftp.ensembl.org/pub/release-106/fasta/homo_sapiens/dna/

... and one level up...

http://ftp.ensembl.org/pub/release-106/fasta/homo_sapiens/

```
>1 dna:chromosome chromosome:GRCh38:1:1:248956422:1 REF
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
...
TTGGTGCCAGTTCCTCCAAGTCGATGGCACCTCCCTCCCTCTCAACCACTTGAGCAAACT
CCAAGACATCTTCTACCCCAACACCAGCAATTGTGCCAAGGGCCATTAGGCTCTCAGCAT
GACTATTTTTAGAGACCCCGTGTCTGTCACTGAAACCTTTTTTGTGGGAGACTATTCCTC
CCATCTGCAACAGCTGCCCCTGCTGACTGCCCTTCTCTCCTCCCTCTCATCCCAGAGAAA
CAGGTCAGCTGGGAGCTTCTGCCCCCACTGCCTAGGGACCAACAGGGGCAGGAGGCAGTC
```

Reference files

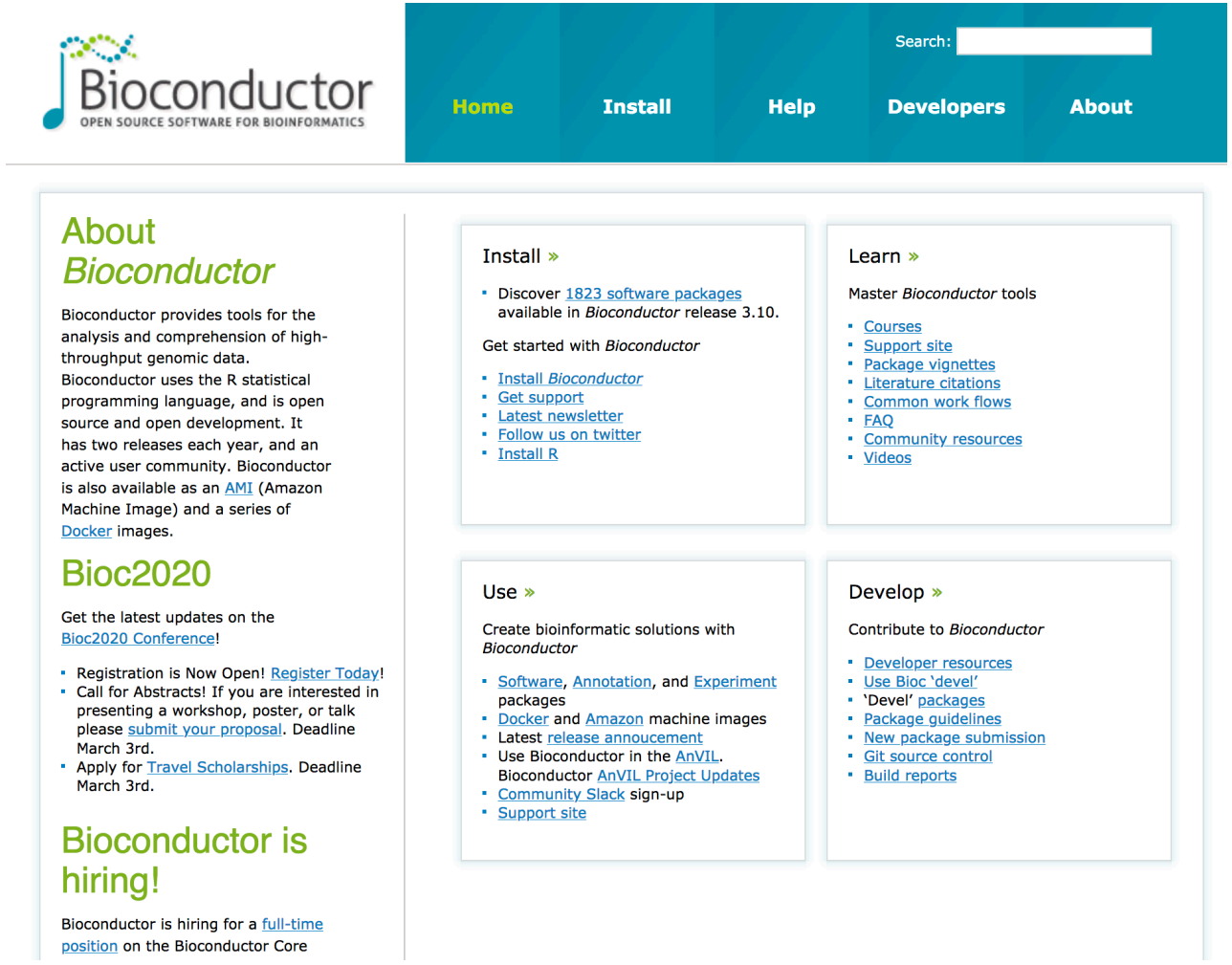
The GTF format

```
chr1    unknown exon    11874   12227   .       +       .       gene_id "DDX11L1"; gene_name "DDX11L1";
transcript_id "NR_046018"; tss_id "TSS16107";
chr1    unknown CDS     3427347 3427466 .       -       2       gene_id "MEGF6"; gene_name "MEGF6";
p_id "P34437"; transcript_id "NM_001409"; tss_id "TSS31177";
```

- One line per "feature" (exon, transcript, gene, CDS, 3'UTR, 5'UTR, ...)
- One feature = 9 columns of data, plus optional track definition lines
- Essential for releasing annotation information

```
seqname - name of chromosome/scaffold
source - data/program source
feature - feature type name
start - positions of the feature
end
score - floating point value
strand - forward or reverse
frame -  0|1|2
attribute - semicolon-separated list of tag-value pairs, providing
additional information
```

Bioconductor



Bioconductor - soon your best friend

- an open source project
- a repository of packages, focused on bioinformatics/computational biology
- a open development platform and community

Currently (July 2024)

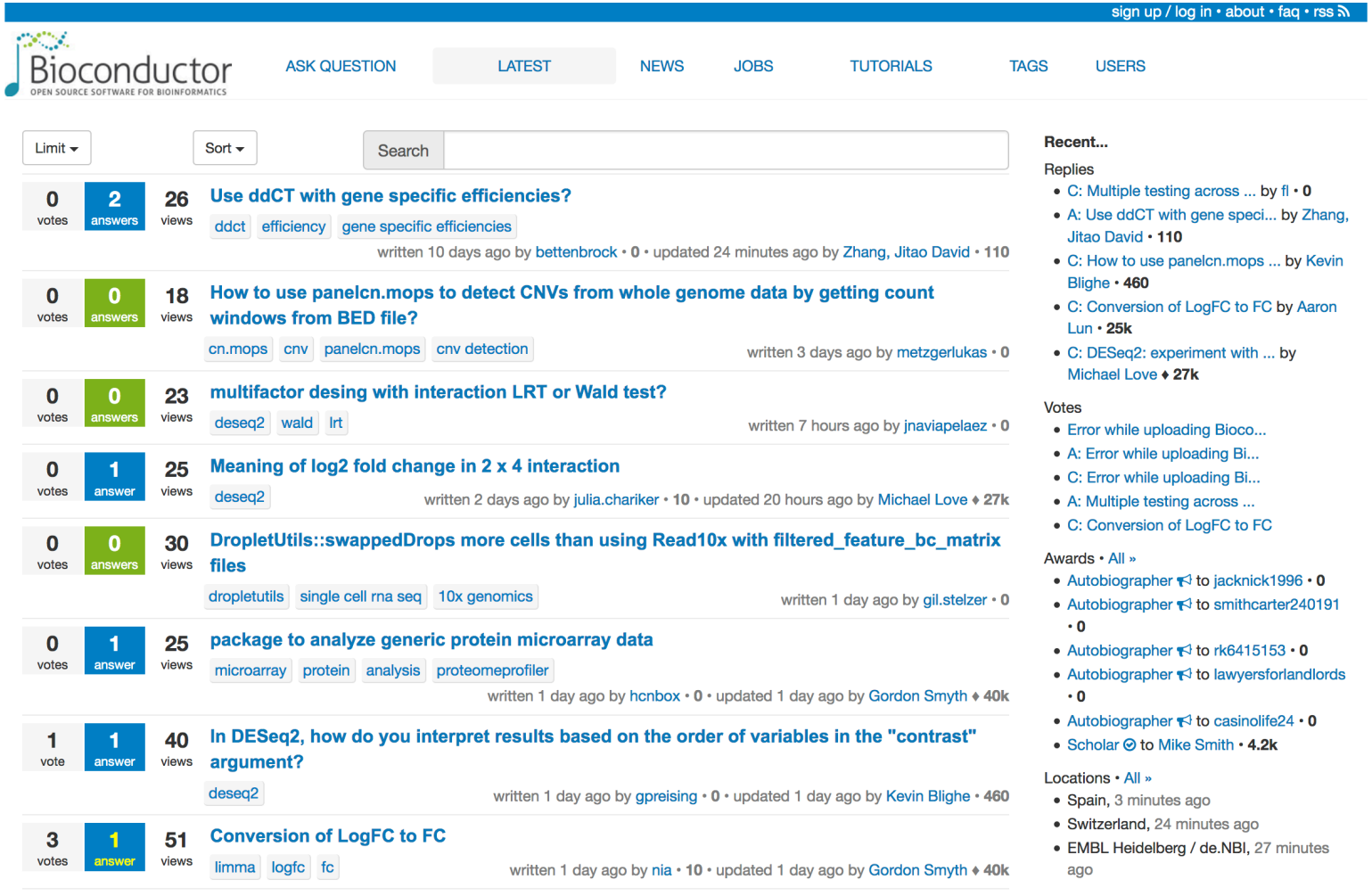
- 2300 software packages
- 926 annotation packages
- 430 experiment packages
- 30 workflows
- 8 books (https://bioconductor.org/help/bioconductor-books/)

Aim: interdisciplinary research, collaboration and rapid development of scientific software

Documentation

- function manual pages, most of them with runnable examples
- package vignettes - mandatory here!
- workflows, documenting full analyses spanning multiple tools
- a very active support site

The real deal: Bioconductor's community



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votes	answers	views			
0	2	26	Use ddCT with gene specific efficiencies?	ddct, efficiency, gene specific efficiencies	written 10 days ago by bettenbrock • 0 • updated 24 minutes ago by Zhang, Jitao David • 110
0	0	18	How to use panelcn.mops to detect CNVs from whole genome data by getting count windows from BED file?	cn.mops, cnv, panelcn.mops, cnv detection	written 3 days ago by metzgerlukas • 0
0	0	23	multifactor desing with interaction LRT or Wald test?	deseq2, wald, lrt	written 7 hours ago by jnaviapelaez • 0
0	1	25	Meaning of log2 fold change in 2 x 4 interaction	deseq2	written 2 days ago by julia.chariker • 10 • updated 20 hours ago by Michael Love ♦ 27k
0	0	30	DropletUtils::swappedDrops more cells than using Read10x with filtered_feature_bc_matrix files	dropletutils, single cell rna seq, 10x genomics	written 1 day ago by gil.stelzer • 0
0	1	25	package to analyze generic protein microarray data	microarray, protein, analysis, proteomeprofiler	written 1 day ago by hcnbox • 0 • updated 1 day ago by Gordon Smyth ♦ 40k
1 vote	1	40	In DESeq2, how do you interpret results based on the order of variables in the "contrast" argument?	deseq2	written 1 day ago by gpreising • 0 • updated 1 day ago by Kevin Blighe • 460
3	1	51	Conversion of LogFC to FC	limma, logfc, fc	written 1 day ago by nia • 10 • updated 1 day ago by Gordon Smyth ♦ 40k

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- C: DESeq2: experiment with ... by Michael Love ♦ 27k

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- Scholar to Mike Smith • 4.2k

Locations • All »
- Spain, 3 minutes ago
- Switzerland, 24 minutes ago
- EMBL Heidelberg / de.NBI, 27 minutes ago

Data processing

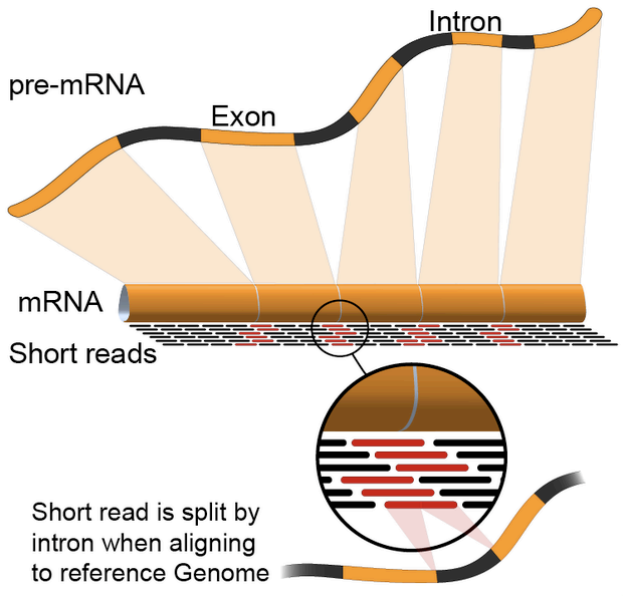
Data processing

Turning millions of text lines into properly structured abundance tables

Our aim: we often want to compare abundance (expression) of genes or other features between conditions

Splice-aware genome alignment vs "direct" transcript mapping and quantification

Alignment: not just simple mapping



For RNA-seq data, we need a splice-aware aligner

Common choices:

- STAR
- HISAT2

Alignment

File-format: sam (compressed into bam)

```
SRR1055095.6079377  353  chr1    11167    0    50M    =    11751    634    CGCCCCTTGCTTGCAGCCGGGCAC
TACAGGACCCGCTTGCTCACGGTGAA    CCCFFFFFHHHHHJJJJJJJJJJJJIGJJJIJJJJJJJJJJJDHJJCEHH
AS:i:-10    XN:i:0    XM:i:2    XO:i:0    XG:i:0    NM:i:2    MD:Z:48C0T0    YT:Z:UU    NH:i:20
CC:Z:chrY    CP:i:59361513    HI:i:0
```

Again: repeat this, one read at a time!

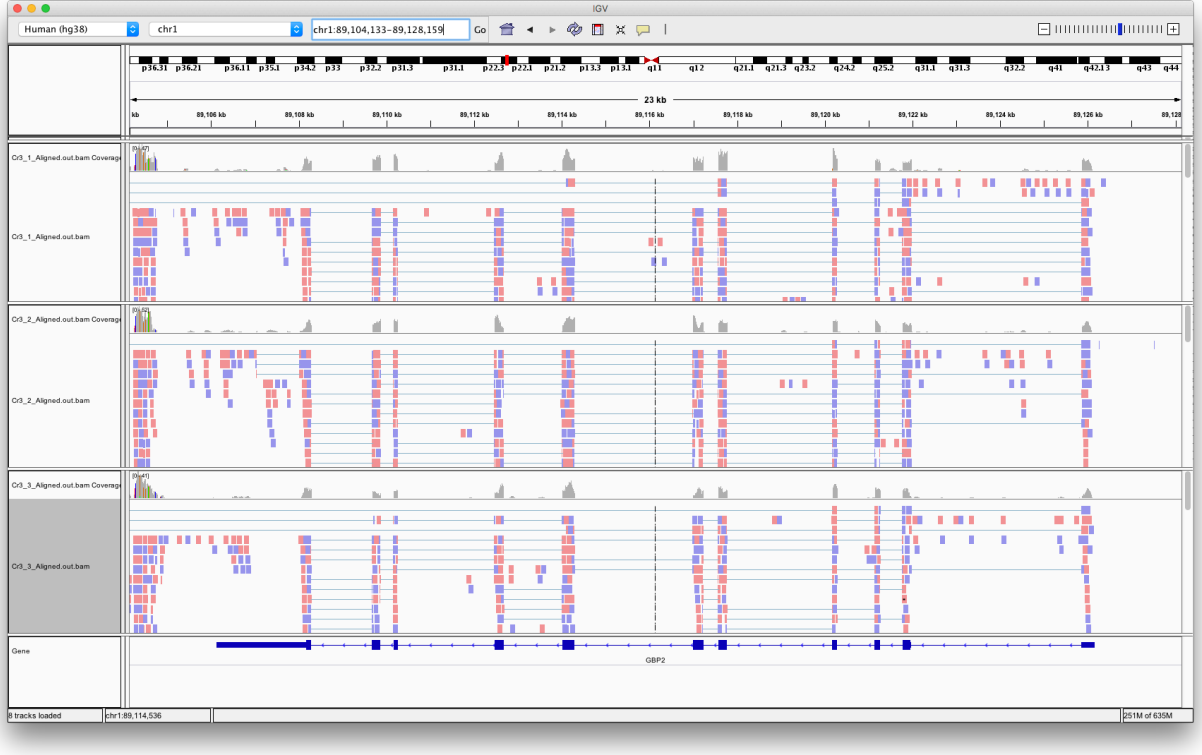
Entries:

```
QNAME - Query NAME of the read or the read pair
FLAG - Bitwise FLAG (pairing, strand, mate strand, etc.)
RNAME - Reference sequence NAME
POS - 1-Based leftmost POSition of clipped alignment
MAPQ - MAPping Quality (Phred-scaled)
CIGAR - Extended CIGAR string (operations: MIDNSHP)
MRNM - Mate Reference NaMe ('=' if same as RNAME)
MPOS - 1-Based leftmost Mate POSition
ISIZE - Inferred Insert SIZE
SEQ - Query SEQuence on the same strand as the reference
QUAL - Query QUALity (ASCII-33=Phred base quality)
Tags: used to store info about alignment
```

Before quantification

… and actually, always: Do visualize your data!

Options: UCSC Genome Browser, IGV, IGB - http://software.broadinstitute.org/software/igv/

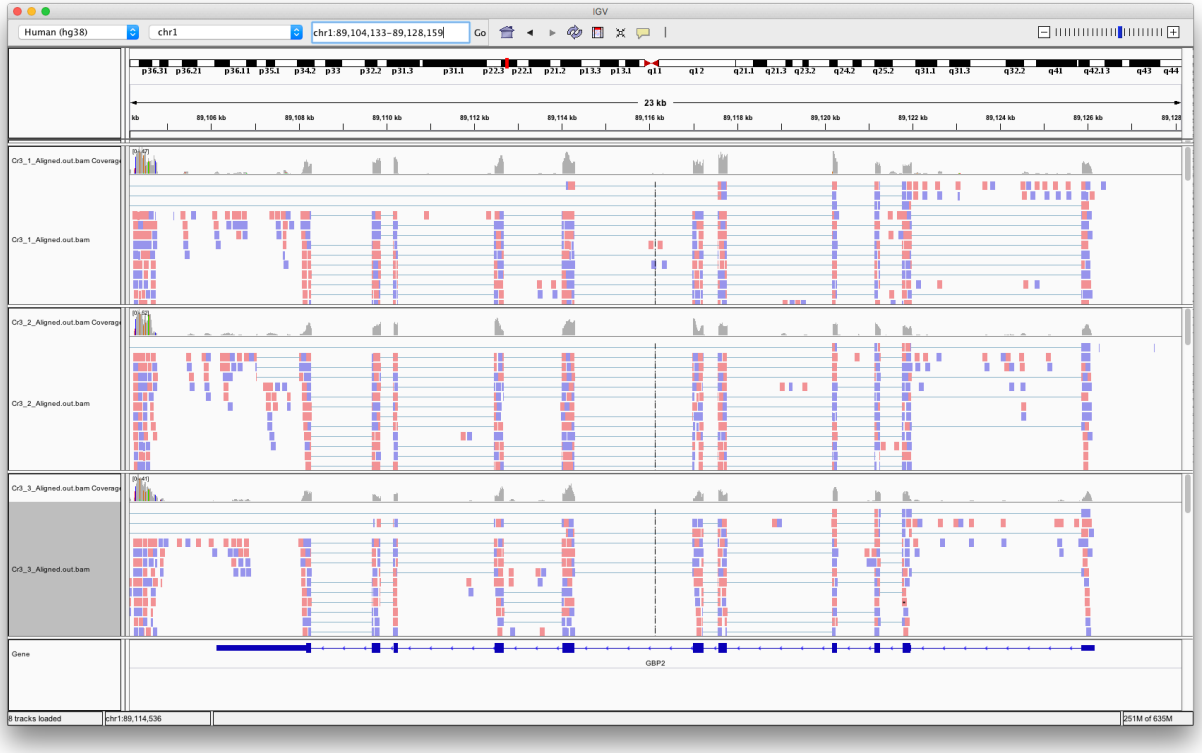


Quantification

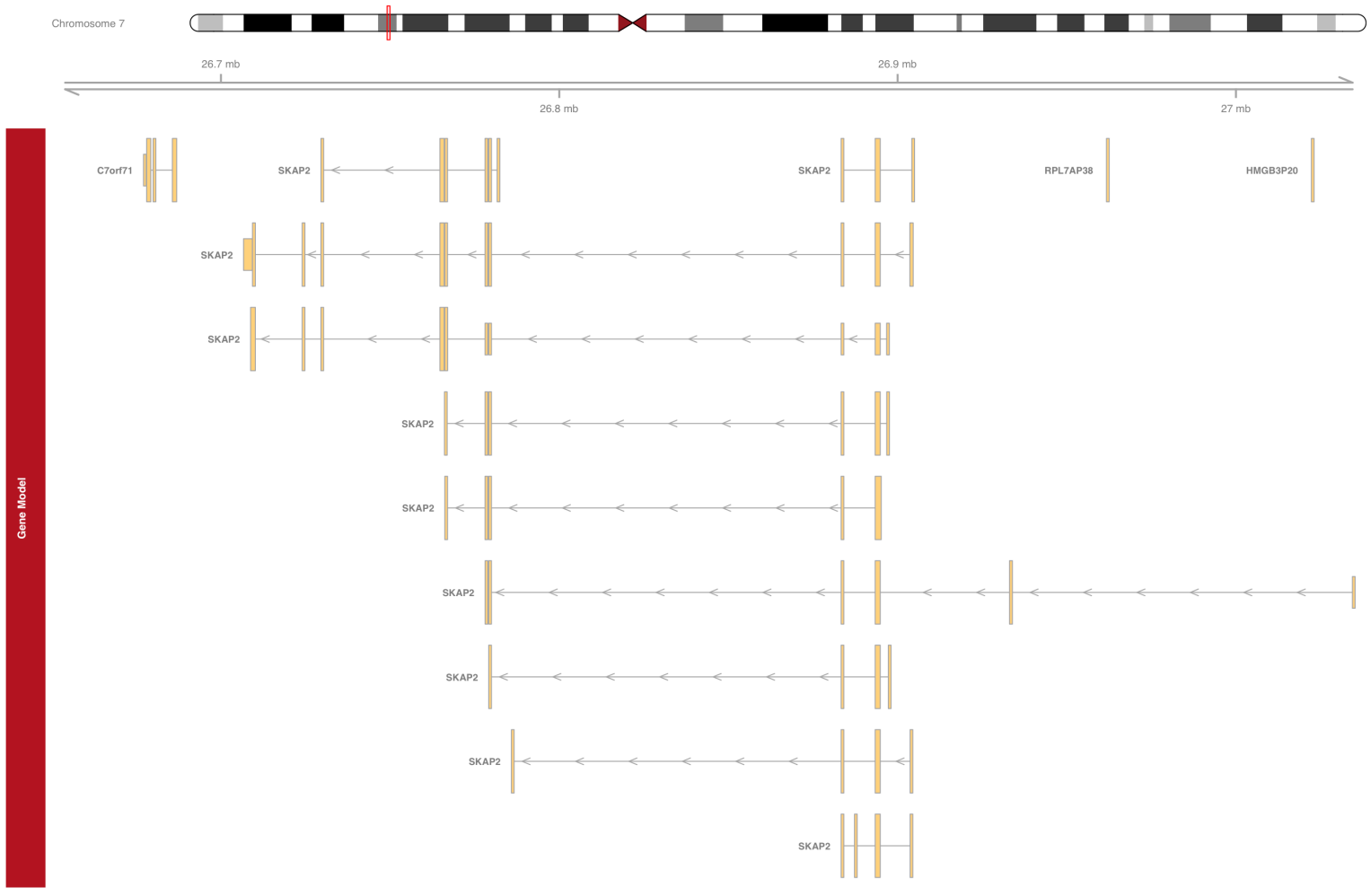
Ingredients: BAM data + GTF annotation file

Output: number of reads overlapping known features (discrete, positive, skewed)

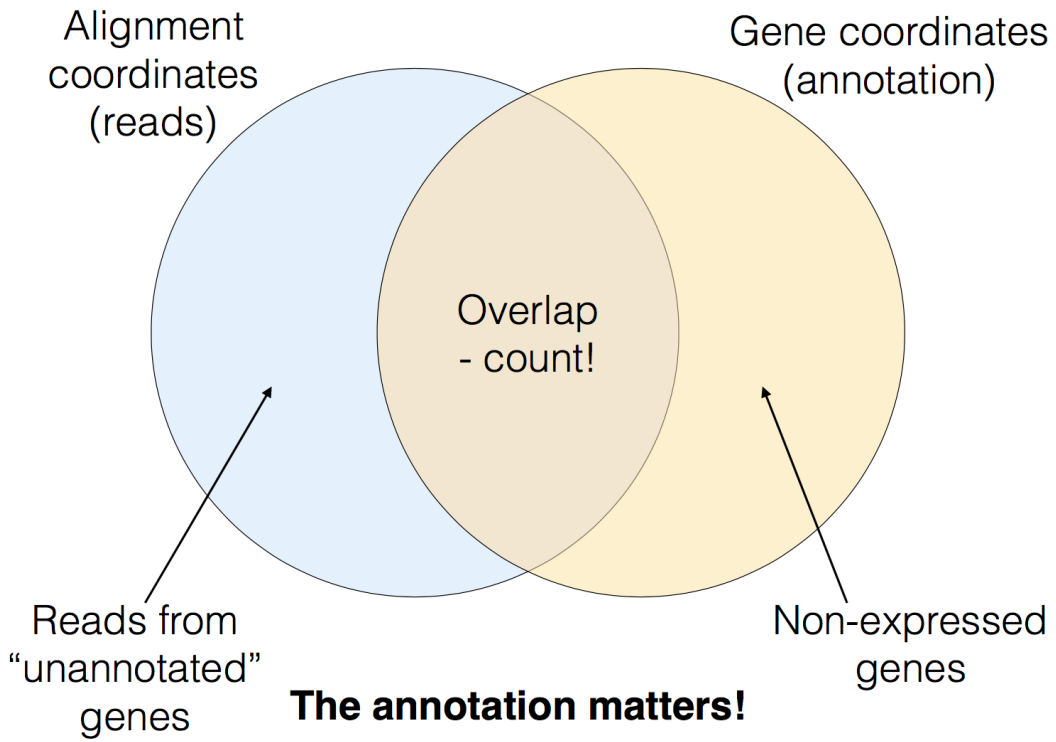
Gene-level counts, often obtained by genome alignment + overlap counting



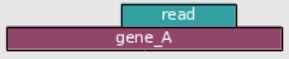
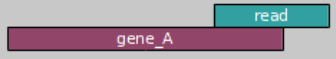
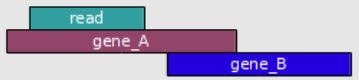
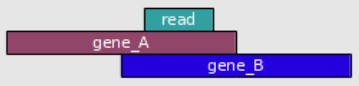
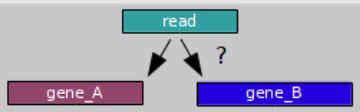
The role of annotation



The annotation matters!



The annotation matters!

	union	intersection_strict	intersection_nonempty
	gene_A	gene_A	gene_A
	gene_A	no_feature	gene_A
	gene_A	no_feature	gene_A
	gene_A	gene_A	gene_A
	gene_A	gene_A	gene_A
	ambiguous (both genes with --nonunique all)	gene_A	gene_A
	ambiguous (both genes with --nonunique all)		
	alignment_not_unique (both genes with --nonunique all)		

Alignment-free quantifications

Some recently developed methods:

- salmon (Patro et al, Nat Methods 2017)
- kallisto (Bray et al, Nat Biotechnol 2016)

return…

- Transcript-level counts and TPM (transcripts-per-million) estimates, which can be summed up to get
- Gene-level counts and TPM estimates

Pros & cons

- considerably faster than traditional alignment+counting -> allow bootstrapping
- more highly resolved estimates (transcripts rather than gene) + can be aggregated
- can use a slightly larger fraction of the reads since multi-mapping reads are not excluded
- don't return precise alignments (bam files, for e.g. visualization in genome browser)

Which way to go?

Based on genome alignment - mainly gene-level quantification: combine exons, "ignoring" splice variants

- Simple, powerful, yet in some cases inaccurate
- Tools:
  - `htseq-count`, `featureCounts` for estimating expression levels (counts)
  - `edgeR`, `DESeq2`, `voom+limma` for statistical modeling

Based on transcriptome mapping - transcript- and gene-level quantification: 'assign' reads (or rather, estimate most likely expression level) based on probabilistic modeling

- Potentially cleaner, but high degree of uncertainty on the transcript level!
- Tools:
  - `bitSeq`, `RSEM`, `salmon`, `kallisto` for (pseudo)alignment/quantification
  - `DESeq2`, `edgeR`, `voom+limma`, `swish`, `DRIMseq`, `DEXSeq`, `sleuth` for modeling (depending on the question of interest)

What it would look like - STAR + featureCounts:

... due to time constraints

Index the genome

```
$ STAR --runThreadN 24 \
       --runMode genomeGenerate \
       --genomeDir my_genome \
       --genomeFastaFiles my_genome.fa \
       --sjdbGTFfile my_genes.gtf \
       --sjdbOverhang 99
```

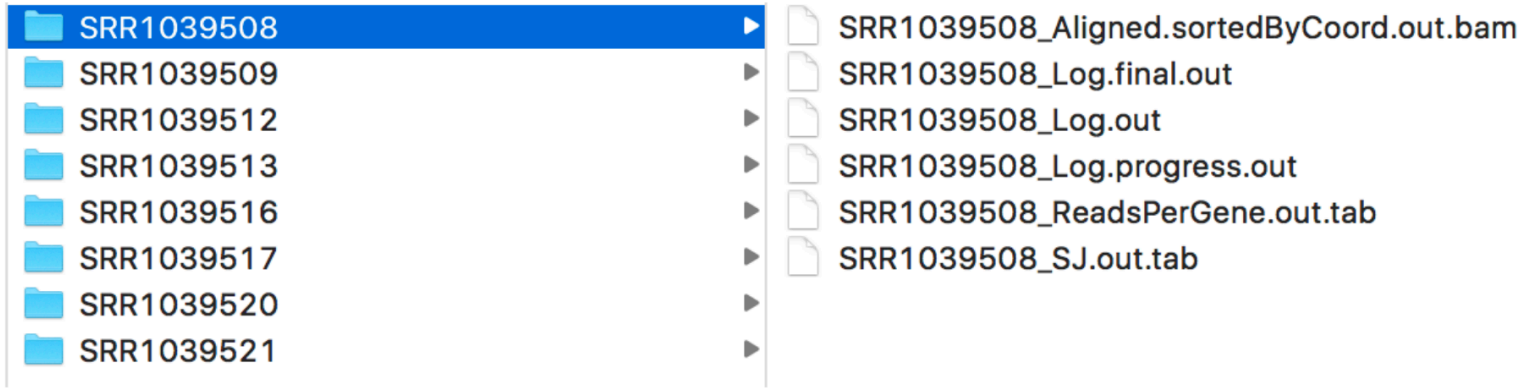
What it would look like - STAR + featureCounts:

... due to time constraints

Map each file

```
$ STAR --runThreadN 24 \
       --runMode alignReads \
       --genomeDir my_genome \
       --readFilesIn my_sample_read1.fastq.gz \
                     my_sample_read2.fastq.gz \
       --readFilesCommand zcat \
       --outFileNamePrefix output/S1/ \
       --outSAMtype BAM SortedByCoordinate \
       --quantMode GeneCounts
```

What it would look like - STAR + featureCounts:



What it would look like - STAR + featureCounts:

... due to time constraints

Quantify

```
featureCounts(files = bamfiles,
              annot.ext = "my_genes.gtf",
              isGTFAnnotationFile = TRUE,
              GTF.featureType = "exon",
              GTF.attrType = "gene_id",
              useMetaFeatures = TRUE,
              isPairedEnd = TRUE,
              strandSpecific = 0)
```

Directly generates a count matrix in your R session.

What it would look like - salmon

... due to time constraints

Create an index of the transcriptome

```
$ salmon index -i my_transcripts.idx \
               -t <(cat my_transcripts.fasta my_genome.fasta) \
               -d chromosome_names.txt
```

The genome acts as a 'decoy' sequence, to collect reads truly arising from intronic or intergenic locations.

What it would look like - salmon

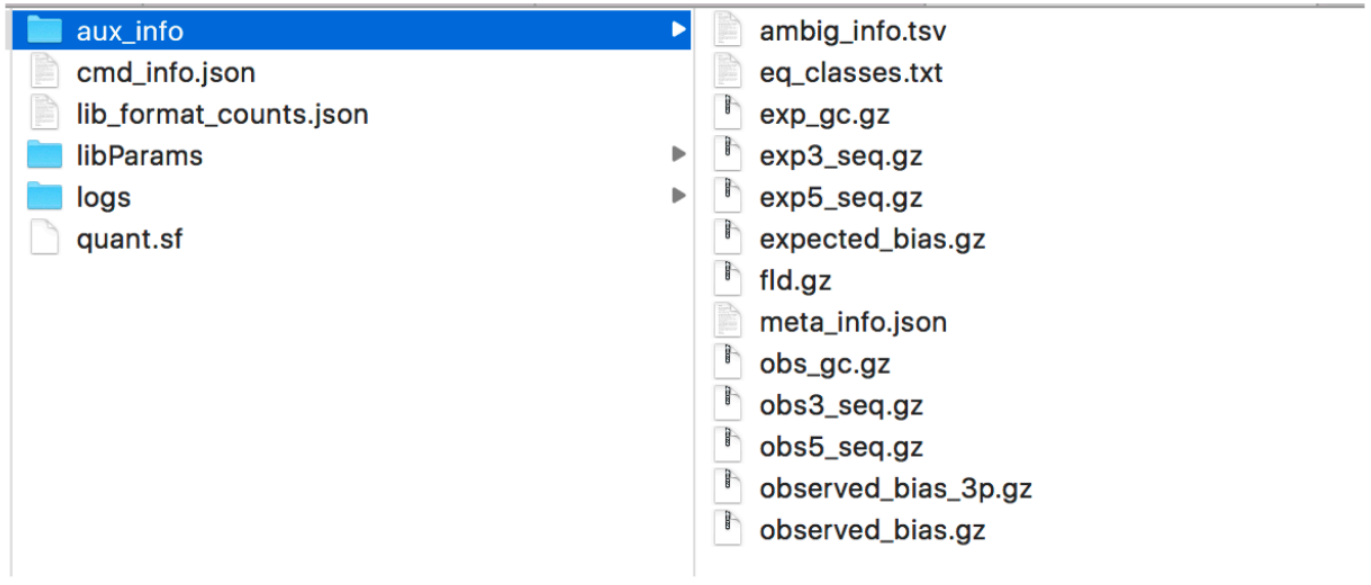
... due to time constraints

Quantify a sample at the transcript level

```
$ salmon quant -i my_transcripts.idx -l A \
               -1 my_sample_read1.fastq.gz -2 my_sample_read2.fastq.gz \
               -p 10 -o results/sample1 --validateMappings \
               --numBootstraps 30 --seqBias --gcBias
```

What it would look like - salmon

… due to time constraints



What it would look like - salmon

... due to time constraints

Salmon

[quant.sf]

Name	Length	EffectiveLength	TPM	NumReads
ENST00000406070	2025	1869.81	0	0
ENST00000446844	2227	2071.81	0.137334	3.71695
ENST00000599620	686	530.936	0	0
ENST00000471557	505	350.256	0.731211	3.3457
ENST00000338761	1456	1300.81	0	0
ENST00000417509	1444	1288.81	7.58582e-08	1.27717e-06
ENST00000484946	610	455.039	2.87905	17.1142
ENST00000490656	660	504.969	1.46703	9.67744
ENST00000439537	1161	1005.81	1.47611	19.3952
ENST00000493251	641	485.994	0.597774	3.79512
ENST00000460127	408	253.708	0	0

Importing salmon quantifications into R

You can follow (offline) the instructions of the `tximport` package - https://bioconductor.org/packages/tximport/

`tximeta`: another precious assistant on the way to be consistent and to keep track of provenance identification (we'll see it in action during the exercises)

What does our data look like now?

	SRR1039508	SRR1039509	SRR1039512	SRR1039513	SRR1039516	SRR1039517	SRR1039520	SRR1039521
ENSG00000000003	679	448	873	408	1138	1047	770	572
ENSG00000000005	0	0	0	0	0	0	0	0
ENSG00000000419	467	515	621	365	587	799	417	508
ENSG00000000457	260	211	263	164	245	331	233	229
ENSG00000000460	60	55	40	35	78	63	76	60
ENSG00000000938	0	0	2	0	1	0	0	0
ENSG00000000971	3251	3679	6177	4252	6721	11027	5176	7995
ENSG00000001036	1433	1062	1733	881	1424	1439	1359	1109
ENSG00000001084	519	380	595	493	820	714	696	704
ENSG00000001167	394	236	464	175	658	584	360	269
ENSG00000001460	172	168	264	118	241	210	155	177
ENSG00000001461	2112	1867	5137	2657	2735	2751	2467	2905
ENSG00000001497	524	488	638	357	676	806	493	475
ENSG00000001561	71	51	211	156	23	38	134	172
ENSG00000001617	555	394	905	415	727	697	618	599
ENSG00000001626	10	2	9	2	10	6	5	5
ENSG00000001629	1660	1251	2259	1079	2462	2514	1888	1660
ENSG00000001630	59	54	66	23	84	87	31	59
ENSG00000001631	729	692	943	475	1034	1163	731	744
ENSG00000002016	201	161	256	99	268	257	160	137
ENSG00000002079	3	0	3	1	4	0	0	1
ENSG00000002330	206	174	184	111	194	260	156	177

Some challenges in RNA-seq data analysis

1 - Choosing an appropriate statistical distribution

2 - Normalization between samples

3 - Few samples available make it difficult to estimate parameters (e.g., variance)

4 - Many genes, many tests - high dimensionality

Some challenges in RNA-seq data analysis - 1

Choosing an appropriate statistical distribution

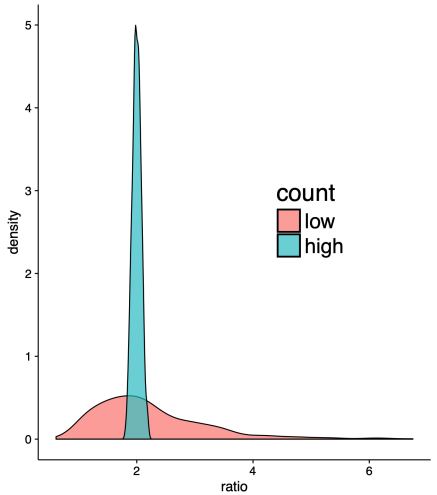
Variance depends on the mean count

Counts are non-negative and often highly skewed
This means you can't just use t-tests, ANOVA - no prob, `glm`s to the rescue!

Poisson -> negative binomial, better captures variability across biological replicates

"Why do we not just take the ratios?"

Fold changes, relative abundances



Ex: ratio between two Poisson distributed variables

Low: mean = 20 vs mean = 10
High: mean = 2000 vs mean = 1000

Which one would you trust more? Why?

This goes back to having appropriate statistical frameworks that nicely model your datasets (and how these get generated)

Some challenges in RNA-seq data analysis - 2

Normalization between samples

Observed counts depend on:

- abundance level
- gene/transcript length
- sequencing depth
- sequencing biases

"As-is" estimates not directly comparable across samples

Normalization aims to ensure our expression estimates are

- comparable across features (genes, isoforms, etc)
- comparable across libraries (different samples)
- on a human-friendly scale (interpretable magnitude)

Most RNA-seq methods (e.g., edgeR, DESeq2, voom) need raw counts (or equivalent) as input
Don’t provide these methods with (e.g.) RPKMs, FPKMs, TPMs, CPMs, log-transformed counts, normalized counts, ...
Read documentation carefully!

Digression: Normalization expression units

- RPKM/FPKM (Reads/Fragments per kilobase of transcript per million reads of library)
    - Corrects for total library coverage
    - Corrects for gene length
    - Comparable between different genes within the same dataset
- TPM (transcripts per million)
    - normalizes to transcript copies instead of reads - gives an idea of the proportion of transcripts
    - Corrects for cases where the total RNA output differs between samples
    - More appropriate for between sample comparisons (the sum of all TPMs in each sample are the same)

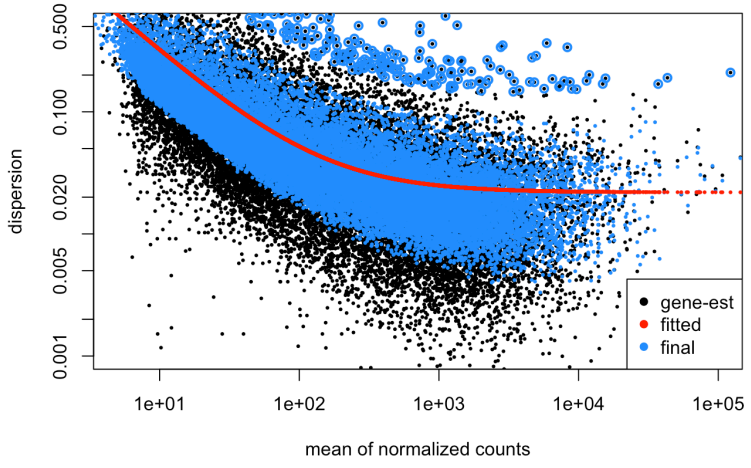
For DE analysis you have to work with discrete counts…

… and for comparisons you can use normalized counts (median ratio/TMM methods are robust across all genes!)

Some challenges in RNA-seq data analysis - 3

Few samples available make it difficult to estimate parameters (e.g., variance)

You can take advantage of the large number of genes



-> Shrink the gene-wise estimates towards a center value defined by the observed distribution of dispersions across...

- all genes (common dispersion estimate)
- genes with similar expression (trended dispersion estimate)

Some challenges in RNA-seq data analysis - 4

Many genes, many tests - high dimensionality

FDR for multiple test correction

Some more ideas:

- filter out genes that have little chance of showing significance (without looking at the test results)
- independent hypothesis weighting

... all nicely implemented for the DESeq framework

Do not test AND filter on logFC post-hoc!

Think of the null you're testing against - by default, useful to adapt if you want to focus on larger effect sizes

Exploratory analysis and visualization

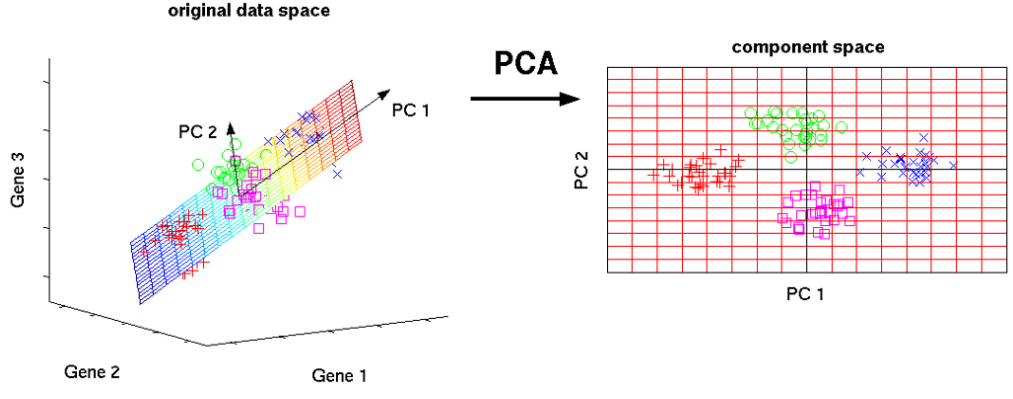
Is the data of good quality?

Quality as "fitness for purpose": DE

Very important: Transforming the data is often required for better further explorations (here: variance stabilization, regularized logarithm…)

One notable example, commonly used: Principal Components Analysis.

```
The data points (here, the samples) are projected onto the 2D plane
such that they spread out in the two directions that explain most
of the differences (the variability)
```



Exploratory analysis and visualization

"Perspective matters"

Gene identifiers

Take for example **GBP2**, guanylate binding protein 2 (4 transcripts available)

Ensembl ID: ENSG00000162645 (human), ENSMUSG00000028270 (mouse)

http://www.ensembl.org/id/ENSG00000162645

Entrez ID: 2634

HGNC ID: 4183

RefSeq ID: NM_004120

UCSC ID: uc001dmz.3

Official symbol: GBP2

Synonyms: - (sometimes makes the ambiguity even bigger!!!)

Gene symbols can change over time!

Typically, no 1:1 mapping between different ID types

Working with Excel

Don't do it - and not just because I do like R

Correspondence | Open Access | Published: 23 June 2004

Mistaken Identifiers: Gene name errors can be introduced inadvertently when using Excel in bioinformatics

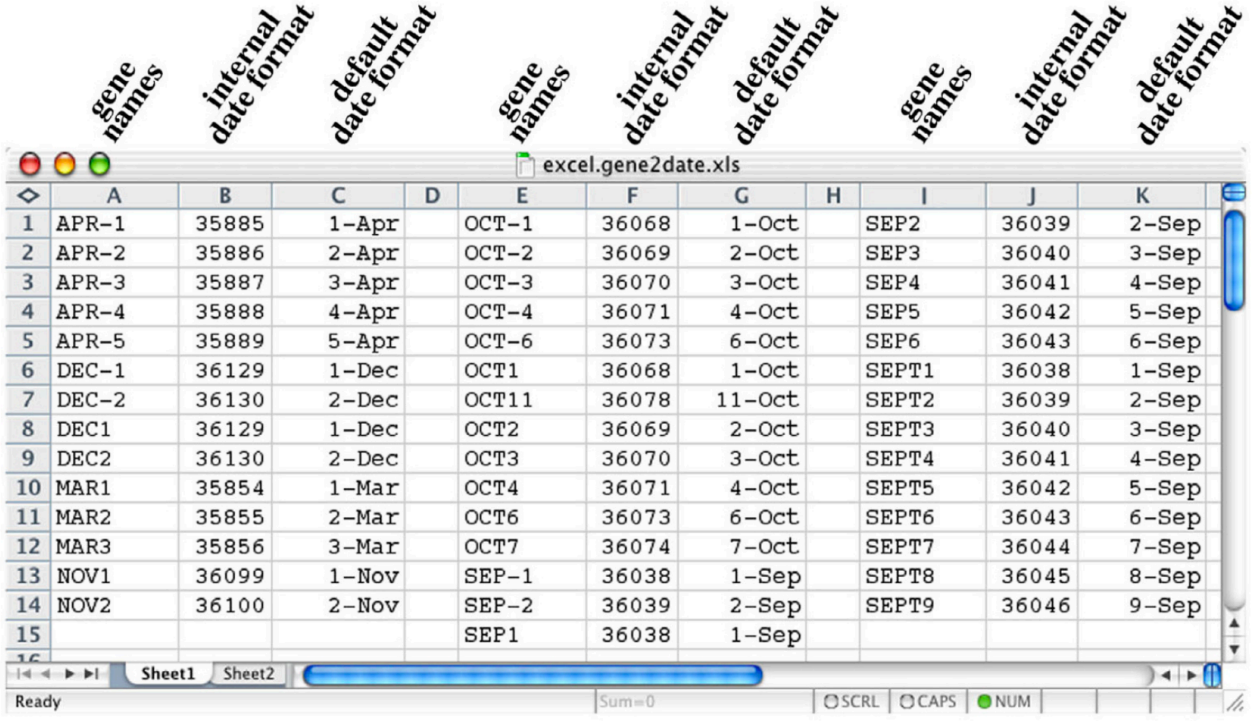
Barry R Zeeberg, Joseph Riss, David W Kane, Kimberly J Bussey, Edward Uchio, W Marston Linehan, J Carl Barrett & John N Weinstein

BMC Bioinformatics **5**, Article number: 80 (2004) | Cite this article

113k Accesses | **43** Citations | **515** Altmetric | Metrics

Working with Excel

There are excellent reasons *not* to do it

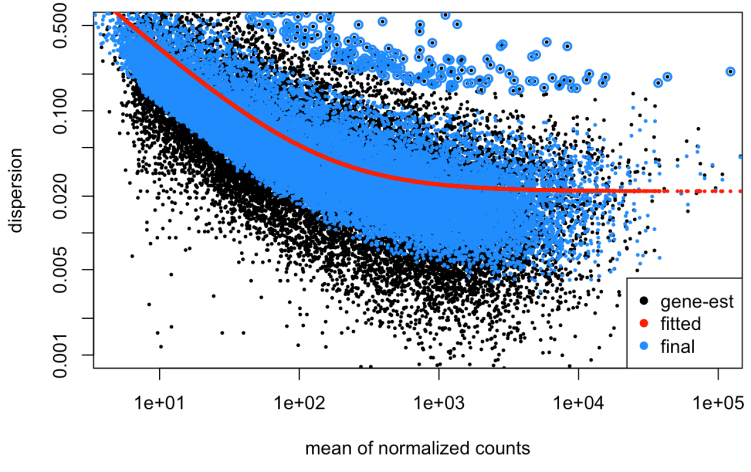


	A	B	C	D	E	F	G	H	I	J	K
1	APR-1	35885	1-Apr		OCT-1	36068	1-Oct		SEP2	36039	2-Sep
2	APR-2	35886	2-Apr		OCT-2	36069	2-Oct		SEP3	36040	3-Sep
3	APR-3	35887	3-Apr		OCT-3	36070	3-Oct		SEP4	36041	4-Sep
4	APR-4	35888	4-Apr		OCT-4	36071	4-Oct		SEP5	36042	5-Sep
5	APR-5	35889	5-Apr		OCT-6	36073	6-Oct		SEP6	36043	6-Sep
6	DEC-1	36129	1-Dec		OCT1	36068	1-Oct		SEPT1	36038	1-Sep
7	DEC-2	36130	2-Dec		OCT11	36078	11-Oct		SEPT2	36039	2-Sep
8	DEC1	36129	1-Dec		OCT2	36069	2-Oct		SEPT3	36040	3-Sep
9	DEC2	36130	2-Dec		OCT3	36070	3-Oct		SEPT4	36041	4-Sep
10	MAR1	35854	1-Mar		OCT4	36071	4-Oct		SEPT5	36042	5-Sep
11	MAR2	35855	2-Mar		OCT6	36073	6-Oct		SEPT6	36043	6-Sep
12	MAR3	35856	3-Mar		OCT7	36074	7-Oct		SEPT7	36044	7-Sep
13	NOV1	36099	1-Nov		SEP-1	36038	1-Sep		SEPT8	36045	8-Sep
14	NOV2	36100	2-Nov		SEP-2	36039	2-Sep		SEPT9	36046	9-Sep
15					SEP1	36038	1-Sep				

Differential expression analysis, with DESeq2

There is one main function, `DESeq`, that does the following

- estimation of size factors
- estimation of the dispersion values for each gene
- fitting of the generalized linear model and performing statistical testing



What do our results look like?

	id	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
1	ENSG00000152583	997.43977	-4.5749187	0.18405609	-24.856111	2.220933e-136	4.003898e-132
2	ENSG00000165995	495.09291	-3.2910618	0.13317370	-24.712551	7.839410e-135	7.066444e-131
3	ENSG00000120129	3409.02938	-2.9478099	0.12143769	-24.274258	3.666925e-130	2.203577e-126
4	ENSG00000101347	12703.38706	-3.7669954	0.15543799	-24.234715	9.583815e-130	4.319425e-126
5	ENSG00000189221	2341.76725	-3.3535799	0.14178235	-23.653014	1.098955e-123	3.962392e-120
6	ENSG00000211445	12285.61515	-3.7304027	0.16583058	-22.495263	4.618318e-112	1.387651e-108
7	ENSG00000157214	3009.26322	-1.9767725	0.08998232	-21.968454	5.769975e-107	1.486016e-103
8	ENSG00000162614	5393.10168	-2.0356649	0.09418231	-21.614091	1.323849e-103	2.983294e-100
9	ENSG00000125148	3656.25278	-2.2109786	0.10564078	-20.929214	2.902397e-97	5.813824e-94
10	ENSG00000154734	30315.13547	-2.3456037	0.11580806	-20.254235	3.259713e-91	5.876611e-88
11	ENSG00000139132	1223.46614	-2.2289030	0.11283944	-19.752872	7.578284e-87	1.242012e-83
12	ENSG00000162493	1099.62587	-1.8912170	0.09577959	-19.745511	8.767336e-87	1.317146e-83
13	ENSG00000134243	5510.95826	-2.1957116	0.11200768	-19.603224	1.451321e-85	2.012647e-82
14	ENSG00000179094	776.59667	-3.1917499	0.16396292	-19.466291	2.120834e-84	2.731028e-81
15	ENSG00000162692	508.17023	3.6926606	0.19018590	19.416059	5.646022e-84	6.785765e-81
16	ENSG00000163884	561.10717	-4.4591282	0.23486369	-18.986027	2.225442e-80	2.507517e-77
17	ENSG00000178695	2649.85015	2.5281746	0.13443012	18.806607	6.667027e-79	7.070186e-76
18	ENSG00000198624	2057.19173	-2.9184357	0.16129634	-18.093626	3.577533e-73	3.583098e-70
19	ENSG00000107562	25136.30757	1.9116698	0.10574810	18.077582	4.786167e-73	4.541317e-70
20	ENSG00000148848	1365.17399	1.8145431	0.10048365	18.058094	6.813522e-73	6.141709e-70

I would like to understand more what is going on here

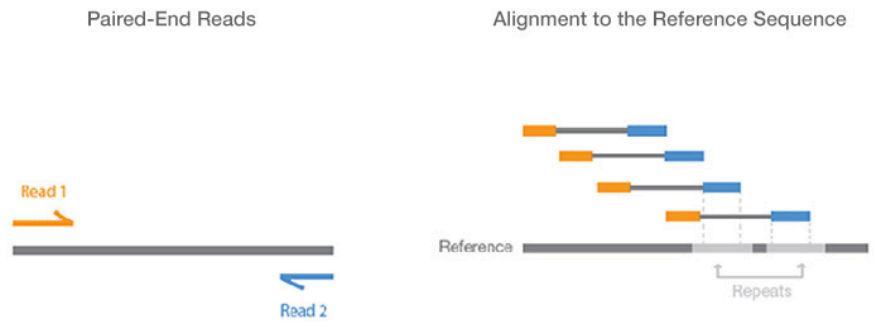
Practical tips for your (next) RNA-seq dataset

Single- vs paired-end sequencing

Each fragment can be sequenced from one end only, or from both ends

Single-end cheaper and faster

Paired-end provide improved ability to localize the fragment in the genome and resolve mapping close to repeat regions - less multimapping reads



Paired-end sequencing enables both ends of the DNA fragment to be sequenced. Because the distance between each paired read is known, alignment algorithms can use this information to map the reads over repetitive regions more precisely. This results in much better alignment of the reads, especially across difficult-to-sequence, repetitive regions of the genome.

Practical tips for your (next) RNA-seq dataset

Strand-specificity

In “standard” protocols, we don’t know from which strand a read stems

Various “strand-specific” protocols allow us to keep this information

Strand-specificity leads to lower number of ambiguous reads (overlapping multiple genes)

Practical tips for your (next) RNA-seq dataset

Sequencing depth and number of replicates

Recommendation: At least 3 biological replicates - Conesa et al, Genome Biol, 2016

Recommendation: At least 6 biological replicates - Schurch

Diminishing returns in increasing the read depth - go for replicates given a fixed budget!

I am not kidding you

Practical tips for your (next) RNA-seq dataset

Bulk or single cell?

That depends on your question!

Did someone say heterogeneity?

What do our results look like?

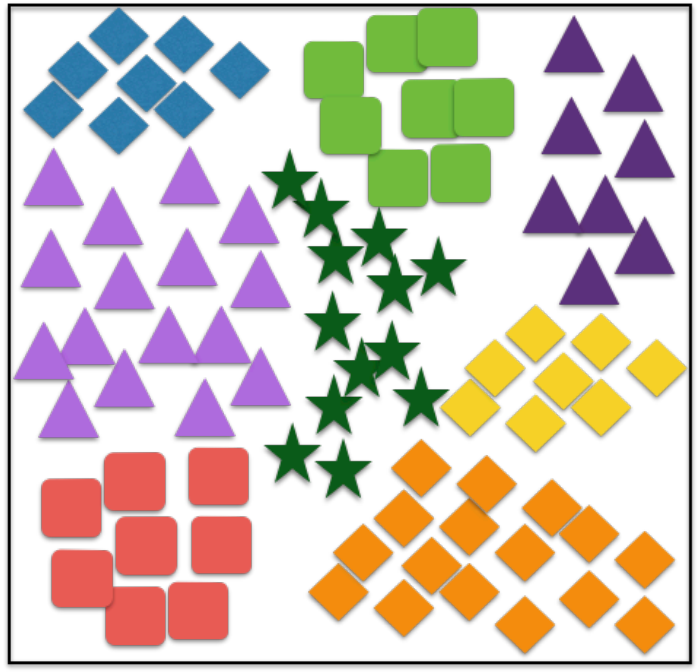
	id	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
1	ENSG00000152583	997.43977	-4.5749187	0.18405609	-24.856111	2.220933e-136	4.003898e-132
2	ENSG00000165995	495.09291	-3.2910618	0.13317370	-24.712551	7.839410e-135	7.066444e-131
3	ENSG00000120129	3409.02938	-2.9478099	0.12143769	-24.274258	3.666925e-130	2.203577e-126
4	ENSG00000101347	12703.38706	-3.7669954	0.15543799	-24.234715	9.583815e-130	4.319425e-126
5	ENSG00000189221	2341.76725	-3.3535799	0.14178235	-23.653014	1.098955e-123	3.962392e-120
6	ENSG00000211445	12285.61515	-3.7304027	0.16583058	-22.495263	4.618318e-112	1.387651e-108
7	ENSG00000157214	3009.26322	-1.9767725	0.08998232	-21.968454	5.769975e-107	1.486016e-103
8	ENSG00000162614	5393.10168	-2.0356649	0.09418231	-21.614091	1.323849e-103	2.983294e-100
9	ENSG00000125148	3656.25278	-2.2109786	0.10564078	-20.929214	2.902397e-97	5.813824e-94
10	ENSG00000154734	30315.13547	-2.3456037	0.11580806	-20.254235	3.259713e-91	5.876611e-88
11	ENSG00000139132	1223.46614	-2.2289030	0.11283944	-19.752872	7.578284e-87	1.242012e-83
12	ENSG00000162493	1099.62587	-1.8912170	0.09577959	-19.745511	8.767336e-87	1.317146e-83
13	ENSG00000134243	5510.95826	-2.1957116	0.11200768	-19.603224	1.451321e-85	2.012647e-82
14	ENSG00000179094	776.59667	-3.1917499	0.16396292	-19.466291	2.120834e-84	2.731028e-81
15	ENSG00000162692	508.17023	3.6926606	0.19018590	19.416059	5.646022e-84	6.785765e-81
16	ENSG00000163884	561.10717	-4.4591282	0.23486369	-18.986027	2.225442e-80	2.507517e-77
17	ENSG00000178695	2649.85015	2.5281746	0.13443012	18.806607	6.667027e-79	7.070186e-76
18	ENSG00000198624	2057.19173	-2.9184357	0.16129634	-18.093626	3.577533e-73	3.583098e-70
19	ENSG00000107562	25136.30757	1.9116698	0.10574810	18.077582	4.786167e-73	4.541317e-70
20	ENSG00000148848	1365.17399	1.8145431	0.10048365	18.058094	6.813522e-73	6.141709e-70

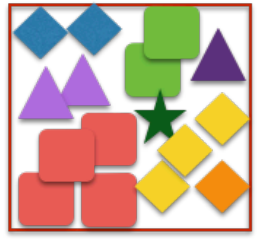
I need to understand more what is going on here

Functional enrichment analysis

Our problem, at a glance

All known genes in a species
(categorized into groups)





DEGs

Functional enrichment analysis

Test whether known biological functions or processes are over-represented (= enriched) in an experimentally-derived gene list, e.g. a list of differentially expressed (DE) genes.

Example: Transcriptomic study, in which 12671 genes have been tested for differential expression between two sample conditions and 529 genes were found DE

Among the DE genes, 28 are annotated to a specific functional gene set, which contains in total 170 genes. This setup corresponds to a 2x2 contingency table:

```
deTable <- matrix(
  c(28, 142, 501, 12000),
  nrow = 2,
  dimnames = list(c("DE", "Not_DE"),
                  c("In_gene_set", "Not_in_gene_set")))
deTable
#        In_gene_set Not_in_gene_set
# DE              28             501
# Not_DE         142           12000
```

```
fisher.test(deTable, alternative = "greater")
#
#     Fisher's Exact Test for Count Data
```

Some terminology: Gene sets, pathways, networks

Gene sets are simple lists of usually functionally related genes without further specification of relationships between genes. **Any** *a priori* classification of genes into biologically relevant groups. Sets do not need to be exhaustive or disjoint

Pathways can be interpreted as specific gene sets, typically representing a group of genes that work together in a biological process.

Gene regulatory networks describe the interplay and effects of regulatory factors (such as transcription factors and microRNAs) on the expression of their target genes.

Is the expression of genes in a gene set associated with the experimental conditions?

Genes categories	Organism-specific Background	DE results	Over-represented?
Functional category 1	35/13000	25/1000	Likely
Functional category 2	56/13000	4/1000	Unlikely
Functional category 3	90/13000	8/1000	Unlikely
Functional category 4	15/13000	10/1000	Likely
…			
…			

Functional enrichment analysis

Primarily developed and applied on transcriptomic data, now extended and applied also in other fields of genomic and biomedical research (proteomic and metabolomic data, genomic regions, ...)

Many methods available!

- Over-representation analysis (ORA) - are differentially expressed (DE) genes in the set more common than expected? Based on (variations) of the 2x2 contingency table method
- Functional class scoring (FCS) - summarize gene set (= functional class) scores by summarizing statistic of DE of genes in a set, and compare to null
- Pathway topology (PT) - explicitly taking into account interactions between genes as defined in signaling pathways and gene regulatory networks

Topology-based methods appear to be most realistic, but: features that are not-detectable on the transcriptional level + insufficient network knowledge

Cautious interpretation of results is required to derive valid conclusions!

Collections of gene sets

Gene Ontology (GO) Annotation (GOA)

- CC Cellular Components
- BP Biological Processes
- MF Molecular Function

Pathways

- MSigDb
- KEGG
- reactome
- PantherDB
- ...

GO and KEGG annotations are most frequently used for the enrichment analysis of functional gene sets - likely due to their long-standing curation and availability for a wide range of species

Software for functional enrichment

- DAVID
- GSEA and its variations
- inside Bioconductor: `reactome`, `topGO`, `pathview`, `GOSeq`, ...
- Ingenuity Pathway Analysis

Not straightforward, but amazing way of helping generating hypothesis for the bench scientist

Friendly tip: wrappers for topGO and goseq are in `pcaExplorer` and in `ideal`!

General recommendations

Never forget to visualize your data!

Pick candidates, sets of candidates, plot instead of guessing!

The choice of a background matters!

Background matters

... with a slight modification of the example above

```
deTable_detected <- matrix(
  c(8, 142, 501, 12000),
  nrow = 2,
  dimnames = list(c("DE", "Not_DE"),
                  c("In_gene_set", "Not_in_gene_set")))
fisher.test(deTable_detected, alternative = "greater")
```

versus...

```
deTable_allgenes <- matrix(
  c(8, 142, 501, 32000),
  nrow = 2,
  dimnames = list(c("DE", "Not_DE"),
                  c("In_gene_set", "Not_in_gene_set")))
fisher.test(deTable_allgenes, alternative = "greater")
```

Data analysis: where's the real bottleneck?

✅ Efficient methods to process your data (entire workflows available, see e.g. the Bioconductor workflow packages)

✅ Compelling ways of visualizing your data (better QC, better hypotheses, better answers see `http://bioconductor.org/packages/iSEE/` & `http://bioconductor.org/packages/iSEEu/`)

✅ Powerful framework to communicate, interactively and reproducibly

🔥 **Data interpretation**

... just not in plain sight

Show me your data...

	A	B	C	D	E	F	G	H	I	J	K
1	SAMEA103885102	SAMEA103885347	SAMEA103885043	SAMEA103885392	SAMEA103885182	SAMEA103885136	SAMEA103885413	SAMEA103884967	SAMEA103885368	SAMEA103885218	SAMEA103885319
2	55,69716914	96,38981386	67,26753412	142,6835597	186,1485543	122,5926771	170,5541862	203,0472731	70,5820175	30,98336005	71,87276974
3	0	0	0	0	0	0	0	0	0	0	0
4	964,8925057	924,83214	1036,106869	1046,57092	1014,635976	1083,422465	969,604741	982,6266614	954,7538272	1035,036116	1099,503914
5	351,9054782	684,638914	387,1752238	820,970061	375,1488073	948,9162924	503,55028	1231,24557	300,7527914	911,6795373	889,3737712
6	677,4205719	221,0877036	276,2026831	97,82962948	395,4077973	175,5987827	179,7898584	133,993082	675,5371526	153,7212284	319,7823597
7	132,782876	198,7016694	662,811222	1522,435008	386,9046409	560,2933294	1257,995559	5812,826356	909,7667115	2395,917116	2048,353449
8	362,6435931	5375,365498	650,3552061	7471,729136	116,9579367	5036,571099	223,3816218	5507,177186	66,70469095	5451,589642	480,5118025
9	5185,999104	4426,618781	5870,757295	5878,363213	3769,061292	3829,066212	2882,333961	3365,101977	3953,382129	3625,84689	4932,212822
10	4064,476563	10213,97627	2388,342021	10237,20614	3863,969772	13282,99842	2921,831249	11699,4626	2120,792061	6985,444675	2949,988682
11	996,2886541	1107,437138	1481,523638	1905,441941	1296,389901	1195,809922	1705,234892	1943,638311	981,0211341	1280,849815	1550,697703
12	78,82859697	78,75996066	104,2567382	130,0656527	75,32368946	90,65332179	69,80311374	172,8622743	73,59486838	47,6298952	86,1652492
13	372,4875108	375,1582833	336,9448447	378,4351496	484,7559515	589,1766985	548,4876464	415,3602864	281,9806048	533,7955594	265,9931667
14	1004,363486	804,9305608	2122,334216	566,7309848	894,7410645	609,4980038	1607,317126	355,0574378	1234,02879	589,4720116	1880,361894
15	47,6088729	190,9341388	47,12634333	206,4662057	145,2740402	422,2343534	150,387129	287,7915443	53,8085684	313,9134375	92,61472368
16	10,2940199	10,73067656	18,42333204	34,72412059	11,87265036	24,24830727	21,76451809	77,31764486	6,892555164	4,824180733	11,23090998
17	8,461862162	0,100956323	6,053534158	14,24249154	8,595001149	0	0,22640779	17,29978818	4,094109524	5,538925825	21,58950506
18	1265,474946	1617,700447	1669,609838	2297,800891	1288,313487	1497,771426	1576,57678	2747,84197	890,4337923	1037,283091	1936,845356
19	2742,714674	1367,003771	5053,573294	2427,122201	1532,174172	1042,918499	2445,006435	1099,595415	1781,289062	913,0693244	2293,72266
20	739,0239221	721,4533065	978,5249905	869,4506791	758,6712728	687,2941888	958,8405469	1131,158723	723,1258083	891,5201189	1172,627644
21	153,0850266	223,6814932	90,69677231	99,67707173	165,2491282	181,6727726	165,8323252	124,8435309	135,0806311	172,4092075	154,5518966
22	0	0	0	10,63374428	1,275177865	4,407102394	0,415747599	0,615201563	4,740536892	0	2,063830113
23	294,9241388	200,997003	193,0178533	259,2214914	272,6918871	292,386033	180,4896341	179,036124	382,9993779	271,7909482	229,4676362
24	10311,29607	40953,9043	14546,93203	48197,23868	12049,09318	61320,54376	15556,91082	48401,85979	6945,254193	62689,66844	24693,50787
25	14252,63718	8840,178525	18210,18028	12299,01927	11043,55437	8344,372672	11131,06854	8353,885536	9092,021277	6232,986277	11739,0848
26	2129,428152	471,0255994	287,0384441	86,35857737	2472,120332	559,3936962	486,4665709	66,4070027	5038,903217	1219,154589	445,1902436
27	0	3,371313144	5,038960845	1,962785986	19,06817927	25,57466411	19,61201231	32,07074799	2,764897276	7,007072669	3,240100076
28	0,4707608	0	0	0	0	0	0	0	0	0	0
29	0,83189235	0,56534712	0,780066877	1,377008507	1,834964003	3,243938183	1,613445267	35,93939273	21,80390352	0,697877051	0,497286622
30	660,247462	697,0714736	376,575126	468,8681819	1120,360629	875,9863134	416,0590986	737,4089693	1126,114691	873,0030589	707,054169
31	15314,39018	12764,13479	5147,168455	7605,221356	17247,28895	19126,9456	5236,812531	11044,68781	16448,27488	19508,61894	5852,803153
32	2828,408307	2687,797465	2800,493773	6277,1227	2275,27131	2454,51924	1339,12723	2779,114942	2188,442779	2400,119315	1476,802287
33	40,23728704	299,899605	56,13562779	568,1032424	385,3034226	2407,236342	279,7861222	2043,400943	69,42871741	1174,143223	83,05725953
34	13078,03109	14673,85241	12869,05451	14992,04765	11871,1049	16338,89938	10146,36181	10380,14905	10500,81202	14966,33552	10032,07621

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	A	B	C	D	E	F	G	H	I	J	K
1	SAMEA103885102	SAMEA103885347	SAMEA103885043	SAMEA103885392	SAMEA103885182	SAMEA103885136	SAMEA103885413	SAMEA103884967	SAMEA103885368	SAMEA103885218	SAMEA103885319
2	55,69716914	96,38981386	67,26753412	142,6835597	186,1485543	122,5926771	170,5541862	203,0472731	70,5820175	30,98336005	71,87276974
3	0	0	0	0	0	0	0	0	0	0	0
4	964,8925057	924,83214	1036,106869	1046,57092	1014,635976	1083,422465	969,604741	982,6266614	954,7538272	1035,036116	1099,503914
5	351,9054782	684,638914	387,1752238	820,970061	375,1488073	948,91					
6	677,4205719	221,0877036	276,2026831	97,82962948	395,4077973	175,59					
7	132,782876	198,7016694	662,811222	1522,435008	386,9046409	560,29					
8	362,6435931	5375,365498	650,3552061	7471,729136	116,9579367	5036,5					
9	5185,999104	4426,618781	5870,757295	5878,363213	3769,061292	3829,0					
10	4064,476563	10213,97627	2388,342021	10237,20614	3863,969772	13282,					
11	996,2886541	1107,437138	1481,523638	1905,441941	1296,389901	1195,8					
12	78,82859697	78,75996066	104,2567382	130,0656527	75,32368946	90,653					
13	372,4875108	375,1582833	336,9448447	378,4351496	484,7559515	589,17					
14	1004,363486	804,9305608	2122,334216	566,7309848	894,7410645	609,49					
15	47,6088729	190,9341388	47,12634333	206,4662057	145,2740402	422,23					
16	10,2940199	10,73067656	18,42333204	34,72412059	11,87265036	24,248					
17	8,461862162	0,100956323	6,053534158	14,24249154	8,595001149						
18	1265,474946	1617,700447	1669,609838	2297,800891	1288,313487	1497,7					
19	2742,714674	1367,003771	5053,573294	2427,122201	1532,174172	1042,9					
20	739,0239221	721,4533065	978,5249905	869,4506791	758,6712728	687,29					
21	153,0850266	223,6814932	90,69677231	99,67707173	165,2491282	181,67					
22	0	0	0	10,63374428	1,275177865	4,4071					
23	294,9241388	200,997003	193,0178533	259,2214914	272,6918871	292,3					
24	10311,29607	40953,9043	14546,93203	48197,23868	12049,09318	61320,					
25	14252,63718	8840,178525	18210,18028	12299,01927	11043,55437	8344,3					
26	2129,428152	471,0255994	287,0384441	86,35857737	2472,120332	559,39					
27	0	3,371313144	5,038960845	1,962785986	19,06817927	25,574					
28	0,4707608	0	0	0	0						
29	0,83189235	0,56534712	0,780066877	1,377008507	1,834964003	3,2439					
30	660,247462	697,0714736	376,575126	468,8681819	1120,360629	875,98					
31	15314,39018	12764,13479	5147,168455	7605,221356	17247,28895	19126					
32	2828,408307	2687,797465	2800,493773	6277,1227	2275,27131	2454,					
33	40,23728704	299,899605	56,13562779	568,1032424	385,3034226	2407,2					
34	13078,03109	14673,85241	12869,05451	14992,04765	11871,1049	16338,					

	A	B	C	D	E	F	G	H
1	id	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	SYMBOL
2	ENSG00000125347	30585,07489	5,560123732	0,218266321	20,89247536	6,26847E-97	1,11616E-92	IRF1
3	ENSG00000162645	36790,59205	6,665803772	0,286573165	19,77088043	5,30422E-87	4,72235E-83	GBP2
4	ENSG00000111181	688,5779196	4,710591861	0,196102275	18,92171759	7,55518E-80	4,48425E-76	SLC6A12
5	ENSG00000174944	1324,397476	9,863903776	0,471512737	18,7988639	7,71512E-79	3,43439E-75	P2RY14
6	ENSG00000100336	2551,478907	8,708550505	0,421409592	18,29229959	9,53083E-75	3,39412E-71	APOL4
7	ENSG00000145365	3637,222141	5,193447761	0,236854233	17,70476176	3,85308E-70	1,14346E-66	TIFA
8	ENSG00000137496	7129,418865	4,05866209	0,177364585	17,24505541	1,21896E-66	3,10068E-63	IL18BP
9	ENSG00000154451	17174,98786	10,37647789	0,551586848	16,9990962	8,33958E-65	1,85618E-61	GBP5
10	ENSG00000129244	682,8305656	7,191566714	0,381506097	16,2292733	3,13145E-59	6,19539E-56	ATP1B2
11	ENSG00000231389	5147,824664	5,593758623	0,28477475	16,13120061	1,53997E-58	2,74208E-55	HLA-DPA1
12	ENSG00000204257	1909,416235	4,071574682	0,190921436	16,08816042	3,08879E-58	4,99991E-55	HLA-DMA
13	ENSG00000100342	4112,437557	7,13529667	0,39590635	15,49683826	3,64388E-54	5,40691E-51	APOL1
14	ENSG00000162654	52292,49435	9,752769157	0,569217591	15,37684234	2,34089E-53	3,2063E-50	GBP4
15	ENSG00000133321	1393,428038	7,448819999	0,419830443	15,36053451	3,01082E-53	3,82933E-50	PLAAT4
16	ENSG00000128284	13363,55118	7,977215573	0,454494945	15,35158013	3,45663E-53	4,10325E-50	APOL3
17	ENSG00000134470	4468,963176	4,300572585	0,220226433	14,98717724	8,90586E-51	9,91111E-48	IL15RA
18	ENSG00000172399	714,5095698	-7,829885987	0,463112753	-14,74778213	3,17943E-49	3,33017E-46	MYOZ2
19	ENSG00000164509	700,3132408	8,395184784	0,536171751	13,79256698	2,82525E-43	2,7948E-40	IL31RA
20	ENSG00000225492	1295,305924	6,141301184	0,374065413	13,74439071	5,50344E-43	5,15759E-40	GBP1P1
21	ENSG00000204267	10442,43781	3,458265659	0,179235202	13,71530609	8,22187E-43	7,31993E-40	TAP2
22	ENSG00000254838	135,9997527	5,591075237	0,335403469	13,68821633	1,19404E-42	1,01244E-39	GVINP1
23	ENSG00000131203	92938,1422	9,545152592	0,644384499	13,26095306	3,89909E-40	3,15578E-37	IDO1
24	ENSG00000213886	2086,328471	10,26938285	0,703626324	13,17372947	1,24302E-39	9,62317E-37	UBD
25	ENSG00000100911	11111,0667	3,358971492	0,179410258	13,14847609	1,73639E-39	1,28825E-36	PSME2
26	ENSG00000168394	38700,36687	5,356767076	0,337264864	12,91793941	3,56563E-38	2,53958E-35	TAP1
27	ENSG00000242574	2265,524717	3,819583423	0,219870422	12,82384142	1,20575E-37	8,25756E-35	HLA-DMB
28	ENSG00000234518	312,0912993	8,276859324	0,576720452	12,61765436	1,68774E-36	1,11303E-33	
29	ENSG00000163568	1455,22973	7,804253083	0,543387364	12,52191997	5,6648E-36	3,60241E-33	AIM2
30	ENSG00000213626	4433,054879	-4,42792907	0,275004543	-12,46499068	1,15885E-35	7,11536E-33	LBH
31	ENSG00000183734	893,1217877	4,237240545	0,260041233	12,4489509	1,41695E-35	8,4101E-33	ASCL2
32	ENSG00000101017	28016,70595	3,478496822	0,200633386	12,3533619	4,67175E-35	2,6834E-32	CD40
33	ENSG00000140105	166892,85	5,82727503	0,391766026	12,32183169	6,91066E-35	3,84535E-32	WARS1
34	ENSG00000117228	98501,16773	7,686166048	0,552174564	12,10879039	9,48831E-34	5,11966E-31	GBP1
35	ENSG00000244731	730,7135805	5,027915974	0,334446879	12,04351489	2,09821E-33	1,09885E-30	C4A
36	ENSG00000019582	53359,71495	4,15277183	0,264525014	11,91861512	9,46691E-33	4,81622E-30	CD74
37	ENSG00000204252	147,6369201	5,782657331	0,402965448	11,86865362	1,72217E-32	8,51804E-30	HLA-DOA
38	ENSG00000270547	221,0902311	-5,370582108	0,370389876	-11,79995024	3,90542E-32	1,87946E-29	LINC01235
39	ENSG00000089041	14232,26023	4,656176735	0,316525516	11,55096999	7,29919E-31	3,42025E-28	P2RX7
40	ENSG00000179583	1521,720208	6,112248838	0,449889179	11,36335141	6,36549E-30	2,90625E-27	CIITA
41	ENSG00000121380	1206,206203	5,996911594	0,439943587	11,35807349	6,76193E-30	3,01008E-27	BCL2L14
42	ENSG00000136436	4841,032355	2,828125249	0,161138537	11,34505304	7,84786E-30	3,40827E-27	CALCOCO2
43	ENSG00000170989	704,6955516	-5,365857478	0,386740233	-11,28886293	1,48951E-29	6,31482E-27	S1PR1
44	ENSG00000168899	715,8817981	4,743931938	0,333167369	11,23739082	2,67162E-29	1,1063E-26	VAMP5
45	ENSG00000153012	528,5976347	-6,370842542	0,483211518	-11,11488931	1,06182E-28	4,297E-26	LGI2
46	ENSG00000140511	4173,841235	7,091736603	0,55079617	11,05987466	1,96372E-28	7,60129E-26	HAPLN3
47	ENSG00000185338	4154,862612	7,689753422	0,604771596	11,06161974	1,92588E-28	7,60129E-26	SOCS1
48	ENSG00000174749	1397,798733	2,925585998	0,174477721	11,03628579	2,55372E-28	9,67481E-26	FAM241A
49	ENSG00000188820	8001,349732	5,681784849	0,426951103	10,96562302	5,59141E-28	2,07418E-25	CALHM6

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	A	B	C	D	E	F	G	H	I	J	K
1	SAMEA103885102	SAMEA103885347	SAMEA103885043	SAMEA103885392	SAMEA103885182	SAMEA103885136	SAMEA103885413	SAMEA103884967	SAMEA103885368	SAMEA103885218	SAMEA103885319
2	55,69716914	96,38981386	67,26753412	142,6835597	186,1485543	122,5926771	170,5541862	203,0472731	70,5820175	30,98336005	71,87276974
3	0	0	0	0	0	0	0	0	0	0	0
4	964,8925057	924,83214	1036,106869	1046,57092	1014,635976	1083,422465	969,604741	982,6266614	954,7538272	1035,036116	1099,503914
5	351,9054782	684,638914	387,1752238	820,970061	375,1488073	948,91					
6	677,4205719	221,0877036	276,2026831	97,82962948	395,4077973	175,59					
7	132,782876	198,7016694	662,811222	1522,435008	386,9046409	560,29					
8	362,6435931	5375,365498	650,3552061	7471,729136	116,9579367	5036,5					
9	5185,999104	4426,618781	5870,757295	5878,363213	3769,061292	3829,0					
10	4064,476563	10213,97627	2388,342021	10237,20614	3863,969772	13282,					
11	996,2886541	1107,437138	1481,523638	1905,441941	1296,389901	1195,8					
12	78,82859697	78,75996066	104,2567382	130,0656527	75,32368946	90,653					
13	372,4875108	375,1582833	336,9448447	378,4351496	484,7559515	589,17					
14	1004,363486	804,9305608	2122,334216	566,7309848	894,7410645	609,49					
15	47,6088729	190,9341388	47,12634333	206,4662057	145,2740402	422,23					
16	10,2940199	10,73067656	18,42333204	34,72412059	11,87265036	24,248					
17	8,461862162	0,100956323	6,053534158	14,24249154	8,595001149						
18	1265,474946	1617,700447	1669,609838	2297,800891	1288,313487	1497,7					
19	2742,714674	1367,003771	5053,573294	2427,122201	1532,174172	1042,9					
20	739,0239221	721,4533065	978,5249905	869,4506791	758,6712728	687,29					
21	153,0850266	223,6814932	90,69677231	99,67707173	165,2491282	181,67					
22	0	0	0	10,63374428	1,275177865	4,4071					
23	294,9241388	200,997003	193,0178533	259,2214914	272,6918871	292,3					
24	10311,29607	40953,9043	14546,93203	48197,23868	12049,09318	61320,					
25	14252,63718	8840,178525	18210,18028	12299,01927	11043,55437	8344,3					
26	2129,428152	471,0255994	287,0384441	86,35857737	2472,120332	559,39					
27	0	3,371313144	5,038960845	1,962785986	19,06817927	25,574					
28	0,4707608	0	0	0	0						
29	0,83189235	0,56534712	0,780066877	1,377008507	1,834964003	3,2439					
30	660,247462	697,0714736	376,575126	468,8681819	1120,360629	875,98					
31	15314,39018	12764,13479	5147,168455	7605,221356	17247,28895	19126					
32	2828,408307	2687,797465	2800,493773	6277,1227	2275,27131	2454,					
33	40,23728704	299,899605	56,13562779	568,1032424	385,3034226	2407,2					
34	13078,03109	14673,85241	12869,05451	14992,04765	11871,1049	16338,					

	A	B	C	D	E	F	G	H
1	id	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	SYMBOL
2	ENSG00000125347	30585,07489	5,560123732	0,218266321	20,89247536	6,26847E-97	1,11616E-92	IRF1
3	ENSG00000162645	36790,59205						
4	ENSG00000111181	688,5779196						
5	ENSG00000174944	1324,397476						
6	ENSG00000100336	2551,478907						
7	ENSG00000145365	3637,222141						
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11	ENSG00000231389	5147,824664						
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38	ENSG00000270547	221,0902311						
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1 Category Term Count % PValue Genes List Total Pop Hits Pop Total Fold Enrichment Bonferroni Benjamini FDR

2 GOTERM_BP_DIRECT GO:0060333~interferon-gamma-mediated signaling pathway 31 3.9440203562340965 1.8403392216558493E-23 HLA-DQB1, HLA-DQB2, NMI, HLA-DRB1, PML, OAS2, B2M, VCAM1, CAMK2D, HLA-DRB5, HLA-DPB1, CIITA, ICAM1, HLA-A, HLA-C, HLA-B, HLA-E, STAT1, TRIM22, HLA-G, HLA-DQA1, HLA-F, IRF7, TRIM31, MT2A, IRF1, JAK2, HLA-DPA1, GBP2, GBP1, HLA-DRA 683 71 16792 10.73458024869569 5.563345467065632E-20 5.563345467065632E-20 3.3166682425802433E-20

3 GOTERM_BP_DIRECT GO:0006955~immune response 69 8.778625954198473 5.268440090526076E-23 IL15, HLA-DMB, CXCL11, HLA-DMA, TAPBP, B2M, CXCL10, FAS, CIITA, GTPBP1, GBP6, PTGER4, ADGRE5, HLA-A, HLA-C, HLA-B, CTSS, CD40, HLA-E, HLA-DQA1, PDCD1LG2, HLA-F, OSM, IGSF6, TNFSF13B, IL12A, HLA-DPA1, EDA, GBP2, HLA-DRA, HLA-DQB1, GPR183, TNFRSF21, HLA-DQB2, CXCL5, HLA-DRB1, IFITM2, CYSLTR2, ENPP2, C3, IFITM3, CXCL9, CCL8, IL32, C1R, OAS2, CCL5, CD74, SLC11A1, HRH2, HLA-DRB5, HLA-DPB1, HLA-DOA, HLA-DOB, CD7, LY75, SECTM1, TLR10, IL1RN, CTLA4, CD1A, TRIM22, CCL15, AIM2, CCL18, TNFSF8, TNFSF10, CCL13, CD274 683 421 16792 4.029477330347113 1.5926494393660327E-19 7.963247196830164E-20 9.494808201969659E-20

4 GOTERM_BP_DIRECT GO:0060337~type I interferon signaling pathway 23 2.926208651399491 2.8522557811781157E-15 IFITM1, IFITM2, IFITM3, HLA-A, RSAD2, HLA-C, OAS2, HLA-B, STAT1, HLA-E, HLA-G, PSMB8, IFI35, ISG20, HLA-F, STAT2, IFIT3, IFIT2, IFI27, IRF7, IRF1, XAF1, GBP2 683 64 16792 8.835468521229869 8.726130928948805E-12 2.9086733022154476E-12 5.206945985491984E-12

5 GOTERM_BP_DIRECT GO:0006954~inflammatory response 51 6.488549618320611 1.6612565037102797E-13 CRHBP, CXCR3, TLR5, IL15, CXCL11, TLR7, MMP25, TLR8, CXCL10, CCRL2, TNFRSF11A, CASP4, TICAM2, FAS, ADAM8, CIITA, GBP5, LYN, C4A, C4B, IL27, ADGRE5, RELB, POLB, CD40, TNFAIP6, RIPK2, ACOD1, AOC3, TNFRSF21, NMI, CXCL5, C3, GSDMD, CXCL9, CCL8, CCL5, CCL7, SLC11A1, MEFV, ZC3H12A, BCL6, LY75, TLR10, CCL15, AIM2, CCL18, GGT5, APOL3, P2RX7, CCL13 683 379 16792 3.3083594416994715 5.020881488349005E-10 1.2552203720872512E-10 2.9932722966918845E-10

6 GOTERM_BP_DIRECT GO:0051607~defense response to virus 31 3.9440203562340965 4.168934089695492E-12 IFITM1, IFITM2, IFITM3, CXCL9, PML, RSAD2, APOBEC3G, IFI44L, OAS2, PMAIP1, CXADR, TLR7, TLR8, CXCL10, ISG20, APOBEC3D, APOBEC3A, NLRC5, SERINC5, DDX60, ZC3H12A, CD40, STAT1, TRIM22, STAT2, IFIT3, IFIT2, IFIT5, IRF1, GBP3, GBP1 683 165 16792 4.619122410044811 1.2602546672901838E-8 2.520509356784828E-9 7.51319007008533E-9

7 GOTERM_BP_DIRECT GO:0002504~antigen processing and presentation of peptide or polysaccharide antigen via MHC class II 12 1.5267175572519083 4.539944388103518E-12 HLA-DQB1, HLA-DQB2, HLA-DRB1, HLA-DRB5, HLA-DPA1, HLA-DPB1, HLA-DMB, HLA-DOA, HLA-DMA, HLA-DOB, HLA-DQA1, HLA-DRA 683 17 16792 17.35457755576608 1.3724190117692103E-8 2.2873650751265018E-9 8.181877397817061E-9

8 GOTERM_BP_DIRECT GO:0002250~adaptive immune response 29 3.689567430025445 8.041234756321517E-12 TNFRSF21, GPR183, KLRK1, TNFSF18, CLEC10A, TNFRSF11A, LILRA1, LILRA2, TAP2, TAP1, ERAP2, CLEC6A, CD7, ITK, SIT1, LYN, CTLA4, CD1C, CD1A, SLAMF7, CTSS, HLA-E, BTN3A1, LAMP3, LILRB3, RIPK2, JAK2, RNF19B, JAK3 683 148 16792 4.817458747180563 2.4308651092042055E-8 3.4726644893012804E-9 1.4491952082806847E-8

9 GOTERM_BP_DIRECT GO:0045087~innate immune response 50 6.361323155216285 6.524367175538553E-11 APOBEC3G, TLR5, TLR7, TLR8, APOBEC3D, B2M, APOBEC3A, NLRC5, NOD2, CASP4, DDX60, TICAM2, LYN, C4A, NCF1, C4B, IL27, RELB, HLA-C, COLEC12, SERPING1, HLA-B, HLA-E, C1QB, TRIM31, RIPK2, IFIH1, GSDMD, KLRK1, PML, C1R, C1S, CLEC10A, CYLD, SERINC5, CLEC6A, C2, ZBP1, ITK, TLR10, SLAMF6, MSRB1, SLAMF1, AIM2,

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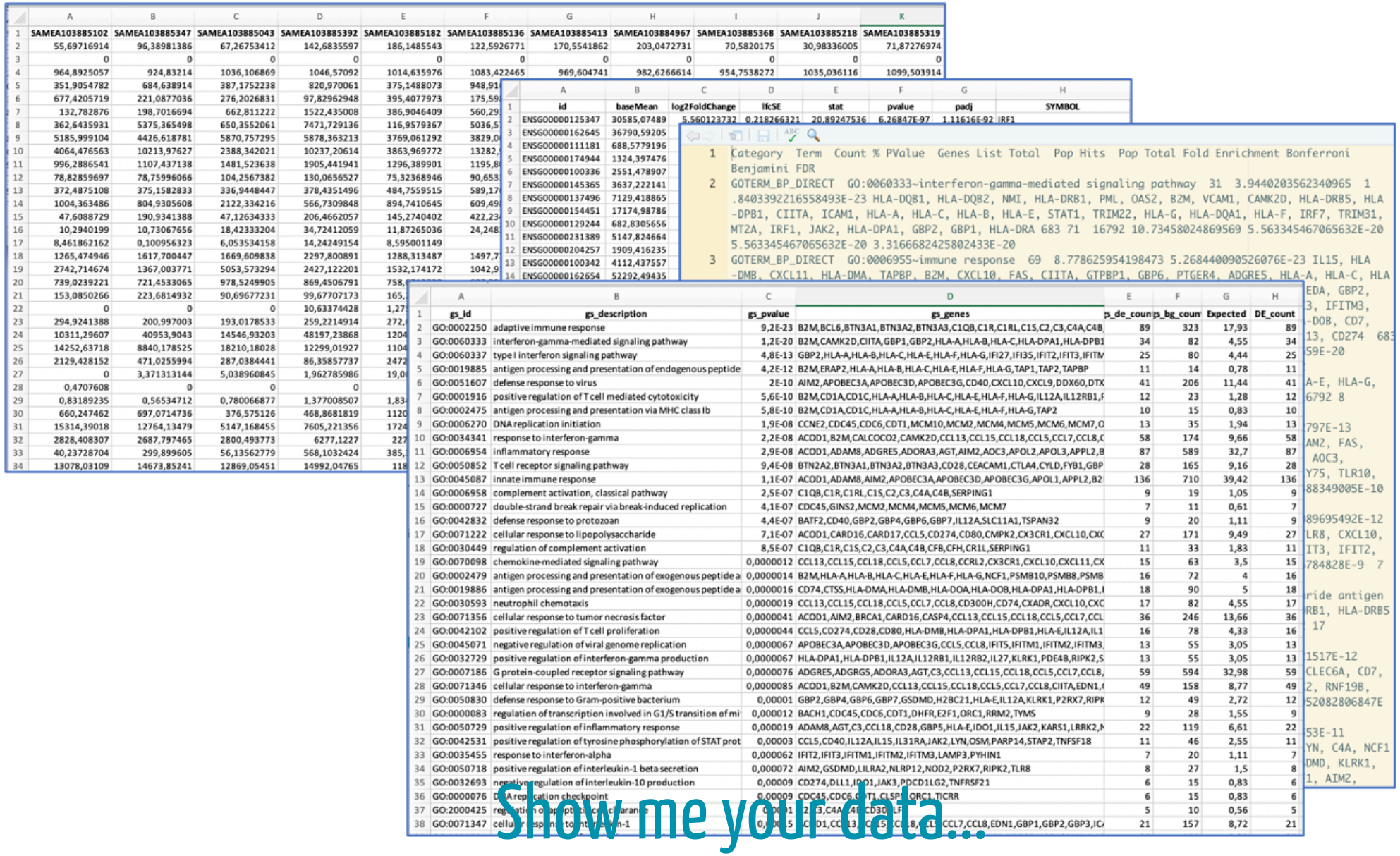

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3	0	0	0	0	0	0	0	0	0	0	0
4	964,8925057	924,83214	1036,106869	1046,57092	1014,635976	1083,422465	969,604741	982,6266614	954,7538272	1035,036116	1099,503914
5	351,9054782	684,638914	387,1752238	820,970061	375,1488073	948,91					
6	677,4205719	221,0877036	276,2026831	97,82962948	395,4077973	175,59					
7	132,782876	198,7016694	662,811222	1522,435008	386,9046409	560,29					
8	362,6435931	5375,365498	650,3552061	7471,729136	116,9579367	5036,5					
9	5185,999104	4426,618781	5870,757295	5878,363213	3769,061292	3829,0					
10	4064,476563	10213,97627	2388,342021	10237,20614	3863,969772	13282,					
11	996,2886541	1107,437138	1481,523638	1905,441941	1296,389901	1195,8					
12	78,82859697	78,75996066	104,2567382	130,0656527	75,32368946	90,653					
13	372,4875108	375,1582833	336,9448447	378,4351496	484,7559515	589,17					
14	1004,363486	804,9305608	2122,334216	566,7309848	894,7410645	609,49					
15	47,6088729	190,9341388	47,12634333	206,4662057	145,2740402	422,23					
16	10,2940199	10,73067656	18,42333204	34,72412059	11,87265036	24,248					
17	8,461862162	0,100956323	6,053534158	14,24249154	8,595001149						
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19	2742,714674	1367,003771	5053,573294	2427,122201	1532,174172	1042,9					
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23	294,9241388	200,997003	193,0178533	259,2214914	272,						
24	10311,29607	40953,9043	14546,93203	48197,23868	1204						
25	14252,63718	8840,178525	18210,18028	12299,01927	1104						
26	2129,428152	471,0255994	287,0384441	86,35857737	2472						
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28	0,4707608	0	0	0							
29	0,83189235	0,56534712	0,780066877	1,377008507	1,83						
30	660,247462	697,0714736	376,575126	468,8681819	1120						
31	15314,39018	12764,13479	5147,168455	7605,221356	1724						
32	2828,408307	2687,797465	2800,493773	6277,1227	227						
33	40,23728704	299,899605	56,13562779	568,1032424	385,						
34	13078,03109	14673,85241	12869,05451	14992,04765	118						

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3	ENSG00000162645	36790,59205						
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14	ENSG00000162654	52292,49435						

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Category  Term  Count % PValue  Genes List Total  Pop Hits  Pop Total Fold Enrichment Bonferroni
Benjamini FDR
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-DPB1, CIITA, ICAM1, HLA-A, HLA-C, HLA-B, HLA-E, STAT1, TRIM22, HLA-G, HLA-DQA1, HLA-F, IRF7, TRIM31,
MT2A, IRF1, JAK2, HLA-DPA1, GBP2, GBP1, HLA-DRA 683 71  16792 10.73458024869569 5.563345467065632E-20
5.563345467065632E-20 3.3166682425802433E-20
GOTERM_BP_DIRECT  GO:0006955~immune response  69  8.778625954198473 5.2684400905260 76E-23 IL15, HLA
-DMB, CXCL11, HLA-DMA, TAPBP, B2M, CXCL10, FAS, CIITA, GTPBP1, GBP6, PTGER4, ADGRE5, HLA-A, HLA-C, HLA
                                                                                    EDA, GBP2,
                                                                                    3, IFITM3,
                                                                                    -DOB, CD7,
                                                                                    13, CD274  683
                                                                                    59E-20

                                                                                    A-E, HLA-G,
                                                                                    6792 8

                                                                                    797E-13
                                                                                    AM2, FAS,
                                                                                     AOC3,
                                                                                    Y75, TLR10,
                                                                                    8834900 5E-10

                                                                                    89695492E-12
                                                                                    LR8, CXCL10,
                                                                                    IT3, IFIT2,
                                                                                    784828E-9  7

                                                                                    ride antigen
                                                                                    RB1, HLA-DRB5
                                                                                     17

                                                                                    1517E-12
                                                                                    CLEC6A, CD7,
                                                                                    2, RNF19B,
                                                                                    52082806847E

                                                                                    53E-11
                                                                                    YN, C4A, NCF1
                                                                                    DMD, KLRK1,
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	A	B	C	D	E	F	G	H
1	gs_id	gs_description	gs_pvalue	gs_genes	s_de_coun	s_bg_coun	Expected	DE_count
2	GO:0002250	adaptive immune response	9,2E-23	B2M,BCL6,BTN3A1,BTN3A2,BTN3A3,C1QB,C1R,C1RL,C1S,C2,C3,C4A,C4B	89	323	17,93	89
3	GO:0060333	interferon-gamma-mediated signaling pathway	1,2E-20	B2M,CAMK2D,CIITA,GBP1,GBP2,HLA-A,HLA-B,HLA-C,HLA-DPA1,HLA-DPB1	34	82	4,55	34
4	GO:0060337	type I interferon signaling pathway	4,8E-13	GBP2,HLA-A,HLA-B,HLA-C,HLA-E,HLA-F,HLA-G,IFI27,IFI35,IFIT2,IFIT3,IFITM	25	80	4,44	25
5	GO:0019885	antigen processing and presentation of endogenous peptide	4,2E-12	B2M,ERAP2,HLA-A,HLA-B,HLA-C,HLA-E,HLA-F,HLA-G,TAP1,TAP2,TAPBP	11	14	0,78	11
6	GO:0051607	defense response to virus	2E-10	AIM2,APOBEC3A,APOBEC3D,APOBEC3G,CD40,CXCL10,CXCL9,DDX60,DTX	41	206	11,44	41
7	GO:0001916	positive regulation of T cell mediated cytotoxicity	5,6E-10	B2M,CD1A,CD1C,HLA-A,HLA-B,HLA-C,HLA-E,HLA-F,HLA-G,IL12A,IL12RB1,F	12	23	1,28	12
8	GO:0002475	antigen processing and presentation via MHC class Ib	5,8E-10	B2M,CD1A,CD1C,HLA-A,HLA-B,HLA-C,HLA-E,HLA-F,HLA-G,TAP2	10	15	0,83	10
9	GO:0006270	DNA replication initiation	1,9E-08	CCNE2,CDC45,CDC6,CDT1,MCM10,MCM2,MCM4,MCM5,MCM6,MCM7,C	13	35	1,94	13
10	GO:0034341	response to interferon-gamma	2,2E-08	ACOD1,B2M,CALCOCO2,CAMK2D,CCL13,CCL15,CCL18,CCL5,CCL7,CCL8,C	58	174	9,66	58
11	GO:0006954	inflammatory response	2,9E-08	ACOD1,ADAM8,ADGRE5,ADORA3,AGT,AIM2,AOC3,APOL2,APOL3,APPL2,B	87	589	32,7	87
12	GO:0050852	T cell receptor signaling pathway	9,4E-08	BTN2A2,BTN3A1,BTN3A2,BTN3A3,CD28,CEACAM1,CTLA4,CYLD,FYB1,GBP	28	165	9,16	28
13	GO:0045087	innate immune response	1,1E-07	ACOD1,ADAM8,AIM2,APOBEC3A,APOBEC3D,APOBEC3G,APOL1,APPL2,B2	136	710	39,42	136
14	GO:0006958	complement activation, classical pathway	2,5E-07	C1QB,C1R,C1RL,C1S,C2,C3,C4A,C4B,SERPING1	9	19	1,05	9
15	GO:0000727	double-strand break repair via break-induced replication	4,1E-07	CDC45,GINS2,MCM2,MCM4,MCM5,MCM6,MCM7	7	11	0,61	7
16	GO:0042832	defense response to protozoan	4,4E-07	BATF2,CD40,GBP2,GBP4,GBP6,GBP7,IL12A,SLC11A1,TSPAN32	9	20	1,11	9
17	GO:0071222	cellular response to lipopolysaccharide	7,1E-07	ACOD1,CARD16,CARD17,CCL5,CD274,CD80,CMPK2,CX3CR1,CXCL10,CXC	27	171	9,49	27
18	GO:0030449	regulation of complement activation	8,5E-07	C1QB,C1R,C1S,C2,C3,C4A,C4B,CFB,CFH,CR1L,SERPING1	11	33	1,83	11
19	GO:0070098	chemokine-mediated signaling pathway	0,0000012	CCL13,CCL15,CCL18,CCL5,CCL7,CCL8,CCRL2,CX3CR1,CXCL10,CXCL11,CX	15	63	3,5	15
20	GO:0002479	antigen processing and presentation of exogenous peptide a	0,0000014	B2M,HLA-A,HLA-B,HLA-C,HLA-E,HLA-F,HLA-G,NCF1,PSMB10,PSMB8,PSMB	16	72	4	16
21	GO:0019886	antigen processing and presentation of exogenous peptide a	0,0000016	CD74,CTSS,HLA-DMA,HLA-DMB,HLA-DOA,HLA-DOB,HLA-DPA1,HLA-DPB1,I	18	90	5	18
22	GO:0030593	neutrophil chemotaxis	0,0000019	CCL13,CCL15,CCL18,CCL5,CCL7,CCL8,CD300H,CD74,CXADR,CXCL10,CXC	17	82	4,55	17
23	GO:0071356	cellular response to tumor necrosis factor	0,0000041	ACOD1,AIM2,BRCA1,CARD16,CASP4,CCL13,CCL15,CCL18,CCL5,CCL7,CCL	36	246	13,66	36
24	GO:0042102	positive regulation of T cell proliferation	0,0000044	CCL5,CD274,CD28,CD80,HLA-DMB,HLA-DPA1,HLA-DPB1,HLA-E,IL12A,IL1	16	78	4,33	16
25	GO:0045071	negative regulation of viral genome replication	0,0000067	APOBEC3A,APOBEC3D,APOBEC3G,CCL5,CCL8,IFIT5,IFITM1,IFITM2,IFITM3,	13	55	3,05	13
26	GO:0032729	positive regulation of interferon-gamma production	0,0000067	HLA-DPA1,HLA-DPB1,IL12A,IL12RB1,IL12RB2,IL27,KLRK1,PDE4B,RIPK2,S	13	55	3,05	13
27	GO:0007186	G protein-coupled receptor signaling pathway	0,0000076	ADGRE5,ADGRG5,ADORA3,AGT,C3,CCL13,CCL15,CCL18,CCL5,CCL7,CCL8,	59	594	32,98	59
28	GO:0071346	cellular response to interferon-gamma	0,0000085	ACOD1,B2M,CAMK2D,CCL13,CCL15,CCL18,CCL5,CCL7,CCL8,CIITA,EDN1,(	49	158	8,77	49
29	GO:0050830	defense response to Gram-positive bacterium	0,00001	GBP2,GBP4,GBP6,GBP7,GSDMD,H2BC21,HLA-E,IL12A,KLRK1,P2RX7,RIPK	12	49	2,72	12
30	GO:0000083	regulation of transcription involved in G1/S transition of mi	0,000012	BACH1,CDC45,CDC6,CDT1,DHFR,E2F1,ORC1,RRM2,TYMS	9	28	1,55	9
31	GO:0050729	positive regulation of inflammatory response	0,000019	ADAM8,AGT,C3,CCL18,CD28,GBP5,HLA-E,IDO1,IL15,JAK2,KARS1,LRRK2,N	22	119	6,61	22
32	GO:0042531	positive regulation of tyrosine phosphorylation of STAT prot	0,00003	CCL5,CD40,IL12A,IL15,IL31RA,JAK2,LYN,OSM,PARP14,STAP2,TNFSF18	11	46	2,55	11
33	GO:0035455	response to interferon-alpha	0,000062	IFIT2,IFIT3,IFITM1,IFITM2,IFITM3,LAMP3,PYHIN1	7	20	1,11	7
34	GO:0050718	positive regulation of interleukin-1 beta secretion	0,000072	AIM2,GSDMD,LILRA2,NLRP12,NOD2,P2RX7,RIPK2,TLR8	8	27	1,5	8
35	GO:0032693	negative regulation of interleukin-10 production	0,00009	CD274,DLL1,IDO1,JAK3,PDCD1LG2,TNFRSF21	6	15	0,83	6
36	GO:0000076	DNA replication checkpoint	0,00009	CDC45,CDC6,CDT1,CLSPN,ORC1,TICRR	6	15	0,83	6
37	GO:2000425	regulation of apoptotic cell clearance	0,0001	C1QB,C1R,C1S,C2,C3,C4A,C4B,CD300LF	5	10	0,56	5
38	GO:0071347	cellular response to interleukin-1	0,00015	ACOD1,CCL13,CCL15,CCL18,CCL5,CCL7,CCL8,EDN1,GBP1,GBP2,GBP3,ICA	21	157	8,72	21

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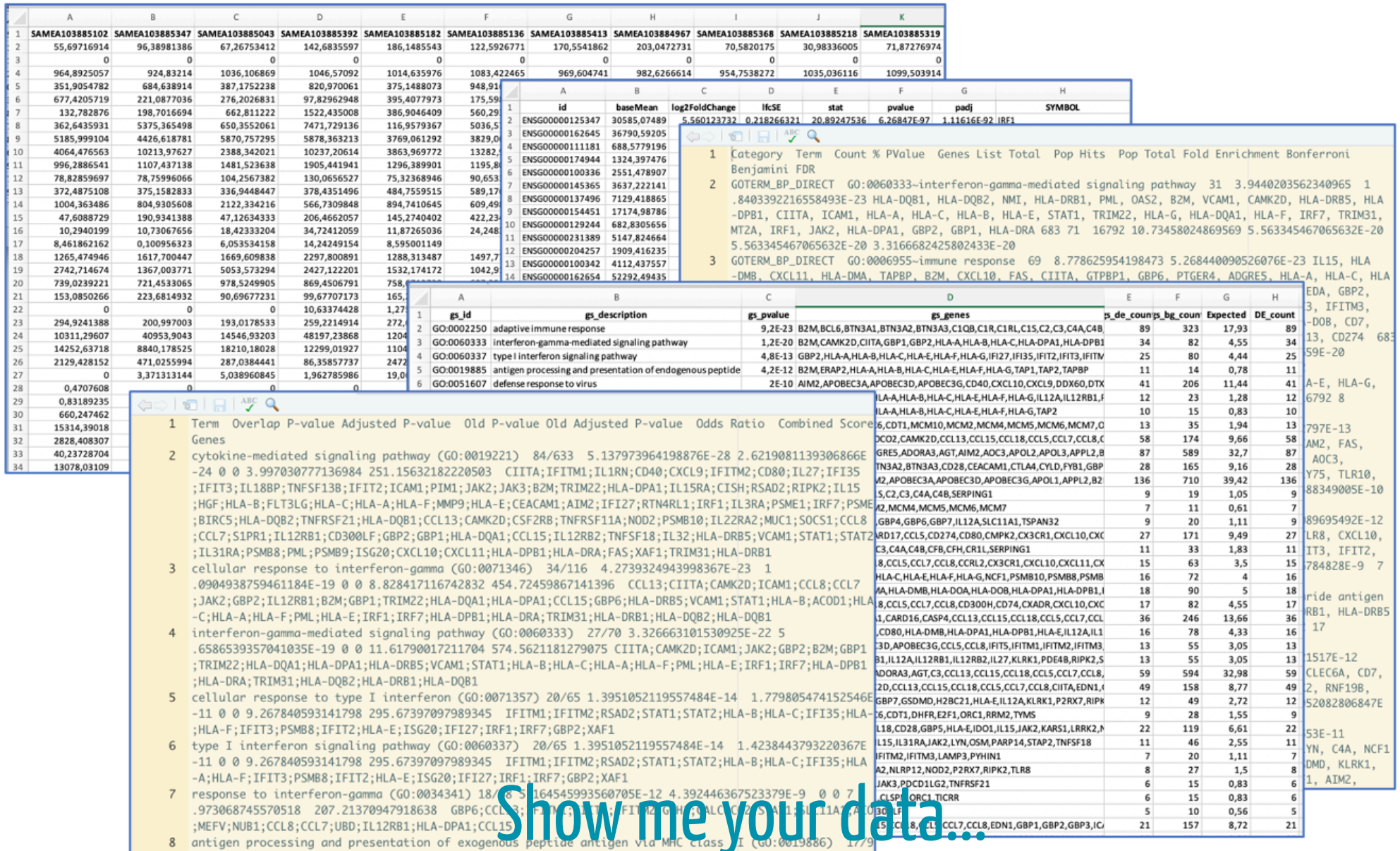
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A cocktail recipe

You might (and should) have a set of standardized objects for your datasets and results (Excel does ***not*** *count)*

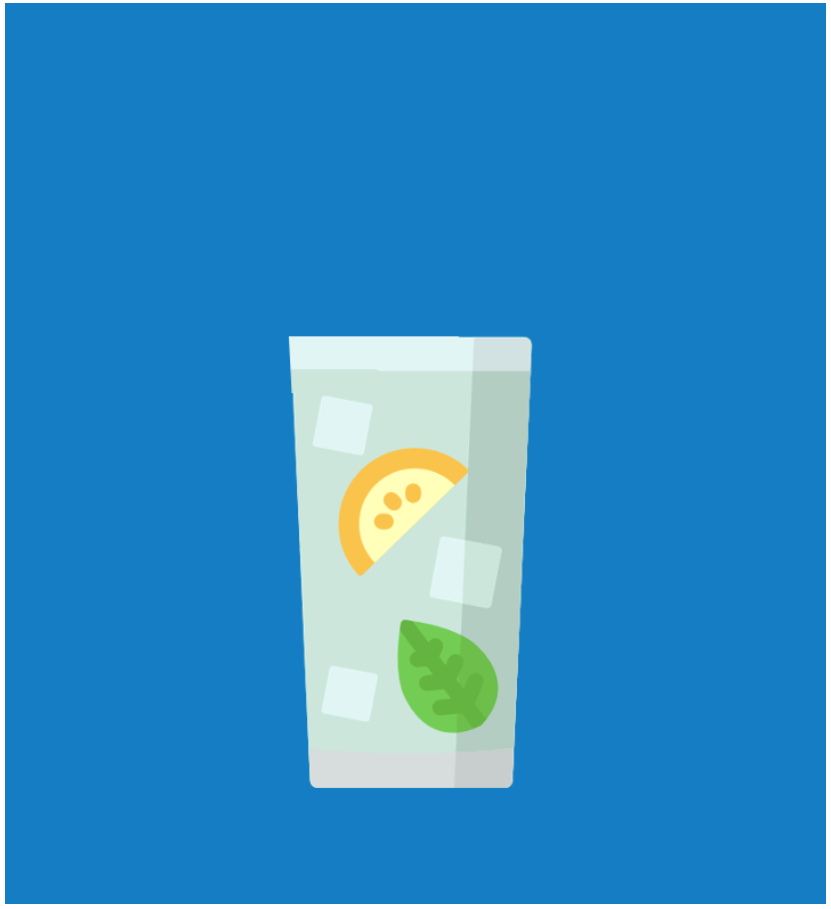
A cocktail recipe

A container for the expression matrix, `dds`

A cocktail recipe

A container for the results of differential expression, `res_de`

A cocktail recipe

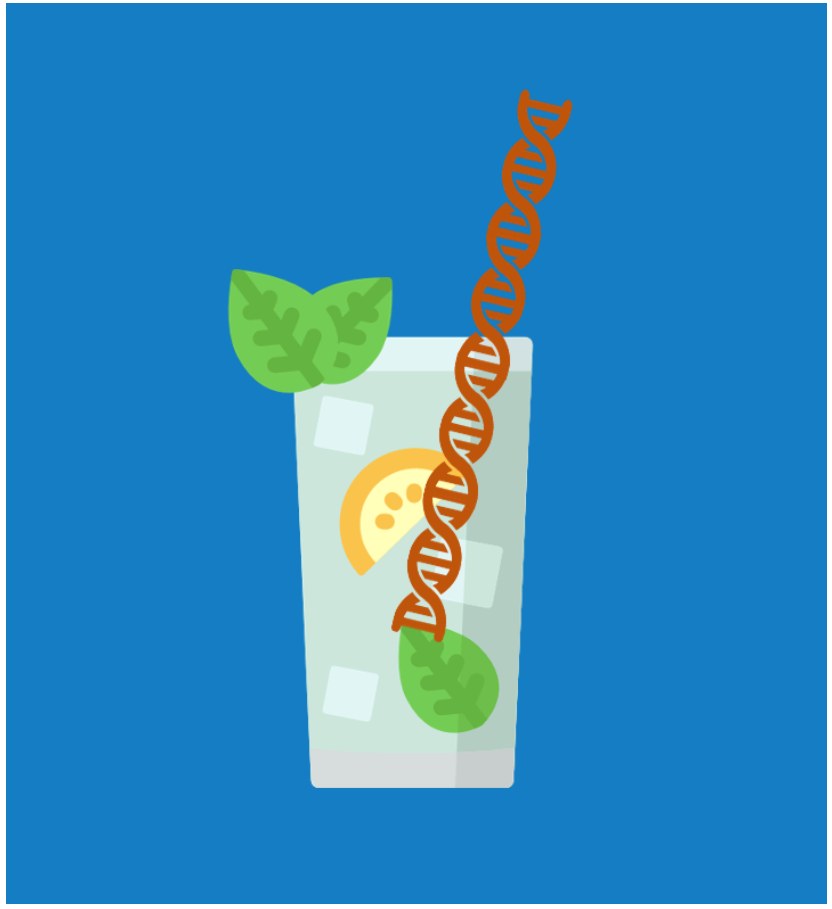


A table for the result of functional enrichment, `res_enrich`

A cocktail recipe

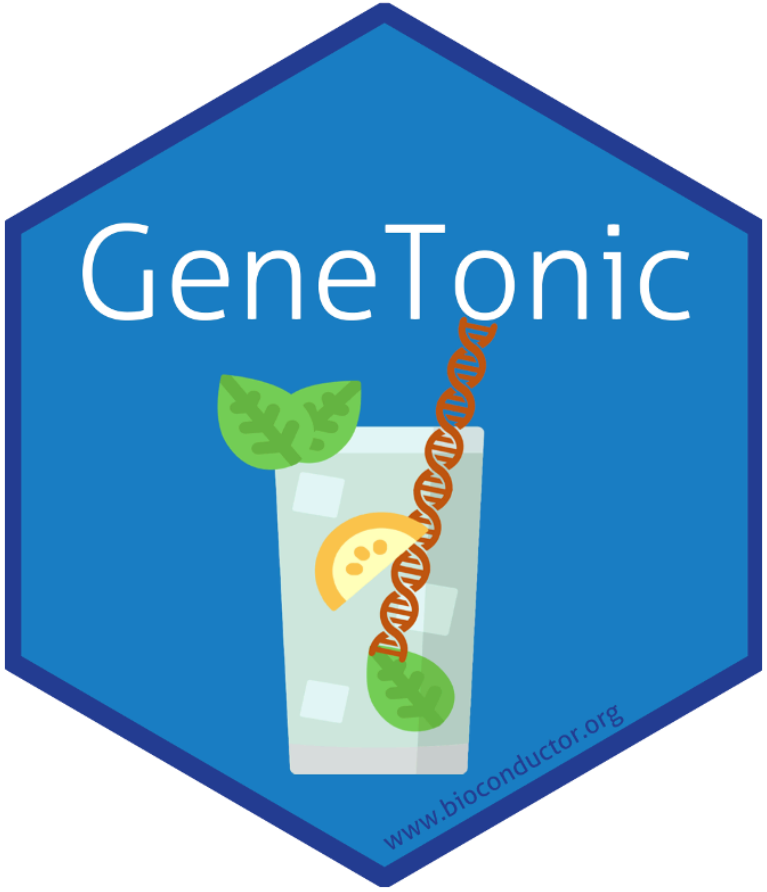
Decorate with an annotation table, `anno_df`

A cocktail recipe

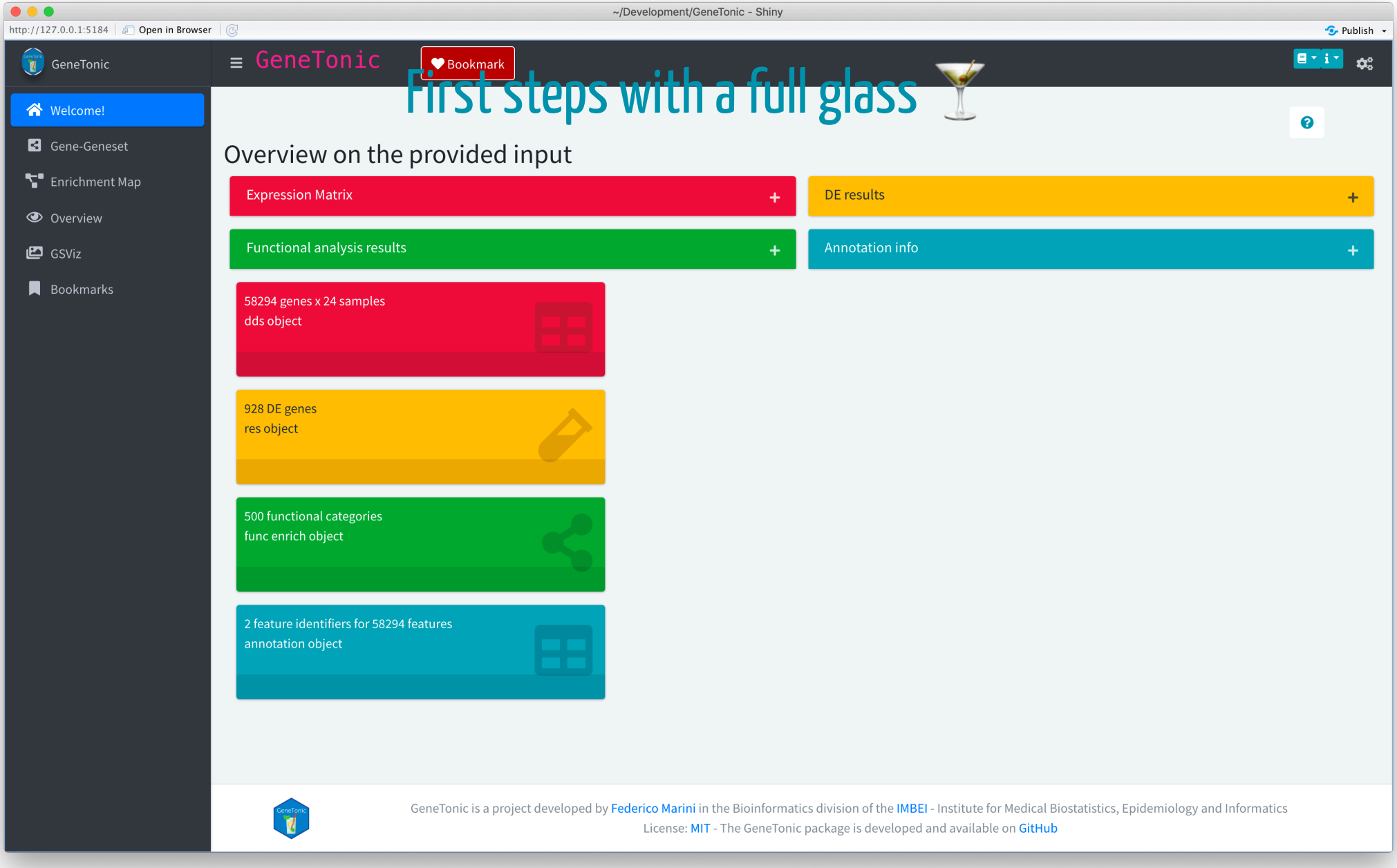


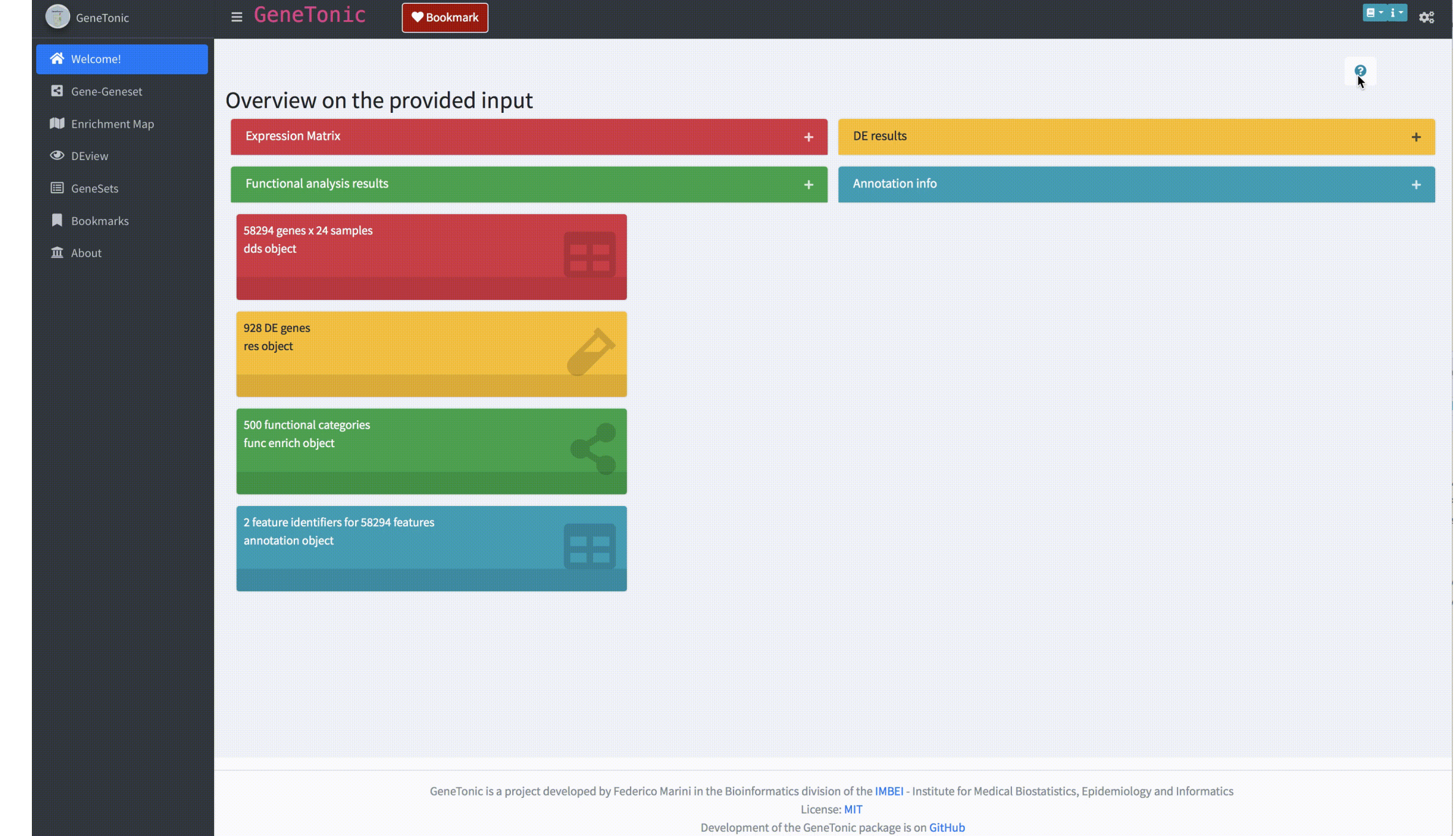
Shaken, not stirred...

A cocktail recipe



`GeneTonic` is now on Bioconductor!


~/Development/GeneTonic - Shiny
http://127.0.0.1:5184
Open in Browser
Publish
GeneTonic
GeneTonic
Bookmark
First steps with a full glass
Welcome!
Gene-Geneset
Enrichment Map
Overview
GSViz
Bookmarks
Overview on the provided input
Expression Matrix
DE results
Functional analysis results
Annotation info
58294 genes x 24 samples
dds object
928 DE genes
res object
500 functional categories
func enrich object
2 feature identifiers for 58294 features
annotation object
GeneTonic is a project developed by Federico Marini in the Bioinformatics division of the IMBEI - Institute for Medical Biostatistics, Epidemiology and Informatics
License: MIT - The GeneTonic package is developed and available on GitHub


GeneTonic
GeneTonic
Bookmark
Welcome!
Gene-Geneset
Enrichment Map
DEview
GeneSets
Bookmarks
About
Overview on the provided input
Expression Matrix
DE results
Functional analysis results
Annotation info
58294 genes x 24 samples
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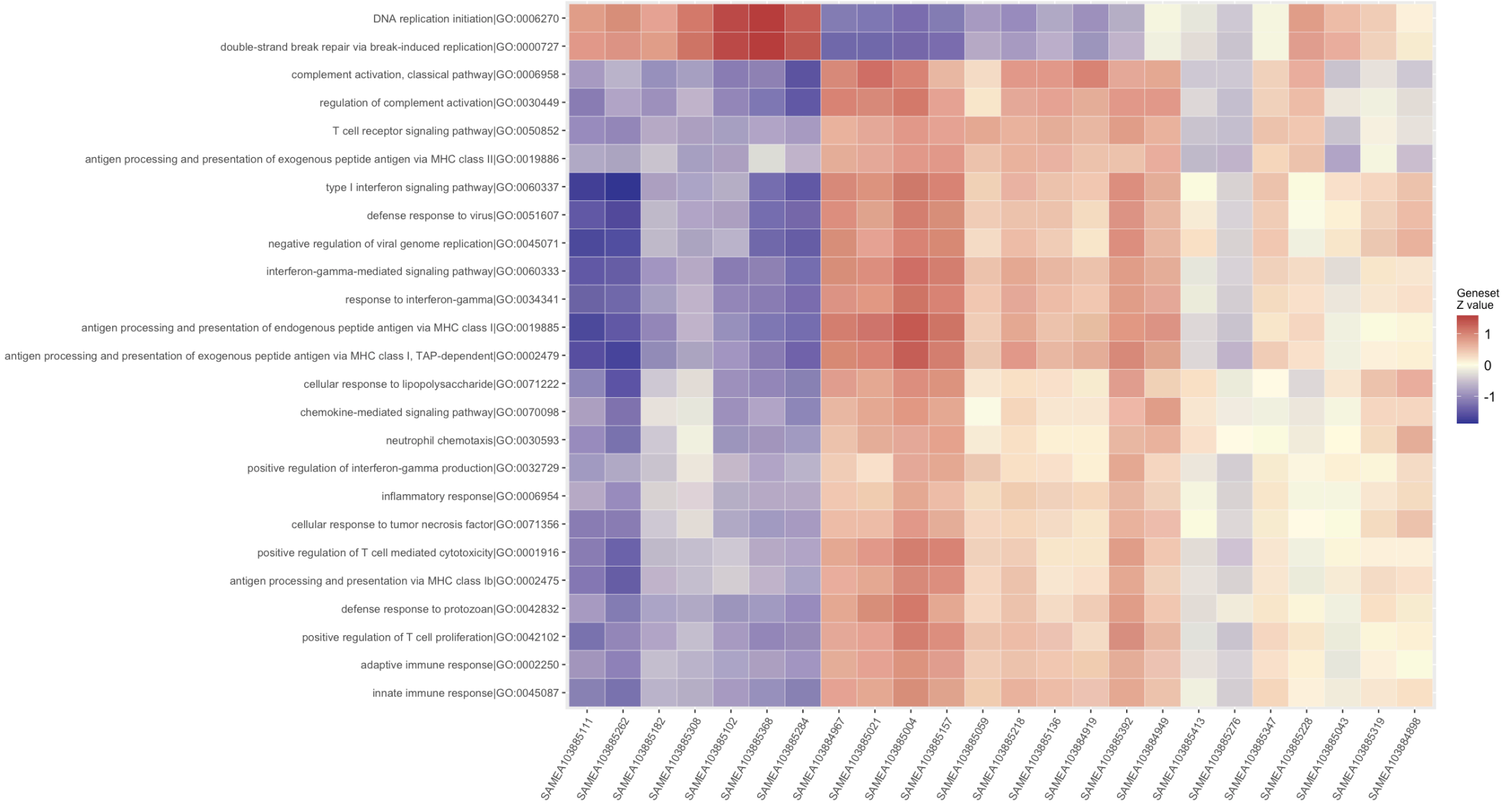
License: MIT
Development of the GeneTonic package is on GitHub


adaptive immune response
interferon-gamma-mediated signaling pathway
type I interferon signaling pathway
antigen processing and presentation of endogenous peptide antigen via MHC class I
defense response to virus
positive regulation of T cell mediated cytotoxicity
antigen processing and presentation via MHC class Ib
DNA replication initiation
response to interferon-gamma
inflammatory response
T cell receptor signaling pathway
innate immune response
complement activation, classical pathway
double-strand break repair via break-induced replication
defense response to protozoan
cellular response to lipopolysaccharide
regulation of complement activation
chemokine-mediated signaling pathway
antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent
antigen processing and presentation of exogenous peptide antigen via MHC class II
neutrophil chemotaxis
cellular response to tumor necrosis factor
positive regulation of T cell proliferation
positive regulation of interferon-gamma production
negative regulation of viral genome replication
Gene set description
0
5
10
15
20
log10 p-value
z_score
5
0
Visual overviews

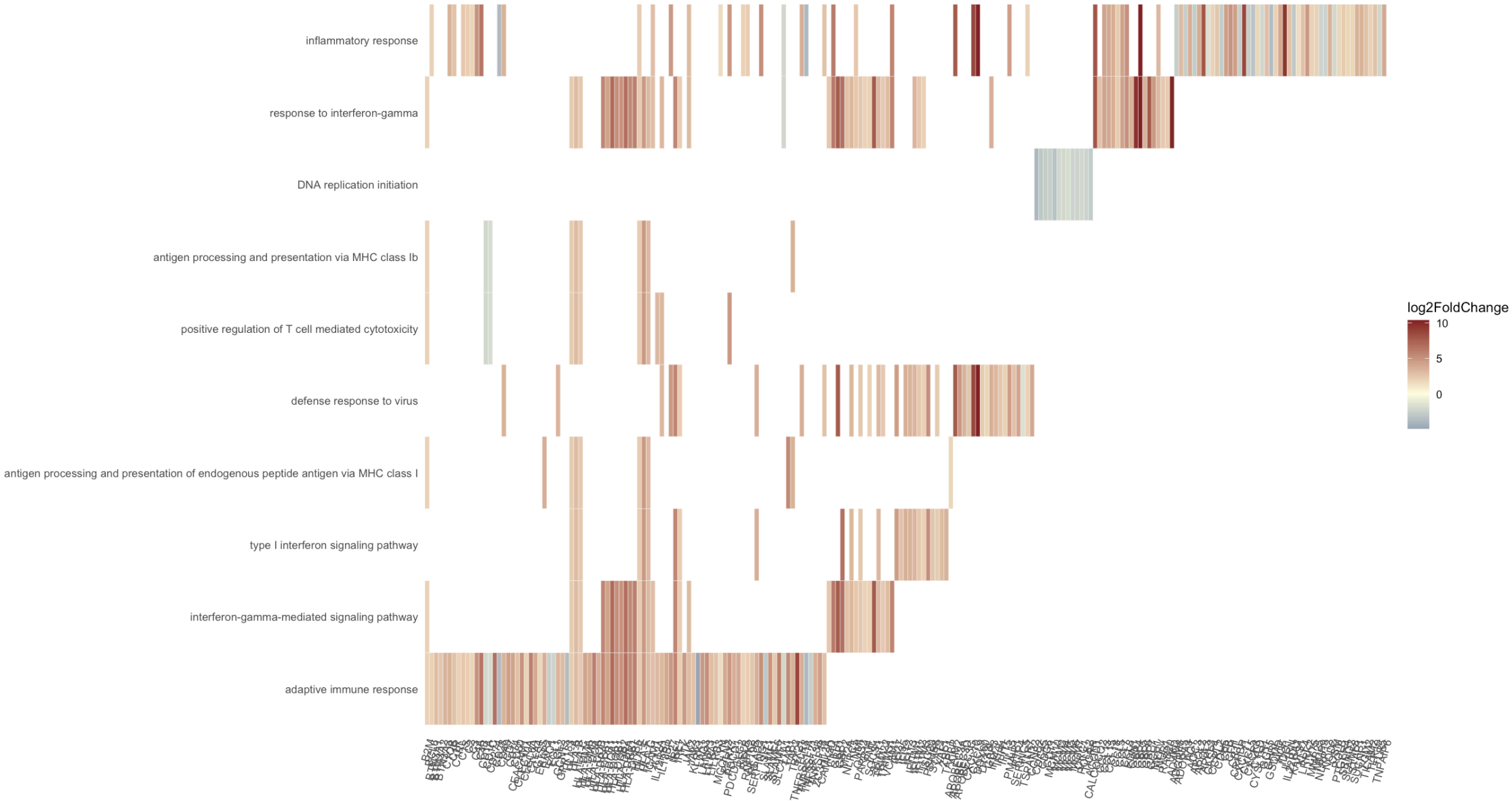
GSViz

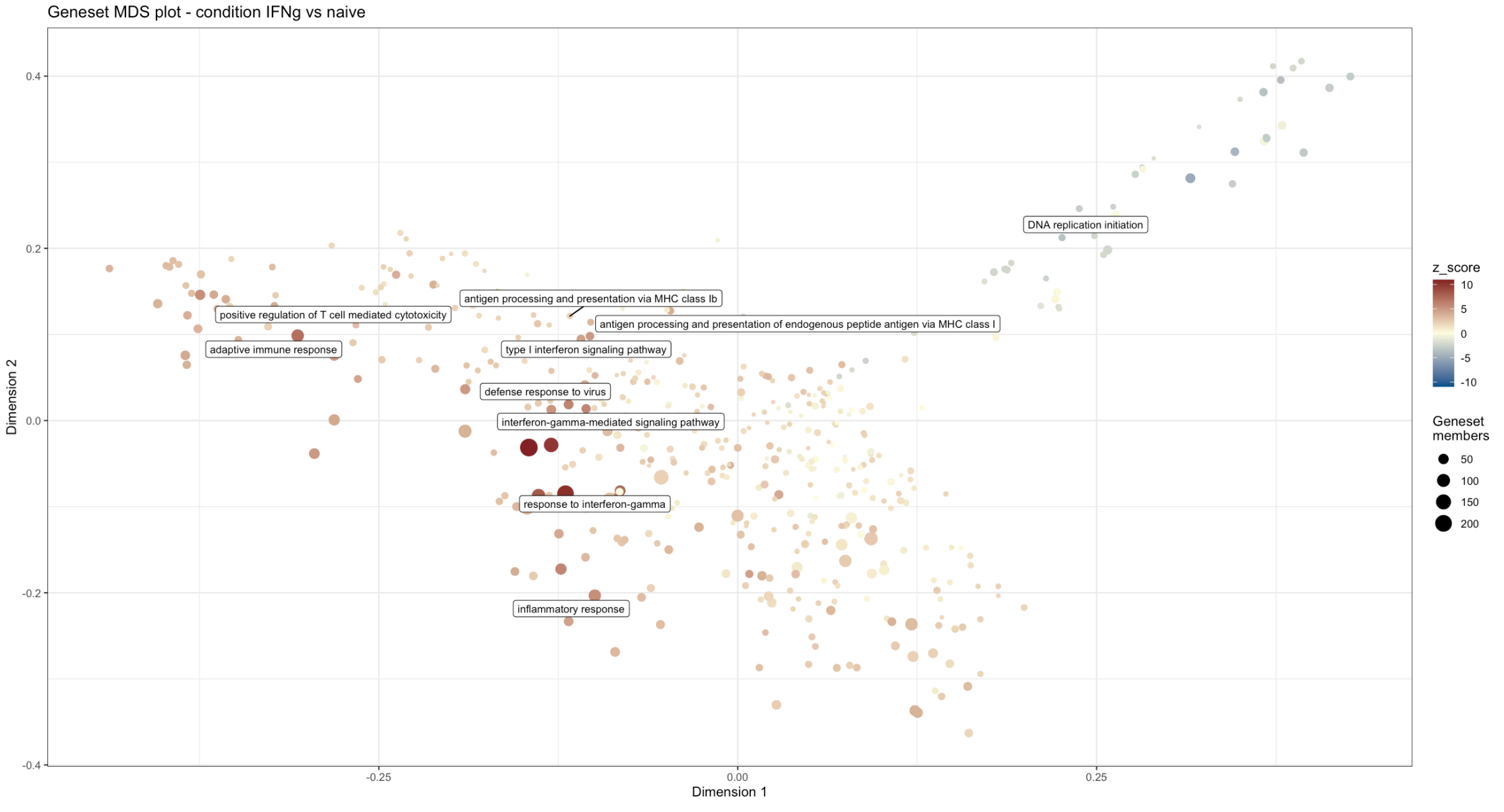
Scores Heatmap | Alluvial Plot | Summary Heatmap | Geneset MDS | Summary Overview | Geneset Radar | Geneset Dendrogram

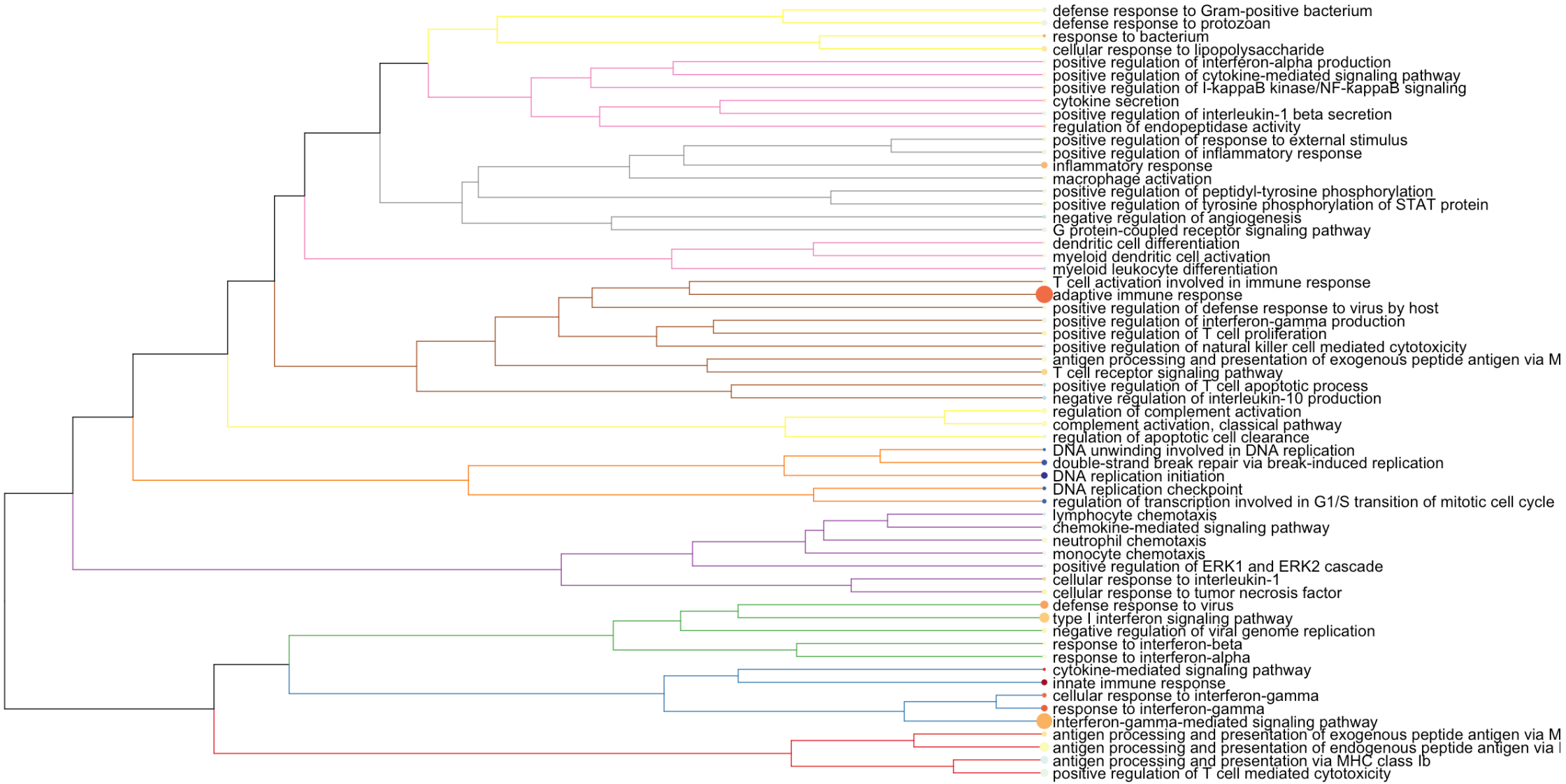


GSViz

Scores Heatmap | Alluvial Plot | **Summary Heatmap** | Geneset MDS | Summary Overview | Geneset Radar | Geneset Dendrogram



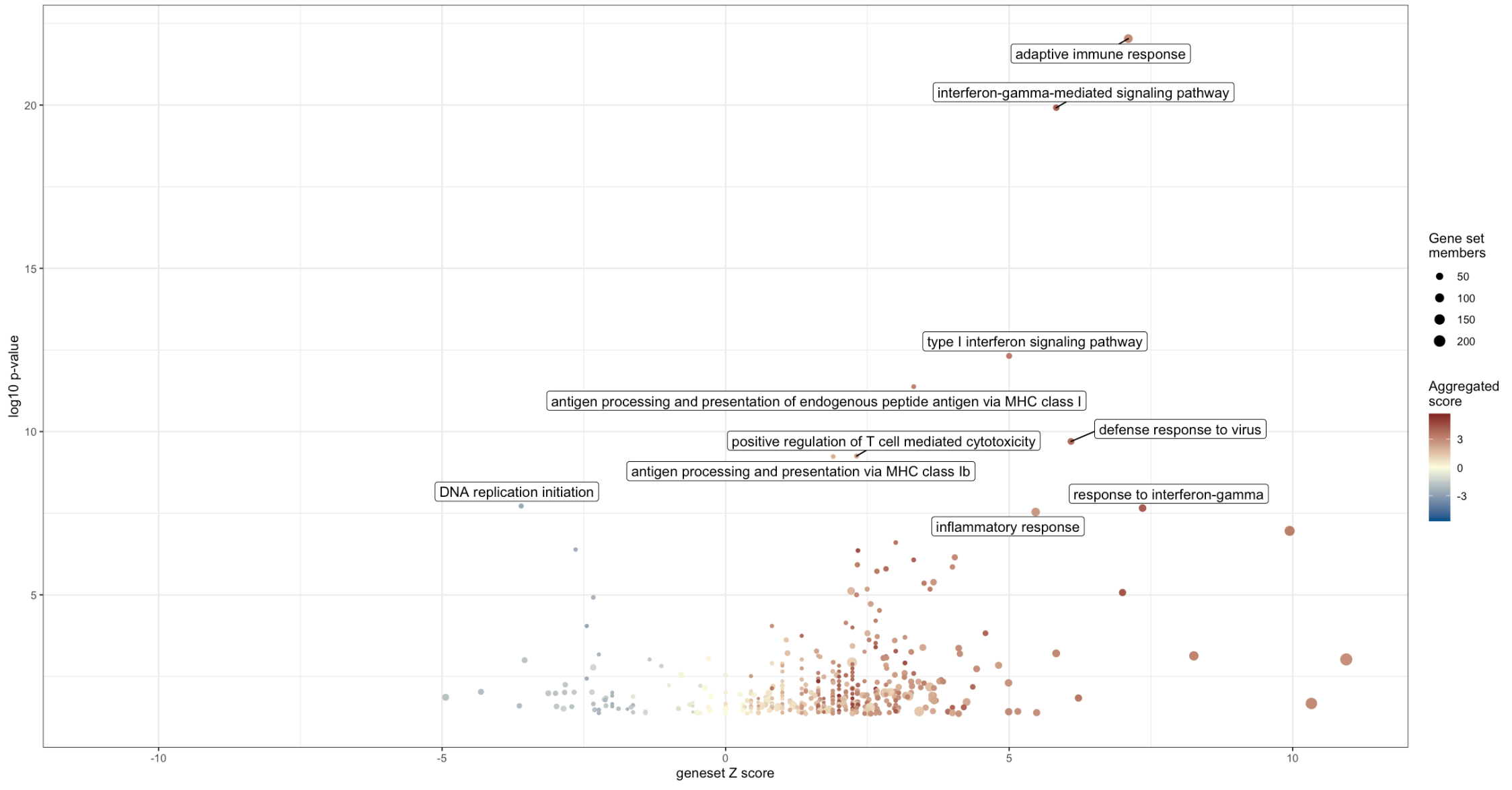

Geneset MDS plot - condition IFNg vs naive
DNA replication initiation
antigen processing and presentation via MHC class Ib
positive regulation of T cell mediated cytotoxicity
antigen processing and presentation of endogenous peptide antigen via MHC class I
adaptive immune response
type I interferon signaling pathway
defense response to virus
interferon-gamma-mediated signaling pathway
response to interferon-gamma
inflammatory response
Dimension 2
Dimension 1
0.4
0.2
0.0
-0.2
-0.4
-0.25
0.00
0.25
z_score
10
5
0
-5
-10
Geneset members
50
100
150
200


defense response to Gram-positive bacterium
defense response to protozoan
response to bacterium
cellular response to lipopolysaccharide
positive regulation of interferon-alpha production
positive regulation of cytokine-mediated signaling pathway
positive regulation of I-kappaB kinase/NF-kappaB signaling
cytokine secretion
positive regulation of interleukin-1 beta secretion
regulation of endopeptidase activity
positive regulation of response to external stimulus
positive regulation of inflammatory response
inflammatory response
macrophage activation
positive regulation of peptidyl-tyrosine phosphorylation
positive regulation of tyrosine phosphorylation of STAT protein
negative regulation of angiogenesis
G protein-coupled receptor signaling pathway
dendritic cell differentiation
myeloid dendritic cell activation
myeloid leukocyte differentiation
T cell activation involved in immune response
adaptive immune response
positive regulation of defense response to virus by host
positive regulation of interferon-gamma production
positive regulation of T cell proliferation
positive regulation of natural killer cell mediated cytotoxicity
antigen processing and presentation of exogenous peptide antigen via M
T cell receptor signaling pathway
positive regulation of T cell apoptotic process
negative regulation of interleukin-10 production
regulation of complement activation
complement activation, classical pathway
regulation of apoptotic cell clearance
DNA unwinding involved in DNA replication
double-strand break repair via break-induced replication
DNA replication initiation
DNA replication checkpoint
regulation of transcription involved in G1/S transition of mitotic cell cycle
lymphocyte chemotaxis
chemokine-mediated signaling pathway
neutrophil chemotaxis
monocyte chemotaxis
positive regulation of ERK1 and ERK2 cascade
cellular response to interleukin-1
cellular response to tumor necrosis factor
defense response to virus
type I interferon signaling pathway
negative regulation of viral genome replication
response to interferon-beta
response to interferon-alpha
cytokine-mediated signaling pathway
innate immune response
cellular response to interferon-gamma
response to interferon-gamma
interferon-gamma-mediated signaling pathway
antigen processing and presentation of exogenous peptide antigen via M
antigen processing and presentation of endogenous peptide antigen via I
antigen processing and presentation via MHC class Ib
positive regulation of T cell mediated cytotoxicity

Overview

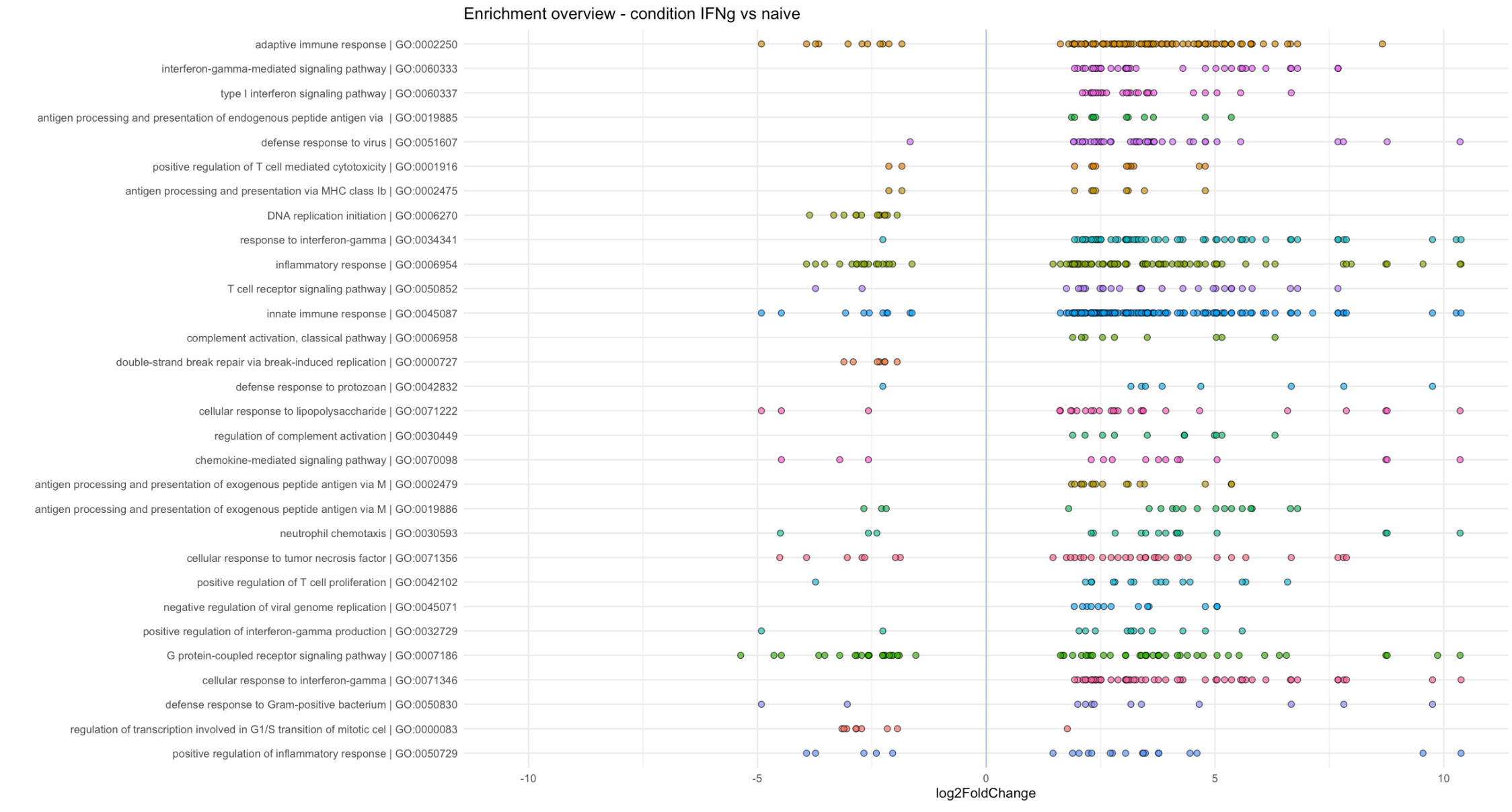
Geneset Volcano | Geneset Volcano - simplified | Enhanced Table | Enhanced Table - interactive

Geneset volcano



Overview

Geneset Volcano | Geneset Volcano - simplified | **Enhanced Table** | Enhanced Table - interactive



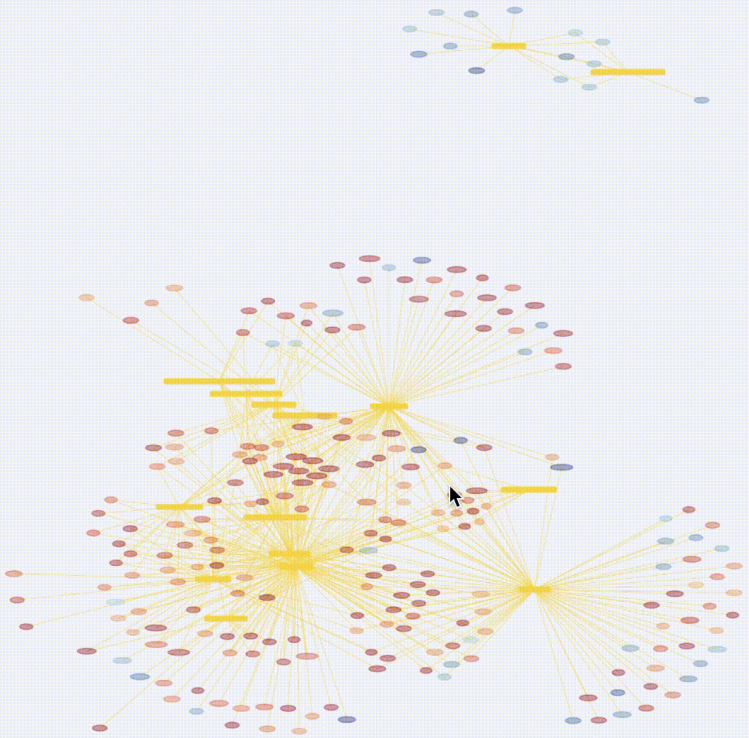
GeneTonic

GeneTonic

Bookmark

- Welcome!
- Gene-Geneset
- Enrichment Map
- DEview
- GeneSets
- Bookmarks
- About

Select by id



Genesetbox

Please select a gene set.

Genebox

Please select a gene/feature.

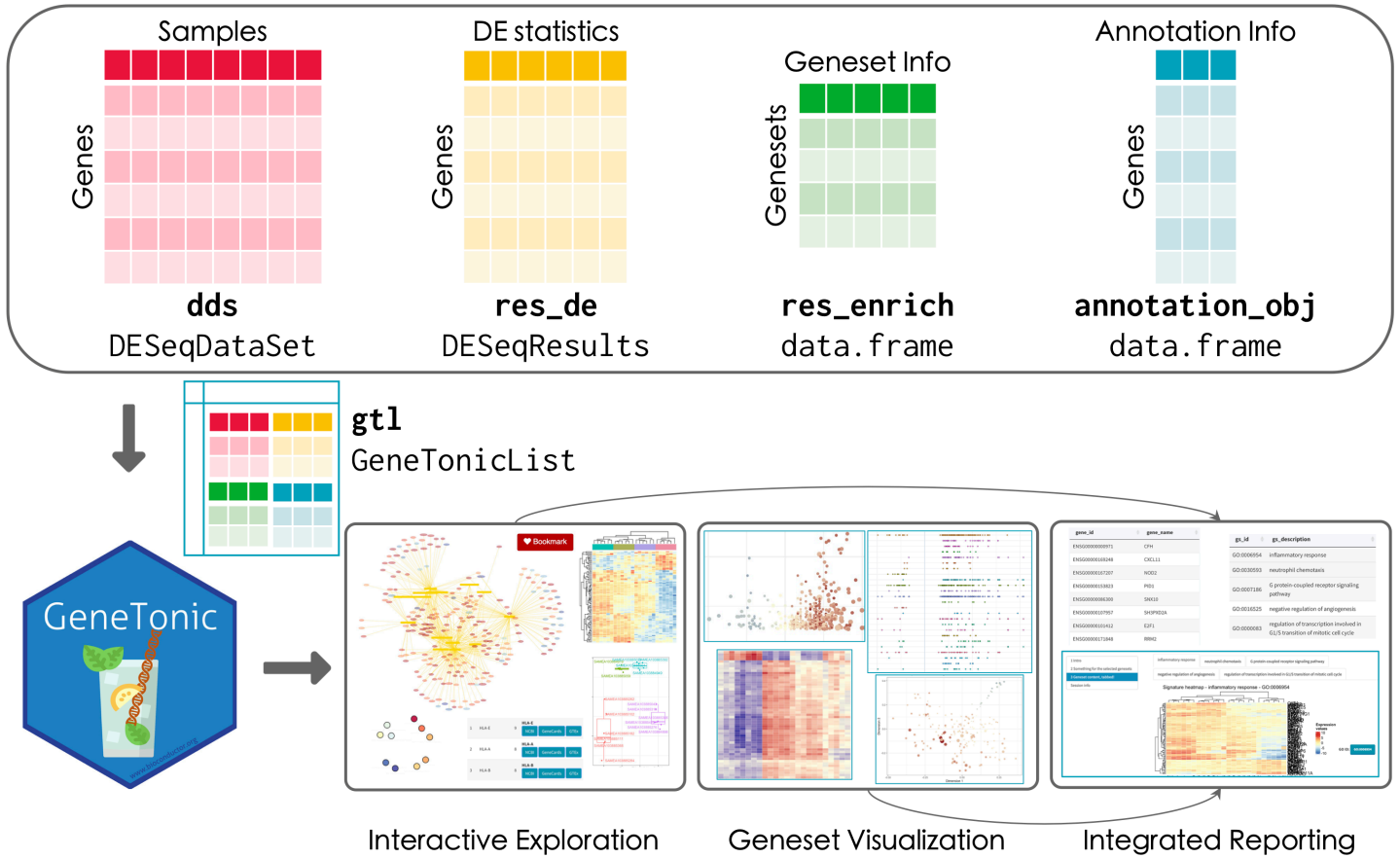
The interplay of genes and genesets

A quick demo

```
BiocManager::install("GeneTonic")
library("GeneTonic")
example(GeneTonic, ask = FALSE)
```

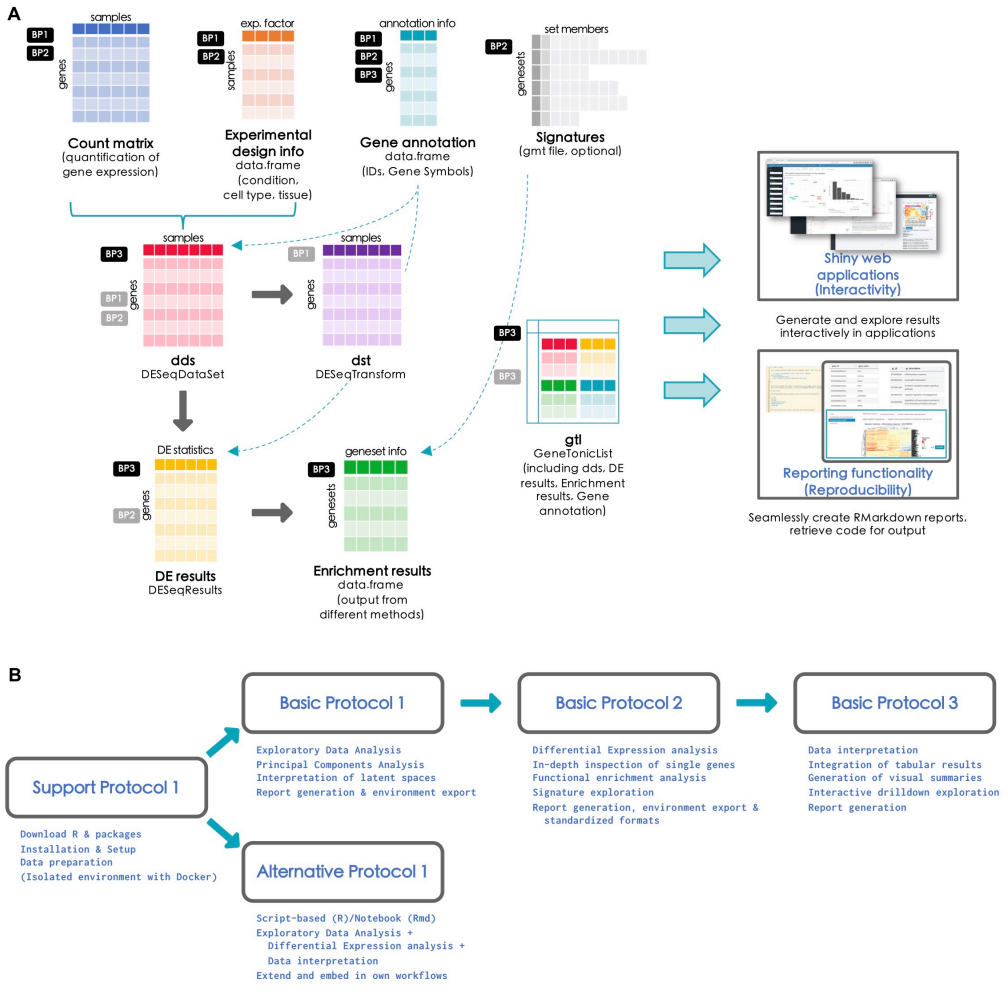
GeneTonic

A way to enjoy the data interpretation...



Suddenly iSEE

Putting it all together



Interfaces for RNA-seq

Systematically evaluating interfaces for RNA-seq analysis from a life scientist perspective FREE

Alicia Poplawski, Federico Marini, Moritz Hess, Tanja Zeller, Johanna Mazur, Harald Binder

Briefings in Bioinformatics, Volume 17, Issue 2, March 2016, Pages 213–223,
https://doi.org/10.1093/bib/bbv036
Published: 23 June 2015 **Article history** ▾

Interfaces have been developed for making such analysis steps accessible to life scientists without extensive knowledge of command line tools.

Systematic search and evaluation of such interfaces

Definition of criteria for evaluation (ease of configuration, documentation, usability, computational demand, reporting)

There's no `one tool fits all` winner!


Does the analysis/exploration/interpretation of your data spark joy?

Lessons learnt for me (so far)

It takes two to tango

Results & Interpretation

Bioinformatician & Experimental scientist

Reproducibility & Interactivity

Stop using Excel for performing Bioinformatics data analysis :)

Our contributions to the Bioconductor project:

`pcaExplorer` - Exploratory Data Analysis

`ideal` - Differential Expression analysis

`GeneTonic` - Interpretation of DE results

`iSEE` - Exploration of single cell datasets

...

`countsimQC` - comparing count data sets

`iCOBRA` - calculation and visualization of performance metrics

`ExploreModelMatrix`

`tximeta` - tx importing with metadata, *for free*

`alevinQC`

The essential - Recap

- Expression quantification
- Data exploration: principal components analysis, gene plots
- Differential analysis: DE modeling, design, effect size, variability, significance
- Functional interpretation: gene sets, pathways, biological themes

Practical session

Check again the README file at https://github.com/imbeimainz/MSE_GenEpi_2024

References:

- Marini and Binder (2016) - Development of Applications for Interactive and Reproducible Research: a Case Study (Genomics Computational Biology) 10.18547/gcb.2017.vol3.iss1.e39
- Marini and Binder (2019) - pcaExplorer: an R/Bioconductor package for interacting with RNA-seq principal components (BMC Bioinformatics) 10.1186/s12859-019-2879-1
- Marini, Linke, Binder (2020) - ideal: an R/Bioconductor package for Interactive Differential Expression Analysis (BMC Bioinformatics) 10.1186/s12859-020-03819-5
- Rue-Albrecht, Marini, Soneson, Lun (2018) - iSEE: Interactive SummarizedExperiment Explorer (F1000 Research) https://doi.org/10.12688/f1000research.14966.1
- Marini, Ludt, Linke, Strauch (2021) - GeneTonic: an R/Bioconductor package for streamlining the interpretation of RNA-seq data (BMC Bioinformatics) https://doi.org/10.1186/s12859-021-04461-5
- Ludt, Ustjanzew, Binder, Strauch, Marini (2022) - Interactive and Reproducible Workflows for Exploring and Modeling RNA-seq Data with pcaExplorer, Ideal, and GeneTonic (Current Protocols) https://doi.org/10.1002/cpz1.411

References: (cont'd)

- Srivastava, Malik, Sarkar, Zakeri, Almodaresi, Soneson, Love, Kingsford, Patro (2020) - Alignment and mapping methodology influence transcript abundance estimation (Genome Biology) https://genomebiology.biomedcentral.com/articles/10.1186/s13059-020-02151-8
- Conesa, Madrigal, Tarazona, Gomez-Cabrero, Cervera, McPherson, Szcześniak, Gaffney, Elo, Zhang, Mortazavi (2016) - A survey of best practices for RNA-seq data analysis (Genome Biology) `https://genomebiology.biomedcentral.com/articles/10.1186/s13059-016-0881-8`

Resources

The whole `rnaseqGene` workflow is detailed here: https://www.bioconductor.org/packages/rnaseqGene/

... thank you for your attention!

marinif@uni-mainz.de - @FedeBioinfo
charlotte.soneson@fmi.ch - @CSoneson