

RNA-Seq analysis using Galaxy

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Correlation with gene expression



RESEARCH ARTICLE

A complex association between DNA methylation and gene expression in human placenta at first and third trimesters

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Correlation with gene expression

- 1st trimester → 3rd trimester
 - ~200 diff. meth. promoter regions
 - ~2200 diff. meth. genes
 - Hypermethylation for > 90%
 - 2400 diff. expressed genes
 - Downregulation for ~80%
 - 25 with diff. Meth. promoter regions

Data download

[https://share.leibniz-fli.de/index.php/s/
mrWaNafRMnLc7be](https://share.leibniz-fli.de/index.php/s/mrWaNafRMnLc7be)

25 DEG with DME

- Data preparation for 22 protein coding genes as listed in GENCODE – Ensembl v92
 - Minimalistic HG38 derived genome representation
 - Genes & 21 x 4kb intergenic regions: Fasta format
 - Minimalistic annotation
 - Genes: GTF format
 - Promoter - 1kb gene upstream regions: BED format

25 DEG with DME

- Data preparation for RNA-Seq
 - Preprocessing & mapping of
 - RNA-Seq data
 - Subsampling to 5%
 - extraction from RAW FASTQ data

25 DEG with DME

- RNA-Seq
 - 1T & 3T @ N=4
 - 1T paired-end (PE)
 - **3T N1,N4 single (SE)**
 - **3T N2,N3 paired-end (PE)**

Galaxy – Upload

Galaxy / Europe

Tools

search tools

Get Data

1

Download from web or upload from disk

Regular

Composite

Collection

Rule-based

Please wait...30 out of 33 remaining.

Name	Size	Type	Genome	Settings	Status
hg38.goi.fa	1.7 MB	Auto-detect	----- Additional Speci...	⚙	100% ✓
hg38.goi.gtf	1.6 MB	Auto-detect	----- Additional Speci...	⚙	100% ✓
hg38.promoter.bed	816 b	Auto-detect	----- Additional Speci...	⚙	100% ✓
rbs_1T.N1.R1.fastq	14.4 MB	Auto-detect	----- Additional Speci...	⚙	Adding to history... ⚙
rbs_1T.N1.R2.fastq	14.4 MB	Auto-detect	----- Additional Speci...	⚙	0% ⚙
rbs_1T.N2.R1.fastq	20.9 MB	Auto-detect	----- Additional Speci...	⚙	0% ⚙
rbs_1T.N2.R2.fastq	20.9 MB	Auto-detect	----- Additional Speci...	⚙	0% ⚙

Type (set all):

Auto-detect

Genome (set all):

----- Additional Species Are B...

Choose local file

Choose FTP file

Paste/Fetch data

Pause

Reset

Start

Close

2

3

Learn how the NASA uses Galaxy!

Quality analysis: FastQC

The screenshot shows the Galaxy web interface for the FastQC tool. On the left, the 'Tools' sidebar contains a search bar with 'fastqc' and a list of tool categories: FASTA/FASTQ, FASTQ Quality Control, and Mapping. The 'FASTQ Quality Control' category is expanded, showing the 'FastQC: Read Quality reports' tool. On the right, the tool's configuration page is shown, titled 'FastQC Read Quality reports (Galaxy Version 0.72)'. It includes a section for 'Short read data from your current history' with a list of files, a 'Contaminant list' section, and a 'Submodule and Limit specifying file' section. A blue 'Execute' button is at the bottom.

1 Tools

fastqc

2 FASTQ Quality Control

FastQC: Read Quality reports

3 FastQC Read Quality reports (Galaxy Version 0.72)

Short read data from your current history

- 33: rna_3T.N4.fastq
- 32: rna_3T.N3.R2.fastq
- 31: rna_3T.N3.R1.fastq
- 30: rna_3T.N2.R2.fastq
- 29: rna_3T.N2.R1.fastq

This is a batch mode input

4 Contaminant list

Nothing selected

tab delimited file with 2 columns: name and sequence.

Submodule and Limit specifying file

Nothing selected

a file that specifies which submodules are to be executed

✓ Execute

SE – Quality trimming: Trimmomatic

1 Search for Trimmomatic

2 FASTQ Quality Control

3 Trimmomatic flexible read trimming tool for Illumina NGS data (Galaxy Version 0.36.5)

4 Input FASTQ file

5 Average quality required

Trimmomatic flexible read trimming tool for Illumina NGS data (Galaxy Version 0.36.5)

Single-end or paired-end reads?

Single-end

Input FASTQ file

- 33: rna_3T.N4.fastq
- 32: rna_3T.N3.R2.fastq
- 31: rna_3T.N3.R1.fastq
- 30: rna_3T.N2.R2.fastq
- 29: rna_3T.N2.R1.fastq
- 28: rna_3T.N1.fastq
- 27: rna_1T.N4.R2.fastq

This is a batch mode input field. Separate jobs will be triggered

Perform initial ILLUMINACLIP step?

Yes No

Cut adapter and other illumina-specific sequences from the read

Trimmomatic Operation

1: Trimmomatic Operation

Select Trimmomatic operation to perform

Sliding window trimming (SLIDINGWINDOW)

Number of bases to average across

4

Average quality required

20

2: Trimmomatic Operation

Select Trimmomatic operation to perform

Drop reads below a specified length (MINLEN)

Minimum length of reads to be kept

20

PE – Quality trimming: Trimmomatic

Tools

trimmomatic

FASTA/FASTQ

[fastp](#) - fast all-in-one preprocessing for FASTQ files

FASTQ Quality Control

[Trimmomatic](#) flexible read trimming tool for Illumina NGS data

Assembly

[Shovill](#) Faster SPAdes assembly of Illumina reads

Workflows

- [All workflows](#)

Trimmomatic flexible read trimming tool Illumina NGS data (Galaxy Version 0.36.5)

Single-end or paired-end reads?

Paired-end (two separate input files)

Input FASTQ file (R1/first of pair)

23: rna_1T.N2.R2.fastq
22: rna_1T.N2.R1.fastq
21: rna_1T.N1.R2.fastq
20: rna_1T.N1.R1.fastq
19: rbs_3T.N4.R2.fastq
18: rbs_3T.N4.R1.fastq

This is a batch mode input field. Separate jobs will be triggered for each file.

Input FASTQ file (R2/second of pair)

9: rbs_1T.N3.R2.fastq
8: rbs_1T.N3.R1.fastq
7: rbs_1T.N2.R2.fastq
6: rbs_1T.N2.R1.fastq
5: rbs_1T.N1.R2.fastq
4: rbs_1T.N1.R1.fastq

This is a batch mode input field. Separate jobs will be triggered for each file.

Perform initial ILLUMINACLIP step?

Yes No

Cut adapter and other illumina-specific sequences from the read

RNA – SE – Mapping: Hisat2

Tools

hisat2

Mapping

HISAT2 A fast and sensitive alignment program

RNA Analysis

StringTie transcript assembly and quantification

Workflows

All workflows

HISAT2 A fast and sensitive alignment program (Galaxy Version 2.1.0+galaxy4)

Source for the reference genome

Use a genome from history

Built-in references were created using default

Select the reference genome

1: hg38.goi.fa

Is this a single or paired library

Single-end

FASTA/Q file

96: Trimmomatic on rbs_1T.N1.R1.fastq (paired)
95: Trimmomatic on rna_3T.N4.fastq
94: Trimmomatic on rna_3T.N1.fastq
33: rna_3T.N4.fastq
32: rna_3T.N3.R2.fastq
31: rna_3T.N3.R1.fastq

This is a batch mode input field. Separate jobs will be t

Must be of datatype "fastqsanger" or "fasta"

Specify strand information

Unstranded

'F' means a read corresponds to a transcript. 'R' means a read corresponds to the
every read alignment will have an XS attribute tag: '+' means a read belongs to a t
strand of genome. (--rna-strandness)

Summary Options

Advanced Options

Execute

RNA – PE – Mapping: Hisat2

Tools

hisat2

Mapping

HISAT2 A fast and sensitive alignment program

RNA Analysis

StringTie transcript assembly and quantification

Workflows

All workflows

HISAT2 A fast and sensitive alignment program (Galaxy Version 2.1.0+galaxy4)

Source for the reference genome

Use a genome from history

Built-in references were created using default options

Select the reference genome

1: hg38.goi.fa

Is this a single or paired library

Paired-end

FASTA/Q file

4

148: Trimmomatic on rna_3T.N2.R1.fastq (R1 paired)

147: Trimmomatic on rna_3T.N2.R2.fastq (R2 unpaired)

146: Trimmomatic on rna_3T.N2.R1.fastq (R1 unpaired)

145: Trimmomatic on rna_3T.N2.R2.fastq (R2 paired)

144: Trimmomatic on rna_3T.N2.R1.fastq (R1 paired)

5

149: Trimmomatic on rna_3T.N2.R2.fastq (R2 paired)

148: Trimmomatic on rna_3T.N2.R1.fastq (R1 paired)

147: Trimmomatic on rna_3T.N2.R2.fastq (R2 unpaired)

146: Trimmomatic on rna_3T.N2.R1.fastq (R1 unpaired)

145: Trimmomatic on rna_3T.N2.R2.fastq (R2 paired)

144: Trimmomatic on rna_3T.N2.R1.fastq (R1 paired)

This is a batch mode input field. Separate jobs will be created for each file.

Must be of datatype "fastqsanger" or "fasta"

FASTA/Q file

5

149: Trimmomatic on rna_3T.N2.R2.fastq (R2 paired)

148: Trimmomatic on rna_3T.N2.R1.fastq (R1 paired)

147: Trimmomatic on rna_3T.N2.R2.fastq (R2 unpaired)

146: Trimmomatic on rna_3T.N2.R1.fastq (R1 unpaired)

145: Trimmomatic on rna_3T.N2.R2.fastq (R2 paired)

144: Trimmomatic on rna_3T.N2.R1.fastq (R1 paired)

This is a batch mode input field. Separate jobs will be created for each file.

Must be of datatype "fastqsanger" or "fasta"

Specify strand information

Unstranded

'FR' means a read corresponds to a transcript. 'RF' means a read corresponds to a transcript. 'FF' means a read corresponds to a transcript. 'RR' means a read corresponds to a transcript. 'FR' means a read corresponds to a transcript. 'RF' means a read corresponds to a transcript. 'FF' means a read corresponds to a transcript. 'RR' means a read corresponds to a transcript.

used, every read alignment will have an XS attribute tag: '+' means a read belongs to the '+' strand of genome. ('-rna-strandness')

Paired-end options

Specify paired-end parameters

See "Alignment Options" section of Help below for information

Select the upstream/downstream mate orientations for a valid paired-end alignment

--fr

Read filter: Samtools view

The screenshot shows the Galaxy interface for the 'samtools view' tool. Red arrows and a red 'X' mark highlight specific features and a missing feature.

- 1**: Points to the 'Tools' menu.
- 2**: Points to the 'samtools view' tool in the search results.
- 3**: Points to the 'Filter SAM or BAM file to filter' section.
- 4**: Points to the 'SAM or BAM file to filter' input field, which contains a list of Bismark Mapper output files.
- 5**: Points to the 'Header in output' section, which has a checkbox for 'Include header'.
- 6**: Points to the 'Minimum MAPQ quality score' input field, which has a value of '1'.
- 7**: Points to the 'Skip alignments with any of these flag bits set' section, which has a checkbox for 'Select/Unselect all'.
- 8**: Points to the 'The alignment or this read is not primary' checkbox, which is checked.

A red 'X' mark is placed over the 'Filter SAM or BAM files on FLAG MAPQ RG LN or by region' link in the left sidebar.

Sort reads: Samtools sort

Tools

samtools sort

FASTA/FASTQ

- [UMI-tools group](#) Extract UMI from fastq files

SAM/BAM

- [Filter pileup](#) on coverage and SNPs
- [Generate pileup](#) from BAM dataset
- [SAM-to-BAM](#) convert SAM to BAM
- [Samtools flagstat](#) tabulate descriptive stats for BAM dataset
- [samtools mpileup](#) multi-way pileup of variants

Samtools sort order of storing aligned sequences (Galaxy Version 2.0.2)

BAM File

203: Filter SAM or BAM, output SAM or BAM on data 176: bam
202: Filter SAM or BAM, output SAM or BAM on data 174: bam
201: Filter SAM or BAM, output SAM or BAM on data 172: bam
200: Filter SAM or BAM, output SAM or BAM on data 170: bam
199: Filter SAM or BAM, output SAM or BAM on data 168: bam
198: Filter SAM or BAM, output SAM or BAM on data 166: bam
197: Filter SAM or BAM, output SAM or BAM on data 164: bam
196: Filter SAM or BAM, output SAM or BAM on data 162: bam
176: Bismark Mapper on data 125, data 124, and data 1: mapped

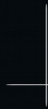
This is a batch mode input field. Separate jobs will be triggered

Primary sort key

coordinate

✓ Execute

Differential Gene expression



RNA – Quantification

- Requirements
 - Experiment strandness information
 - Prediction needs annotation in BED format

GTF to BED

The screenshot displays the Galaxy web interface. On the left, the 'Tools' panel is visible, featuring a search bar with the text 'gtf to bed' (marked with a red triangle and the number 1). Below the search bar, under the 'Convert Formats' section, three tools are listed: 'Convert gffC... to BED for ... results' (marked with a red triangle and the number 2), 'Convert GTF to BED12', and 'GTF-to-BEDGraph converter'. On the right, the configuration page for the 'Convert GTF to BED12 (Galaxy Version ...)' tool is shown. This page includes a 'GTF File to convert' section with file selection icons and a text box containing '2: hg38.goi.gtf' (marked with a red triangle and the number 3). Below this, the 'Advanced options' section contains a 'Use default options' button and a description: 'Advanced options for gtfToGenePred.' At the bottom of the configuration page is a blue 'Execute' button with a checkmark icon.

RNA – SE – Infer experiment

Tools

infer_experiment

RNA Analysis

Infer Experiment speculates how RNA-seq were configured

FPKM Count calculates raw read count, FPM, and FPKM for each gene

BAM to Wiggle converts all types of RNA-seq data from .bam to .wig

RPKM Count calculates raw count and RPKM values for transcript at exon, intron, and mRNA level

Workflows

- All workflows

Infer Experiment speculates how RNA-seq were configured (Galaxy Version 2.6.4.1)

Input .bam file

153: HISAT2 on data 94 and data 1: aligned reads (BAM)

(--input-file)

Reference gene model

177: Convert GTF to BED12 on data 2

(--refgene)

Number of reads sampled from SAM/BAM file (default = 200000)

200000

(--sample-size)

Minimum mapping quality

0

Minimum mapping quality for an alignment to be considered as "uniquely mapped" (--mapq)

This is SingleEnd Data

Fraction of reads failed to determine: 0.0000

Fraction of reads explained by "++,-": 0.5033

Fraction of reads explained by "+-,-+": 0.4967

RNA – PE – Infer experiment

Tools

infer_experiment

RNA Analysis

[Infer Experiment](#) speculates how RNA-seq were configured

[FPKM Count](#) calculates raw read count, FPM, and FPKM for each gene

[BAM to Wiggle](#) converts all types of RNA-seq data from .bam to .wig

[RPKM Count](#) calculates raw count and RPKM values for transcript at exon, intron, and mRNA level

Workflows

- [All workflows](#)

Infer Experiment speculates how RNA-seq were configured (Galaxy Version 2.6.4.1)

Input bam file

155: HISAT2 on data 129, data 128, and data 1: aligned reads (BAM)

(--input-file)

Reference gene model

177: Convert GTF to BED12 on data 2

(--refgene)

Number of reads sampled from SAM/BAM file (default = 200000)

200000

(--sample-size)

Minimum mapping quality

0

Minimum mapping quality for an alignment to be considered as "uniquely mapped" (--mapq)

This is PairEnd Data
Fraction of reads failed to determine: 0.0000
Fraction of reads explained by "1++,1--,2+-,2-+": 0.4941
Fraction of reads explained by "1+-,1-+,2++,2--": 0.5059

RNA – SE – Quantify: featurecounts

Tools

featurecounts

Annotation

[goseq](#) tests for overrepresented gene categories

RNA Analysis

[limma](#) Perform differential expression with limma-voom or limma-trend

[edgeR](#) Perform differential expression of count data

[DESeq2](#) Determines differentially expressed features from count tables

[featureCounts](#) Measure gene expression in RNA-Seq experiments from SAM or BAM files.

[Remove Unwanted Variation](#) from RNA-seq data

Workflows

- [All workflows](#)

featureCounts Measure gene expression in RNA-Seq experiments from SAM or BAM files. (Galaxy Version 1.6.3+galaxy2)

Alignment file

157: HISAT2 on data 137, data 136, and data 1: aligned reads (BAM)
156: HISAT2 on data 133, data 132, and data 1: aligned reads (BAM)
155: HISAT2 on data 129, data 128, and data 1: aligned reads (BAM)
154: HISAT2 on data 95 and data 1: aligned reads (BAM)
153: HISAT2 on data 94 and data 1: aligned reads (BAM)

This is a batch mode input field. Separate jobs with a new line.

The input alignment file(s) where the gene expression has to be counted. The input can be a single file or a list of files. If you are using a Gene annotation file in the History, these files must have the same prefix.

Specify strand information

Unstranded

Indicate if the data is stranded and if strand-specific read counting should be performed. If the data is unstranded, the output will be the same regardless of the strand of the mapped BAM input(s) (-s)

Gene annotation file

in your history

Gene annotation file

2: hg38.goi.gtf

The program assumes that the provided annotation file is in GTF format. The output will be tabular, select the preferred columns here

Output format

Gene-ID "\t" read-count (MultiQC/DESeq2/edgeR/limma-voom compatible)

The output format will be tabular, select the preferred columns here

Count multi-mapping reads/fragments

Disabled; multi-mapping reads are excluded (default)

If specified, multi-mapping reads/fragments will be counted (ie. a multi-mapping read will be counted once for each mapping location). Use the '-M' tag to find multi-mapping reads. (-M)

Minimum mapping quality per read

0

The minimum mapping quality score a read must satisfy in order to be counted. (-q)

Exon-exon junctions

Yes No

If specified, reads supporting each exon-exon junction will be counted (-J)

Long reads

Yes No

If specified, long reads such as Nanopore and PacBio reads will be counted. (-L)

Count reads by read group

Yes No

If specified, reads are counted for each read group separately. The 'RG' tag is required. (-g)

Largest overlap

Yes No

If specified, overlapping reads/fragments will be assigned to the target that has the largest overlap. (-O)

Minimum bases of overlap

10

Specify the minimum required number of overlapping bases between a read and a feature. (-b)

RNA – PE – Quantify: featurecounts

The screenshot displays the Galaxy web interface for the **featureCounts** tool. The interface is divided into a left sidebar with navigation links and a main workspace with input fields and options. Red arrows numbered 1 through 8 highlight specific elements:

- 1** Points to the **Alignment** input field, which contains a list of BAM files (e.g., 160: HISAT2 on data 149, data 148, and data 1: aligned reads (BAM)).
- 2** Points to the **Gene annotation file** input field, which contains the file **2: hg38.goi.gtf**.
- 3** Points to the **Specify strand information** dropdown menu, which is set to **Unstranded**.
- 4** Points to the **Gene annotation file** input field, which contains the file **2: hg38.goi.gtf**.
- 5** Points to the **Gene annotation file** input field, which contains the file **2: hg38.goi.gtf**.
- 6** Points to the **Create gene-length file** dropdown menu, which is set to **No**.
- 7** Points to the **Count multi-mapping reads/fragments** dropdown menu, which is set to **Disabled; multi-mapping reads are excluded (default)**.
- 8** Points to the **Minimum bases of overlap** input field, which is set to **10**.

The main workspace also includes a **Count multi-mapping reads/fragments** section with a **Minimum mapping quality per read** input field (set to 0) and a **Long reads** section with a **Count reads by read group** dropdown menu (set to **No**). The **Options for paired-end reads** section includes a **Count fragments instead of reads** dropdown menu (set to **Enabled; fragments (or templates) will be counted instead of reads**).

RNA – Diff. expression: DESeq2

The screenshot shows the Galaxy DESeq2 tool interface. On the left is a 'Tools' sidebar with a search bar containing 'deseq2'. Below the search bar are sections for 'Annotation', 'RNA Analysis', 'Peak Calling', and 'Workflows'. The 'RNA Analysis' section is highlighted, showing 'DESeq2 Determines differentially expressed features from count tables'. On the right is the main tool configuration panel titled 'DESeq2 Determines differentially expressed features from count tables (Galaxy Version 2.11.40.6)'. It contains several input fields and sections. Red arrows with numbers 1 through 9 point to specific elements: 1 points to the 'Tools' search bar; 2 points to the 'DESeq2' tool description; 3 points to the 'Factor' section header; 4 points to the 'Specify a factor name' field; 5 points to the 'Factor level' section header; 6 points to the 'Specify a factor level' field; 7 points to the 'Factor level' section header; 8 points to the 'Specify a factor level' field; 9 points to the 'Factor level' section header. The 'Factor' section includes a '1: Factor' subsection with a 'Specify a factor name' field containing 'placenta'. Below this is a 'Factor level' subsection with a 'Specify a factor level' field containing '3t'. The 'Count file(s)' section shows a list of files, including '188: featureCounts on data 2 and data 157: Counts'. The '2: Factor level' subsection is also visible, with a 'Specify a factor level' field containing '1t' and a 'Count file(s)' section showing a list of files, including '182: featureCounts on data 2 and data 154: Counts'.

Tools

deseq2

Annotation

[Annotate DE\(X\)Seq results](#)

RNA Analysis

[edgeR](#) Perform differential expression analysis of count data

[DESeq2](#) Determines differentially expressed features from count tables

[Annotate DESeq2/DEXSeq output tables](#) Append annotation from GTF to differential expression tool outputs

[featureCounts](#) Measure gene expression in RNA-Seq experiments from SAM or BAM files.

[Remove Unwanted Variation](#) from RNA-seq data

[StringTie](#) transcript assembly and quantification

Peak Calling

[PEAKachu](#) Calls Peaks in CLIP data

[DiffBind](#) differential binding analysis of ChIP-Seq peak data

Workflows

- All workflows

DESeq2 Determines differentially expressed features from count tables (Galaxy Version 2.11.40.6)

how

Select datasets per level

Factor

1: Factor

Specify a factor name, e.g. `subjects_drug_x` or `cancer_markers`

placenta

Only letters, numbers and underscores will be retained in this field

Factor level

1: Factor level

Specify a factor level, e.g. `tumor`, `normal`, `treated` or `control`

3t

Only letters, numbers and underscores will be retained in this field

Count file(s)

188: featureCounts on data 2 and data 157: Counts
187: featureCounts on data 2 and data 156: Summary
186: featureCounts on data 2 and data 156: Counts
185: featureCounts on data 2 and data 155: Summary
184: featureCounts on data 2 and data 155: Counts

2: Factor level

1: Factor level

Specify a factor level, e.g. `tumor`, `normal`, `treated` or `control`

1t

Only letters, numbers and underscores will be retained in this field

Count file(s)

182: featureCounts on data 2 and data 154: Counts
181: featureCounts on data 2 and data 153: Summary
180: featureCounts on data 2 and data 153: Counts
177: Convert GTF to BED12 on data 2
3: hg38.promoter.bed

+ Insert Factor level

+ Insert Factor

(Optional) provide a tabular file with additional batch factors to include in the model.

Cut columns: cut

Tools

cut

Text Manipulation

- [Replace Text in entire line](#)
- [Text reformating with awk](#)
- [Cut columns from a table \(cut\)](#)
- [Cut columns from a table](#)

Filter and Sort

- [Filter sequences by ID from a tabular file](#)
- [Select lines that match an expression](#)

FASTA/FASTQ

- [Filter by quality](#)
- [fastp - fast all-in-one preprocessing for FASTQ files](#)

Cut columns from a table (cut) (Galaxy Version 1.1.0)

File to cut

247: Compute on data 246

Operation

Keep

Delimited by

Tab

Cut by

fields

List of Fields

Select/Unselect all

Column: 1 Column: 3

(-f)

✓ Execute

Tag results

Edit dataset attributes

Attributes

Convert

Datatypes

Permissions

Edit attributes

Auto-detect

Save

Name

DGE.tsv

Info

Annotation

History

search datasets

Rostock

245 shown, 8 [deleted](#), 8 [hidden](#)

868.02 MB

253: Cut on data 249

22 lines

format: **tabular**, database: ?

1

2

ENSG00000110934

2.23208401512721

ENSG00000136859

1.40905050600934

ENSG00000143850

-1.89543026151967

ENSG00000137868

-2.06915939568834

ENSG00000166444

-1.71738157515273