

バイオインフォマティクスへの招待 ～高速シーケンサーと RNA-Seq～
Invitation to Bioinformatics: High-Throughput Sequencers and RNA-Seq
(Lecture and Practice in English/Japanese)

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この講義・実習では、高速シーケンサーによる塩基配列の読み取りの原理を概説し、得られた膨大な塩基配列データから遺伝子の発現解析（RNA-Seq）を行う手法について、実際にマルチメディアセンターの PC を使ってハンズオンで学ぶ。

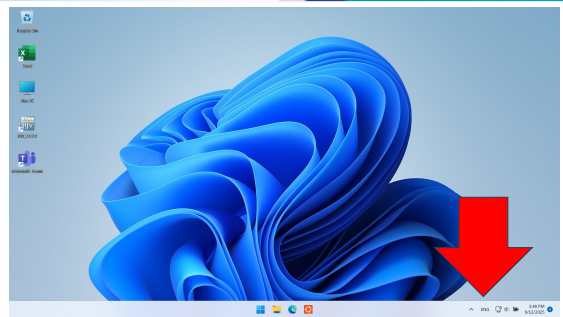
This lecture and practical course outlines the principles of sequencing using high-throughput sequencers. Furthermore, participants will receive hands-on training on how to analyze gene expression (RNA-Seq) from the massive amount of sequence data obtained, using the PCs in the Multimedia Center.

Introduction of Bioinformatics

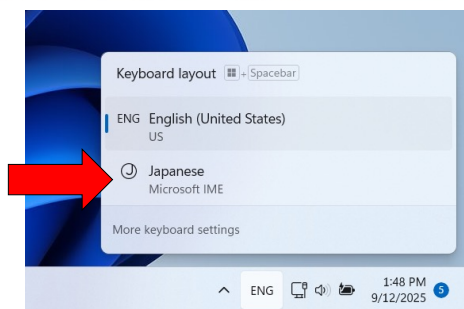
: High throughput Sequencer and RNAseq



Keyboard layout



Keyboard layout

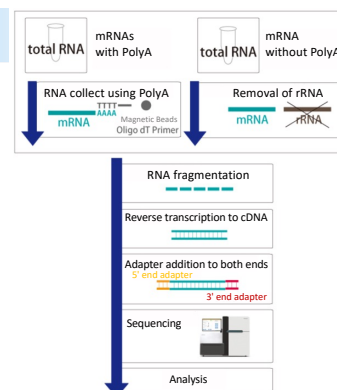


What is RNAseq?

- Sequencing of RNA (mainly mRNA) by High-speed sequencer
- Quantitative analysis of gene expression
- Compare with qPCR
 - gene expression can be analyzed comprehensively.
- Splicing analysis is also available

Examples of analysis

- Comparison of differences in gene expression in normal and cancerous tissues
 - Identification of up- or down-regulated genes
- Comparison of splicing differences between normal and cancerous tissues
 - Identification of used variants



Yakukensha Co., Ltd. Contract
Online
<https://www.yakukensha.co.jp/jtk/det.php?i=745>

Flow of RNAseq analysis

50 YEARS

Sample Preparation

- mRNA extraction
- Reverse transcription to cDNA

High-speed sequencing

- Fastq file
 - Sequencing data

Expression Analysis

- quality check
- Mapping
- Expression Analysis
- visualization

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Preparation of the environment required for RNAseq

50 YEARS

- Installation of software required for analysis
 - Free UNIX-based software
 - Linux or Mac

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WSL(Windows Subsystem for Linux)

50 YEARS

- Windows Subsystem for Linux (WSL) is a feature of Windows that allows you to run a Linux environment on your Windows machine, without the need for a separate virtual machine or dual booting. WSL is designed to provide a seamless and productive experience for developers who want to use both Windows and Linux at the same time.



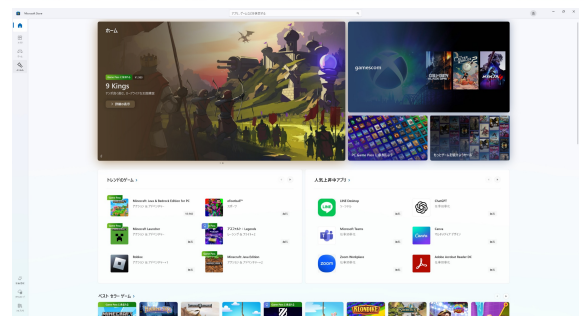
Microsoft Learn

<https://learn.microsoft.com/en-us/windows/wsl/about>

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Microsoft Store

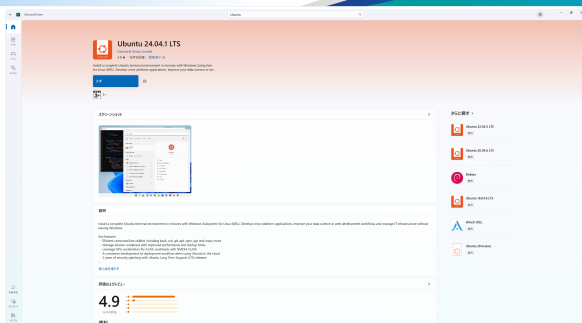
50 YEARS



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Ubuntu

50 YEARS



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Ubuntu

50 YEARS



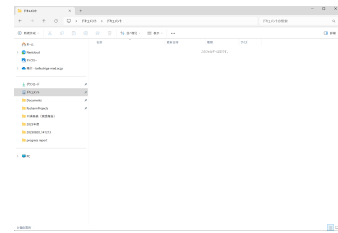
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CUI (Character User Interface)



- A method of operating a computer through character input using a keyboard, which allows the user to interact with the computer in a character-based manner.

GUI (Graphical User Interface)



- Open Document folder

Let's operate by CUI



- Directory (folder) operations
 - pwd : Show current directory
 - cd : Moving Directories
 - ls : Listing of folders and files
 - mkdir : create folder

pwd : print working directory



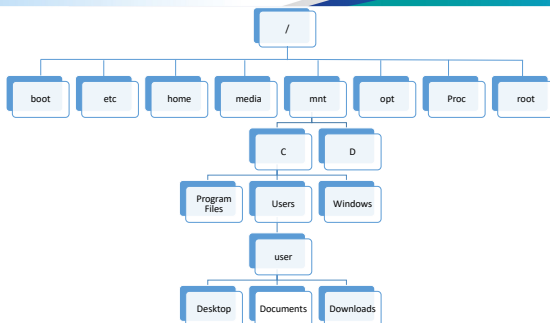
\$ pwd



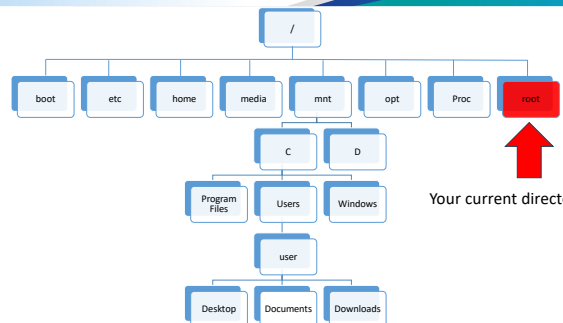
The following slides will show commands in this style. Ignore the dollar mark (\$) and enter the command that follows.

```
root@Kadota-Lenovo-WS2:~# pwd
/root
```

Let's operate by CUI

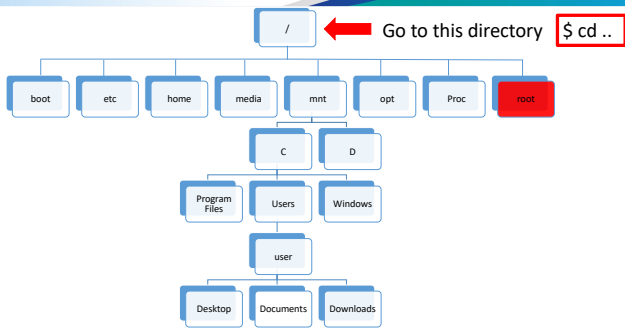


Let's operate by CUI



Your current directory is this.

Let's operate by CUI



cd

\$ cd ..

This command means "go to upper directory"

There is "space" here.

```
root@Kadota-Lenovo-WS2:~# cd ..
root@Kadota-Lenovo-WS2:/# pwd
/
```

Let's operate by CUI



cd

\$ cd mnt

```
root@Kadota-Lenovo-WS2:/# cd mnt
root@Kadota-Lenovo-WS2:/mnt# pwd
/mnt
```

Let's operate by CUI



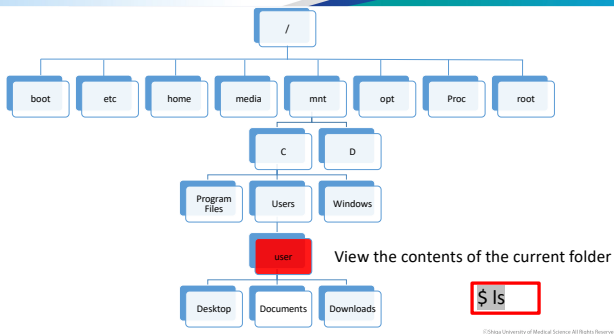
cd

\$ cd c
\$ cd Users
\$ cd user

```
root@Kadota-Lenovo-WS2:/mnt# cd c
root@Kadota-Lenovo-WS2:/mnt/c# cd Users
root@Kadota-Lenovo-WS2:/mnt/c/Users# cd user
```

```
root@Kadota-Lenovo-WS2:/mnt/c/Users/shiga# pwd
/mnt/c/Users/ user
```

Let's operate by CUI



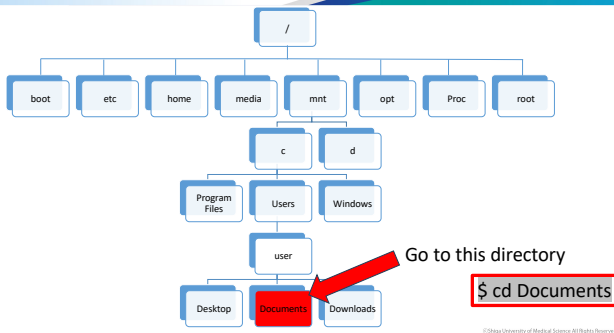
ls : list segments

`$ ls`

```

root@DESKTOP-UEN7AMJ: /mnt/c/Users/shiga# ls
AppData
Application Data
Contacts
Cookies
Desktop
Documents
Downloads
Favorites
Links
Local Settings
Music
My Documents
NetHood
OneDrive
Pictures
PrintHood
Recent
Saved Games
Searches
SendTo
Templates
Videos
スタート メニュー
    
```

Let's operate by CUI

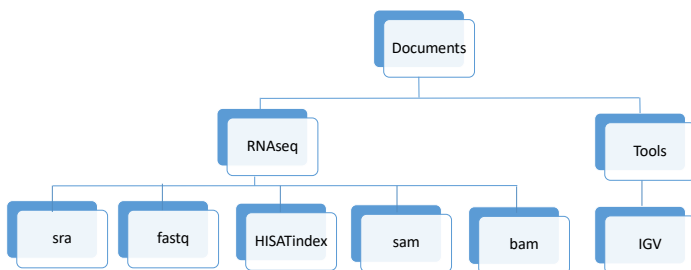


mkdir : create folder

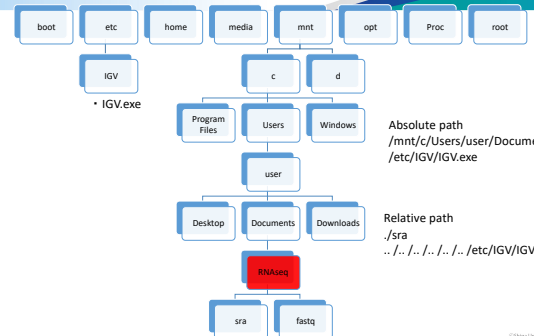
`$ mkdir RNAseq`



mkdir : create folder

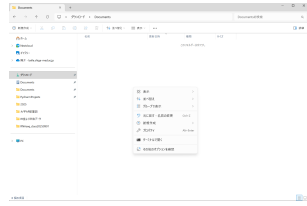


absolute path and relative path



Make text file

- Use GUI
 - Open Document folder
 - Right click → New → Text Document
 - Change name "test.txt"
 - Open "test.txt" (double click)
 - Write something to test.txt
 - For example : your name
 - Save



Let's operate by CUI



cat : View all of the text files

```
$ cat test.txt
```

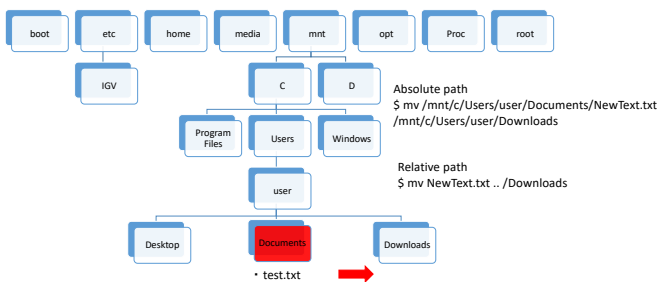
less : View some of the text files

For large text file

```
$ less command.txt
```

Press 'q' to quit.

absolute path and relative path



mv: Move file or directory

- Absolute path

```
$ mv /mnt/c/Users/user/Documents/test.txt /mnt/c/Users/user/Downloads
```

- Relative path

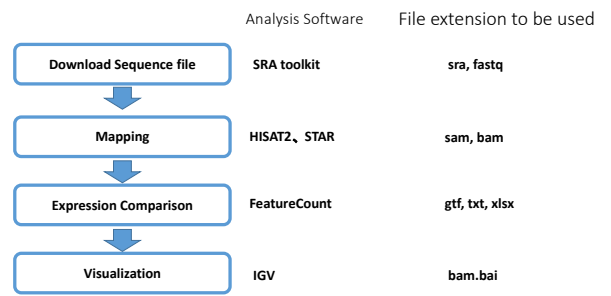
```
$ mv test.txt ../Downloads
```

CUI commands

- **Commands**
 - pwd : print working directory
 - cd : change directory
 - ls : list segments
 - mkdir : make directory
 - cat : View all of the text files
 - less : View some of the text files
- mv : Moving files and folders
- cp : Copying files and folders

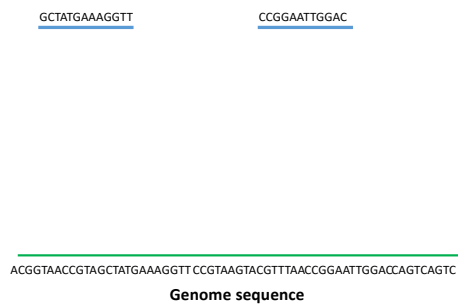
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RNAseq flowchart



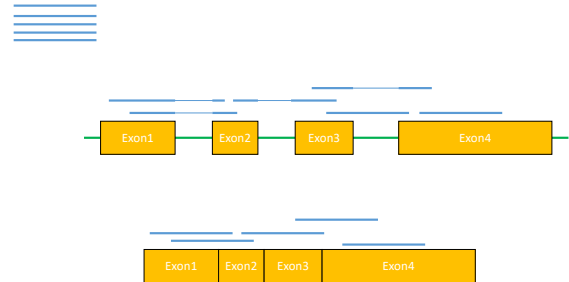
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mapping



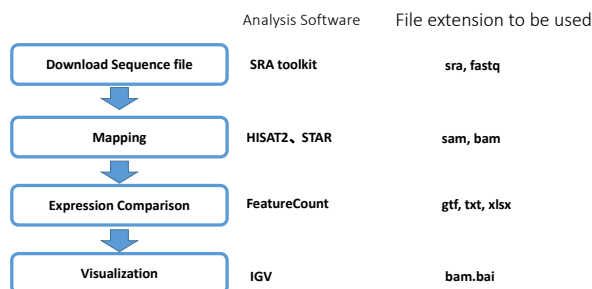
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mapping



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RNAseq flowchart



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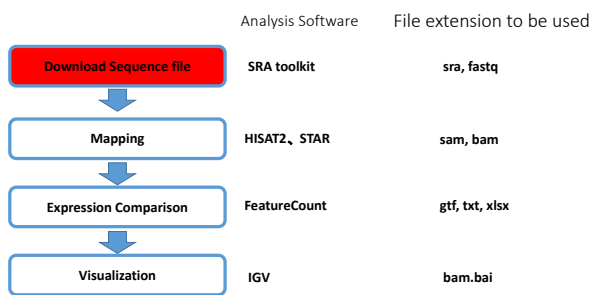
(filename) extension

- sra
 - Archive of FASTQ files
- fastq
 - Sequence data (text format)
- am
 - Mapping data (text format)
- bam
 - Mapping data (binary format)
- gz
 - compressed file

Filename.fastq.gz

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RNAseq flowchart



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International Nucleotide Sequence Database

• INSDC(International Nucleotide Sequence Database Collaboration)

- INSDC is a global cooperative of independent government agencies or non-profit organizations that manage sequence databases, collect sequence information and annotations, preserve them in the scientific record, and create a comprehensive collection of such data that can be widely shared.

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International Nucleotide Sequence Database

- The National Library of Medicine(NLM)
 - National Center of Biotechnology Information(NCBI)
 - [NCBI Sequence Read Archive\(SRA\)](#)
- The European Molecular Biology Laboratory(EMBL)
 - European Bioinformatics Institute(EBI)
 - [EBI Sequence Read Archive\(ERA\)](#)
- The Research Organization of Information and System
 - DNA Data Bank of Japan(DDBJ)
 - [DDBJ Sequence Read Archive\(DRA\)](#)

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International Nucleotide Sequence Database

- All sequence data registered in DDBJ are issued with DDBJ accession numbers. The accession number is assigned to each nucleotide sequence data registered in the database and is unique to that sequence data. Data registered in DDBJ are sent to NCBI and EBI at the time of publication, and are common worldwide. More than 99% of all registrations from Japan are made through DDBJ.

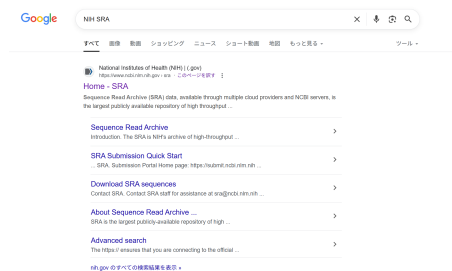
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Find an accession number

- Pubmed
 - Srsf7 Establishes the Juvenile Transcriptome through Age-Dependent Alternative Splicing in Mice
- DRP004930

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NIH SRA



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DRP004930



CLICK



Download Sequence file

- SRA-Toolkit

```
$ apt install sra-toolkit
```

Download archive file

```
$ prefetch DRR144888
```

Download Sequence file

- SRA-Toolkit

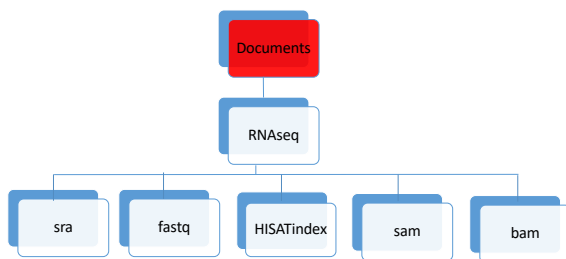
```
$ apt install sra-toolkit
```

Download archive file

```
$ prefetch DRR144888
```

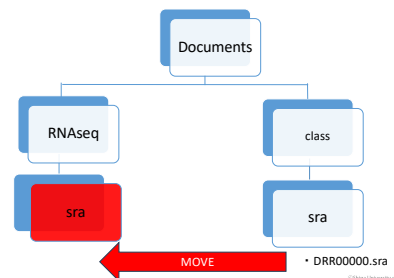
Ctrl + C ➡ Force quit

COPY cd : change directory

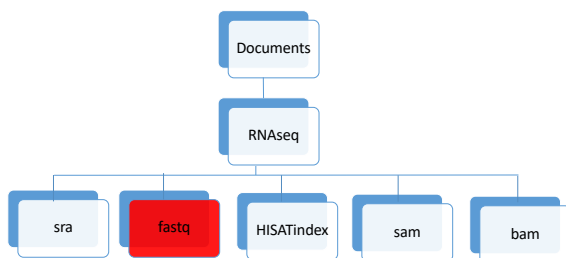


COPY Copy the ready-to-use file

```
$ mv class/sra/DRR144888.sra RNAseq/sra
```



cd : change directory



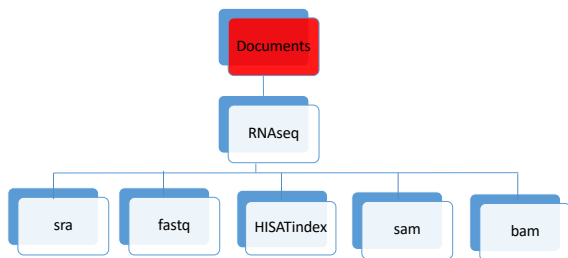
Fastq file

Convert archive files to Fastq format

```
$ fastq-dump ../SRA/DRR144879.sra
```

COPY

cd : change directory



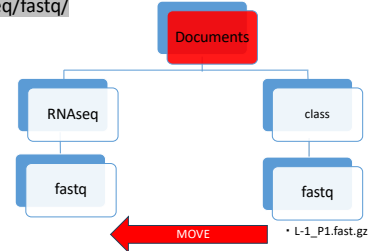
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COPY

Copy the ready-to-use file



\$ mv class/fastq/L-1_P1.fastq.gz RNAseq/fastq/



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fastq



\$ less RNAseq/fastq/L-1_P1.fastq.gz

```
@MG00HS09:723:C9A15ACXX:7:1101:1483:1919 1:N:0:CGATGT
NGACCCGCTGAATTTAAGCATATTAGTCAGCGGAGGAAAAGAACTAACCA
+
#11B?D@8DAF?DGGECHFDF?4ACFB?GEGGB6)?69DE;FGE=4@F)=C
```

- 1 : @Sequence ID and additional information
- 2 : Nucleotide sequence
- 3 : + Sequence ID and additional information
- 4 : sequence quality

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ASC2

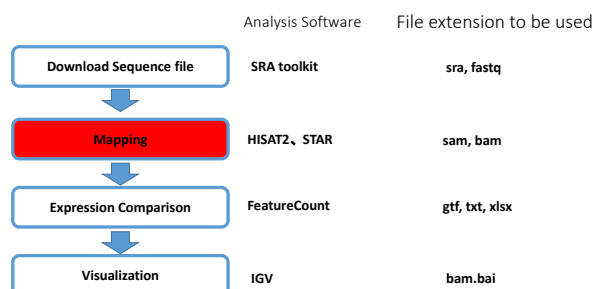


33!	45-	57 9	69E	81Q
34"	46.	58:	70F	82R
35#	47/	59;	71G	83S
36\$	48 0	60<	72H	84T
37%	49 1	61=	73I	85U
38&	50 2	62>	74J	86V
39'	51 3	63?	75K	87W
40(	52 4	64@	76L	88X
41)	53 5	65A	77M	89Y
42*	54 6	66B	78N	90Z
43+	55 7	67C	79O	
44,	56 8	68D	80P	

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ASCII character	ASCII code	Quality score	Probability of incorrect bases	ASCII character	ASCII code	Quality score	Probability of incorrect bases
!	33	0	1.0 (100%)	6	54	21	0.00794 (0.794%)
"	34	1	0.794 (79.4%)	7	55	22	0.00631 (0.631%)
#	35	2	0.631 (63.1%)	8	56	23	0.00501 (0.501%)
\$	36	3	0.501 (50.1%)	9	57	24	0.00398 (0.398%)
%	37	4	0.398 (39.8%)	:	58	25	0.00316 (0.316%)
&	38	5	0.316 (31.6%)	;	59	26	0.00251 (0.251%)
'	39	6	0.251 (25.1%)	<	60	27	0.00199 (0.199%)
(	40	7	0.199 (19.9%)	=	61	28	0.00158 (0.158%)
)	41	8	0.158 (15.8%)	>	62	29	0.00126 (0.126%)
*	42	9	0.126 (12.6%)	?	63	30	0.00100 (0.1%)
+	43	10	0.100 (10%)	@	64	31	0.000794 (0.0794%)
,	44	11	0.0794 (7.94%)	A	65	32	0.000631 (0.0631%)
-	45	12	0.0631 (6.31%)	B	66	33	0.000501 (0.0501%)
.	46	13	0.0501 (5.01%)	C	67	34	0.000398 (0.0398%)
/	47	14	0.0398 (3.98%)	D	68	35	0.000316 (0.0316%)
0	48	15	0.0316 (3.16%)	E	69	36	0.000251 (0.0251%)
1	49	16	0.0251 (2.51%)	F	70	37	0.000199 (0.0199%)
2	50	17	0.0199 (1.99%)	G	71	38	0.000158 (0.0158%)
3	51	18	0.0158 (1.58%)	H	72	39	0.000126 (0.0126%)
4	52	19	0.0126 (1.26%)	I	73	40	0.000100 (0.01%)
5	53	20	0.0100 (1.0%)				

RNAseq flowchart



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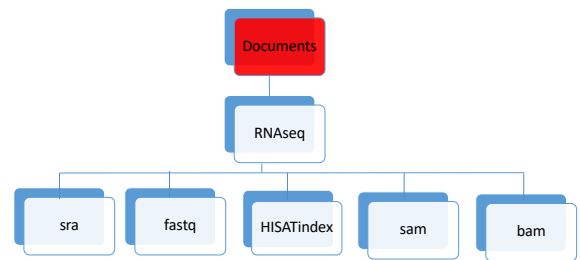
Download Index file of the genome

- <http://daehwankimlab.github.io/hisat2/download/#m-musculus>

HL, Ensembl	
• GRCh38	
genome	https://genome-dls.us.amsresearch.com/blast/ht2l_genome.tar.gz
genome_snp	https://genome-dls.us.amsresearch.com/blast/ht2l_snp.tar.gz
genome_snp_bim	https://genome-dls.us.amsresearch.com/blast/ht2l_bim.tar.gz
genome_snp_bim_2.0.0	https://genome-dls.us.amsresearch.com/blast/ht2l_snp_bim_2.0.0.tar.gz
genome_snp_bim_2.0.0	https://genome-dls.us.amsresearch.com/blast/ht2l_snp_bim_2.0.0.tar.gz
• UCSC hg38	
genome	https://genome-dls.us.amsresearch.com/blast/ht2l_genome.tar.gz
genome_snp	https://genome-dls.us.amsresearch.com/blast/ht2l_snp.tar.gz
genome_snp_bim	https://genome-dls.us.amsresearch.com/blast/ht2l_bim.tar.gz
• UCSC hg19	
genome	https://genome-dls.us.amsresearch.com/blast/ht2l_genome.tar.gz
genome_snp	https://genome-dls.us.amsresearch.com/blast/ht2l_snp.tar.gz
genome_snp_bim	https://genome-dls.us.amsresearch.com/blast/ht2l_bim.tar.gz
• UCSC hg19	
genome	https://genome-dls.us.amsresearch.com/blast/ht2l_genome.tar.gz
genome_snp	https://genome-dls.us.amsresearch.com/blast/ht2l_snp.tar.gz
genome_snp_bim	https://genome-dls.us.amsresearch.com/blast/ht2l_bim.tar.gz
HL, Musculus	
• GRCh38	
genome	https://blast.broadinstitute.org/blast/ht2l_genome.tar.gz
genome_snp	https://blast.broadinstitute.org/blast/ht2l_snp.tar.gz
genome_snp_bim	https://blast.broadinstitute.org/blast/ht2l_bim.tar.gz

COPY

cd : change directory

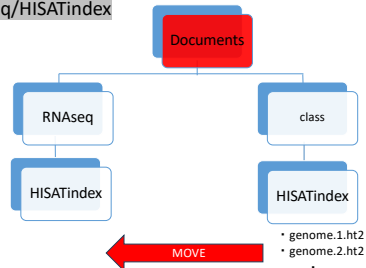


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COPY

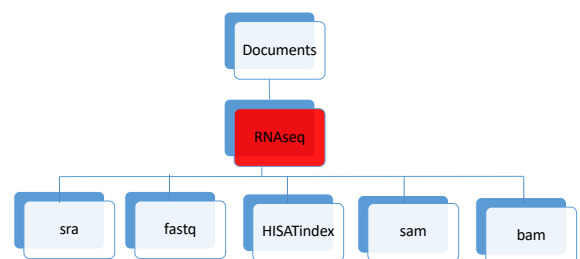
Copy the ready-to-use file

\$ mv class/HISATIndex/genome* RNAseq/HISATIndex



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cd : change directory



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Mapping

- Install hisat2
 - \$ sudo apt install hisat2
- hisat2 -p [number of CPU] -x [genome index] -U [input file] -S [output file]
- Mapping by using hisat2. Make sam file.
 - \$ hisat2 -p 2 -x HISATIndex/genome -U fastq.gz/L-1_P1.fastq.gz -S sam/L1.sam

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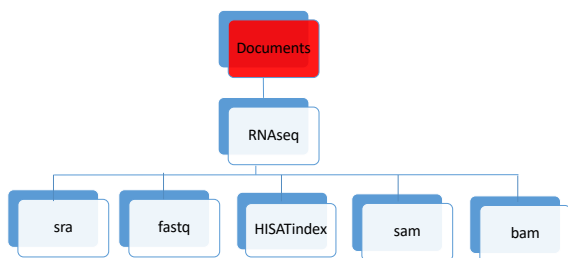
HISAT result

26650646 reads; of these:
 26650646 (100.00%) were unpaired; of these:
 2936747 (11.02%) aligned 0 times
 18286578 (68.62%) aligned exactly 1 time
 5427321 (20.36%) aligned >1 times
 88.98% overall alignment rate

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COPY

cd : change directory



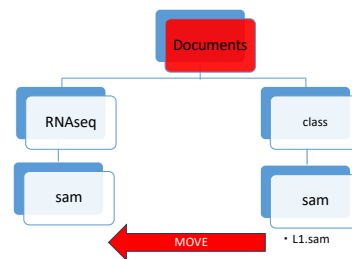
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COPY

Move the ready-to-use file

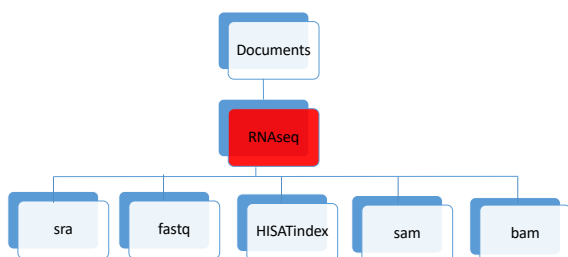


`$ mv class/sam/L-1.sam RNAseq/sam/`



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cd : change directory



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Convert sam file to bam file



- Install samtools
 - `$ sudo apt install samtools`

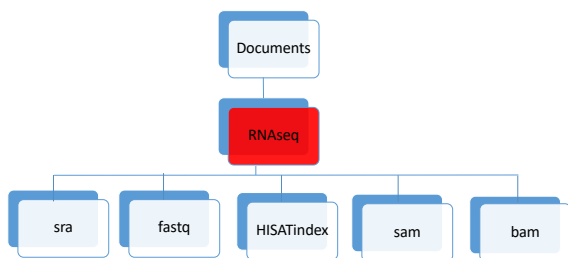
Convert sam file to bam file

`$ samtools view -@ [cpu number] -bS [input sam file] > [output bam file]`

`$ samtools view -@ 2 -bS sam/L1.sam > bam/L1.bam`

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cd : change directory

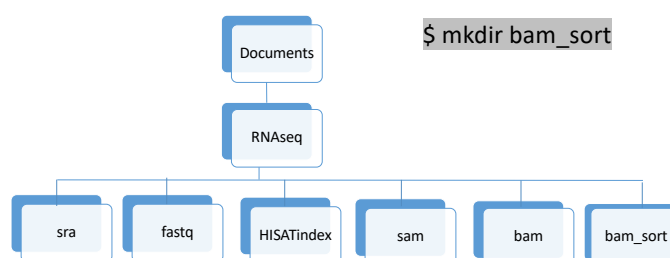


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mkdir : make directory



`$ mkdir bam_sort`



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```
$ samtools sort -@ [cpu number] -o [output sorted bam file] [input bam file]
```

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L1.sort.bam.bai

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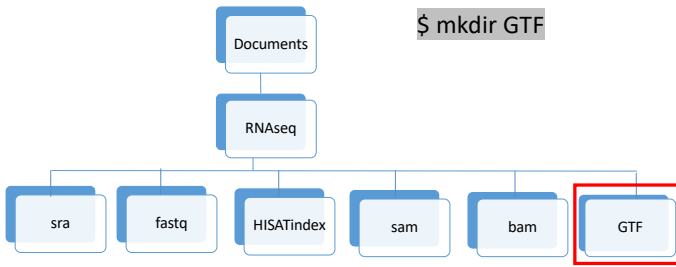
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mkdir : make directory



```
$ mkdir GTF
```



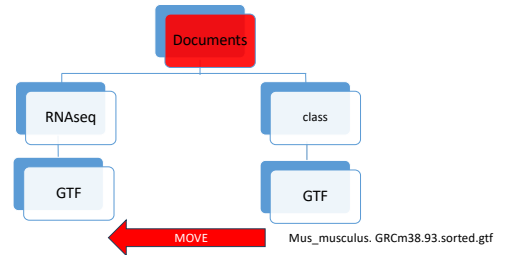
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COPY

Move the ready-to-use file

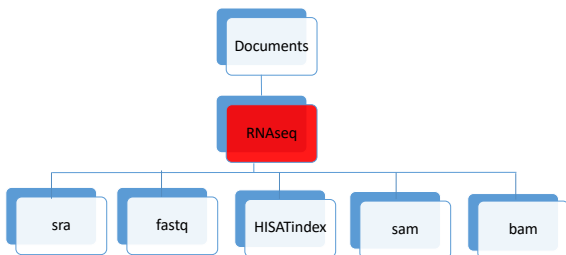


```
$ mv class/GTF/Mus_musculus.GRCm38.93.sorted.gtf RNAseq/GTF
```



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cd : change directory

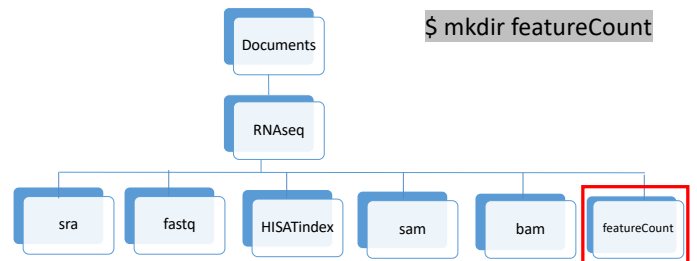


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mkdir : make directory



```
$ mkdir featureCount
```



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Expression Comparison



- Install FeatureCounts

```
$ sudo apt install subread
```

```
featureCounts -T [number of CPU] --extraAttributes gene_name -a [GTF file] -o [output file] [input file1] [input file2] [input file3]
```

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Expression Comparison



- Count the number of reads mapped to each gene

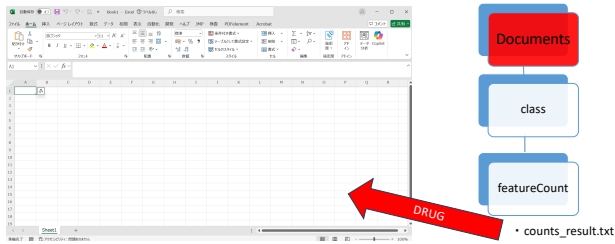
```
$ featureCounts -T 8 --extraAttributes gene_name -a GTF/Mus_musculus.GRCm38.93.sorted.gtf -o featureCount/counts_result.txt bam_sort/L1.sort.bam
```

```
$ featureCounts -T 8 --extraAttributes gene_name -a GTF/Mus_musculus.GRCm38.93.sorted.gtf -o featureCount/counts_result.txt bam_sort/L1.sort.bam bam_sort/L2.sort.bam bam_sort/L3.sort.bam bam_sort/L4.sort.bam bam_sort/L5.sort.bam bam_sort/L6.sort.bam bam_sort/L7.sort.bam bam_sort/L8.sort.bam bam_sort/L9.sort.bam
```

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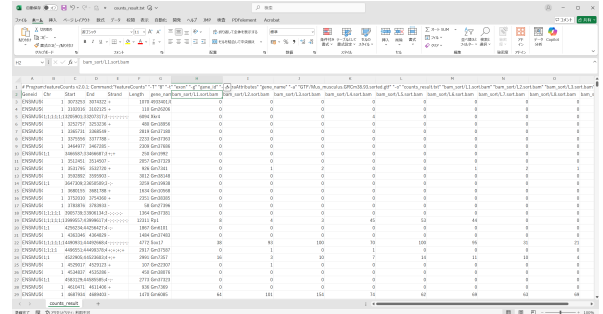
Open file by Excel

50



Open counts_result.txt file by Excel

50



Sample detail

50

No.	Accession No.	dev_stage	replicate	tissue_type	Sample Name
1	DRR144888	postnatal day1	biological replicate 1	liver	L1.bam
2	DRR144889	postnatal day1	biological replicate 2	liver	L2.bam
3	DRR144890	postnatal day1	biological replicate 3	liver	L3.bam
4	DRR144891	Postnatal Day7	biological replicate 1	liver	L4.bam
5	DRR144892	Postnatal Day7	biological replicate 2	liver	L5.bam
6	DRR144893	Postnatal Day7	biological replicate 3	liver	L6.bam
7	DRR144894	Postnatal Day56	biological replicate 1	liver	L7.bam
8	DRR144895	Postnatal Day56	biological replicate 2	liver	L8.bam
9	DRR144896	Postnatal Day56	biological replicate 3	liver	L9.bam

Expression Comparison

50

Gene expression ≠ Number of reads mapped on the gene

- These biases need to be corrected before comparing expression levels.
 - Longer genes have more reads mapped (**intergenic bias**)
 - The more samples (amount of cDNA), the more reads mapped (**sample bias**)

Normalize

50

- TPM (transcripts per million)

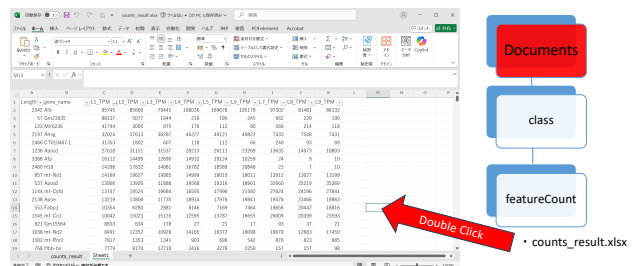
$$A_i = \frac{q_i}{l_i} * 10^3$$

$$TPM_i = A_i * \frac{1}{\sum_j A_j} * 10^6$$

q : The number of reads mapped
l : The transcript length

Open file by Excel

50



Open counts_result.xlsx

Documents/class/featureCount_result/counts_result.xlsx

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	Length	gene_name	L1_TPM	L2_TPM	L3_TPM	L4_TPM	L5_TPM	L6_TPM	L7_TPM	L8_TPM	L9_TPM		
2	2342	Alb	95745	85660	79445	168036	169678	195179	97302	81481	88132		
3	57	Gm23935	86137	5077	1844	216	196	245	652	220	190		
4	123	Mir6236	41744	3005	975	176	112	90	569	214	118		
5	2197	Ahog	32024	37613	36287	46227	49121	49822	7433	7548	7431		
6	2466	CTO10467.1	31553	1802	607	118	112	66	240	93	69		
7	1236	Appa1	27618	31151	31537	28213	26111	23269	13635	14073	10803		
8	3368	Alp	16112	14499	12690	14932	19124	10259	24	9	10		
9	2460	H19	14298	17832	14081	16782	18589	20848	23	7	10		
10	957	mt-Nd1	14169	19627	19985	14999	18010	18011	13912	13027	13199		
11	537	Appa2	13906	13926	11888	19568	19316	18901	32660	35219	35369		
12	1144	mt-Cytb	13747	19524	19684	16595	17996	21492	27624	24196	27841		
13	2136	Apoe	13219	13808	11735	18914	17976	19841	19478	23456	19883		
14	553	Fabp1	10354	6280	2981	8146	7169	7464	19656	20442	18816		
15	1545	mt-Co1	10042	15023	15135	12595	13787	16655	26009	20399	23593		
16	821	Gm15564	8833	634	178	27	21	17	93	37	21		
17	1038	mt-Nd2	8491	12352	10528	14165	16372	18098	18679	12683	17459		
18	1582	mt-Rnr2	7817	1353	1341	903	699	542	876	823	885		
19	268	Hbb-bh	7776	8170	17310	3416	3778	2945	157	157	68		

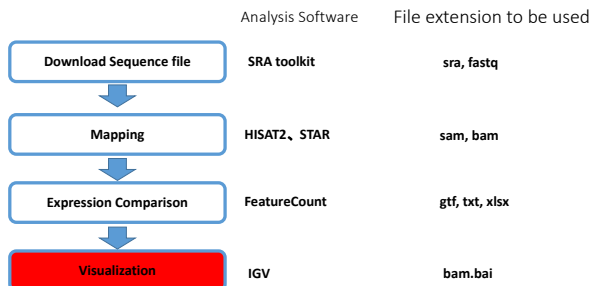
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Sample detail

No.	Accession No.	dev_stage	replicate	tissue_type	Sample Name
1	DRR144888	postnatal day1	biological replicate 1	liver	L1.bam
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3	DRR144890	postnatal day1	biological replicate 3	liver	L3.bam
4	DRR144891	Postnatal Day7	biological replicate 1	liver	L4.bam
5	DRR144892	Postnatal Day7	biological replicate 2	liver	L5.bam
6	DRR144893	Postnatal Day7	biological replicate 3	liver	L6.bam
7	DRR144894	Postnatal Day56	biological replicate 1	liver	L7.bam
8	DRR144895	Postnatal Day56	biological replicate 2	liver	L8.bam
9	DRR144896	Postnatal Day56	biological replicate 3	liver	L9.bam

rel

RNAseq flowchart



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Visualization

- IGV (Integrative Genomics Viewer)

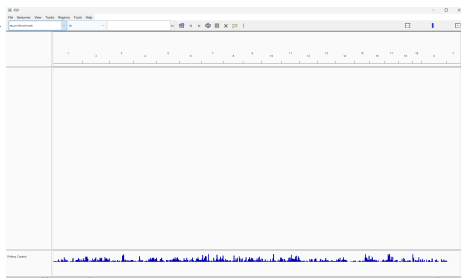


IGV_2.17.4

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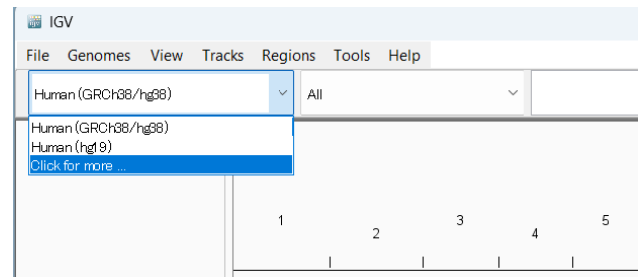
IGV

Look here



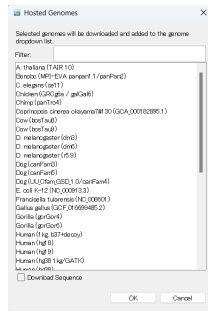
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Selection of the animal species



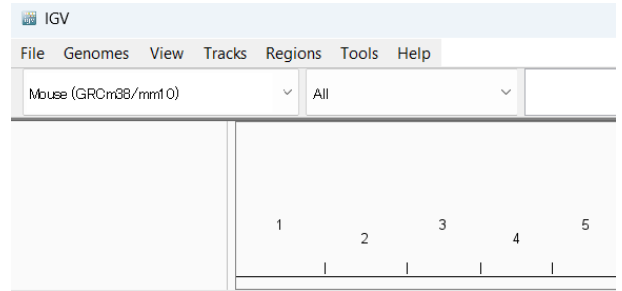
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Select Genomes

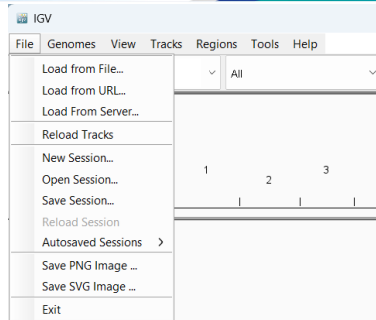


mm10

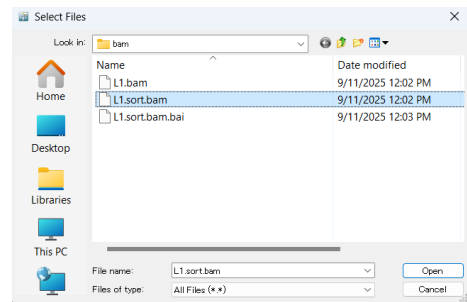
Mouse(GRCm38/mm10)



Load from file

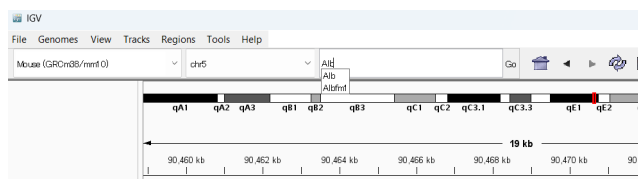


Open L1.sort.bam

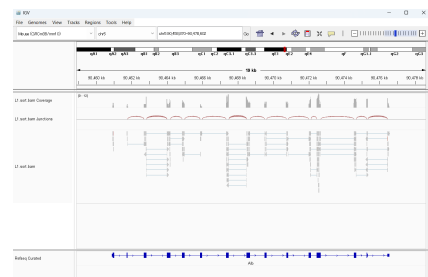


Alb

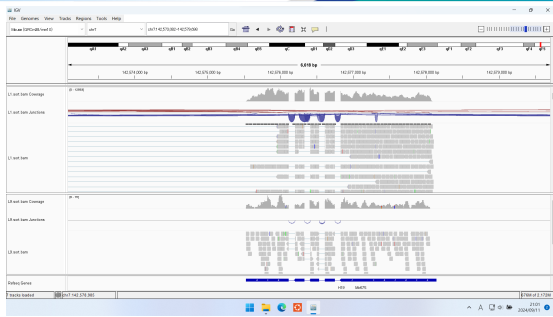
Alb



Alb

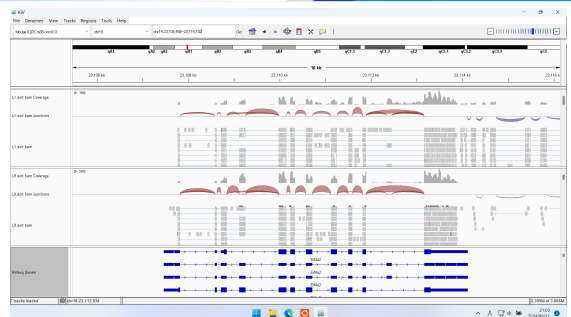


Visualization



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Visualization



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Summary



- The basic workflow and analysis methods of RNA-seq.
- The process from data acquisition to mapping, expression analysis, and visualization.
- The fundamentals of UNIX environments and CUI operations.
- How to use analysis software and databases.

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Thanks for Listening



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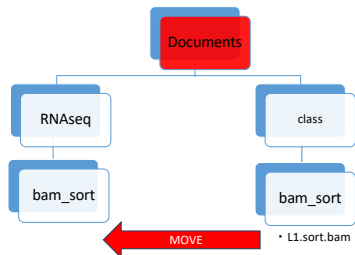
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COPY

Move the ready-to-use file



```
$ mv class/bam_sort/L-1.sort.bam RNAseq/ bam_sort/
```



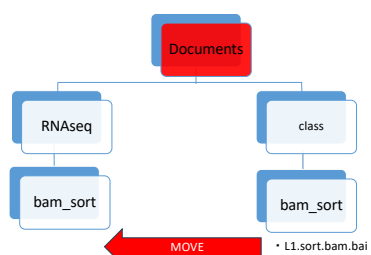
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COPY

Move the ready-to-use file



```
$ mv class/bam_sort/L-1.sort.bam.bai RNAseq/bam_sort
```



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