NGS read mapping

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NGS read mapping

- Introduction to NGS read mapping
- Short read mappers
- Specificity of RNA-seq read mapping
- *Exercise 1 : Mapping of RNA-seq data using Galaxy*
- Alignment and related file formats
- Alignment visualization
- *Exercise 1 : Interpretation of results*
- *Exercise 2 : Whole dataset alignment visualization*
- Quality control of RNA-seq data based on alignments
  - *Exercise 3 : QC on alignments*

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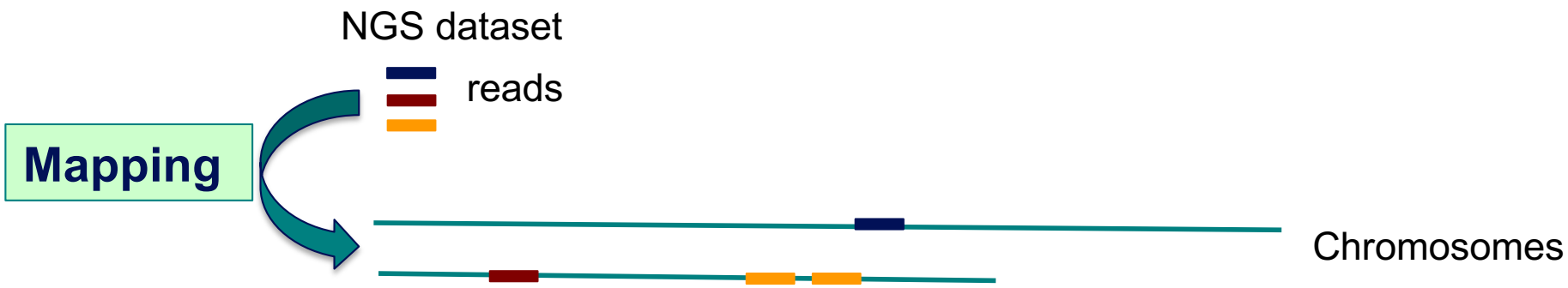
What is mapping ?

- Map reads against a reference genome
  = Predict the locus from which a read originates
  ➔ Find the loci with sufficient similarity



- Sufficient similarity
  ➔ Less mismatches / indels

Alignment

reference genome `CACGTACC` `CACGTA_CC` `CACGTACC`

reads `CACGTTCC` `CACGTATCC` `CACGT_CC`

mismatch indels (insertion/deletion)

Challenges of short read mapping

- Reference sequence can be large (~3 Gb for human)
- Short reads ➔ several, equally likely places in reference sequence from which they could have been read
  e.g. repetitive regions
- The genome from which reads have been generated may be different from the reference genome
  ➔ Need to allow mismatches and indels
- Need to tolerate sequencing errors in reads
- Need to do that for each of the millions of reads !

➔ Too long with traditional mappers such as BLAST or BLAT

➔ Specialized read mappers with highly efficient algorithms

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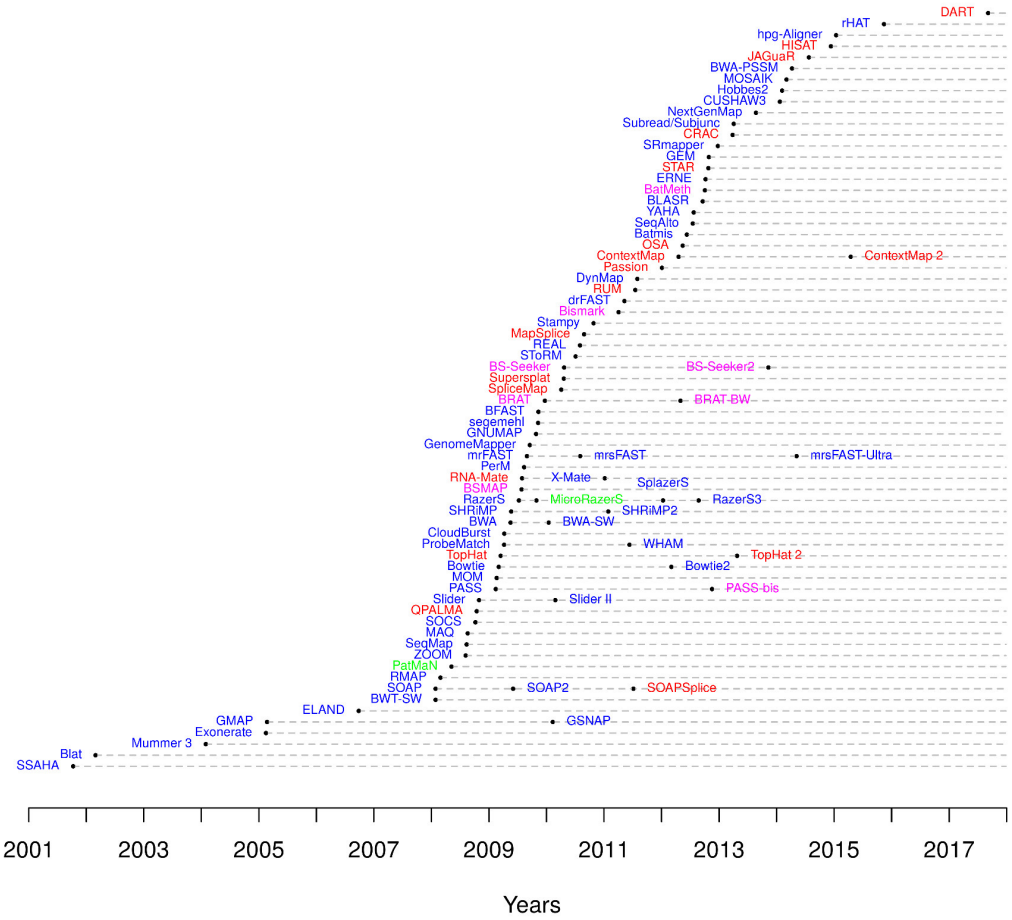
Computational strategies

- Indexing
  - Like the index at the end of a book
    ➔ an index of a large DNA sequence allows one to rapidly find shorter sequences embedded within it
- Transforming
  - Uses a technique originally developed for compressing large files called the Burrows-Wheeler transform (BWT)
    ➔ The transformed human genome fits into memory
- Example : Bowtie2 (*Langmead et al. Nature Methods 2012)*
  - To rapidly narrow the number of possible alignments that must be considered
    - Begins by extracting substrings (“seeds”) from each read and its reverse complement
    - Aligning them in an ungapped fashion using an index

    → Trade-off between speed and sensitivity can be adjusted by setting the seed length, the interval between extracted seeds and the number of mismatches in seed
  - Extend seeds to full reads alignment (allowing gaps)

A lot of tools developed …

- More than 90 mapping tools



DNA mappers
RNA mappers
miRNA mappers
bisulfite mappers

http://wwwdev.ebi.ac.uk/fg/hts_mappers/

How to choose a mapper ?

- Main criteria to take into account
  - Sensitivity
    - Ability to align a large fraction of reads with errors and variants
  - Accuracy
    - If an aligner aligns a large fraction of reads, but most alignments are wrong, this is useless !
  - Type of data (DNA, RNA), support of paired-end
  - Read length limits
  - Quality aware
  - Multi-mapping reporting
  - Speed
  - Memory requirements

- Feature comparison
  - Fonseca et al. Bioinformatics 2012;28 (24): 3169-3177

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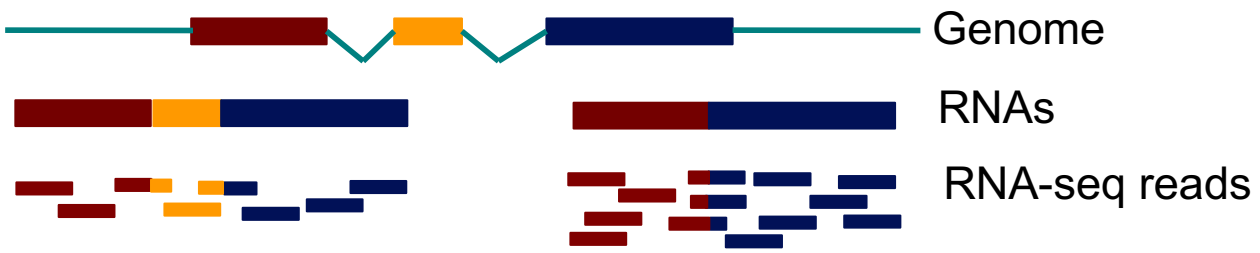
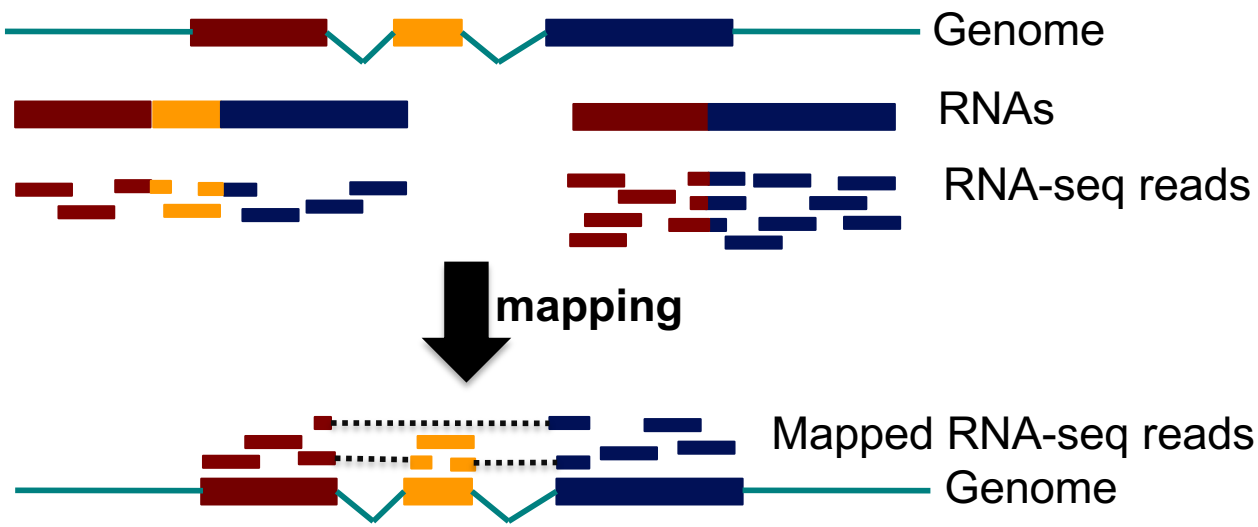
Specificity of RNA-seq reads



➔ In an RNA-seq library, several reads span exon junctions

Spliced mapping

- Allows mapping of reads across splice junctions



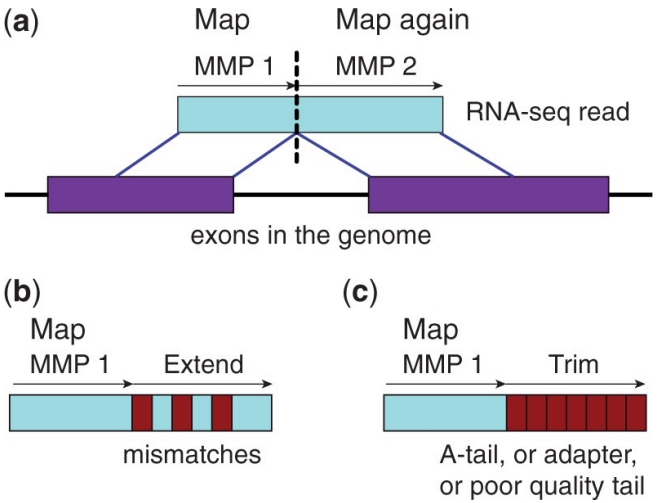
- Spliced alignment programs comparison
  - Engström et al. Nature Methods 2013
  - Baruzzo et al. Nature methods 2017

STAR
Spliced Transcripts Alignment to a Reference

1. Searching for seeds

- For every read : searches for the longest sequence that exactly matches one or more locations on the reference genome : Maximal Mappable Prefix (MMP)
  → MMP1 (seed 1)
- Searches for only the unmapped portion of the read to find the next longest sequence that exactly matches the reference genome → MMP2
- MMP search enables finding mismatches or tails :

  If MMP search does not reach the end of a read
  → MMPs serve as anchors in the genome that can be extended
  → If the extended alignment is not good : tail is soft-clipped



2. Stitching all seeds

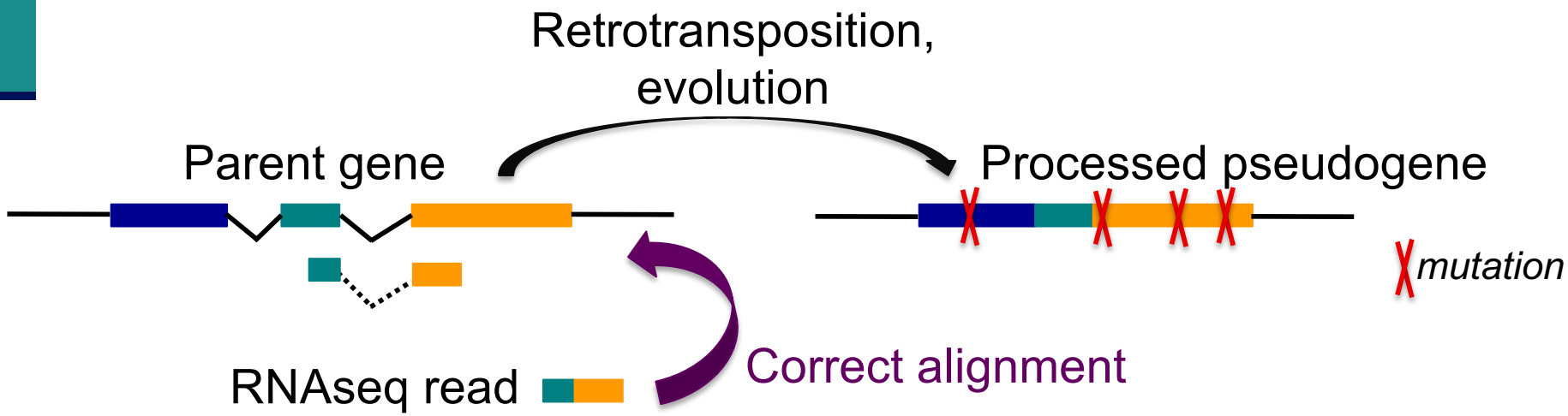
→ alignment of the entire read sequence

Dobin et al. Bioinformatics 2013

Main problems of RNA-seq aligners

- Difficult to accurately detect splicing events involving short sequence overhangs on the donor or acceptor side of a junction

- Alignments biased toward processed pseudogenes



Use of annotations in spliced mapping

- Use splice junctions annotations to mitigate this problem
- STAR
  - Option to provide annotations
  - Incorporates annotated junction sequences into the suffix array
  - Searches the seeds that cross the junctions simultaneously with the seeds that map contiguously to the genome

Genome annotations

- Ensembl project (www.ensembl.org)
  - Goal : automatically annotate genomes, integrate this annotation with other available biological data and make all this publicly available
  - Includes manual curation (by HAVANA) for some species : human, mouse, zebrafish, rat
  - Ensembl data is released on an approximately three-month cycle
- Ensembl genome annotations available on
  - ftp://ftp.ensembl.org/pub/
  - Important to use the same annotation version throughout a project, access to old versions via · View in archive site
- The main Ensembl site focuses on vertebrate genomes and some other representative species (http://www.ensembl.org/info/about/species.html), other sites are dedicated to plants, fungi, bacteria (cf “Our sister sites” links at the bottom of www.ensembl.org)
- Other annotation sources
  - e.g., ordered from most to least complex : AceView, Ensembl, UCSC, Refseq Genes (Wu et al. BMC Bioinformatics 2013 ;14 Suppl 11:S8)

Genome annotations

- Generally provided in a GTF (Gene Transfert Format) / GFF (General Feature Format) file
- GTF file :
  - Tab-delimited text file format
  - Each line correspond to an annotation or feature
  - Specifications :
    - http://www.ensembl.org/info/website/upload/gff.html
  - e.g. human Ensembl 105 GTF file
    - ftp.ensembl.org/pub/release-105/gtf/homo_sapiens/Homo_sapiens.GRCh38.105.chr.gtf.gz
  - Caution : use annotations corresponding to the version of genome assembly you are working on
    - **GRCh38 (1 - 22, X, Y, MT)** / hg38 (chr1 - chr22, chrX, chrY, chrM)

Genome annotations

- Generally provided in a GTF (Gene Transfert Format) file
  - Nine columns :

Seqid	Source	Type	Start	End	Score	Strand	Phase	Attributes
2	ensembl_havana	gene	227813842	227817564	.	+	.	
2	havana	transcript	227813842	227817564	.	+	.	
2	havana	exon	227813842	227813987	.	+	.	
2	havana	CDS	227813912	227813987	.	+	0	
2	havana	start_codon	227813912	227813914	.	+	0	
2	havana	exon	227815457	227815568	.	+	.	
2	havana	CDS	227815457	227815568	.	+	2	

gene_id "ENSG00000115009"; gene_version "11"; transcript_id "ENST00000409189"; transcript_version "7"; exon_number "1"; gene_name "CCL20"; gene_source "ensembl_havana"; gene_biotype "protein_coding"; havana_gene "OTTHUMG00000133189"; havana_gene_version "3"; transcript_name "CCL20-001"; transcript_source "havana"; transcript_biotype "protein_coding"; …

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Exercise 1
Mapping of RNA-seq data using Galaxy

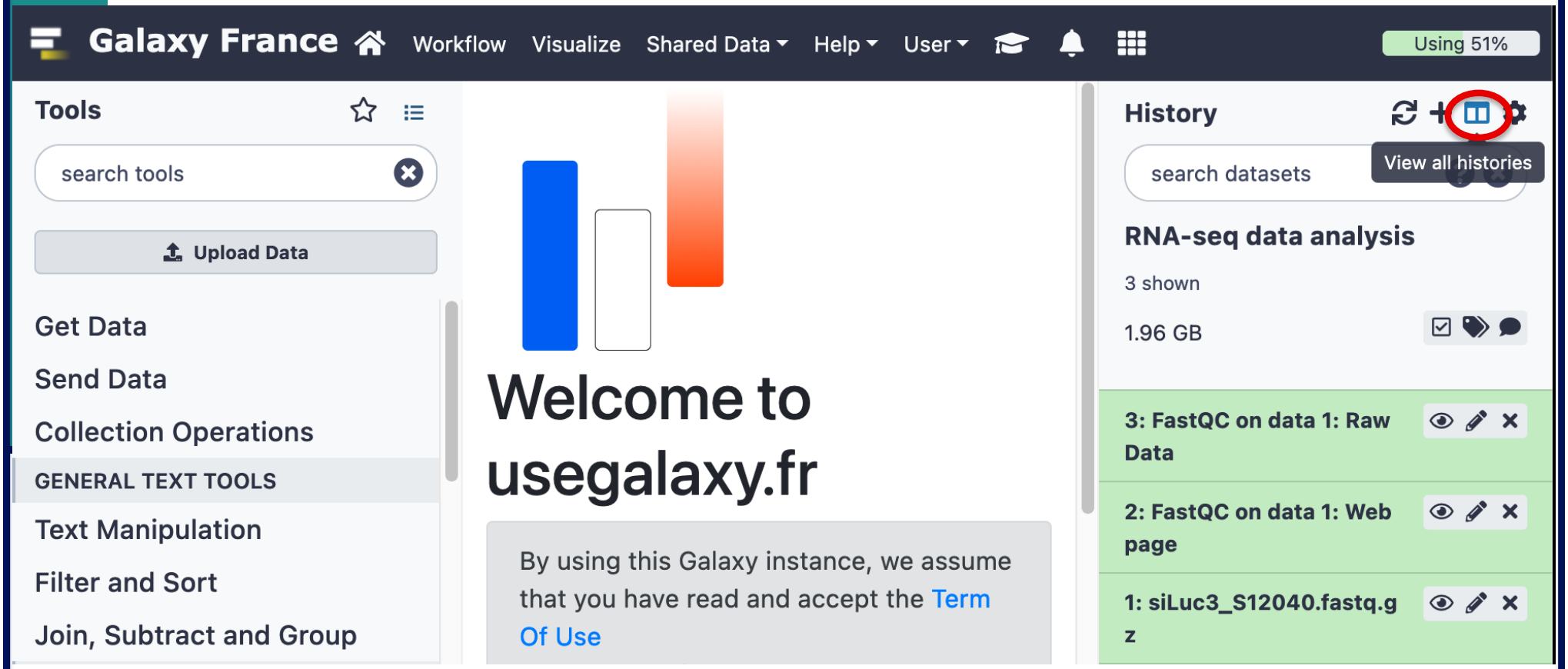
- Map **1 million** reads from siLuc2 mRNA-seq sample using STAR
  1. Copy to your history
     - The corresponding FASTQ file :

       2: siLuc2_1000000.fastq.gz
     - The GTF annotation file :

       3: Homo_sapiens.GRCh38.105.chr.gtf.gz
  2. Launch STAR on this FASTQ file using
     - **homo_sapiens (GRCh38)** reference genome
     - Homo_sapiens.GRCh38.105.chr.gtf.gz GTF annotation file

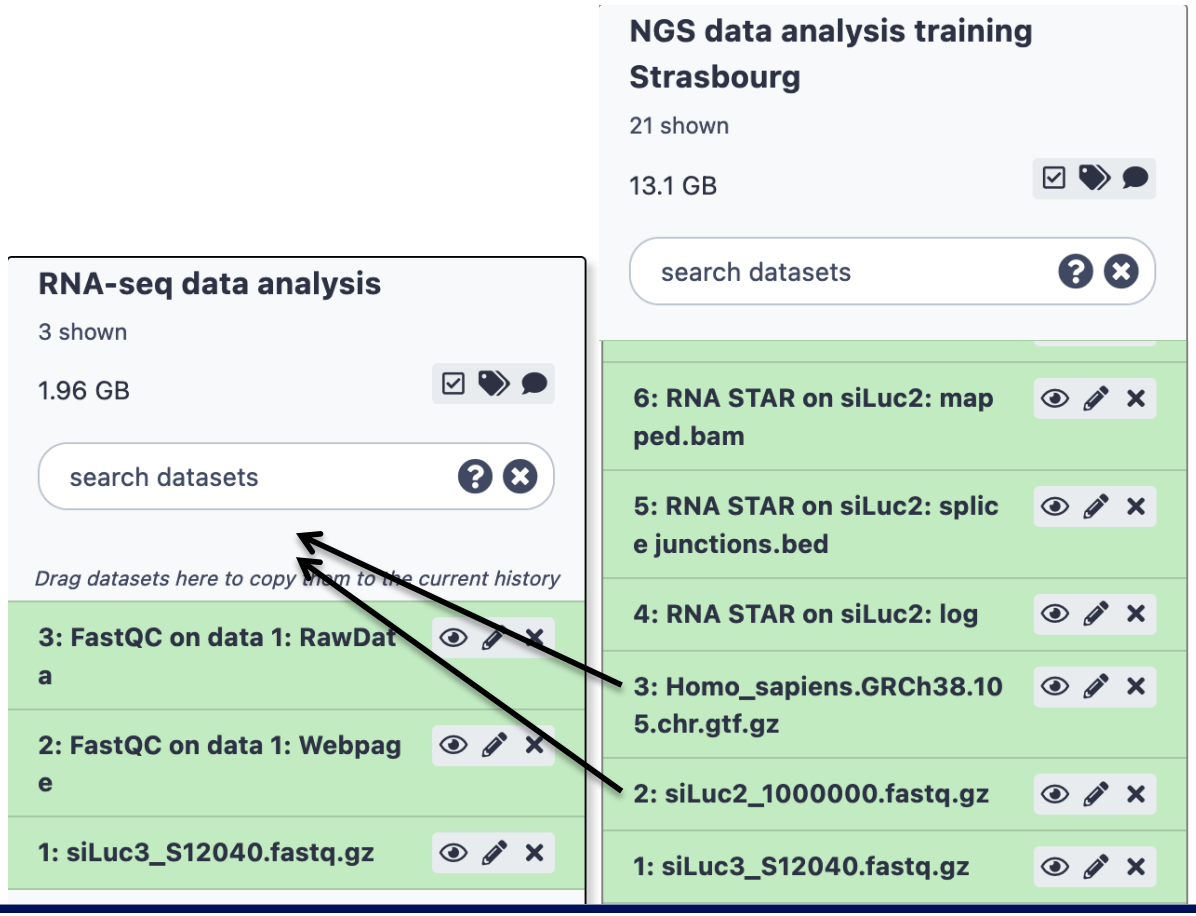
Exercise 1
1. Copy files to your history

- Click on “View all histories”



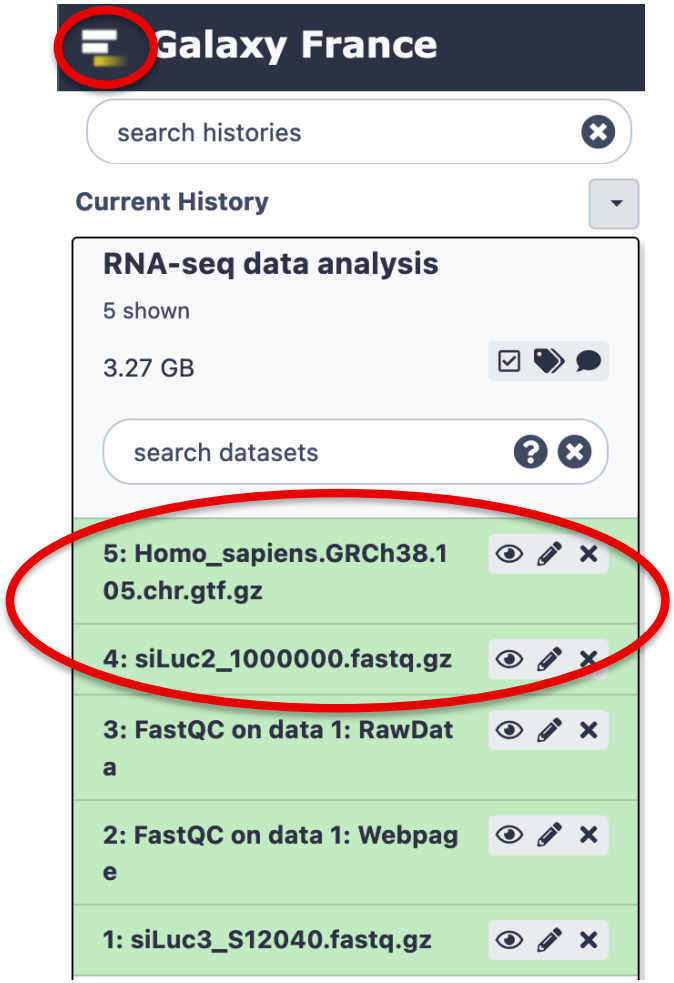
Exercise 1
1. Copy files to your history

- Drag datasets 2 and 3
  - from “NGS data analysis training Strasbourg” history
  - to “RNA-seq data analysis” history

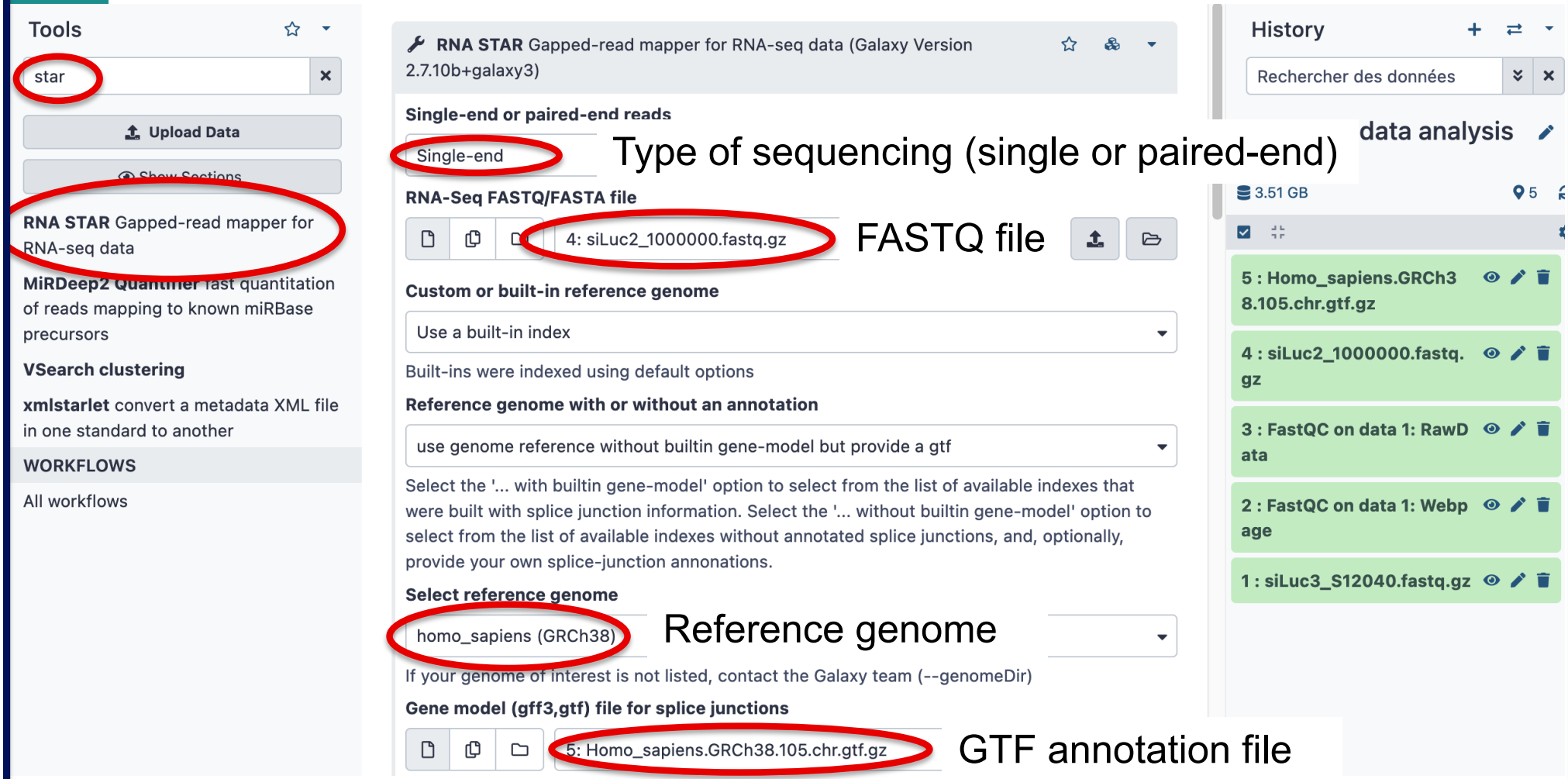


Exercise 1
1. Copy files to your history

- You have now in your history all files needed to launch STAR :



Exercise 1
2. Launch STAR



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Alignment file format : SAM

- Sequence Alignment/Map format → standard alignment format
- Text file containing all information about an alignment
- SAM format specifications
  - Li et al., Bioinformatics 2009;25(16):2078-9.
  - http://samtools.github.io/hts-specs/SAMv1.pdf

- Header section
  - Generic information regarding the SAM file, not required
  - Each line starts with @ and is tab-delimited
  - @HD : SAM file version, whether the file is sorted
  - @SQ : Name + length of reference sequences used for alignment
  - …

Header section example :

```
@HD VN:1.4 SO:coordinate
@SQ SN:1 LN:248956422
@SQ SN:10 LN:133797422
@SQ SN:11 LN:135086622
@SQ SN:12 LN:133275309
```

…

Alignment file format : SAM

- Alignment section : 11 mandatory fields + optional fields
- Mandatory fields :

Col	Field	Type	N/A Value	Description
1	QNAME	string	mandatory	The query/read name.
2	FLAG	int	mandatory	The record's flag.
3	RNAME	string	*	The reference name.
4	POS	32-bit int	0	1-based position on the reference.
5	MAPQ	8-bit int	255	The mapping quality.
6	CIGAR	string	*	The CIGAR string of the alignment.
7	RNEXT	string	*	The reference of the next mate/segment.
8	PNEXT	string	0	The position of the next mate/seqgment.
9	TLEN	string	0	The observed length of the template.
10	SEQ	string	*	The query/read sequence.
11	QUAL	string	*	The ASCII PHRED-encoded base qualities.

Alignment section example :

```
HWI-ST1136:225:HS140:8:1313:4696:85038  256 1   14406   0   1S49M   *   0   0   GTCTGCTCAGTTCTTTATTGATTGGTGTGCCGTTTTCTCTGGAAGCCTCT
CCCFFFFFHHHHHJJJJJJJIJJJJHIHIIJJIJJJIJJJJJIJJJJJJJ  NH:i:8 HI:i:5 AS:i:48 nM:i:0
HWI-ST1136:225:HS140:8:2308:6999:65454  0   1   14453   1   50M *   0   0   CTTAAGAACACAGTGGCGCAGGCTGGGTGGAGCCGTCCCCCCATGGAGCA
@@@DDBFFGHHDFADFABBGGAHGDBB??00?DDB?FHHIIE=BD;;@>?  NH:i:3 HI:i:1 AS:i:49 nM:i:0
HWI-ST1136:225:HS140:8:2109:9064:22156  0   1   14467   0   50M *   0   0   GGCGCAGGCTGGGTGGAGCCGTCCCCCCATGGAGCACAGGCAGACAGAAG
```

Alignment file format : SAM

- Flag (number)

  Describes the alignment

  e.g. reverse strand, not primary alignment, unmapped

  Explain SAM flags in plain English :

  https://broadinstitute.github.io/picard/explain-flags.html

- Mapping quality (number)

  Indicates whether the read is correctly mapped to this location in the reference genome

  - STAR mapping quality
    - 60 by default on Galaxy for uniquely mapped reads
    - int(-10*$\log_{10}$(1-1/N_{map})) for multi-mapping reads

      N_{map} : the number of loci a read maps to

N_{map}	MAPQ
2	3
3-4	1
≥ 5	0

Alignment file format : SAM

- CIGAR (string)
  - M : alignment (can be a sequence match or mismatch)
  - I : insertion to the reference
  - D : deletion from the reference
  - N : skipped region from the reference
  - S : soft clipping (clipped sequences present in SEQ)
    - Bases of the read that are not aligned
  - H : hard clipping (clipped sequences not present in SEQ)
    - Bases of the read that are not aligned and that have been removed from the read sequence in the SAM file

Alignment file format : SAM

- CIGAR example
  - Alignment :

```
Reference ➔ C A T A C T _ G A A C T G A C T A A C
    Read ➔       A C T A G A A _ T G G C T
```

  - CIGAR :

`3M1I3M1D5M`

    - 3M : the first 3 bases in the read sequence align with the reference
    - 1I : the next base in the read does not exist in the reference
    - 3M : then 3 bases align with the reference
    - 1D : the next reference base does not exist in the read sequence
    - 5M : then 5 more bases align with the reference
      - Note that among these bases one is different from the reference but it still counts as an M since it aligns to that position

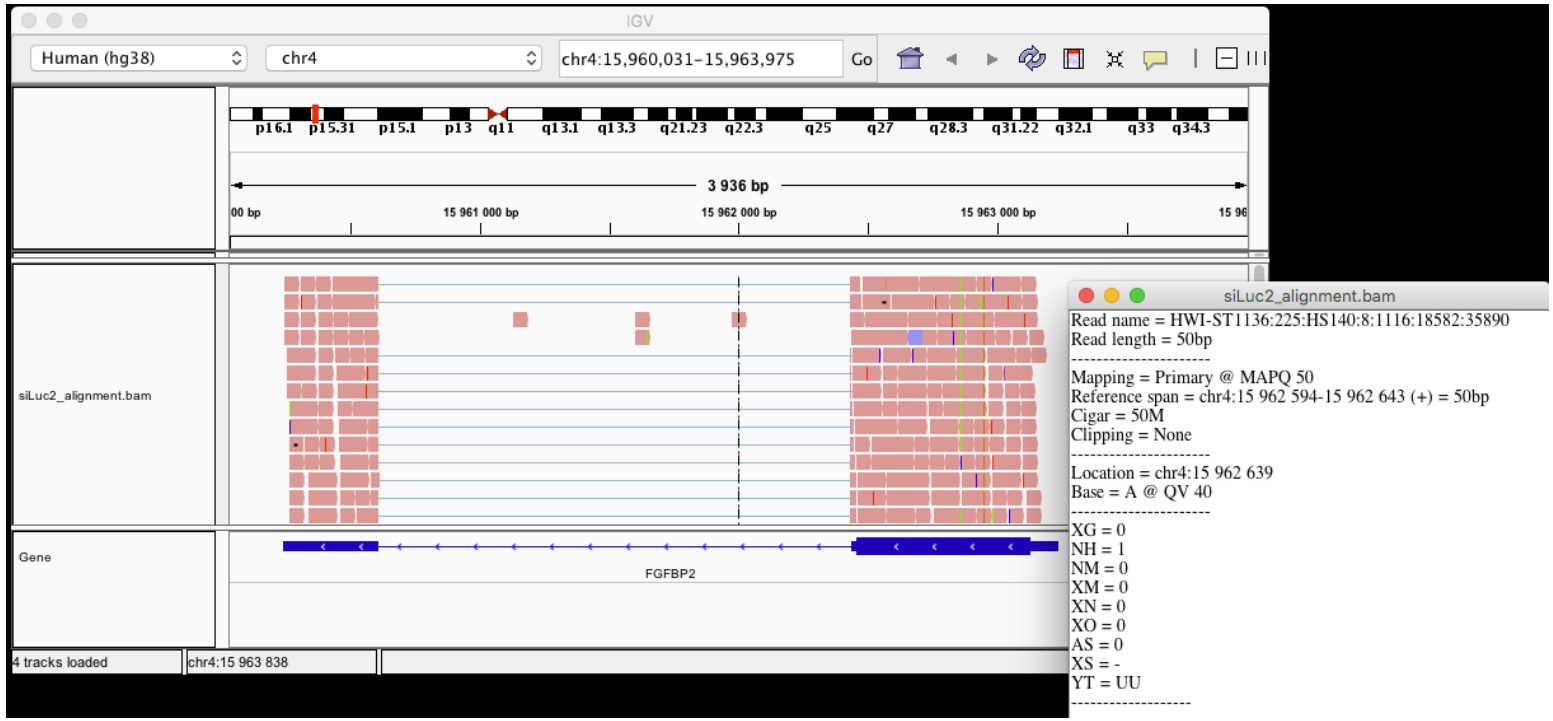
Alignment file format : SAM

- Additional tags (format tag:type:value)

Tag[4]	Type	Description
X?	?	Reserved fields for end users (together with Y? and Z?)
AM	i	The smallest template-independent mapping quality of segments in the rest
AS	i	Alignment score generated by aligner
BC	Z	Barcode sequence, with any quality scores stored in the QT tag.
BQ	Z	Offset to base alignment quality (BAQ), of the same length as the read sequence. At the i-th read base, $\mathrm{BAQ}_i = Q_i - (\mathrm{BQ}_i - 64)$ where Q_i is the i-th base quality.
CC	Z	Reference name of the next hit; '=' for the same chromosome
CM	i	Edit distance between the color sequence and the color reference (see also NM)
CO	Z	Free-text comments
CP	i	Leftmost coordinate of the next hit
CQ	Z	Color read quality on the original strand of the read. Same encoding as QUAL; same length as CS.
CS	Z	Color read sequence on the original strand of the read. The primer base must be included.
CT	Z	Complete read annotation tag, used for consensus annotation dummy features[5].
E2	Z	The 2nd most likely base calls. Same encoding and same length as QUAL.
FI	i	The index of segment in the template.
FS	Z	Segment suffix.
FZ	B,S	Flow signal intensities on the original strand of the read, stored as (uint16_t) round(value * 100.0).
LB	Z	Library. Value to be consistent with the header RG-LB tag if @RG is present.
H0	i	Number of perfect hits
H1	i	Number of 1-difference hits (see also NM)
H2	i	Number of 2-difference hits
HI	i	Query hit index, indicating the alignment record is the i-th one stored in SAM
IH	i	Number of stored alignments in SAM that contains the query in the current record
MC	Z	CIGAR string for mate/next segment
MD	Z	String for mismatching positions. *Regex*: [0-9]+(([A-Z]|\^[A-Z]+)[0-9]+)*[6]
MQ	i	Mapping quality of the mate/next segment
NH	i	Number of reported alignments that contains the query in the current record
NM	i	Edit distance to the reference, including ambiguous bases but excluding clipping

Alignment file format : BAM

- Binary file
- Compressed version of SAM format
- BAM files can be sorted and indexed
  - Makes accessing data very fast
- BAI (extension .bai) : index for a BAM file
  - sample.bam.bai index for sample.bam file



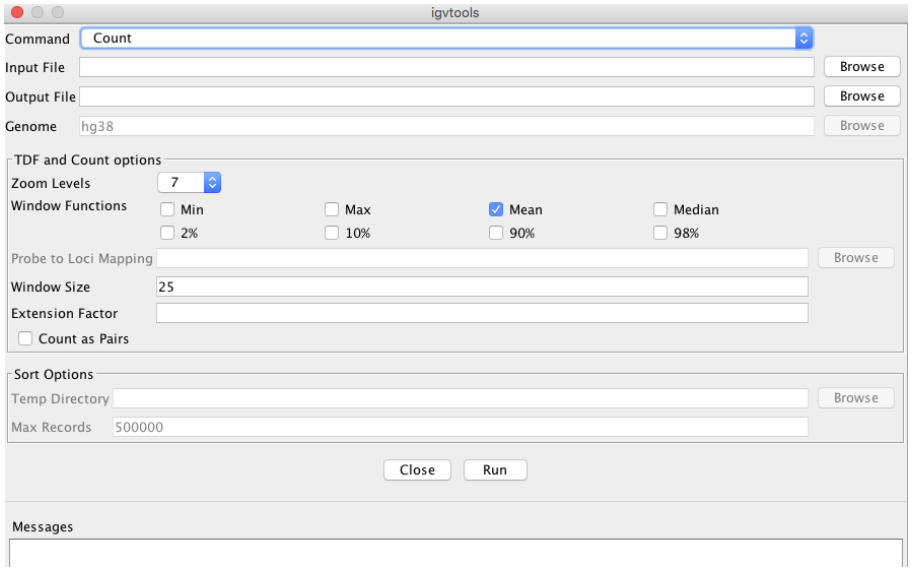
Utilities to manipulate SAM/BAM files

- Samtools (http://www.htslib.org/)
  - Various utilities for manipulating alignment in SAM format (SAM <> BAM conversion, calculating statistics on alignments, …) – available on Galaxy

- Igvtools (http://software.broadinstitute.org/software/igv/)
  - sort, index, …
  - Integrative Genomics Viewer

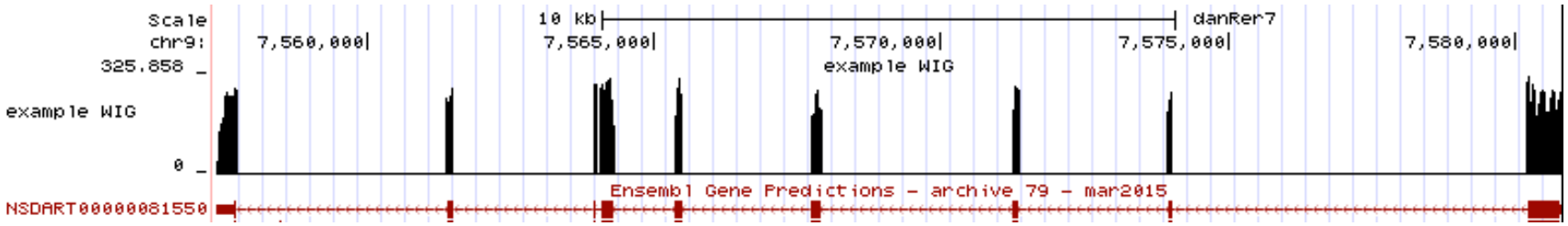
    → Tools menu

    → run igvtools



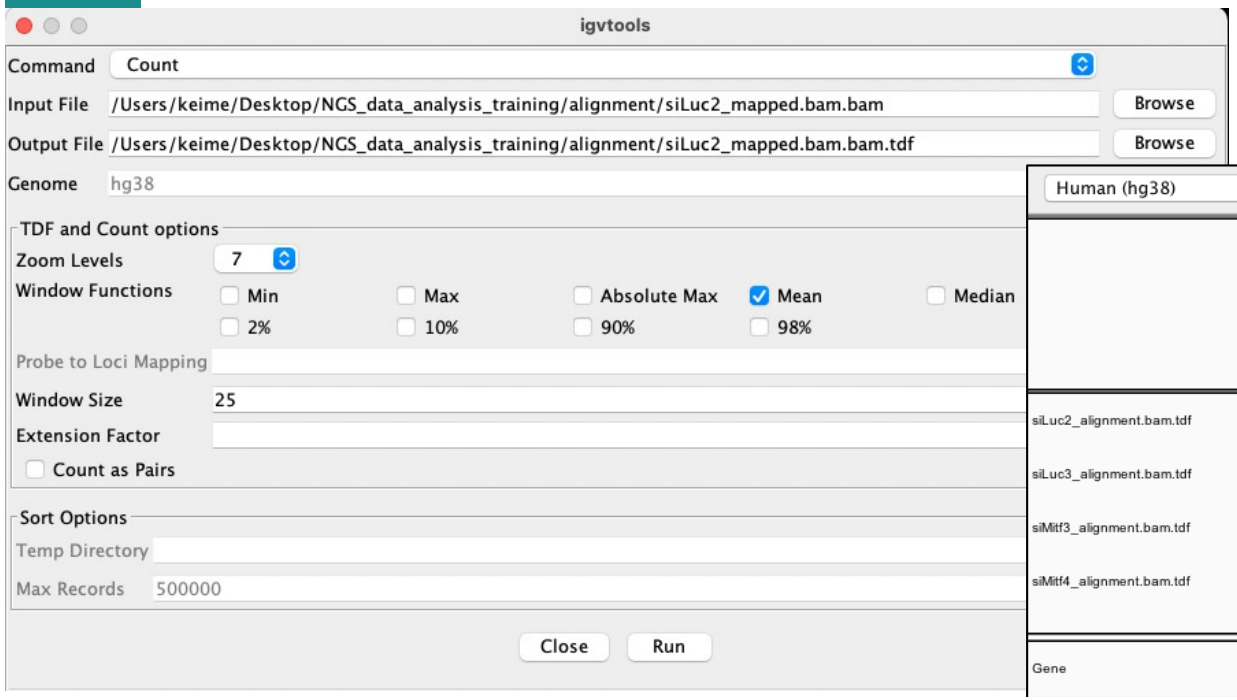
Wiggle (WIG) file format

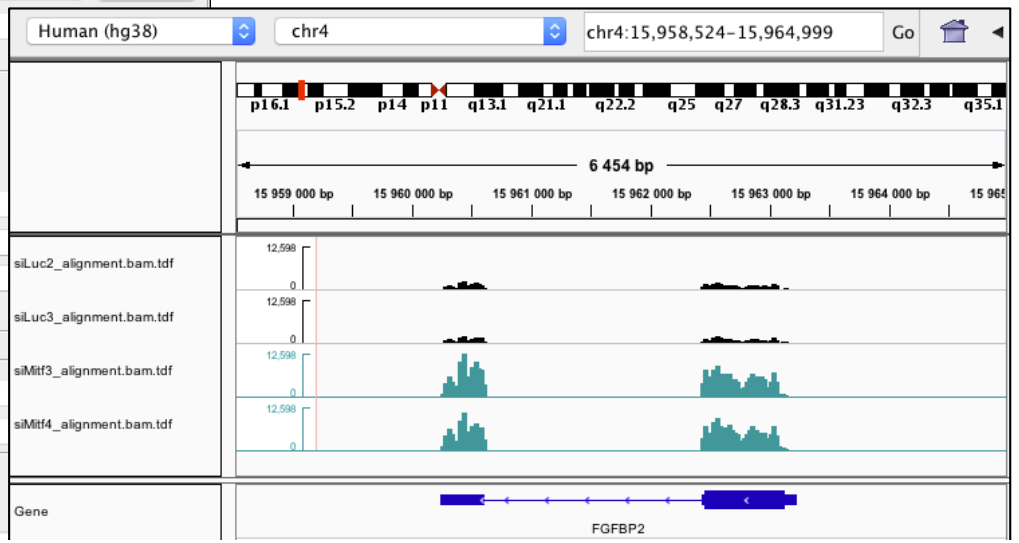
- Tab-delimited text file
- For dense continuous data
  - e.g. coverage : “summary” generated from an alignment
    → only density information
- Each line represents a portion of a chromosome
- Columns :
  - Chromosome
  - Start
  - End
  - Value
- More precise definition and examples
  - http://genome.ucsc.edu/goldenPath/help/wiggle.html
- Compressed binary indexed file derived from a WIG file : bigWig



TDF file format

- Tiled data file
- Binary file
- Read count density
  - Pre-processed data for faster display in IGV
- TDF file can be computed from a BAM file using igvtools
  - IGV Tools menu → run igvtools → Count



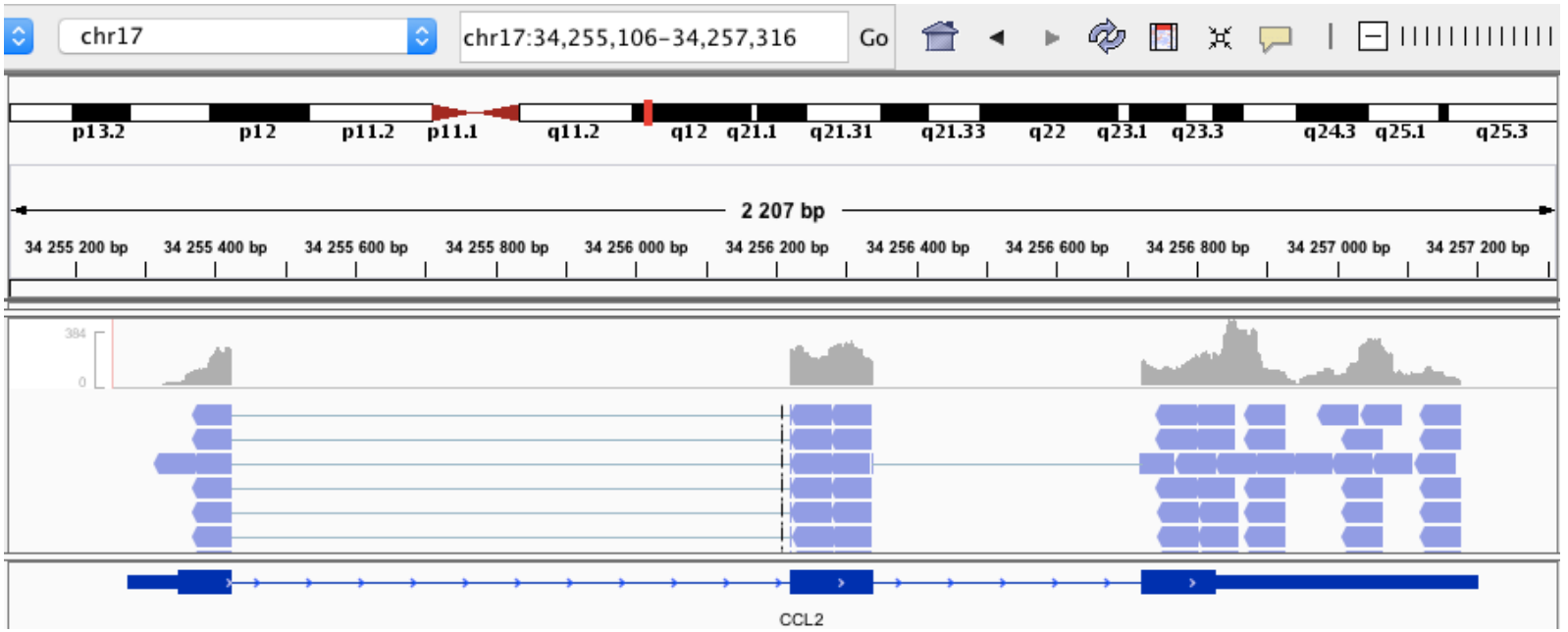


Coverage vs alignment

Coverage

Alignment

Annotation

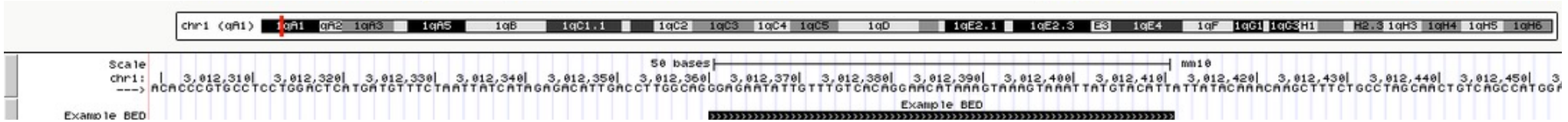


Browser Extensible Data (BED) format

- Tab-delimited text file
- For genomic intervals
- From 3 to 12 columns (always in this order) :
  - Chromosome
  - Start
  - End
  - Name
  - Score
  - Strand (+ or -)
  - …

  Chromosome, Start, End: required

  Most common : 6 columns
- More precise definition and examples
  - http://genome.ucsc.edu/FAQ/FAQformat.html#format1
- Manipulation of BED files
  - BEDTools (different tools available on Galaxy) : https://bedtools.readthedocs.io



Gene Transfert Format (GTF)

- GTF files can be visualized using IGV
  - e.g. Ensembl 105 annotations

downloaded from
http://ftp.ensembl.org/pub/release-105/gtf/homo_sapiens/Homo_sapiens.GRCh38.105.chr.gtf.gz

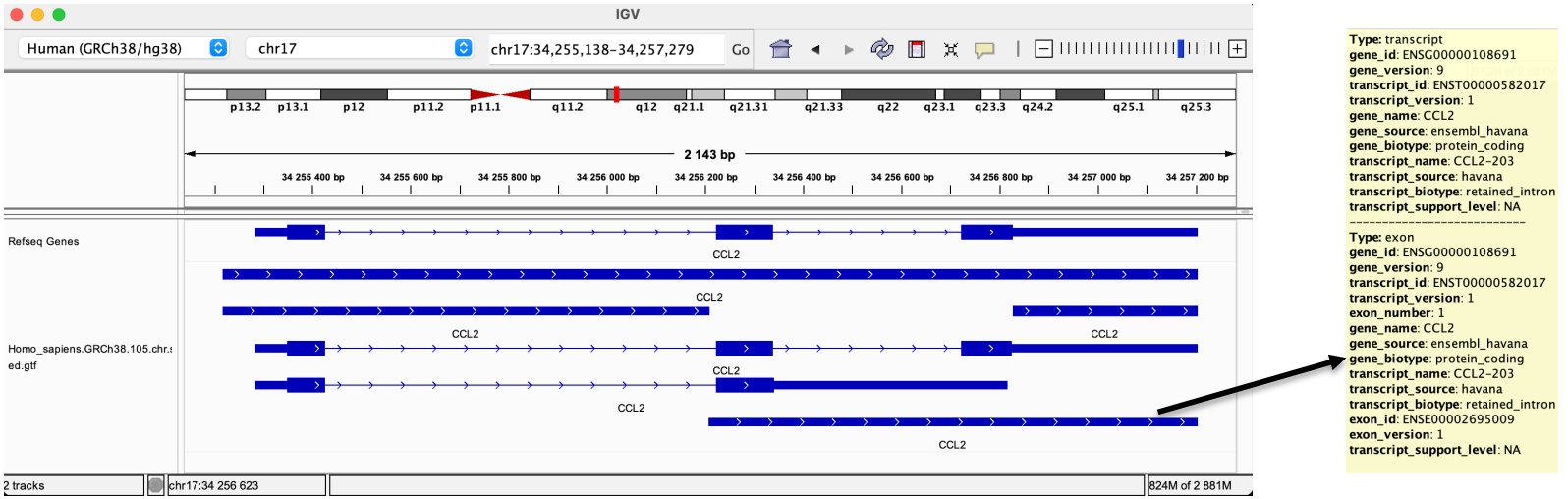
- Sort (by start position) and index for faster display
  - Tools → Run igvtools → Sort

→ Homo_sapiens.GRCh38.105.chr.sorted.gtf

  - Tools → Run igvtools → Index

→ Homo_sapiens.GRCh38.105.chr.sorted.gtf.idx (in the same directory)

  - File → Load from file and choose Homo_sapiens.GRCh38.105.chr.sorted.gtf



Main NGS file formats : summary

- FASTQ
  - Raw data
- SAM / BAM
  - alignment
- WIG / bigWig / TDF
  - coverage
- BED
  - Genomic intervals
- GTF
  - annotations

text
binary

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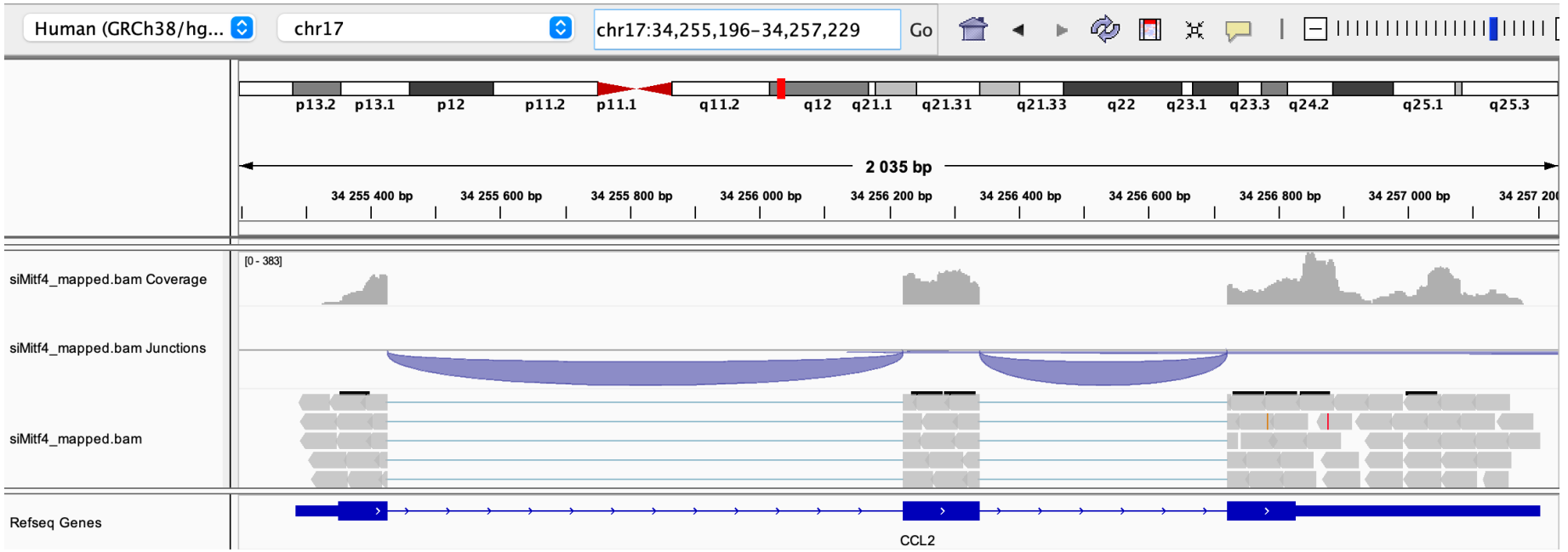
- Quality control of RNA-seq data based on alignments
  - *Exercise 3 : QC on alignments*

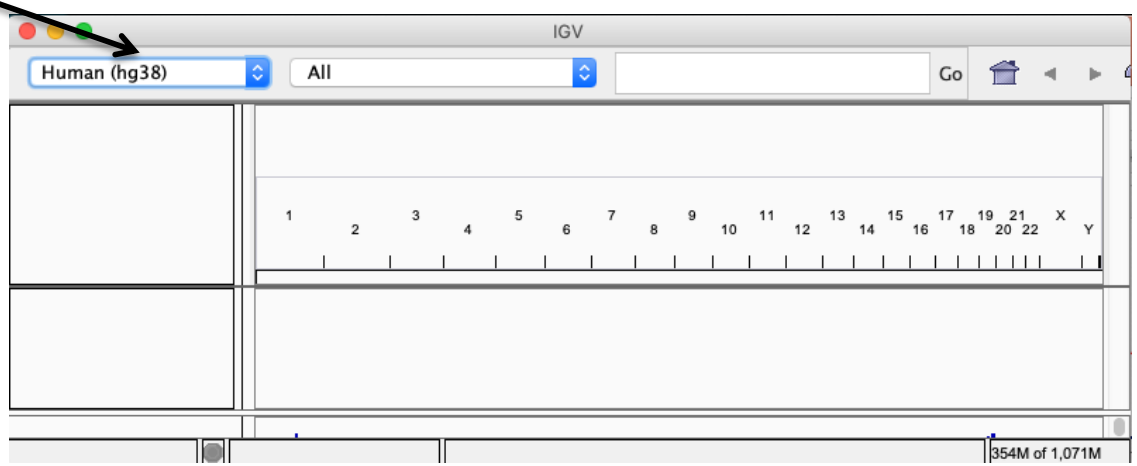
Alignment visualization

- Using a Genome Browser
  - A lot of available genome browsers
    - Ensembl, UCSC, Jbrowse, IGB, IGV, …
  - During this training we will use Integrative Genomics Viewer
    - http://www.broadinstitute.org/igv/



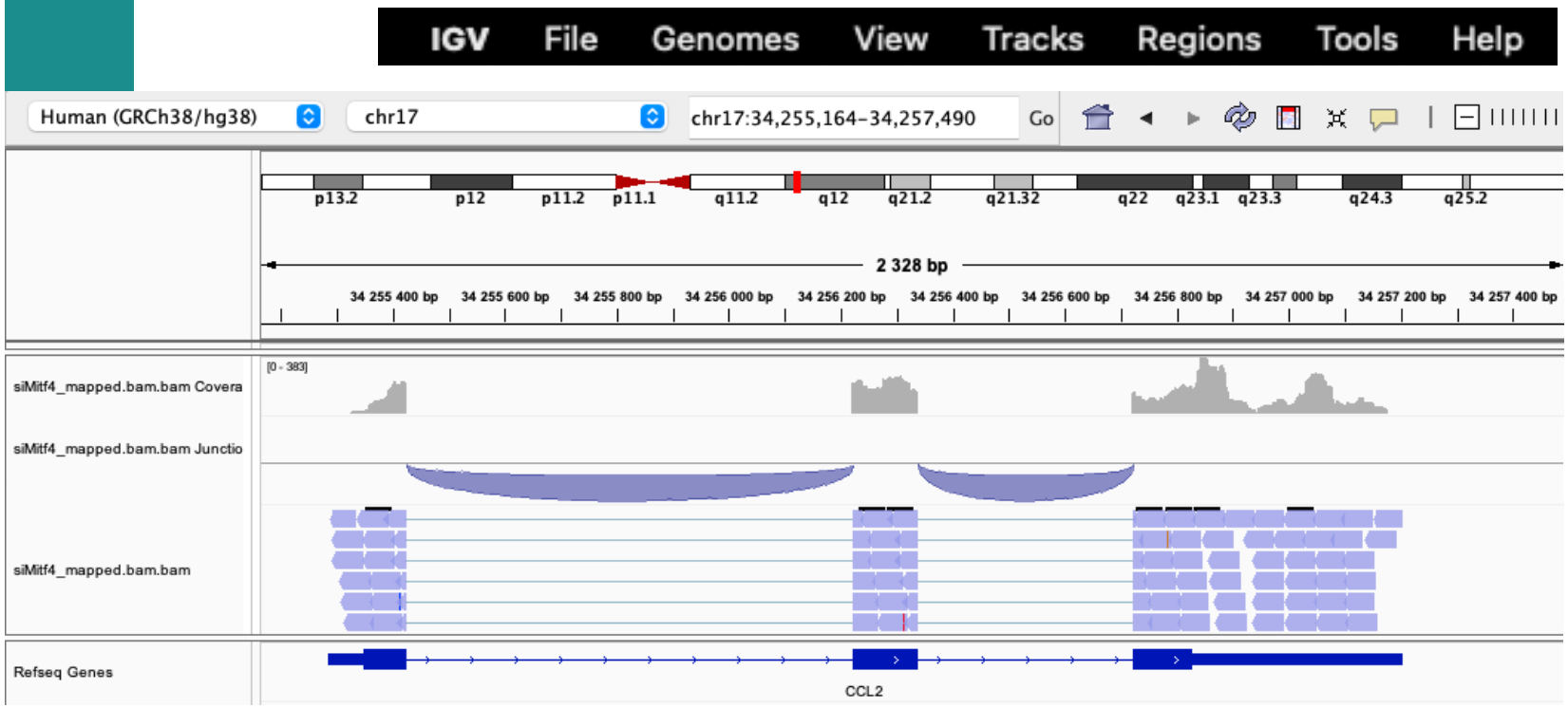
Using IGV : basic steps

- Select a reference genome



- Load data
  - File → load from file
  - File → load from server
  - Many tracks from different formats can be visualized on the same window (but they must correspond to the same assembly !)
- Navigate through the data

IGV



Menu

Tool bar

Chromosome ideogram

Data tracks

Annotation tracks

IGV menu : main features

- File
  - Load files into IGV
  - Manage sessions (e.g. save your current settings to a named session file)
  - Save an image
- Genome
  - Manage genomes available on IGV data server (http://software.broadinstitute.org/software/igv/Genomes)
  - Create new genomes (required : FASTA file, optional : annotation file, …)
- View
  - Preferences : customize the display
- Tools
  - Run igvtools : count (→ tdf), sort, index

IGV tool bar : main features

Chromosome location being shown
To scroll to a different location,
enter the gene name or locus
e.g. CCL2 or chr17:34,255,087-34,257,385
and click Go

Genome assembly

Chromosome

Move backward and forward through views of the genome like the back and forward buttons in a web browser

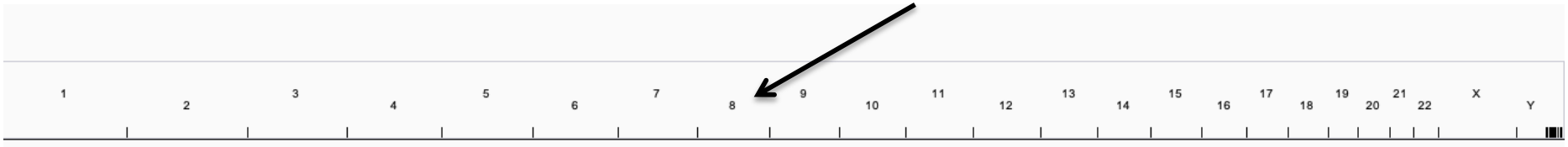
Zoom in and out on a chromosome

Modify popup text behaviour in data panels

- Show details on hover
- Show details on click

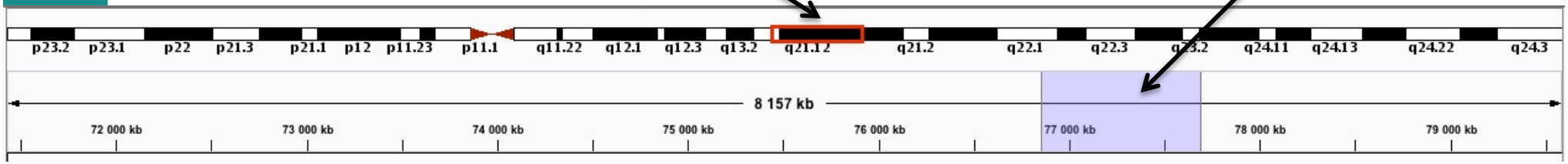
IGV : chromosome ideogram

Click on a chromosome number to jump to this chromosome



Chromosome location being shown

Click and drag to define a region to zoom in

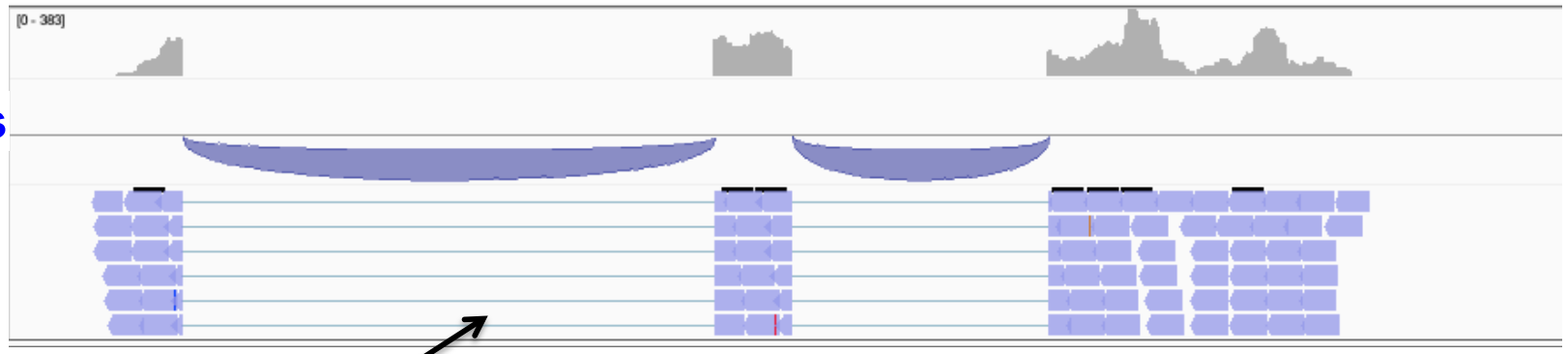


IGV : Data track

Coverage

Splice junctions

Reads



By default a sample of the alignments, to use less memory
(can be changed in View → Preferences → Alignments)

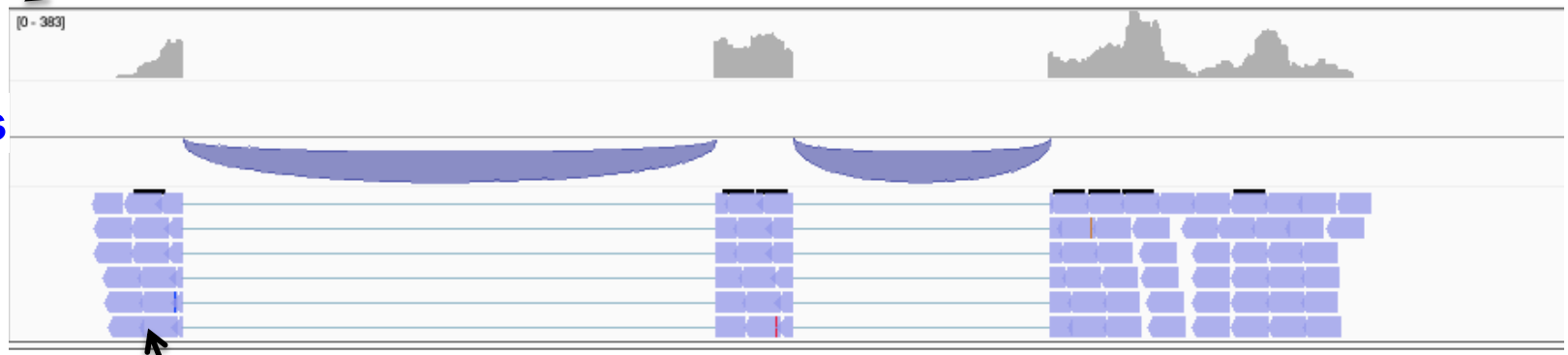
IGV : Data track

Data range (can be changed by right-clicking on track name)

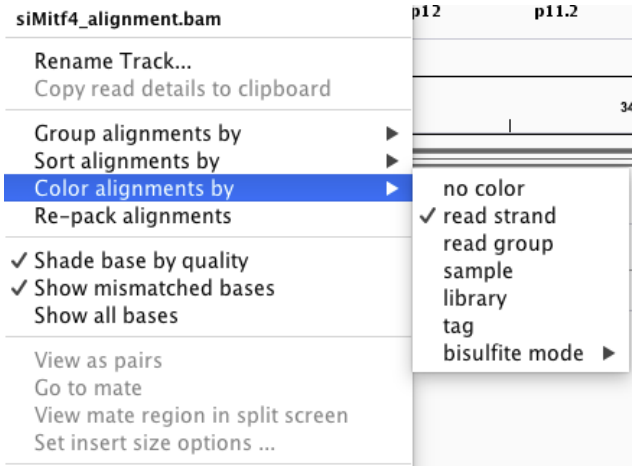
Coverage

Splice junctions

Reads



Read color can be changed by right-clicking on track name

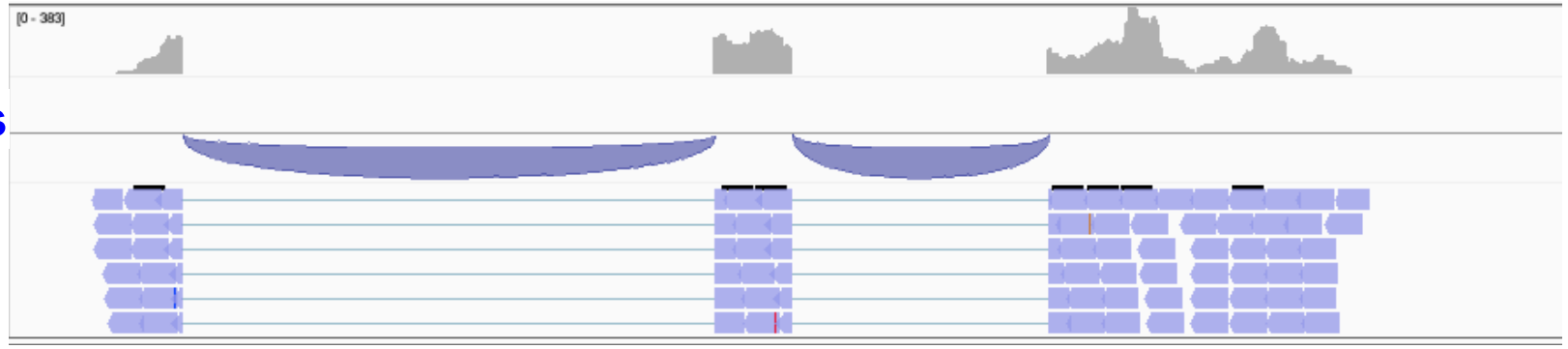


IGV : Data track

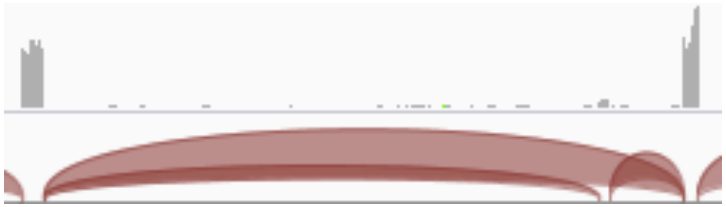
Coverage

Splice junctions

Reads



- Display of splice junctions
  - Color → strand
  - Thickness → depth of coverage
    - All junctions with more than 50 reads have the same thickness

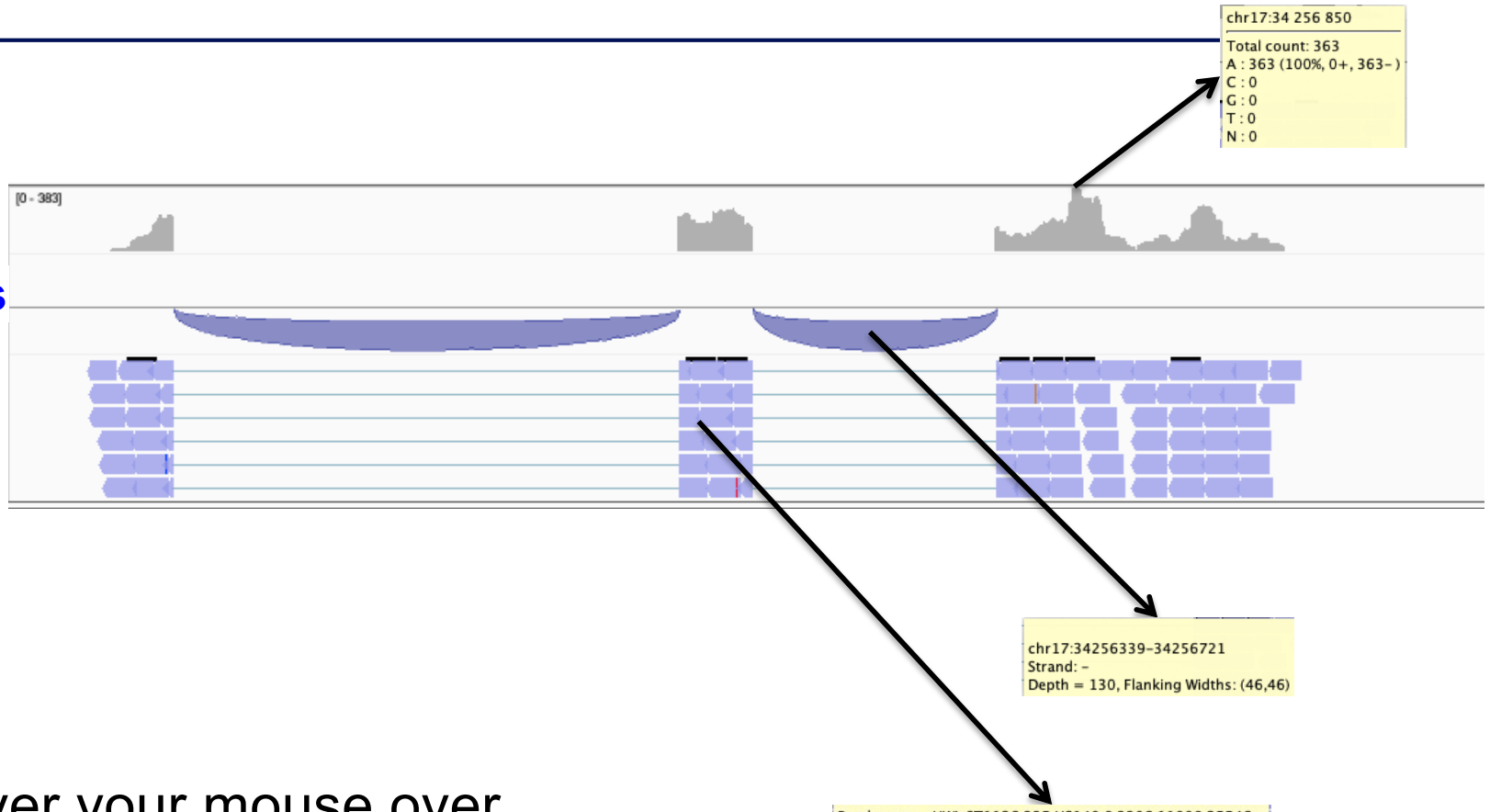


IGV : Data track

Coverage

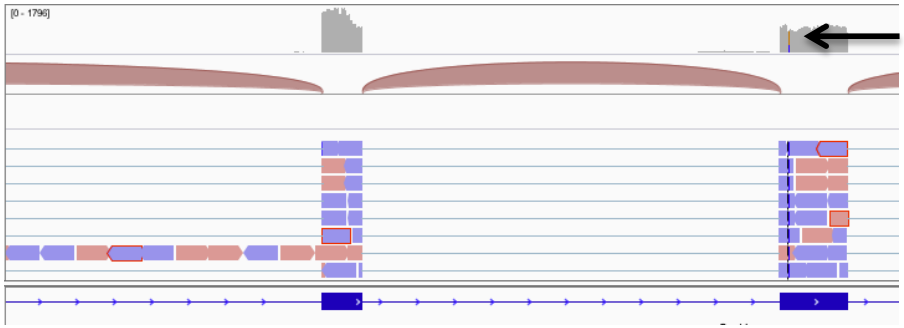
Splice junctions

Reads



→ Hover your mouse over images : pop-up windows provide additional information

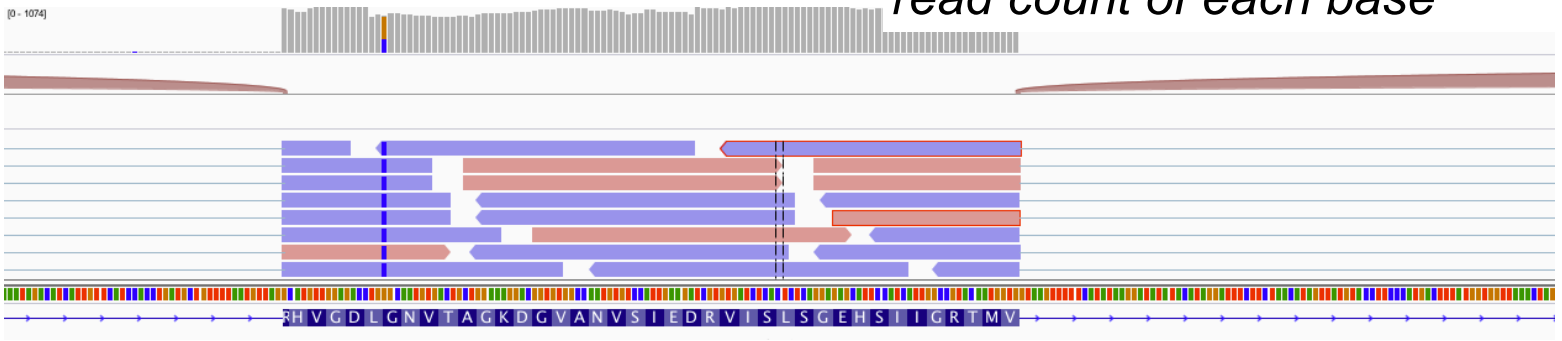
IGV data track differences vs reference genome



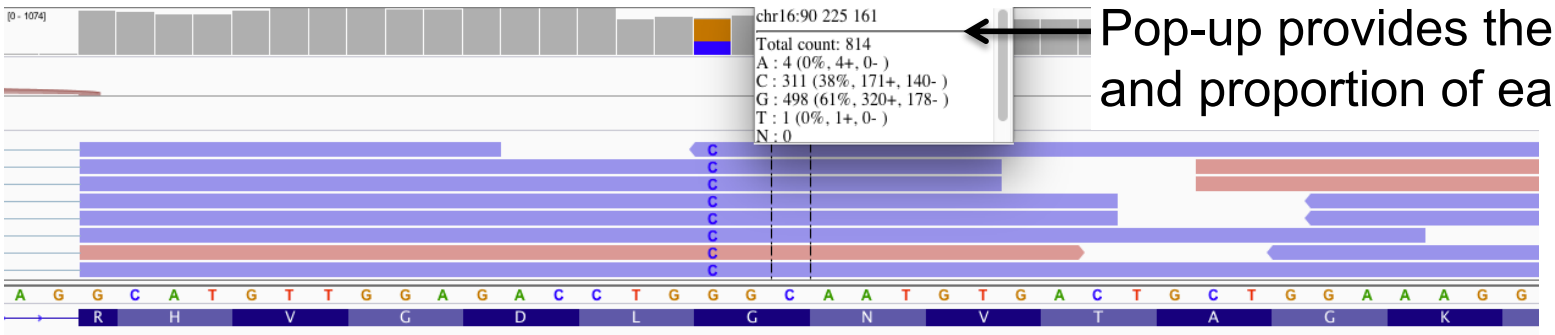
Nucleotide difference compared to the reference genome

If a nucleotide differs from the reference sequence in greater than 20% of reads, IGV colors the bar in proportion to the read count of each base*

Zoom in



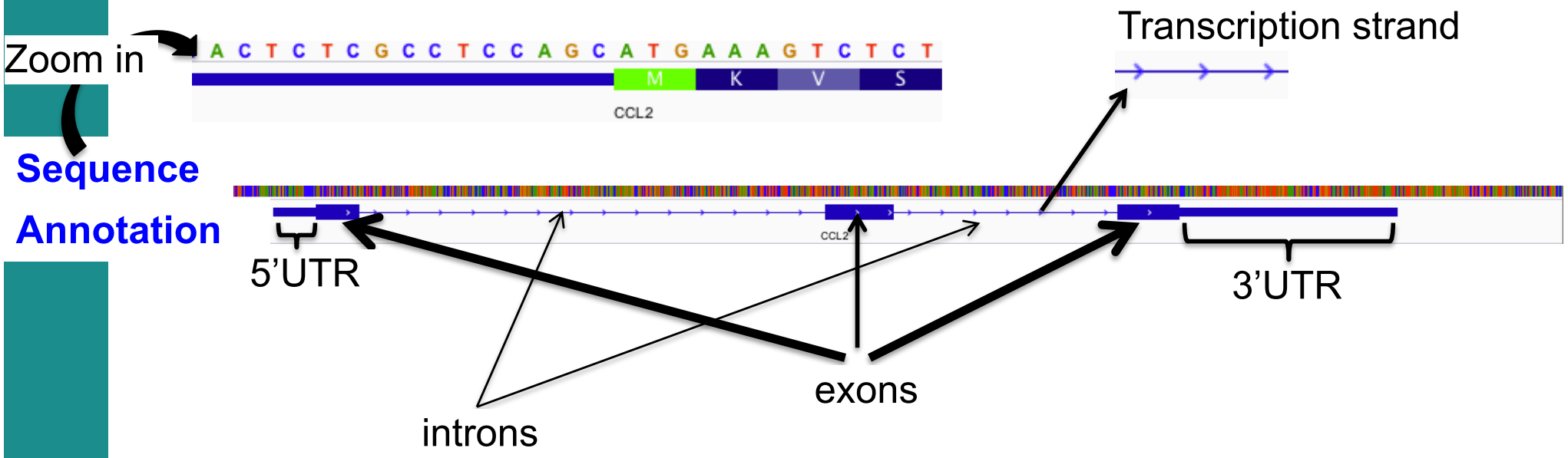
Zoom in



Pop-up provides the number and proportion of each base

* Default threshold, can be changed in
View → Preferences → Alignment → Coverage allele-fraction threshold

IGV annotation track



→ Hover your mouse over images, pop-up windows provide additional information :

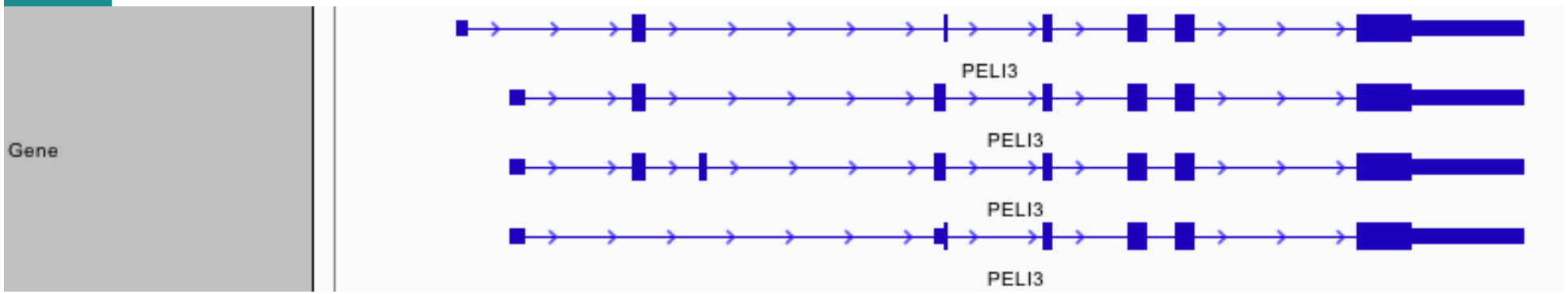
CCL2
chr17:34255277-34257201
id = NM_002982

Exon number: 2
Amino acid coding number: 51
chr17:34256222-34256339

IGV annotation track

Default : collapsed

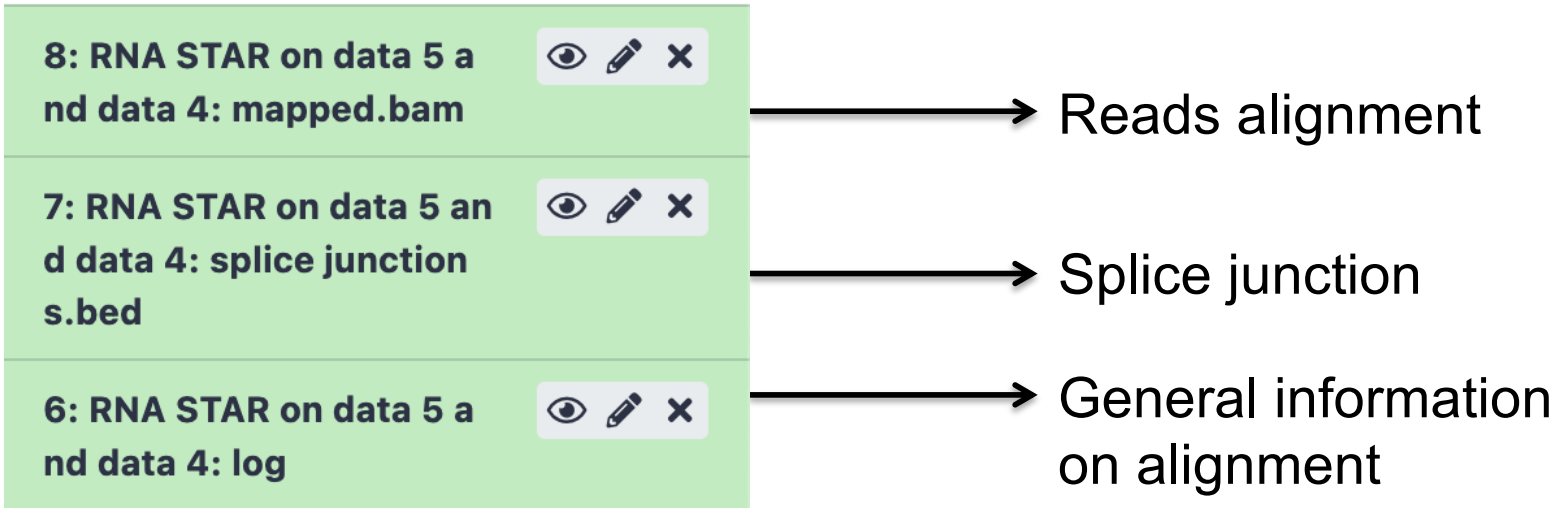
Right click on track name → Expanded
To see all isoforms



NGS read mapping

- Introduction to NGS read mapping
- Short read mappers
- Specificity of RNA-seq read mapping
- *Exercise 1 : Mapping of RNA-seq data using Galaxy*
- Alignment and related file formats
- Alignment visualization
- *Exercise 1 : Interpretation of results*
- *Exercise 2 : Whole dataset alignment visualization*
- Quality control of RNA-seq data based on alignments
  - *Exercise 3 : QC on alignments*

Exercise 1 : results



Exercise 1 : interpretation of results

1. Log file
   - What is the proportion of uniquely mapped reads ?

2. Alignment file
   - Which alignment file format is provided by STAR ?
   - Download this file and the index, visualize this alignment using IGV
   - Look at reads mapped on the junction between the 2 last exons of *Park7* gene. How many reads span this junction ? Look at the CIGAR string of one of these reads
   - Visualize the strand specificity of the reads, for example on *Park7* and *Chmp2a* genes (color alignments by strand)
   - Look at reads aligned on *Actb* gene (color alignments by number of reported alignments : tag=NH). What do you observe ?

NGS read mapping

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Exercise 2 : whole dataset alignments

- STAR results for all samples from Mitf project are available on Galaxy
  - Datasets 4 to 15:

Exercise 2 : whole dataset alignments

1. What is the proportion of uniquely mapped reads in all samples ?

- To save time, the corresponding BAM, BAI and tdf files are already available on your computer (RNAseq/alignment folder)
- Start a new IGV session (File → new session)
- In View → Preferences → Tracks tab, select “Normalize coverage data”
- Load the 4 tdf files on IGV
- Right-click on all track names and choose “Group Autoscale”

2. We are interested in *Idh1* gene.
   Is this gene differentially expressed between siLuc and siMitf samples ?

Exercise 2 : whole dataset alignments

In IGV preferences (View → Preferences) “Alignments” tab

- In “Track Display” section, check “Show junction track”
- In “Splice Junction Track” section, choose “Minimum junction coverage”: 10

Open a new session (File → New session), then load the 4 BAM files

3. What do you observe in exons 11 and 13 of *Eef2* gene ?
4. What do you observe at position chr4:6707961 ?
5. Which transcript isoforms do you observe in region chr20:44,935,294-44,939,521 ?

Notes :

- To see all annotated isoforms right click on an annotation track and select Expanded
- You can perform a Sashimi-plot for a better visualization of isoforms : Right-click on a BAM track → Sashimi plot
  → Select Alignment Tracks : all alignments

Exercise 2 : whole dataset alignments

6. The same RNA samples have been processed with a different RNA-seq protocol. The corresponding alignment file for siLuc2 sample is available on your computer :

   RNAseq/other_protocol/siLuc2_other_protocol_mapped.bam

   What do you think about this protocol ?

   Look for example at *Park7* gene

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Quality control of RNA-seq data based on alignments

- Proportion of mapped, uniquely and multiple mapped reads in all samples within a project
- Read distribution relative to known annotations
- Read coverage over genes
- Strand information (directional protocol)
- For paired-end sequencing : distance between reads

http://rseqc.sourceforge.net/

RSeQC tools available on Galaxy

RSeQC input :
alignment (BAM/SAM) and annotation (BED) files

Read distribution relative to known annotations

- How mapped reads are distributed over genomic features (CDS, UTR, intron, intergenic regions)

- RSeQC read distribution
  - Assigns mapped reads to a genomic feature
  - When genomic features overlap, they are prioritized as:
    - CDS > UTR > Introns > Intergenic regions
  - Does not assign reads located beyond TSS upstream 10Kb or TES downstream 10Kb

CDS : Coding DNA Sequence
UTR : UnTranslated Region
TSS : Transcription Start Site
TES : Transcription End Site

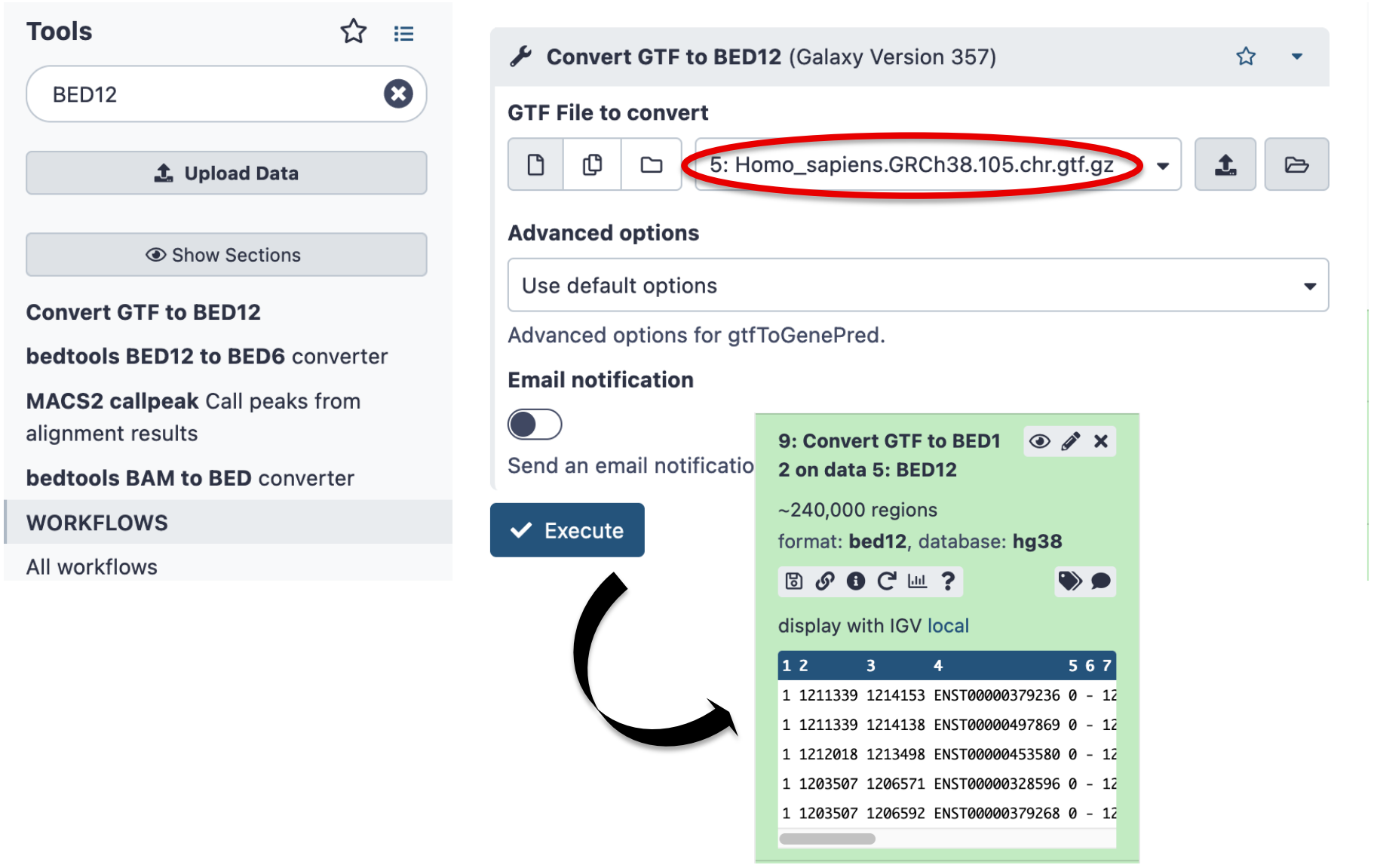
Exercise 3 – Question 1

1. Convert GTF annotation file to BED file using **Convert GTF to BED12** tool
   - Annotation file to use (already imported)

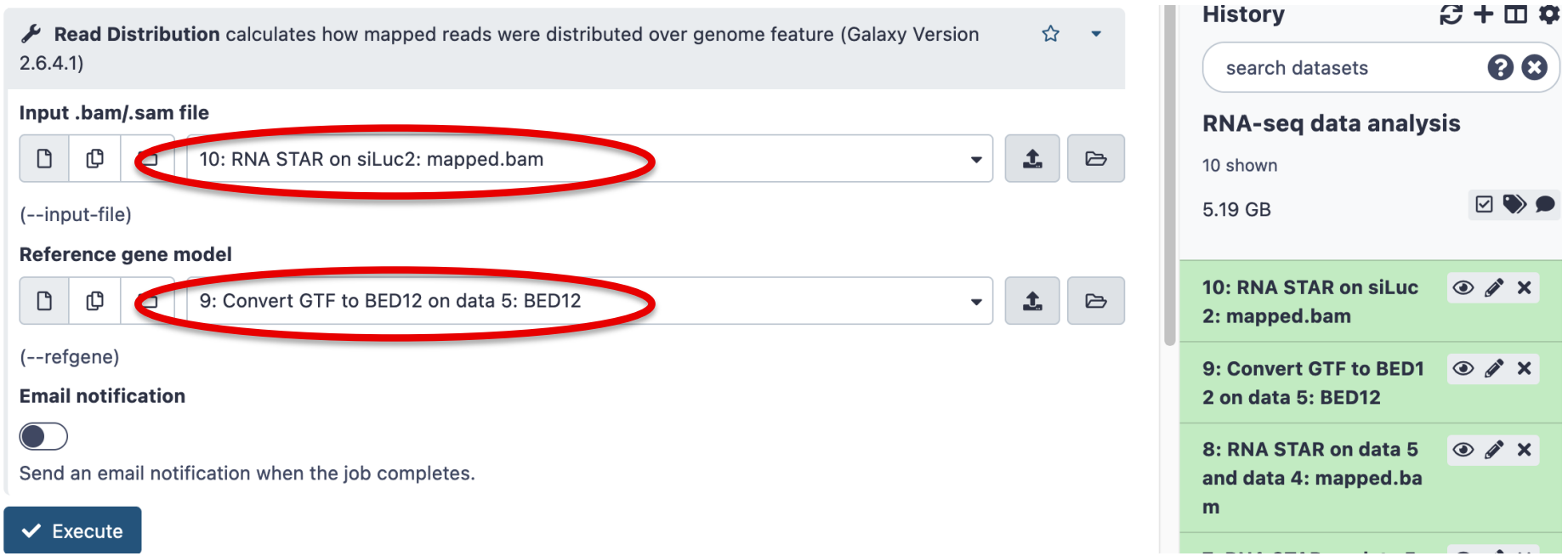
     Homo_sapiens.GRCh38.105.chr.gtf.gz

2. Launch **Read distribution** on the mapping results from siLuc2 sample
   - Alignment file to import
     - 6: RNA STAR on siLuc2: mapped.bam
   - Annotations
     - Bed file obtained during step 1

Exercise 3 – Question 1



Exercise 3 – Question 1



Exercise 3 – Question 1

- Read distribution

```
Total Reads                     43080660
Total Tags*                     49982200
Total Assigned Tags°            46821353
=======================================================================
Group                 Total_bases           Tag_count             Tags/Kb
CDS_Exons             35875018              30475010              849.48
5'UTR_Exons           48312525              2562470               53.04
3'UTR_Exons           75444264              10416230              138.07
Introns               1613277549            3000938               1.86
TSS_up_1kb            28388776              36020                 1.27
TSS_up_5kb            126596357             66649                 0.53
TSS_up_10kb           225614841             90707                 0.40
TES_down_1kb          30986381              124903                4.03
TES_down_5kb          133535951             201514                1.51
TES_down_10kb         233464669             275998                1.18
=======================================================================
```

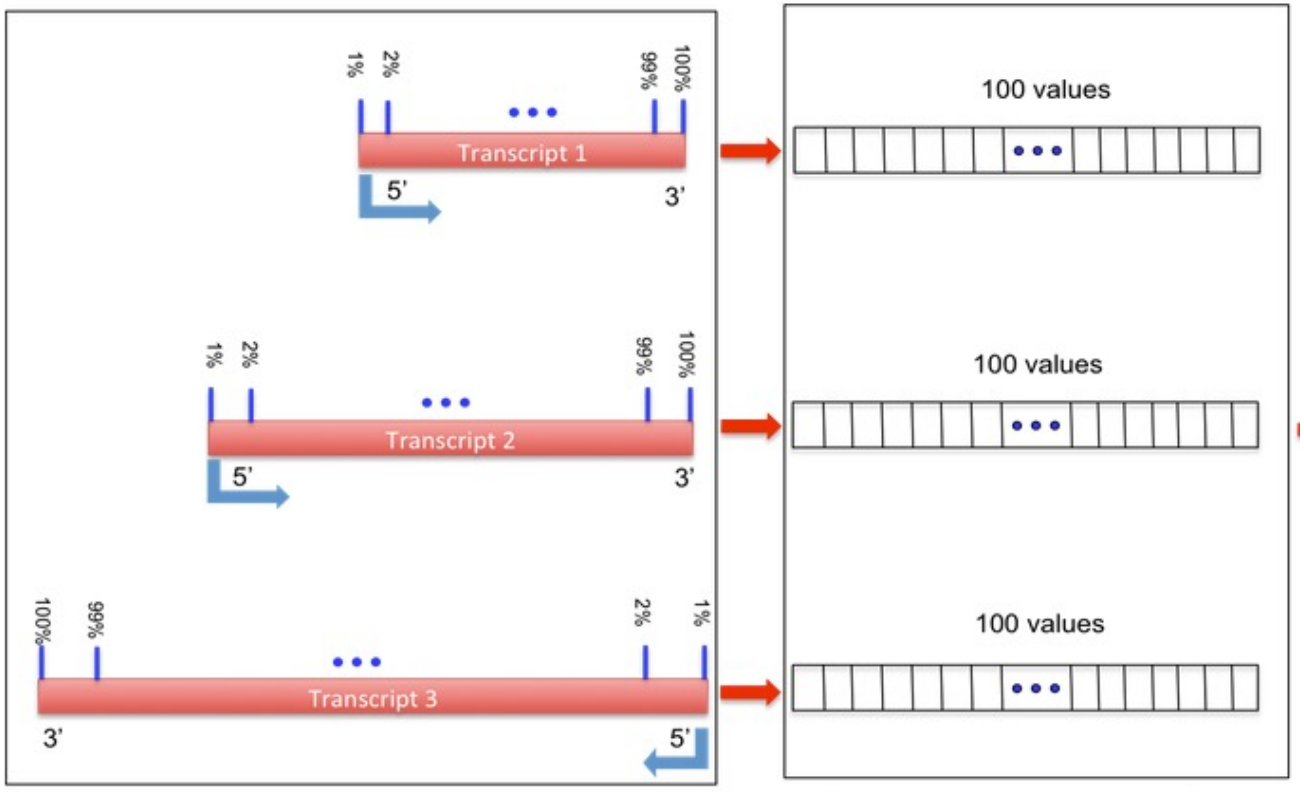
* reads spliced once are counted as 2 tags, reads spliced twice are counted as 3 tags, …

° number of tags that can be assigned to the 10 groups

Tags assigned to “TSS_up_1kb” are also assigned to “TSS_up_5kb” and “TSS_up_10kb”
Tags assigned to “TSS_up_5kb” are also assigned to “TSS_up_10kb”

Read coverage over genes

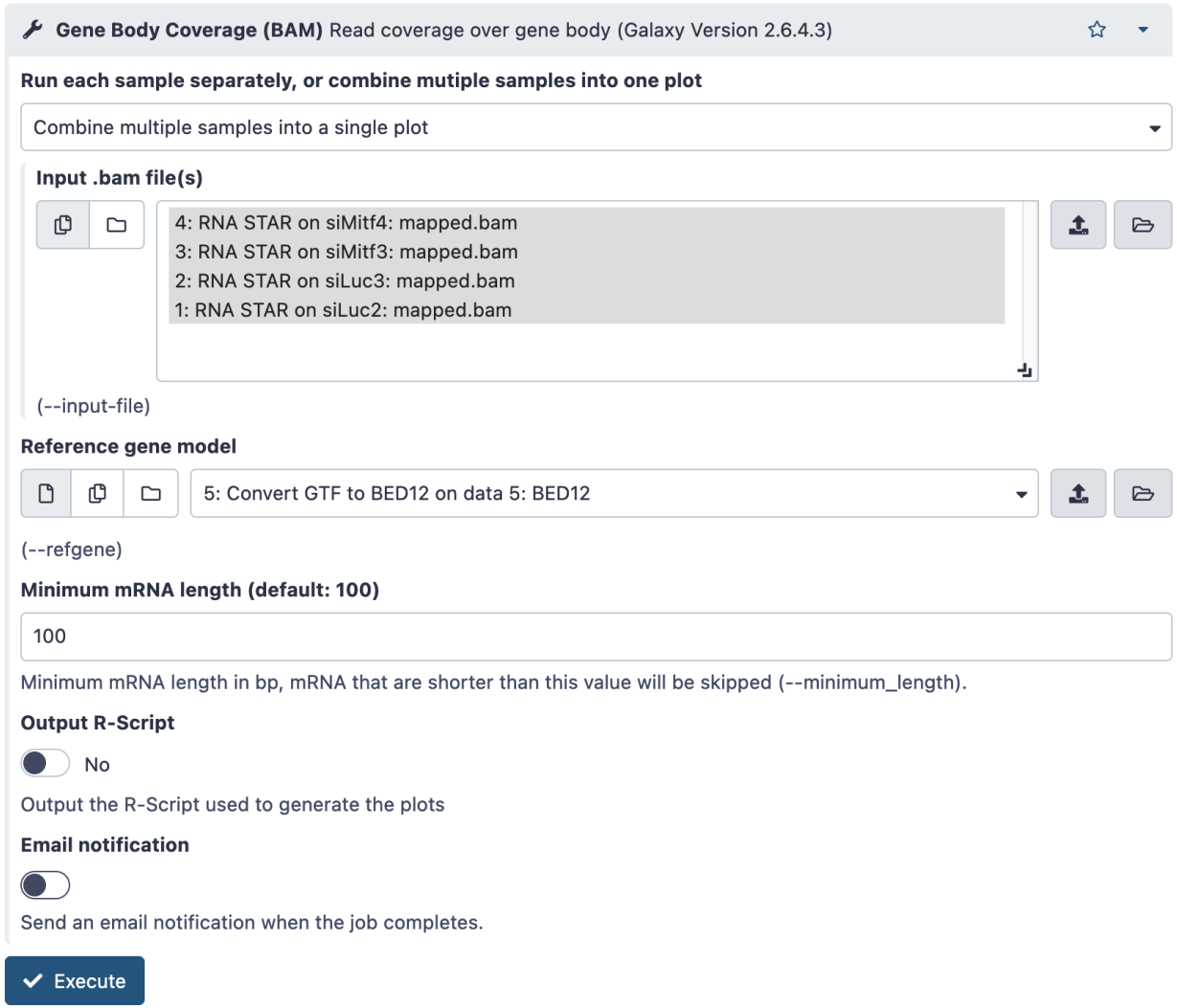
- To identify any bias in read coverage over genes
- RSeQC Gene Body Coverage



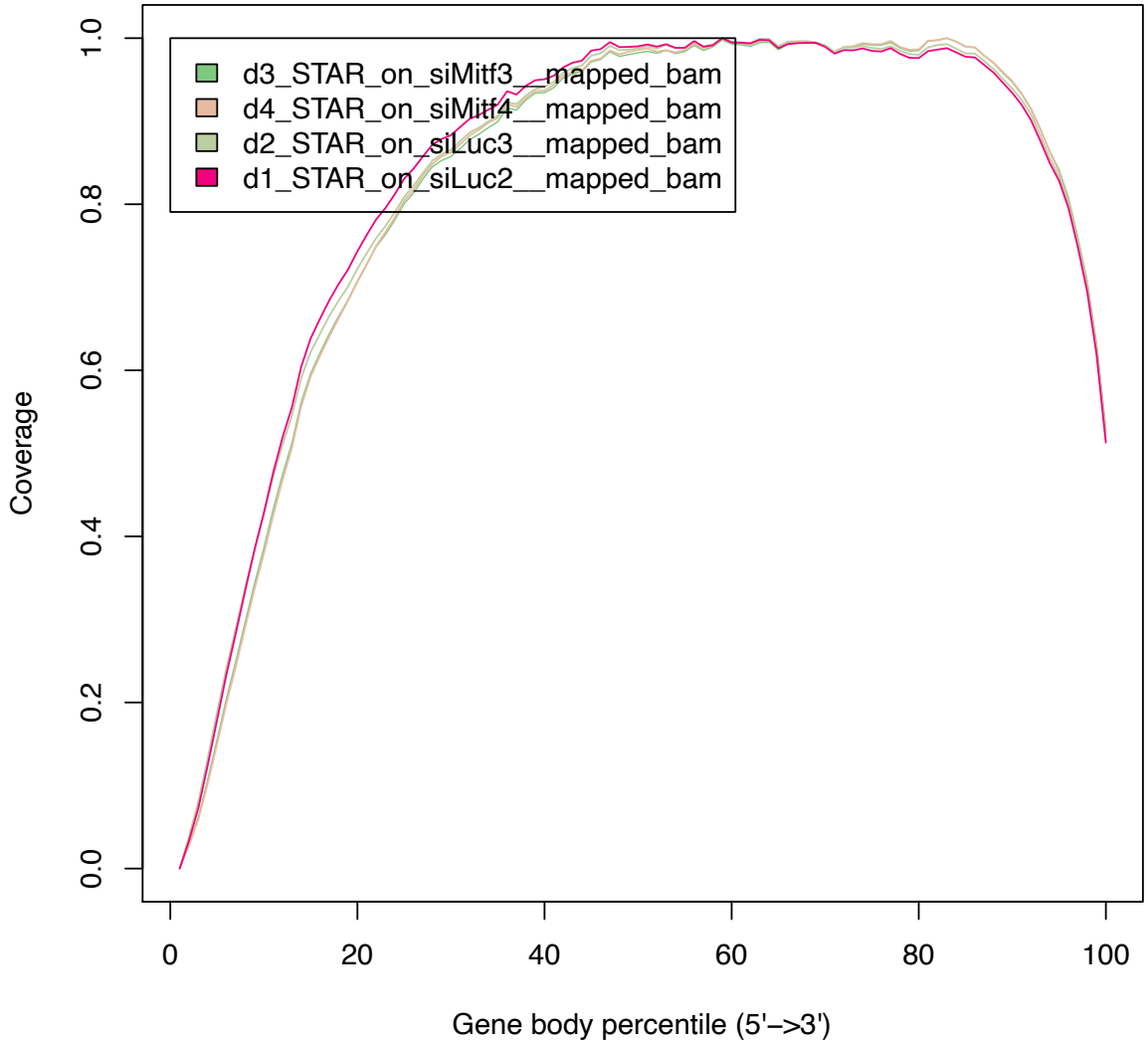
From http://rseqc.sourceforge.net/

Read coverage over genes : Galaxy

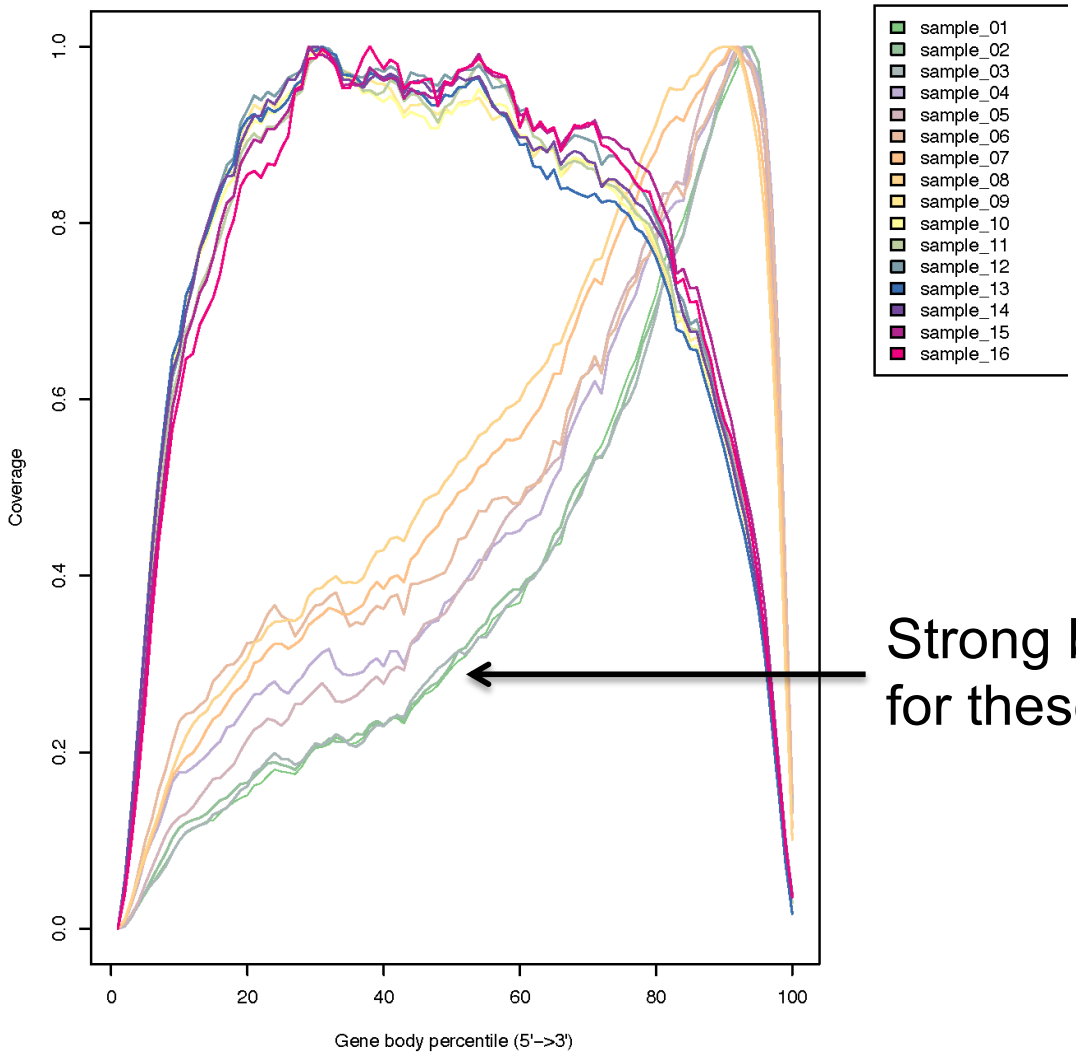
Don’t perform this analysis today



Read coverage over genes : result



Read coverage over genes : example with biased samples



Strong bias in read coverage for these samples

Strand information (directional protocol)

- To infer how reads were stranded for strand-specific RNA-seq data
  - Compare the “strandness of reads” with the “strandness of transcripts”
  - The “strandness of reads” is determined from alignment
  - The “strandness of transcripts” is determined from annotation

- RSeQC infer experiment
  - Calculates the proportion of reads corresponding to :
    - ++,- -
    - +-,-+

	Annotated gene on + strand	Annotated gene on - strand
Read mapped to + strand	++	+-
Read mapped to - strand	-+	--

Exercise 3 – Question 2

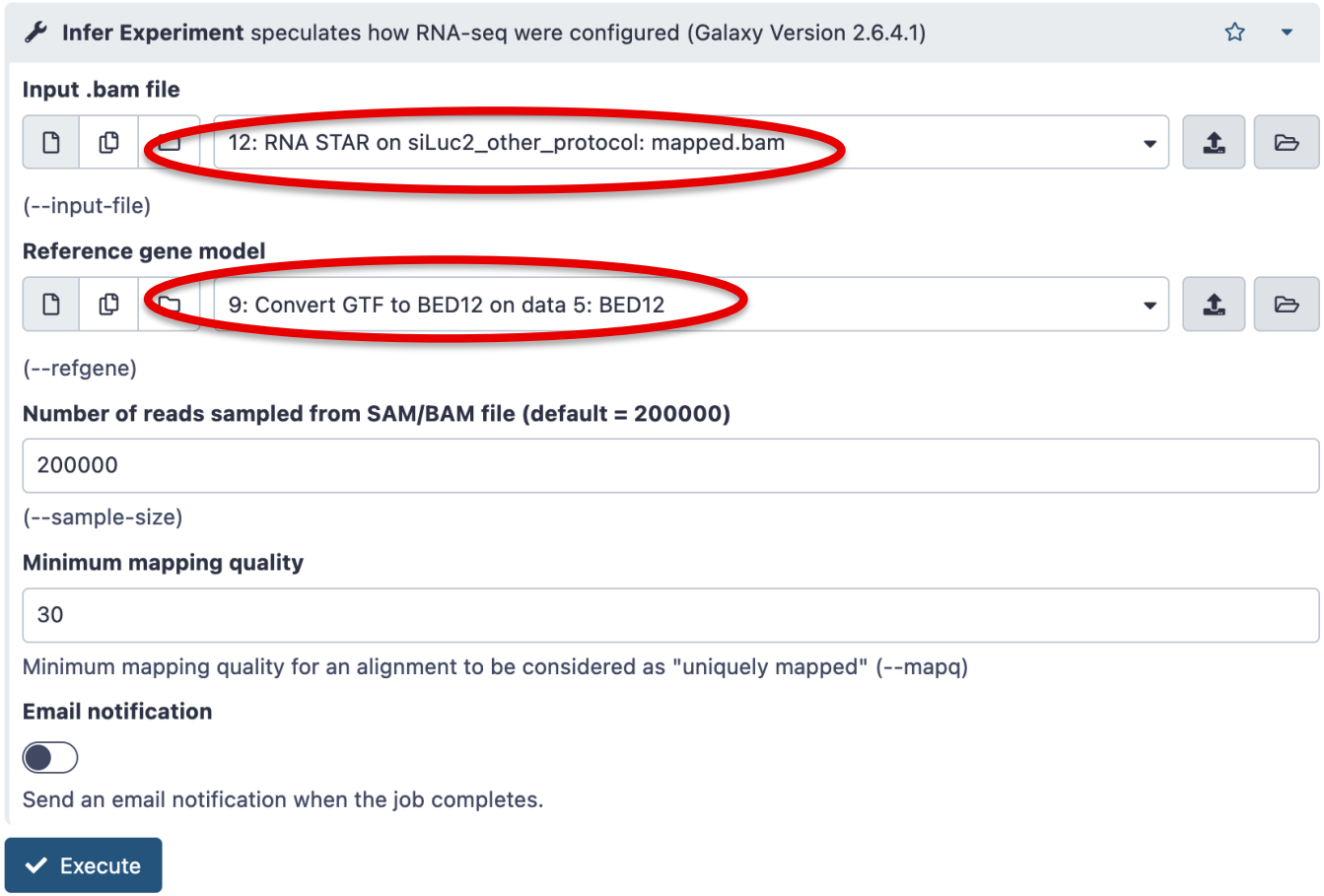
- Launch **Infer experiment** on the mapping results obtained on siLuc2 data from the two different protocols and compare the two results
  - Alignment files
    - RNA STAR on siLuc2: mapped.bam (already imported)
    - 16: RNA STAR on siLuc2_other_protocol: mapped.bam (to import)
  - Annotations
    - Bed file obtained during the previous exercise

Exercise 3 – Question 2

- Infer experiment on siLuc2 mapping results :

Exercise 3 – Question 2

- Infer experiment on siLuc2 mapping results from the library prepared with another protocol :



Exercise 3 – Question 2

- Infer experiment
  - on siLuc2 library prepared with a directional protocol :

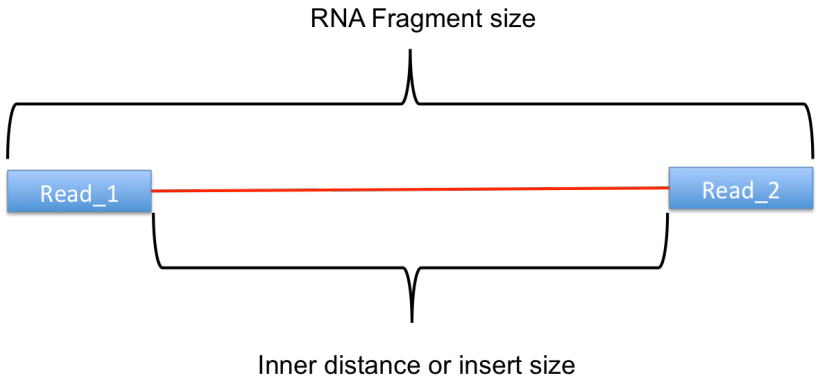
```
This is SingleEnd Data
Fraction of reads failed to determine: 0.1034
Fraction of reads explained by "++,--": 0.0078
Fraction of reads explained by "+-,-+": 0.8887
```

  - On siLuc2 library prepared with a non directional protocol :

```
This is SingleEnd Data
Fraction of reads failed to determine: 0.1446
Fraction of reads explained by "++,--": 0.4278
Fraction of reads explained by "+-,-+": 0.4277
```

Distance between reads (paired-end sequencing)

- To know inner distance (insert size) between paired reads
  - The distance is the mRNA length between two paired fragments



- RSeQC Inner Distance
  - Determines the genomic (DNA) size between two paired reads: D_size = read2_start - read1_end
    - if 2 paired reads map to the same exon or a non-exonic region
      - inner_distance = D_size
    - if 2 paired reads map to different exons
      - inner_distance = D_size - intron_size
  - The inner_distance might be a negative value if 2 fragments overlapped

RSeQC inner distance : example of result