NGS, from **data** to **table** to **figure**

From downloading data to final publication ready figures

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Object

1. I hope everyone can follow me, and I ensure you will meet these problems if you do NGS analysis or data analysis.
2. If you have any question, please **don't hesitate** to ask a question, actually some of the materials are very difficult to me.
3. Although it may take some time to learn, but it will save a lot of time in the future.
4. I hope after each class, you can make some practice and read the supplement files.

Object

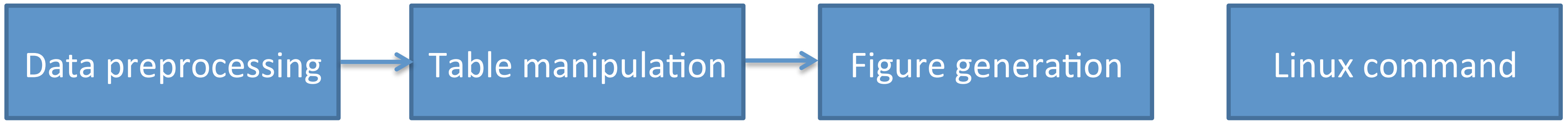
5. All the course martials can be download from
https://github.com/regSNPs/from_data_2_table_2_figure

6. After this report, you will get basic understanding of NGS analysis and data analysis, and try to practice it.

Workflow of the report

- 1. Data preprocessing (First we need to know where we can got the data and how to preprocess the data)
- 2. Table manipulation (We usually don't use the raw data directly, we need process them into a desired format for further investigation)
- 3. Figure generation (Human beings can't read thousands of rows in table simultaneously, we need figure to visualize them)
- 4. Linux (Besides R, we also need Linux to facilitate some work)

Let's learn together



The required knowledge include:

Biology (understand the biology process of the problem)

Programming skill (understand the code)

Statistic graphic (understand the figure)

The example problem

We will follow a problem during the course.

Problem to solve: RNA binding protein binding gene analysis.

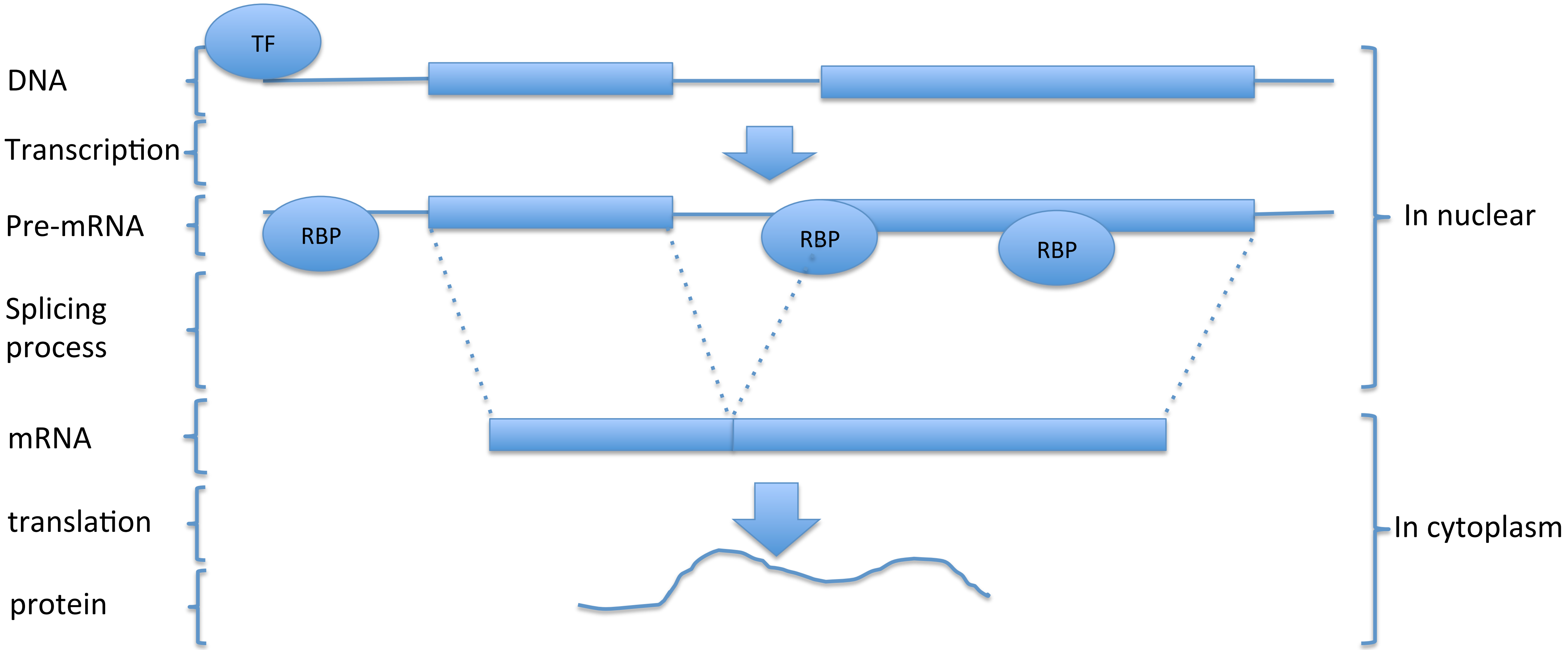
We want to study the relationship between multi RBPs and gene expression. The object is to study which RBP enhance gene expression, and which RBP repress gene expression.

The biology process of this

Although I'm not very good at biology, but **the most important thing is understanding the biology process of the problem**. which will make the analysis meaningful.

I will try to explain the biology process of this problem first.

The biological process of gene expression



We all know the TF can affect the gene expression, but **will the RBPs regulate the gene expression through regulating the transcription of pre-mRNA**? I don't know either, let's do it!!

The analysis process

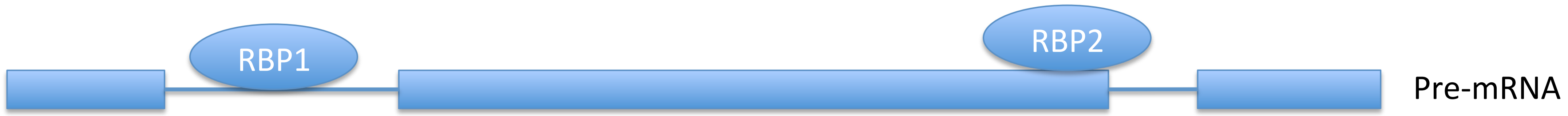
- 1. We need data to know where the RBP binds? This data can be got from **CLIP-seq technique.**

- 2. We need to know how the gene expressed in a cell line. This data can be got from **RNA-seq technique**.

- 3. We need to ensure that both two kinds of dataset **come from the same cell line**. After we got the above information, our final analysis is doing **correlation** between RBP binding count and gene expression.

The CLIP-seq technique

First we need to know where the RBP binds? The below is a demonstration of RBP binding region, **in reality, we don't know where the RBP binds.**



The technique is called CLIP-seq, which can locate the target of RBP binding sites in the pre-mRNA.

The CLIP-seq experiment

Got from Wikipedia

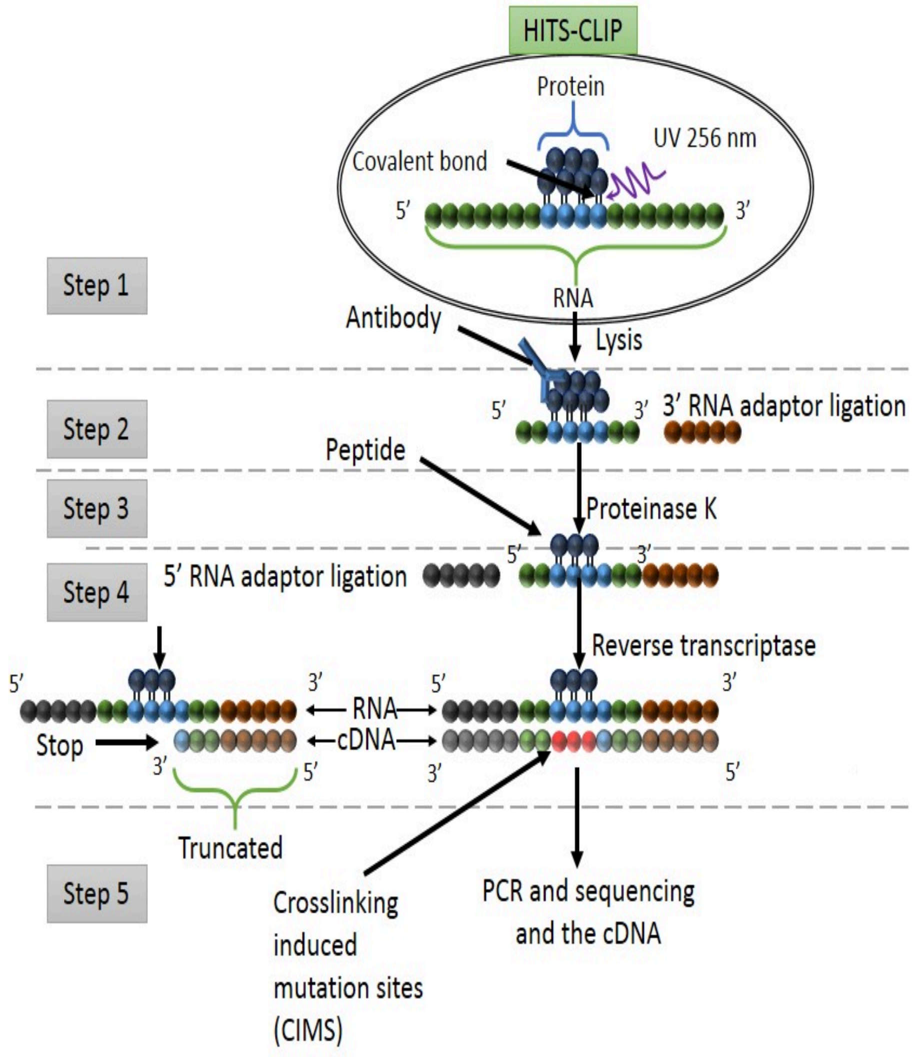


Figure 2: HITS-CLIP

Step 1
HITS-CLIP begins with the in-vivo cross-linking of RNA-protein complexes using ultraviolet light. The cell is lysed and the protein of interest is isolated using immunoprecipitation.

Step 2
Washing is performed to remove free RNA, and RNA adaptors are ligated at the 3′ ends.

Step 3
Proteinase K digestion is performed. This leaves a peptide at the cross-link site that modifies the chemical structure of the nucleotide.

Step 4
5` RNA adaptors are ligated and cDNA is synthesized using reverse transcription.

Step 5
PCR and sequencing of the cDNA.

CLIP-seq workflow

It is a technique just like the CHIP-seq, whereas work on pre-mRNA and RBPs.

The goal of the technique is extracting the sequence in the RBP binding sites.

We just need to know that the reads we will see later come from the binding sites.

I will show you the standard protocol later.

Sequence Database and annotation database

- There are **two kinds of biology database**. We need sequence database to get the sequence and annotation database to get gene annotation.

- 1. The sequence database store the sequence from sequencer, this kind of database is very very large.

- 2. The annotation database stores the genome annotation.

The sequence database

- Once we decide the data (**CLIP-seq** dataset) we need. Next step is selecting the database that we are going to extract the data.

- There are various kinds of sequence database, eg: **Encode, modEncode, GEO, SRA, EBI, GTEx, TCGA, 1000 Genome Project, ESP6500**

- I will give a brief introduction for these databases.

The annotation database

- There are many annotation database available which include: UCSC, refseq, and Ensembl, Uinprot, PDB et.al.
- I will give a bries introduction to these databases.

- Usually, refseq is more accurate while Ensembl is more comprehensive.
- For refseq hg19 (GRCh37), it contains around 50000 transcripts.
- For ensembl hg19 (GRCh37), it contains around 200000 transcripts.

Encode&UCSC

- Encode
- Encode dataset matrix:
- https://www.encodeproject.org/matrix/?type=Experiment&x.limit=

- UCSC
- Table browser, download various kinds of annotation from different assemble.

Encode&UCSC database

- I will show you the two databases.

- One is the encode database, another is the UCSC database.
- **For encode, I will show how to get CLIP data for SRSF1 in K562.** (SRSF1.web) https://www.encodeproject.org/experiments/ENCSR432XUP/

- **For UCSC, I will show how to get the annotation, which will be used in various kinds of NGS analysis.** (e.g. How to use the **table browser**, how to use **download**)

UCSC

- Besides the web interface, UCSC also provide two kinds of methods to extracting data from their database.
- 1. DAS server
  [E.g.http://genome.ucsc.edu/cgi-bin/das/hg19/dna?segment=chr4:35654,35695](http://genome.ucsc.edu/cgi-bin/das/hg19/dna?segment=chr4:35654,35695)
- 2. mysql client:

   Users can also use various kinds of mysql client (e.g. mysql workbench, JDBC et.al.)

```
Mysql –user=genome –host=genom-mysql.cse.ucsc.edu -A
```

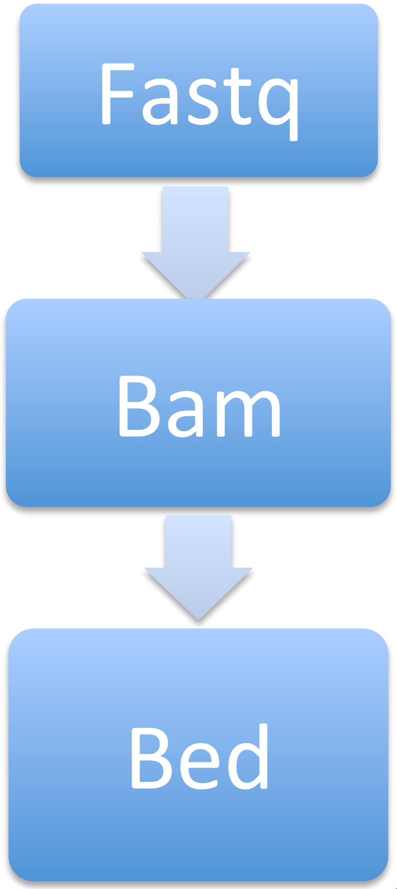
Data formats

The download eCLIP data from Encode can be many different formats.

Including:
fastq format (original reads),
bam format (alignment file),
bed format (peaks region file).

Here we assume the raw format is fastq format, I will show you the above formats.
(UCSC format help:
http://genome.ucsc.edu/FAQ/FAQformat.html)

Data preprocessing workflow



From fastq to bam

- The main command here is **read alignment**.

```
   e.g  bowtie2 [options]* -x <bowtie-index> {-1 read1 -2 read2 | -U
<r>} [-S <sam>]
```

- But there are possibly other command, eg:

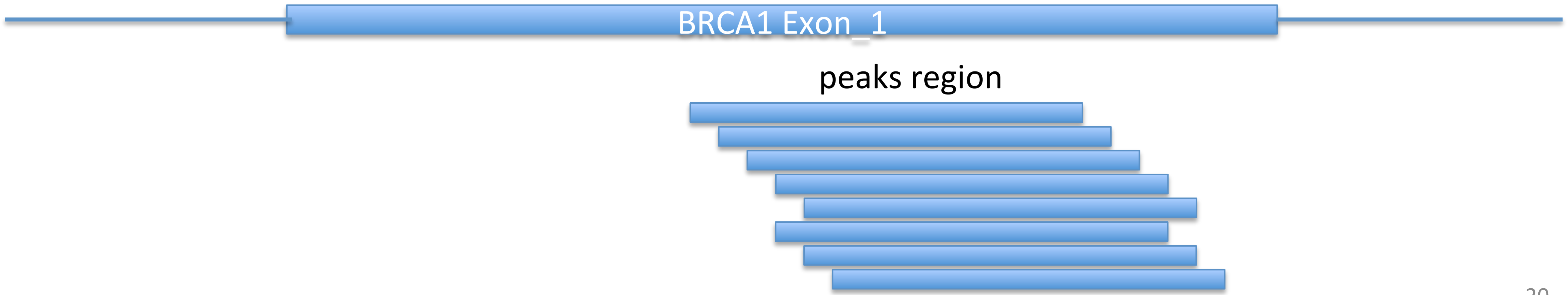
1. Demultiplexing (sometimes, multi samples are sequenced once a time, multiplexing is a technique to index them)
2. Cutadaptor (adaptor is used to do sequencing, we need to remove the adapters from head or tail of the adaptor)
3. FastQC (quality control)
4. rmRep (remove repetitive elements)

The BAM file we got

A bam file looks like below:

```
chr1    13232    33421    ATAAFCFAD    &%dfd2$@$  …
chr2    61323    62121    ATCTTGCGT    *&dfd2)a#  …

…. .
```

The bam file can be viewed through IGV viewer.
I will show you a IGV image.



Quality control

- Usually, we need to check the quality of the result bam file.

- The possible criterion include:
- % of reads mapped to the genome.
- % of reads mapped to the exon region.
- % of reads mapped to the positive strand.
- % of reads mapped to the negative strand.

From bam to bed

- Next we need to call peaks in the **bam** file. It extracted the regions that contain the reads.
- The main command is the **Clipper**.

- It also include:
- Samtools sort (sort the bam file by position)
- Samtools index (index the bam file to do fast retrieve)
- BigbedTobed (convert Bigbed to bed file)

The full pipeline here

- eCLIP_analysisSOP_v1.P.pdf. (the computation pipeline)

- eCLIP_SOP_v1.P_110915.pdf. (the experiment pipeline)

- I will show you the standard analysis pipeline in the supplement file.

The data we got (SRSF_rep1.bed)

```
chr1 15212    15250    ENSG00000227232.4_0_3    15   -   -1   -1   0.0292794233248   15230
chr1 16239    16287    ENSG00000227232.4_1_4    19   -   -1   -1   0.0104649442093   16263
chr1 16441    16485    ENSG00000227232.4_2_5    25   -   -1   -1   0.00256249972915 16462
chr1 17451    17517    ENSG00000227232.4_3_10   63   -   -1   -1   4.50322291742e-07        17481
chr1 90235    90280    ENSG00000239945.1_0_4    19   -   -1   -1   0.0105097488596   90256
chr1 90240    90275    ENSG00000238009.2_0_4    20   -   -1   -1   0.00879079737544 90256
chr1 109158   109192   ENSG00000238009.2_1_5    29   -   -1   -1   0.00115927397829 109175
chr1 113824   113881   ENSG00000238009.2_2_8    49   -   -1   -1   1.21214630854e-05        113848
chr1 115708   115784   ENSG00000238009.2_3_22   145  -   -1   -1   2.6562287759e-15  115737
chr1 116372   116428   ENSG00000238009.2_4_13   74   -   -1   -1   3.4175167505e-08  116397
chr1 135196   135226   ENSG00000237683.5_0_3    15   -   -1   -1   0.0270886550409   135213
chr1 135196   135226   ENSG00000268903.1_0_3    15   -   -1   -1   0.0274502908066   135213
```

Each row is a peak region. **Now we know where the RBP binds!**

Column 9 is the p-value. We can filter some peaks using p-value.

Column 4 is the Ensembl gene id. The gene of the peaks locate.

Basic quality control

- Usually, we need to check the file to ensure the file we got is right, this can be done by basic Linux command.

- Some possible criterion is:
- The number of peaks in the files.
- How many chromosomes in the file.
- The number of peaks in the chromosome 1.
- The p-values of the peaks.

Most used Linux command

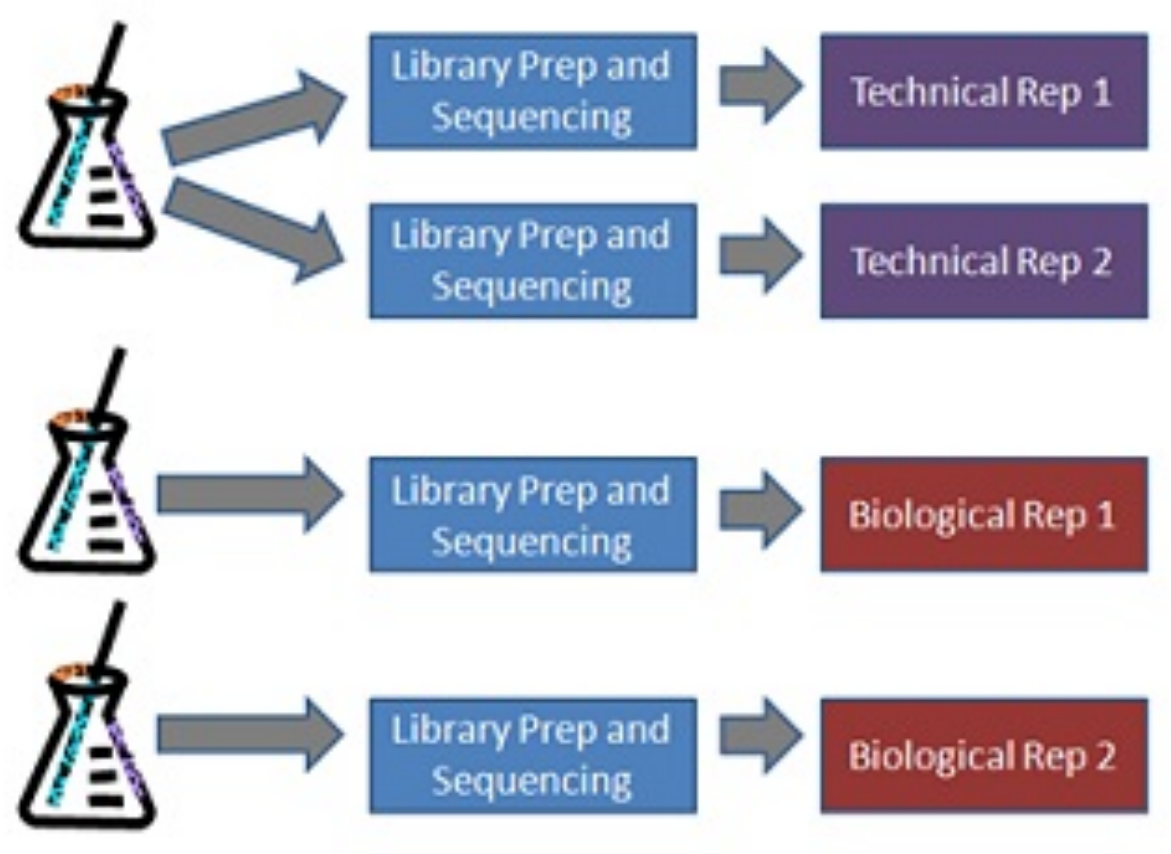
```
#Count how many peaks in the file:
wc –l SRSF1_rep1.bed; (39116)

#Count how many peaks in chr12:
cut –f 1 SRSF1_rep1.bed | grep chr12 | wc –l (2133)

#Get the p-value column:
cut –f 9 SRSF1_rep1.bed > SRSF1_rep1_pvalue.txt
```

Most used Linux command

```
#how many chromosome in it
cut –f 1 SRSF1_rep1.bed | sort –u | wc –l

#remove the ‘chr’ in each line
cut –c 4- SRSF1_rep1.bed >SRSF1_rep1_noChr.bed

#count the peaks in each of the file and output to a new file
for i in `ls *.bed`
do
   `wc –l $i >> peaks_for_each_file.txt`;
done;
```

Biology replicate

- Usually we use at least one biological replicate to ensure the peaks we found are not due to variance.


Library Prep and Sequencing
Technical Rep 1
Library Prep and Sequencing
Technical Rep 2
Library Prep and Sequencing
Biological Rep 1
Library Prep and Sequencing
Biological Rep 2

Overlap of two biological replicates

- One possible solution is finding the overlapped regions between the two biology replicates, and **treat the overlapped region as peaks**.

- But how to find the overlapped region between two bed files?

The two bed files

Bed_rep_1

```
chr1 14924	14954	ENSG00000227232.4_0_3	15	-	-1	-1	0.0286089808565	14938
chr1 17454	17567	ENSG00000227232.4_1_15	95	-	-1	-1	2.52804288143e-10	17488
chr1 89861	89871	ENSG00000238009.2_0_3	16	-	-1	-1	0.020756022461	89862
chr1 89861	89871	ENSG00000239945.1_0_3	16	-	-1	-1	0.0211020769049	89862
chr1 89871	89904	ENSG00000238009.2_1_3	16	-	-1	-1	0.0246792521386	89885
chr1 89871	89904	ENSG00000239945.1_1_3	15	-	-1	-1	0.0258746724881	89885
```

Bed_rep_2

```
chr1 15212	15250	ENSG00000227232.4_0_3	15	-	-1	-1	0.0292794233248	15230
chr1 16239	16287	ENSG00000227232.4_1_4	19	-	-1	-1	0.0104649442093	16263
chr1 16441	16485	ENSG00000227232.4_2_5	25	-	-1	-1	0.00256249972915	16462
chr1 17451	17517	ENSG00000227232.4_3_10	63	-	-1	-1	4.50322291742e-07	17481
chr1 90235	90280	ENSG00000239945.1_0_4	19	-	-1	-1	0.0105097488596	90256
chr1 90240	90275	ENSG00000238009.2_0_4	20	-	-1	-1	0.00879079737544	90256
```

The general problem of overlapping

Here we need to find the overlap between the two bed files, actually sometimes we also need to overlap with different kinds of files.

The most common file formats we encountered are:

BAM, BED, GTF, VCF

They are all interval range formats, i.e. they all contain a chromosome name and at least a start position. A **generic solution to this problem** is need.

Find overlap of biology replicates

- R package GenomicRanges:
- Bed file is table range format, also some other file formats like VCF, GFF, GTF. We need efficient methods to overlap between these kinds of files.
- 1. One Linux solution is using **Tabix**, while a java solution a **tribble**.
- 2. A more powerful solution is using R package **GenomicRanges**. It can efficiently map between ranges format files. GenomicRangesIntroduction.pdf

VCF, BED, GTF file format

- I will show you the above VCF, BED, GTF file formats in UCSC, and show that they are all the general chromosome, coordinate format.

- https://genome.ucsc.edu/FAQ/FAQformat.html#format10.1

R package GenomicRanges

To try to use it, First we need to read the file and then converting Bed, VCF, GFF, GTF to **GenomicRange** objects.
Reference:

Bed format is 0-based and its left coordinate is inclusive, while the right coordinate is exclusive

```
Codes:
#First read bed file into table biology_rep_
SRSF1_rep_1_bed<-read.table("Bed_rep_1")

SRSF1_rep_1_range<-with(SRSF1_rep_1_bed,Granges(seqnames=chr,
+ranges=IRanges(start=start+1,end=end),strand=strand)

SRSF1_rep_2_range<-with(SRSF1_rep_2_bed,Granges(seqnames=chr,
+ranges=IRanges(start=start+1,end=end),strand=strand)
```

R package GenomicRanges

Find the peaks which overlap between the two biology replicates.

```
Codes:
##overlap the two biological replicates.
SRSF1_overlap_mch<-findoverlaps(SRSF1_rep_1_range, SRSF1_rep_2_range);
##get the overlap in replicate 1.
SRSF1_rep_1_overlap<-
SRSF1_rep_1_range[unique(queryHits(SRSF1_overlap_mch)),];
##get the overlap in replicate 2.
SRSF1_rep_2_overlap<-
SRSF1_rep_2_range[unique(subjectHits(SRSF1_overlap_mch)),];
```

What the overlap result looks like

```
#RBP_overlap_mtch is revord the line number of each overlap.
>RBP_overlap_mtch
      queryHits      subjectHits
 [1,]       2             1
 [2,]       3             1
 [3,]       4             1
 [4,]       5             2
 [5,]       6             2
```

What we got currently

What we got is the overlapped peak regions between the two biological replicates.

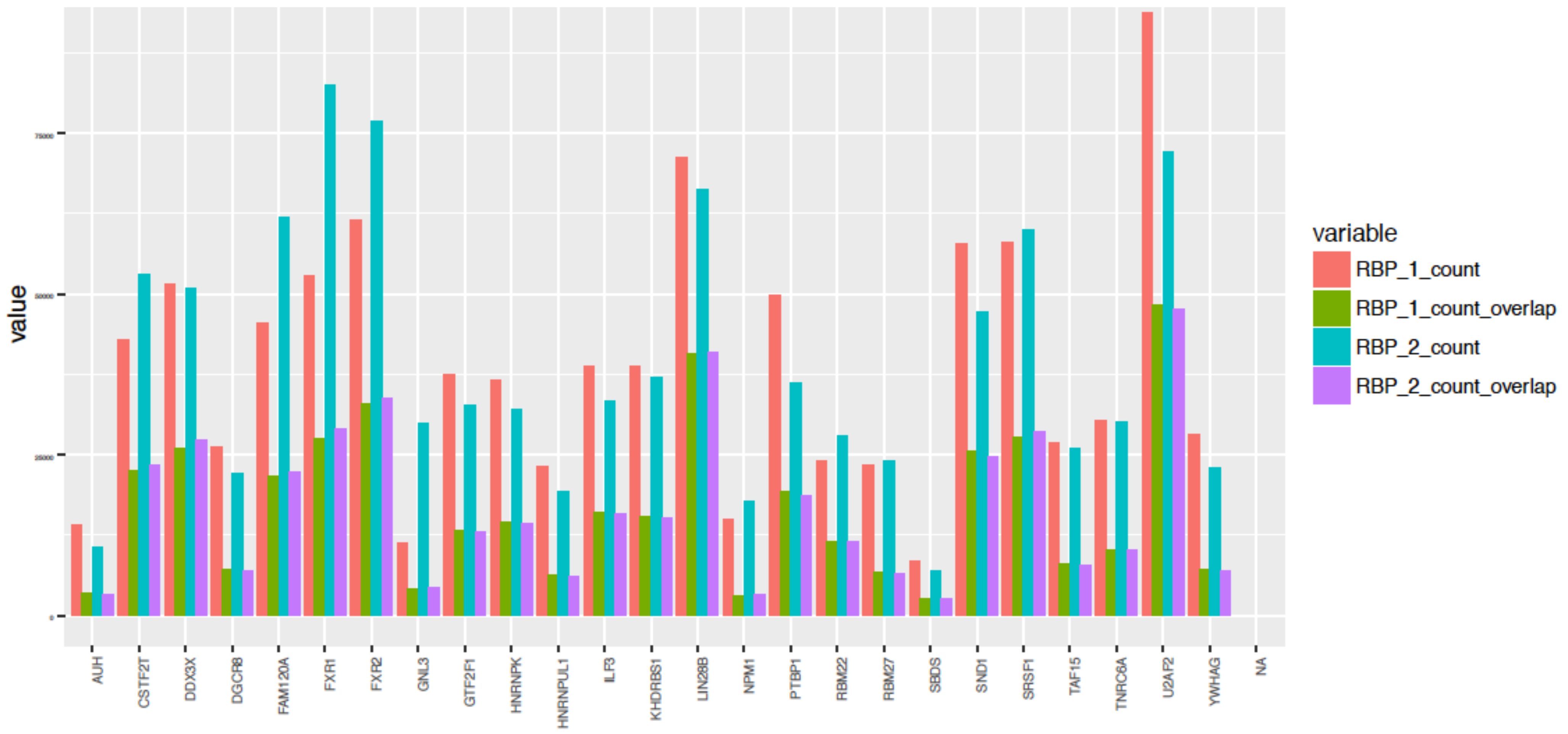
```
chr1        1212        1321
chr2        1312        1521
chr1        2212        3321
…
```

How the result influence the result

How much the biological replicate influence the peaks region? The details of below program will be elucidate later.

```
##The number of overlapped peaks in each replicate.
overLapNum1<-length(unique(queryHits(oneMtch)));
overLapNum2<-length(unique(subjectHits(oneMtch)));
##combine the information
RBPs_replicate_overlap_frame<-c(rbpsnum1,overLapNum1,rbpsnum2,
overLapNum2)
##melt the data frame
RBPs_replicate_overlap_melt<-
melt(RBPs_replicate_overlap_frame,id.vars="RBP");
##plot
p<-ggplot(RBPs_replicate_overlap_melt, aes(x=RBP,
y=value,fill=variable)) + geom_bar(stat="identity",position="dodge”)
```

How many peaks are overlapped between the two biological replicates



R package GenomicRanges

Besides overlap between two genome interval regions. GenomicRanges also include many additional functions. These include:

```
Set operations: ()
  union, setdiff, intersect;
Interval operation: (resize the interval, change the flank region)
  resize, flank, width, shift;
Overlap operations: (overlap with a region)
  countOverlaps,subsetByOverlaps,findOverlaps;
Split operations:split, c(); split a GenomicRanges into multi ones.
Basic operations:tail,head,rev; get subset of the genomicRanges
```

Multi RBPs CLIP-seq bed files

Sometimes, we need to analysis two or more RBPs to study their interaction. We need **GRangesList** structure. Here we take HNRNPK as example.

```
##Get the overlap regions of two HNRNPK replicates
HNRNPK_rep_1_overlap<-
HNRNPK_rep_1_range[unique(queryHits(HNRNPK_overlap_mch)),];


##Combine HNRNPK and SRSF1 into a single GRangesList object
RBPs_range_list<-GRangesList(SRSF1_rep_1_overlap,
HNRNPK_rep_1_overlap);
```

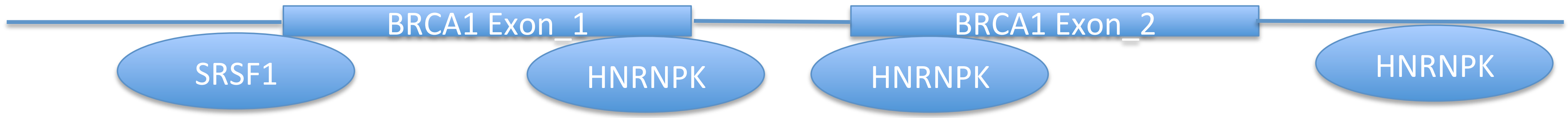
Count RBP binding sites in each gene

Currently we have **two RBPs (SRSF1, HNRNPK) in a GRangeList object**. Next, we want to count RBP binding sites in each transcript and ensure the information we got is enough to do further analysis. We need human genome annotation, here we use hg19. GTF which can be download from UCSC table Browser.
File: Table Browser

GTF is 1-based system and both the left and right coordinate are inclusive.

```
1   pseudogene gene      11869          14412  .   +   .   gene_id "ENSG00000223972";
2   processed_transcript    transcript  11869  14409  .   +   .   gene_id
......
......
```

Count RBP binding sites in each gene



Example: BRCA1 contain 3 HNRNPK binding sites and 1 SRSF1 binding sites.

```
Codes:
##read the GTF file into R
gtf_frame<-read.table("hg19_ens.gtf",header=F,as.is=T);
##construct a GenomicRange object for the GTF
gtf_range<-with(gtf_frame,Granges(seqnames=chr,
+ranges=IRanges(start=start,end=end),strand=strand)
```

Overlap with RBPs binding sites and genome annotation

Next we overlap the **RBPs_range_list** with **gRBP_range** to get number of RBPs in each gene.

```
Code:
GeneName_RBPName<-data.frame(gene_name,RBP_name) #store the result
for(i in 1:2){##for each RBP.
    RBP_overlap_mtch<-
      findOverlaps(RBPs_range_list[[i]], gtf_range);#RBP overlap GTF
  RBP_gtf_overlap<-
    RBPs_range_list[[i]][queryHits(RBP_overlap_mtch),];#overlapped RBP

    Gtf_RBP_overlap<-
        gtf_range[[1]][subjectHits(RBP_overlap_mtch),];#overlapped GTF
```

Overlap with RBPs binding sites and genome annotation

```
Code:
    gene_names<-seqnames(GRBP_RBP_overlap);#overlapped RBP names
    RBP_names<-names(RBPs_range_list)[i];#overlapped gene names
    GeneName_RBPName<-
       cbind(GeneName_RBPName,c(gene_names, RBP_names) );#store the result
}

>GeneName_RBPName
#each line represent a overlap
   Gene_names      RBP_names
   TP53            SFRS1
   TP53            SFRS1
   TP53            HNRNPK
…...
```

An intro to bioconductor

- R package GenomicRanges is part of Bioconductor project, which contains many packages related to bioinformatics, including NGS analysis, microarray analysis and so on.
- Besides the **GenomicRanges**, another general used package I found very useful is the **BSGenome**, which provide different annotation for many genomes.
- There are also other packages in it which target on different aspect, like Chip-seq, RNA-seq (EdgeR) and so on.
- I list two papers which describe the bioconductor packages in the supplement files.

Bioconductor

Many papers are published in Bioconductor by successfully build a package in it for specific use.

R Package reshape2

Next we need to do transformation to count each gene's binding sites.

Here, we first introduce the **long table format** and **short table format**.

Long table and short table

Short Table format

Gene_name	RBP_name
TP53	HNRNPK
TP53	HNRNPK
BRCA1	SRSF1
TP53	HNRNPK
TP53	SRSF1

As you can see,
The data in the short table is redundant. I use more cell to store the same data.

Column name become header

Long Table format

Gene_name	HNRNPK	SRSF1
TP53	xxx	xxx
BRCA1	xxx	xxx

The tidy data

- Usually, we think long table format is more tidy than short table format.

- **A tidy table means each row is a observation and each column is a variable.**

R package reshape2

R package reshape contain two fundamental function:

melt: Convert **long table to short table**.

cast: Convert **short table to long table**.

R package reshape2

```
>GeneName_RBPName
#each line represent a overlap
   Gene_names     RBP_names
   TP53           SFRS1
   TP53           SFRS1
   TP53           HNRNPK

Codes:
#convert the short table format to long table format
GeneName_RBPName_cast<-
Cast(GeneName_RBPName_melt,Gene_names~RBP_names,length)
#Gene_names~RBP_names is folumar, where the left side is #row id and left side is column label
#Where length is the function to count.
```

R package reshape2

```
>GeneName_RBPName_cast

Gene_name     HNRNPK     SRSF1
TP53          3          1
BRCA1         1          3

#Now we have the table we need, we got each gene's RBP #binding sites
for each RBP.
#
#
#
#
```

From table to figure

Human beings can't read hundreds of rows simultaneously, we need visualization method to check the dataset.

We now have the table, we want to represent it using figures to do visualization. Maybe it is not very useful for small table, but for large table, it means a lot.

We need R package ggplot2 to convert table to figure.

R package ggplot2

ggplot2 **usually receive short table format** data and convert it into **statistic figures**. Its main advantage is productive and high versatile. It make use of **graphic grammar.**

```
Codes:
#ggplot2 receive short table format, we need to melt the #table.

>GeneName_RBPName_cast

Gene_name    HNRNPK    SRSF1
TP53              3        1
BRCA1             1        3
```

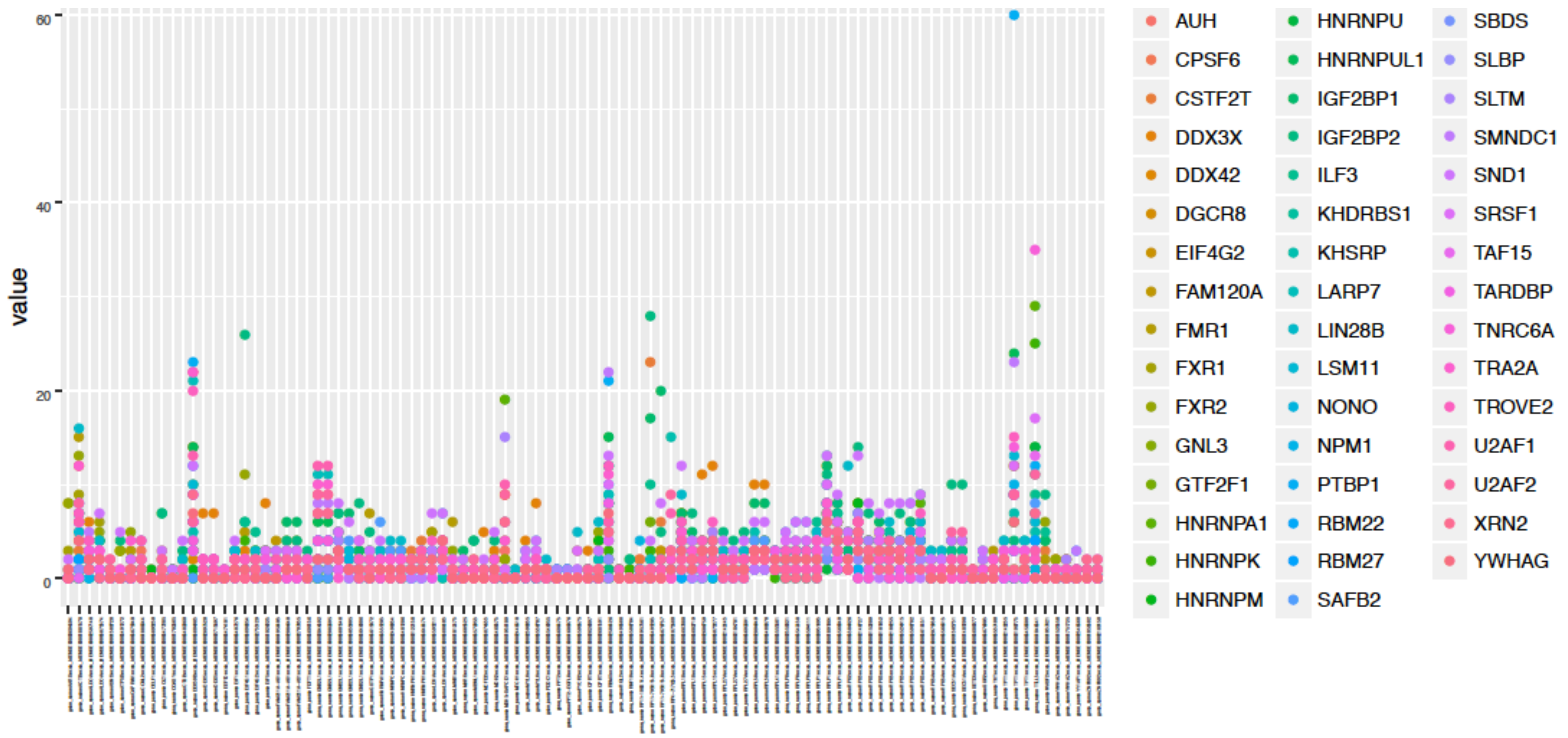
melt long table to short table format

```
Codes:
##convert the long to short fromat
GeneName_RBPName_melt<-
melt(GeneName_RBPName_cast);

##melt result
>GeneName_RBPName_melt

Gene_name      RBP_name       count
TP53           HNRNPK         3
TP53           SRSF1          1
BRCA1          HNRNPK         1
BRCA1          SRSF1          3
```

Give some ggplot figure example

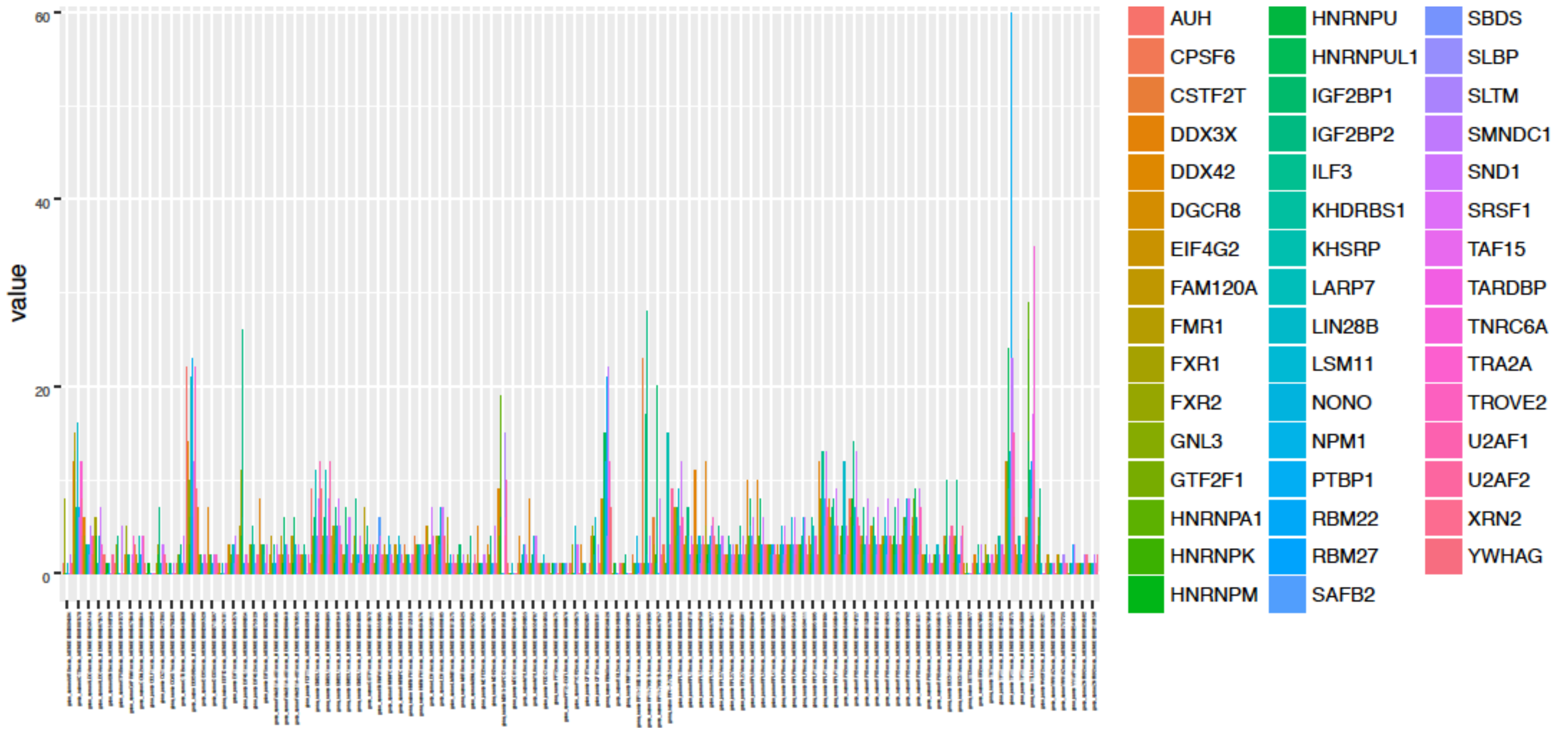
```
Codes:

##count the number RBP binding in each gene’s exon region.
GeneName_RBPName_point2<-ggplot(se_target_data_melt)+
   geom_point( aes(x=gene_name,y=value,color=variable) )

##plot
GeneName_RBPName_bar<-ggplot(se_target_data_melt)+
geom_bar( aes(x=gene_name,y=value,fill=variable),stat="identity",posit
ion="dodge" )+

GeneName_RBPName_tile<-ggplot(se_target_data_melt)+
   geom_tile( aes(x=gene_name,y=variable, fill=value) )

print(GeneName_RBPName_p1);
```

Figure_1



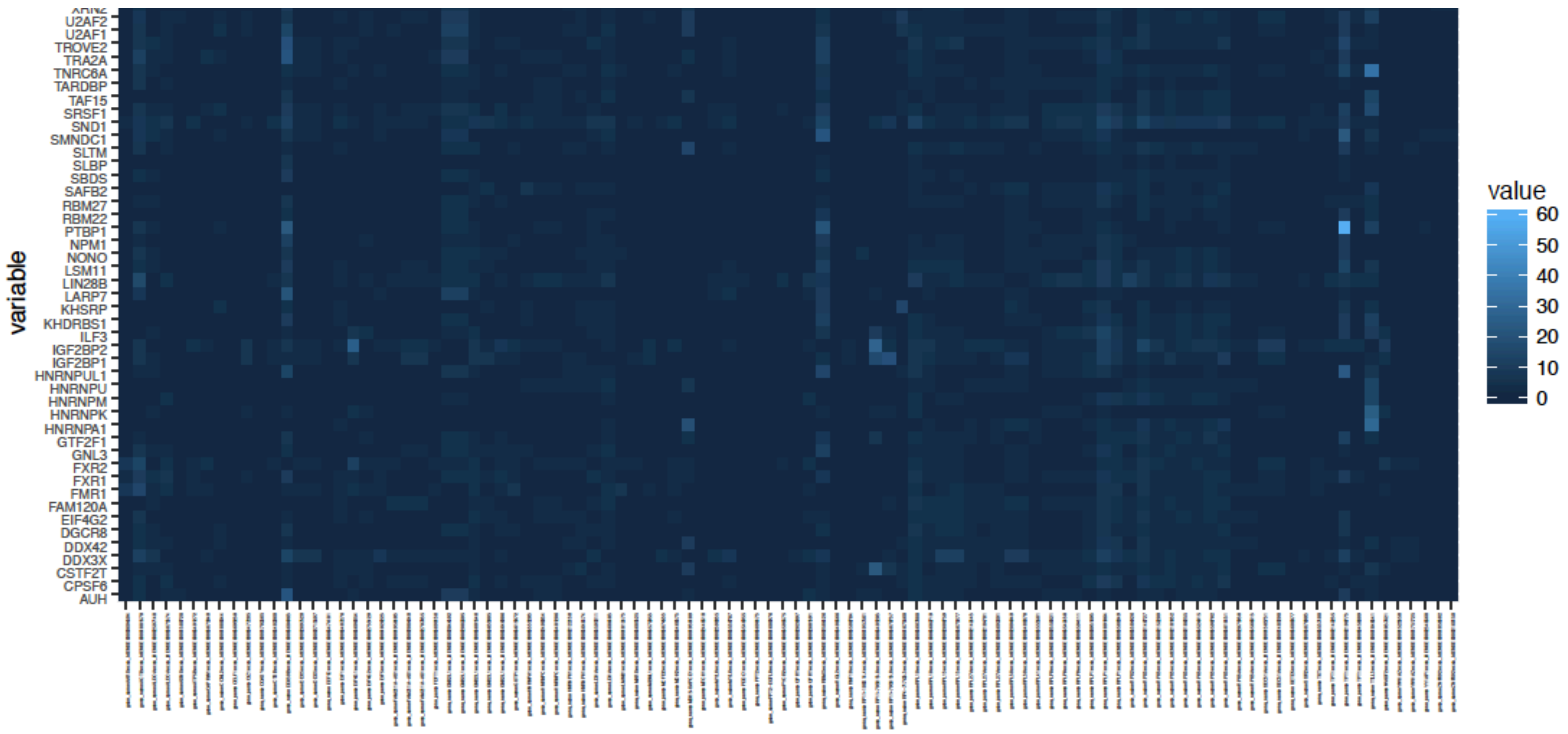
value
60
40
20
0
AUH
CPSF6
CSTF2T
DDX3X
DDX42
DGCR8
EIF4G2
FAM120A
FMR1
FXR1
FXR2
GNL3
GTF2F1
HNRNPA1
HNRNPK
HNRNPM
HNRNPU
HNRNPUL1
IGF2BP1
IGF2BP2
ILF3
KHDRBS1
KHSRP
LARP7
LIN28B
LSM11
NONO
NPM1
PTBP1
RBM22
RBM27
SAFB2
SBDS
SLBP
SLTM
SMNDC1
SND1
SRSF1
TAF15
TARDBP
TNRC6A
TRA2A
TROVE2
U2AF1
U2AF2
XRN2
YWHAG

Figure_2



value
60
40
20
0
AUH
CPSF6
CSTF2T
DDX3X
DDX42
DGCR8
EIF4G2
FAM120A
FMR1
FXR1
FXR2
GNL3
GTF2F1
HNRNPA1
HNRNPK
HNRNPM
HNRNPU
HNRNPUL1
IGF2BP1
IGF2BP2
ILF3
KHDRBS1
KHSRP
LARP7
LIN28B
LSM11
NONO
NPM1
PTBP1
RBM22
RBM27
SAFB2
SBDS
SLBP
SLTM
SMNDC1
SND1
SRSF1
TAF15
TARDBP
TNRC6A
TRA2A
TROVE2
U2AF1
U2AF2
XRN2
YWHAG

Figure_3



variable
XRN2
U2AF2
U2AF1
TROVE2
TRA2A
TNRC6A
TARDBP
TAF15
SRSF1
SND1
SMNDC1
SLTM
SLBP
SBDS
SAFB2
RBM27
RBM22
PTBP1
NPM1
NONO
LSM11
LIN28B
LARP7
KHSRP
KHDRBS1
ILF3
IGF2BP2
IGF2BP1
HNRNPUL1
HNRNPU
HNRNPM
HNRNPK
HNRNPA1
GTF2F1
GNL3
FXR2
FXR1
FMR1
FAM120A
EIF4G2
DGCR8
DDX42
DDX3X
CSTF2T
CPSF6
AUH
value
60
50
40
30
20
10
0

Which one is better?

- **I will show you the raw PDF here**, which is much clear than this one.
- Scatter plot is very easy to check which gene contain highest count, but if two RBP has same count in the gene, they will overlap. So scatter plot is wrong.
- Bar plot avoid the overlap problem by putting each point beside each other, but the 47 colors is very hard to distinguish.
- So I think the best one is the **heatmap**.

R package ggplot2

You can see the power of ggplot2. only change a few codes can do different kinds of plots, very efficient and productivity.

R package ggplot2

ggplot is multi layers figure, as you can see, each '+' add a layer to the final figure.

aes: map variable to figure variable (color, x-axis, y-axis, shape, fill).

geom: The figure type, eg. Bar_plot, histogram, scatter plot.

stat: the statistic transformation, eg. bin, identity.

R package ggplot2

- I list the figure types that support by ggplot2.

  Histogram, boxplot, barplot, contour, density, scatter, tileplot, hex plot, violin, pieplot, line and so on.

- If you want to combine two or ggplot2 object in one figure, you may need R function **multiplot.r**, which can be search and got from web.

R package ggplot2

- Besides productivity, ggplot is very versatile.
- These include:
- geom_xx (different kinds of figures)
- stat_xx (different kinds of statistics transformation)
- Scales (change the scales like color, legend, fill et.al.)
- Coordiante (cartesian, polar, flip, trans)
- Faceting (multi plots in one figure)
- Position adjustments (dodge, fill, identity, stack, jitter)
- Annotation (custom, logticks, map, raste)
- Fortify
- Themes (font, size, label orientation et.al.)
- Aesthetics (map from variable to axis)
- Others ()

Calculate the number of gene for each RBP

We want to summarize which RBP binds most genes.

```
>GeneName_RBPName
#each line represent a overlap
   Gene_names     RBP_names
   TP53           SRSF1
   TP53           SRSF1
   TP53           HNRNPK
```

Generalize the problem

The generalize of the problem is **split-aggregate** problem, what we want is split the data.frame into multi small ones, and do some operation on each group.

There is a R package called ‘plyr’ which can be used to do these kinds of job.

The 'for' loop equivalent

R's **for loop** is seldom used in practice. But R provide other function do to iteration:

sapply: apply a function to each element in list.

apply: apply a function to row or column.

lapply: apply a function to each element in list.

R package plyr

Although R provide some functions for doing iteration. But they are not that powerful enough for some problems, **the main problem is that the input and output format is ambiguous**. While R package plyr solved this problem.

R package plyr

It includes many functions:

Where d means table, l means list, _ means nothing, a means vector, so:

d_ply means input is data.frame, output nothing.

ddply means input is data.frame, output data.frame.

dlply means input is data.frame, output list.

daply means input is data.frame, output vector.

R package plyr

R package plyr is a more powerful solution for this kind of analysis. **It group the table and apply a function. (also called split+modify+aggregate)**

For our problem, we need to **treat each RBP a separate group and count the number of genes in each group**.

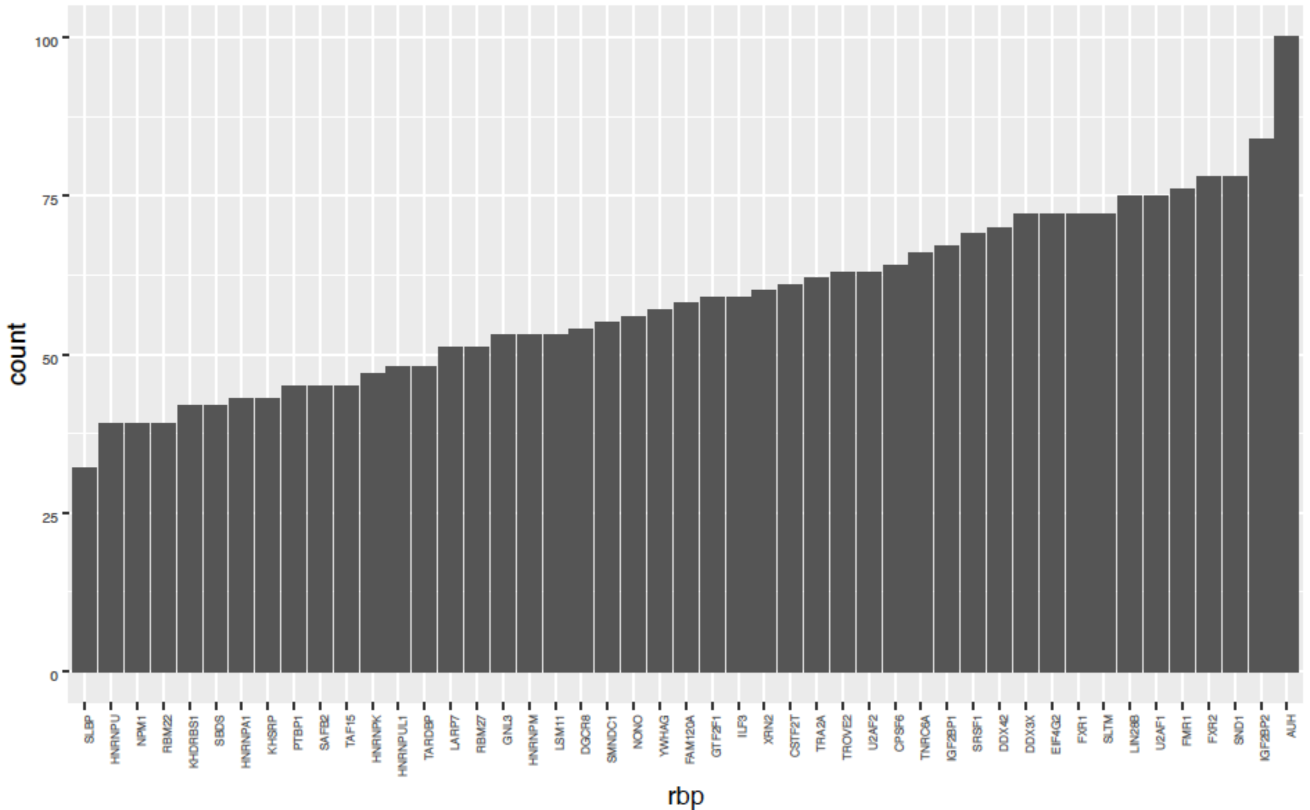
R package plyr

```
RBP_bind_fre<-ddply(GeneName_RBPName,.(RBP_names),nrow)
#each line represent a overlap
   Gene_names     RBP_names
   TP53           SRSF1
   TP53           SRSF1
   TP53           HNRNPK



>RBP_bind_fre
RBP_names         Var1
SRSF1             2
HNRNPK            2
```

Calculate number of gene for each RBP

With a little ggplot2, we can do the below figure.

```
rbp_gene_count_plot<-ggplot(rbp_gene_count)+
  geom_bar(aes(x=rbp,y=count),stat="identity")+
```



R package plyr

- Besides group analysis, R package plyr can also do .
- 1. data.frame operations, like rbind.fill, progress bar,
- 2.generalized for loop, executing a function multi times and return something (like data.frame).

The gene expression data

- Next we add the gene expression data, which can be got from **RNA-seq technique**, and do the correlation between gene expression and RBP binding information

- The key things to know is **RNA-seq can quantatively determine the expression level of gene.**

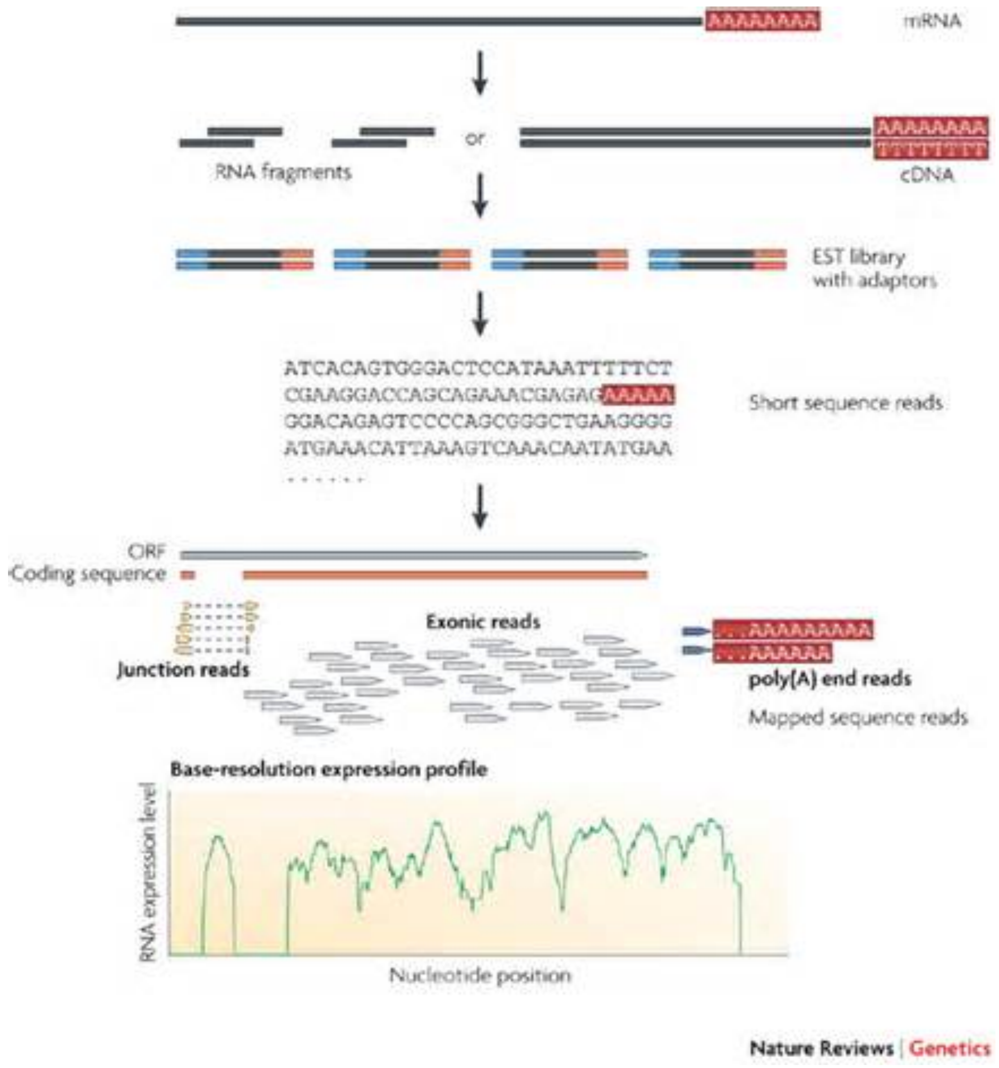
Add gene expression

Here, we use **NGSUtils** to calculate each sample's FPKM, to avoid the bias due to a single sample. We use 115 samples here and take the median FPKM as the gene's expression level.

We have 115 samples and we need to write a small **pipeline** to do it.

RNA-seq workflow

Got from Cufflinks paper



There are many kinds of RNA-seq technique which I also don't know very much.

Different sequencer will also give different result.

The RNA-seq data in Encode

The RNA-seq data set also **come from the ENCODE project**, we also assume the download format is fastq format.

Unlike sequence alignment for CLIP-seq, for RNA-seq, **we must also consider the junction reads to get the bam file**.

One software is the **tophat2**.

The RNA-seq data set

To ensure the expression data we got doesn't come from big variance, we use 104 samples' RNA-seq dataset from a same cell line (K562).

We use a tool called NGSUtils which can calculate the gene expression data from bam file.

A small Linux pipeline

After we got the bam files, we will try to call the **FPKM** values from the BAM files.

For large samples (like 104 samples here), we usually use pipeline to do batch process.

A lot of language can be used to write pipeline, here, I use bash shell, I think writing pipeline in **bash shell** is a nature way (bash.quickref.pdf).

The pipeline for **NGSUtils** here

```
for i in `ls –d dirContainBAMFiles`
do
  echo $i;
  sample=`basename $i`;
  my i_dir=${i/.bam};
  mkdir $i_dir;

  samtools sort $i $i.sort;
  samtools index $i.sort;

  script="bamutils count –gRBP Homo_sapiens.GRCh37.75_4.gRBP -rpkm -norm all $i.sort > 
$sample.count";
  echo $script > $sample.count.job;

  `$script`
  #qsub -l nodes=1:ppn=1,walltime=4:00:00,mem=4gb -M li487@iupui.edu -d /N/dc2/scratch/liulab/
limeng/RBP_network/ -m ae -N $sample.count $sample.count.job
```

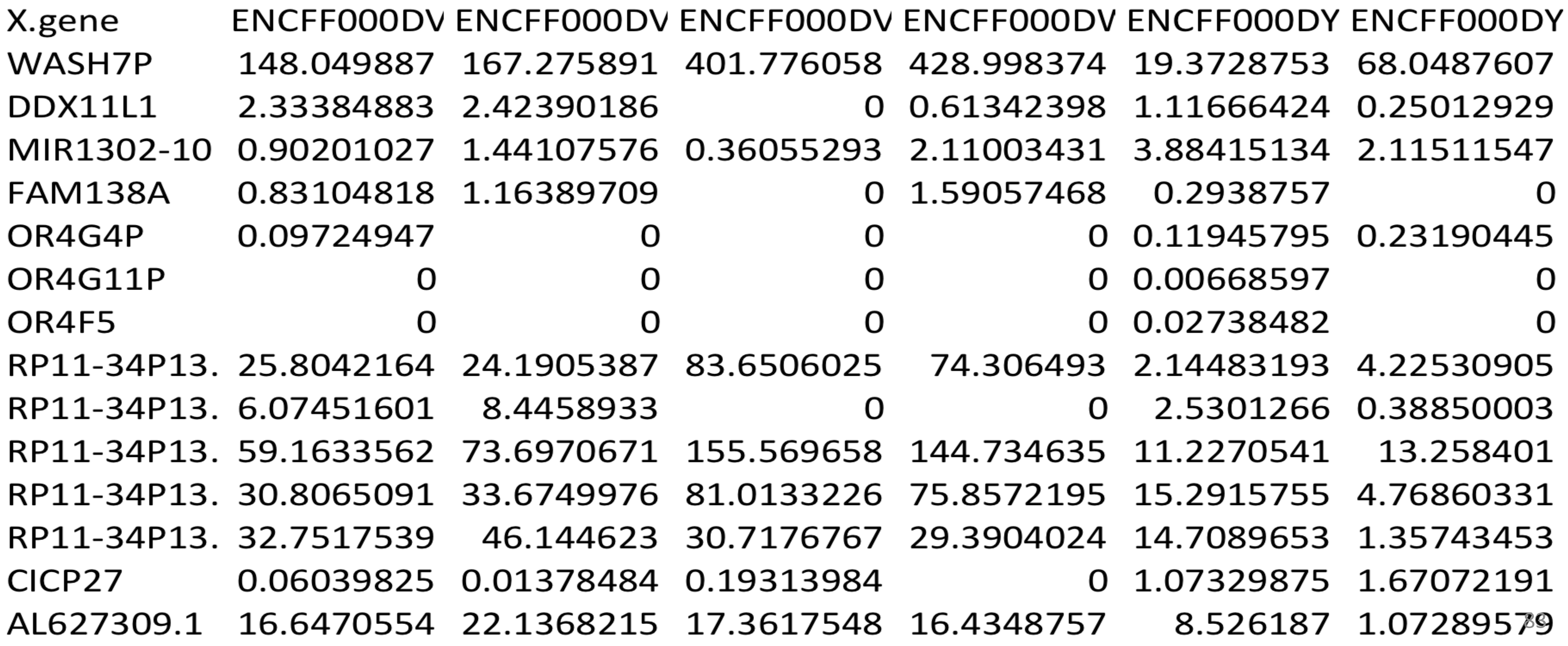
Process Gene expression

For each sample, now we got a **gene expression table** .

```
#gene   geneid  isoid   chrom   strand  txstart txend   length  count   count (CPM)     RPKM
WASH7P  ENSG00000227232 ENSG00000227232 chr1    -       14362   29806   2073    309     1.76326905472   0.850588063058
DDX11L1 ENSG00000223972 ENSG00000223972 chr1    +       11868   14412   1756    41      0.23396126616   0.133235345194
MIR1302-10      ENSG00000243485 ENSG00000243485 chr1    +       29553   31109   1021    65      0.370914202449  0.363285212977
FAM138A ENSG00000237613 ENSG00000237613 chr1    -       34553   36081   1219    224     1.27822740536   1.04858687889
OR4G4P  ENSG00000268020 ENSG00000268020 chr1    +       52472   54936   947     10      0.0570637234537 0.0602573637315
OR4G11P ENSG00000240361 ENSG00000240361 chr1    +       62947   63887   940     0       0.0     0.0
OR4F5   ENSG00000186092 ENSG00000186092 chr1    +       69090   70008   918     3       0.0171191170361 0.0186482756385
RP11-34P13.7    ENSG00000238009 ENSG00000238009 chr1    -       89294   133566  3569    2050    11.698063308    3.2776865531
RP11-34P13.8    ENSG00000239945 ENSG00000239945 chr1    -       89550   91105   1319    561     3.20127488575   2.42704691869
```

Process Gene expression

Next, we need **to combine multi sample's gene expression into one table**. This can be done in multi ways since each file is already ordered by the gene name, including using R read each file. Use linux command cut and paste.



X.gene	ENCFF000DV	ENCFF000DV	ENCFF000DV	ENCFF000DV	ENCFF000DY	ENCFF000DY
WASH7P	148.049887	167.275891	401.776058	428.998374	19.3728753	68.0487607
DDX11L1	2.33384883	2.42390186	0	0.61342398	1.11666424	0.25012929
MIR1302-10	0.90201027	1.44107576	0.36055293	2.11003431	3.88415134	2.11511547
FAM138A	0.83104818	1.16389709	0	1.59057468	0.2938757	0
OR4G4P	0.09724947	0	0	0	0.11945795	0.23190445
OR4G11P	0	0	0	0	0.00668597	0
OR4F5	0	0	0	0	0.02738482	0
RP11-34P13.	25.8042164	24.1905387	83.6506025	74.306493	2.14483193	4.22530905
RP11-34P13.	6.07451601	8.4458933	0	0	2.5301266	0.38850003
RP11-34P13.	59.1633562	73.6970671	155.569658	144.734635	11.2270541	13.258401
RP11-34P13.	30.8065091	33.6749976	81.0133226	75.8572195	15.2915755	4.76860331
RP11-34P13.	32.7517539	46.144623	30.7176767	29.3904024	14.7089653	1.35743453
CICP27	0.06039825	0.01378484	0.19313984	0	1.07329875	1.67072191
AL627309.1	16.6470554	22.1368215	17.3617548	16.4348757	8.526187	1.07289579

Gene expression quality control

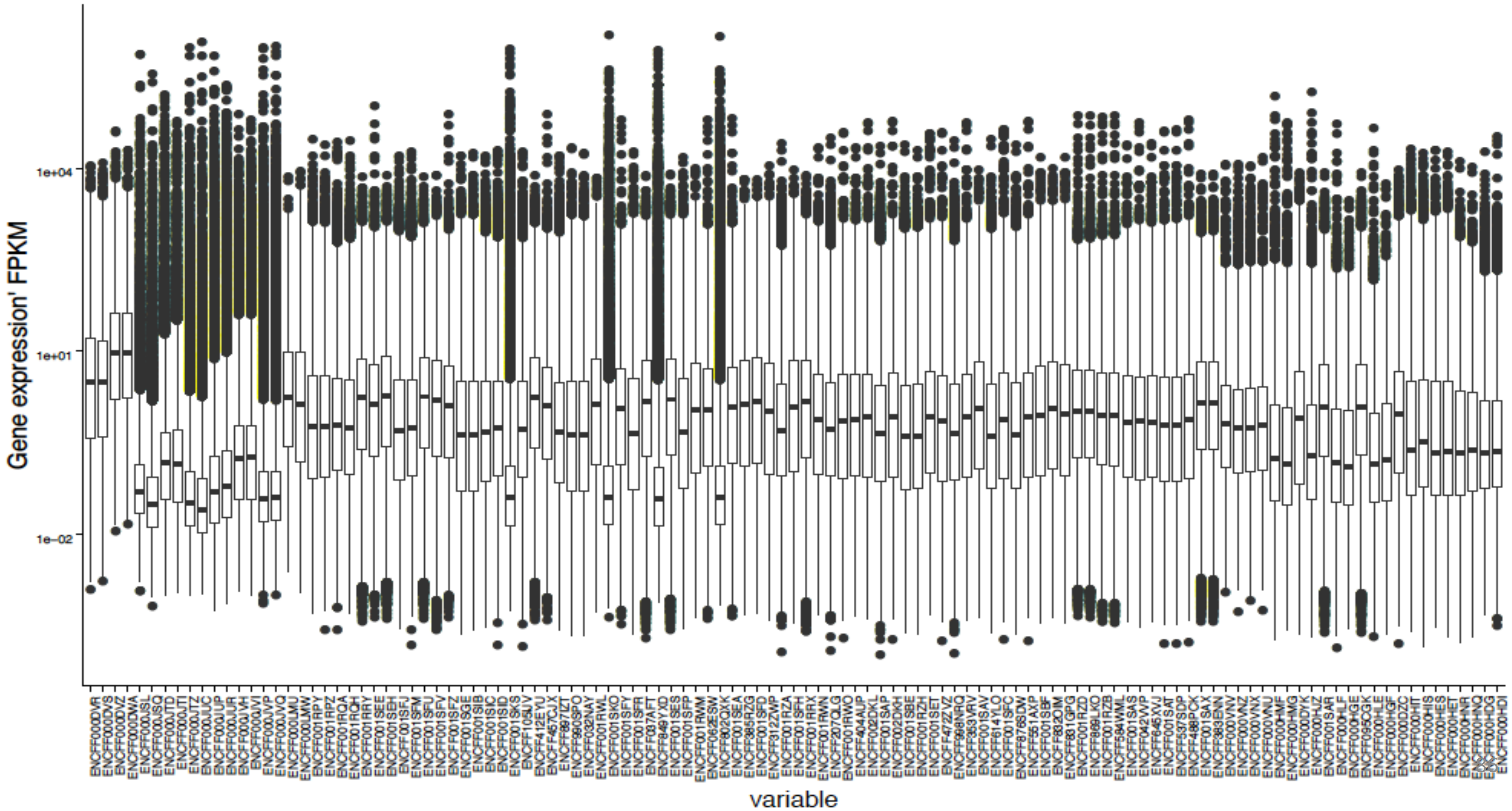
Usually, before we do a experiment, we need to validate the result we got and the feasibility of next step. (quality control)

Here we use heatmap and boxplot to do quality control.

Boxplot code

```
gene_expression_tbl<-
read.table(“gene.expression.tsv”,sep=“\t”,as.is=TRUE);

#ggplot receive short format table
gene_expression_tbl_melt<-melt(gene_expression_tbl);

gene_exp_box_plot<-ggplot(gene_expression_tbl_melt)+
 #geom_boxplot are used to plot box plot.
  geom_boxplot(aes(x=variable, y=value  ) ) +
  theme_classic()+scale_y_log10();
```

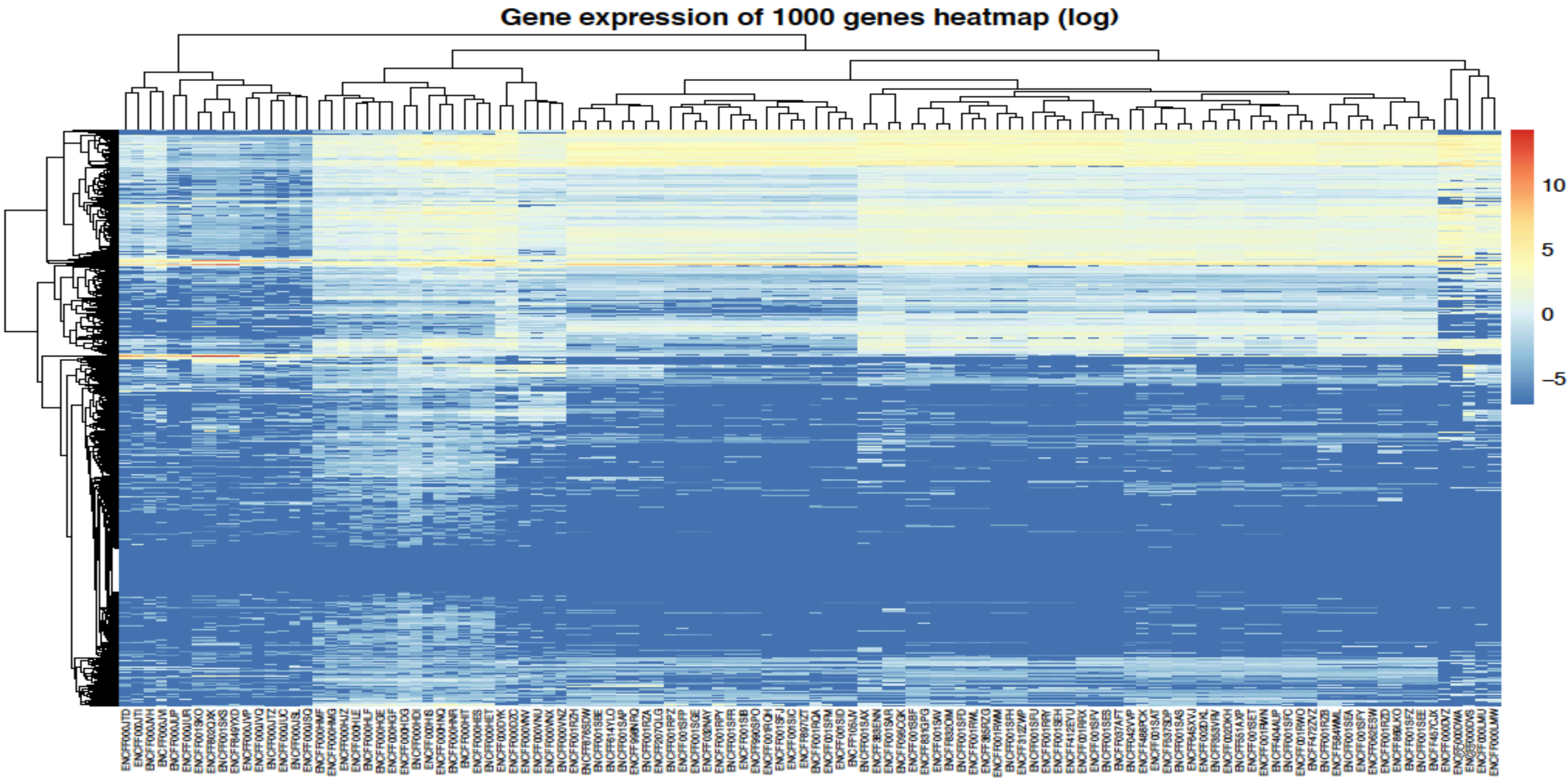
Figure



heatmap

A more generalize way to check gene expression is using heatmap, however, ggplot2 doesn’t provide this, here I use R package ‘pheatmap’.

Figure



What we can see from the figures?

Seems the there are **two different groups of samples**, although they come from only one cell line 'K562'. It turns out the some of the samples come from a independent culture 'K562'.

Gene Expression

Now we take the median of gene expression among samples.

```
And the gene expression.
Gene_name   Sample1  Sample2  Sample3  …
TP53        113.0    313      112
BRCA1       313      113.1    132
…...
```

```
And the gene expression.
Gene_name   Median(FPKM)   …
TP53        213.4
BRCA1       313.5
…...
```

Combine the RBP and gene expression table

```
Now we have a table contain RBP binding information.
Gene_name   SRSF1    BX1
TP53        1        3
BRCA1       3        1
…...
```

```
And the gene expression.
Gene_name   Median(FPKM)   …
TP53        213.4
BRCA1       313.5
…...
```

Generalize of the problem

- **Combine two tables** is a daily task for SQL, and it include many kinds of operations, like left_join, inner_join, right_join and full_join.
- Left_join: keep all the data in left table in the result table.
- Right_join: keep all the data in the right table in the result table.
- Inner_join: keep the data in both the tables.
- Full_join:keep the data in either the table.

R package dplyr

Here we use R package dplyr. Table manupilation language. A very powerful tool to process tables in R. It implements the above join operations in R, and make multi tables analysis more easily.

R package dplyr

Its function include:

filter – select a subset of the rows of a data frame.

arrange – works similarly to filter, except that instead of filtering or selecting rows, it reorders them.

select – select columns of a data frame.

mutate – add new columns to a data frame that are functions of existing columns.

summarize – summarize values.

group_by – describe how to break a data frame into groups of rows.

SQL JOIN – sql style joining multi tables together.

Join two tables

```
>geneName_RBPName_cast
Gene_name    SRSF1   BX1
TP53         1           3
BRCA1        3           1


>gene_expression_tbl_melt
Gene_name    Median(FPKM)…
TP53         213.4
BRCA1        313.5


Gene_RBP_exp_join<-
inner_join(geneName_RBPName_cast,
gene_expression_tbl_melt,by=c(“Gene_name”=“Gene_name”));
```

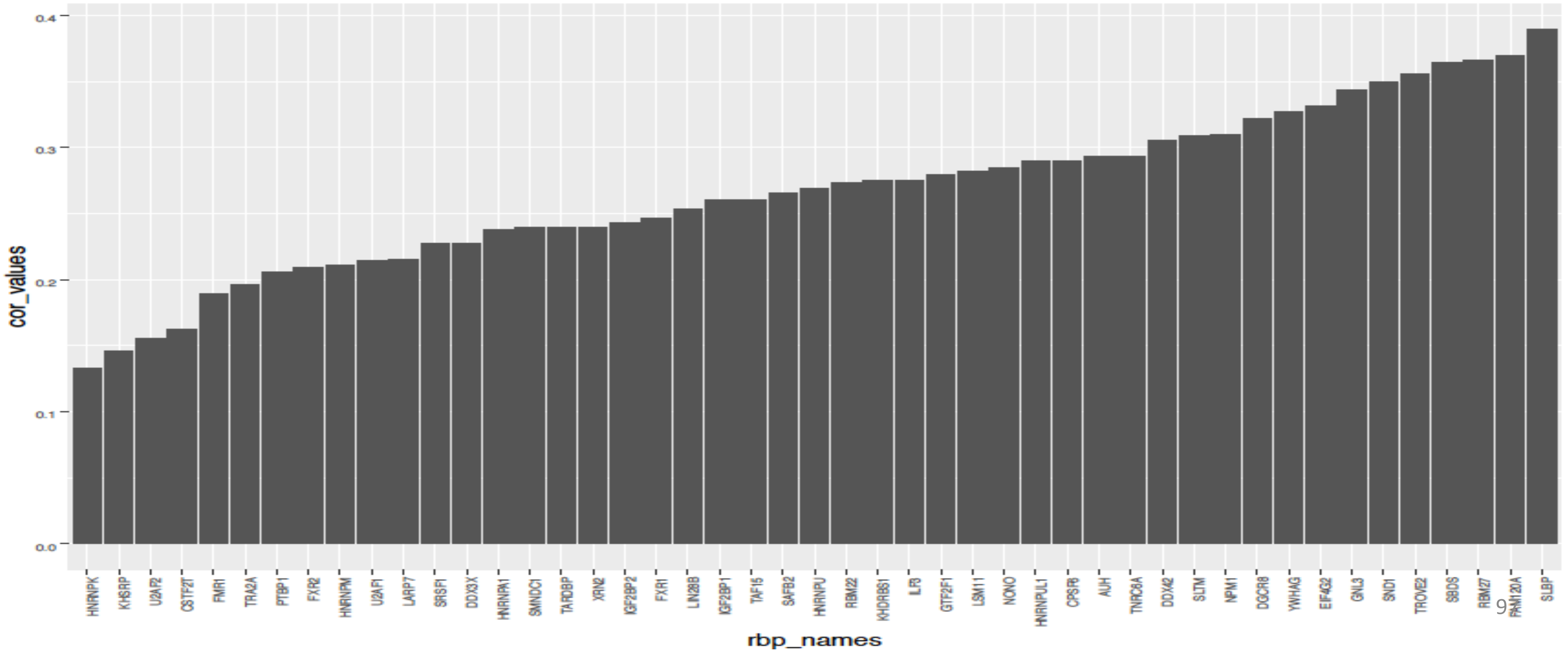
Join two tables

```
Gene_name   Median(FPKM)   SRSF1    BX1
TP53        213.4               1           3
BRCA1       313.5               3           1
…...
```

Now we have all the data in one table.

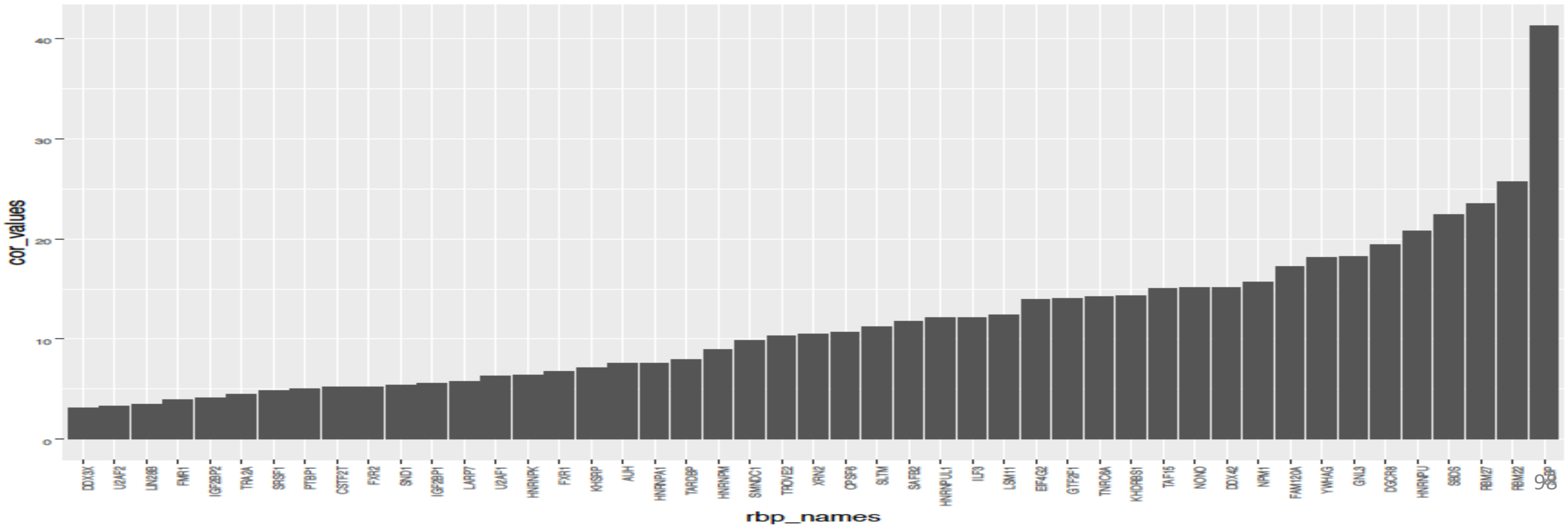
Correlation of RBP and Gene expression

```
With a little ggplot:
rbp_cor_values_data_plot<-ggplot(rbp_exp_data)
+geom_bar(aes(x=rbp_names,y=cor_values),stat="identity”)
```



Linear regression of RBP and Gene expression

Another consideration is longer gene region has higher possibility to contain more RBPs, so we need to consider the gene's length.



The pipe

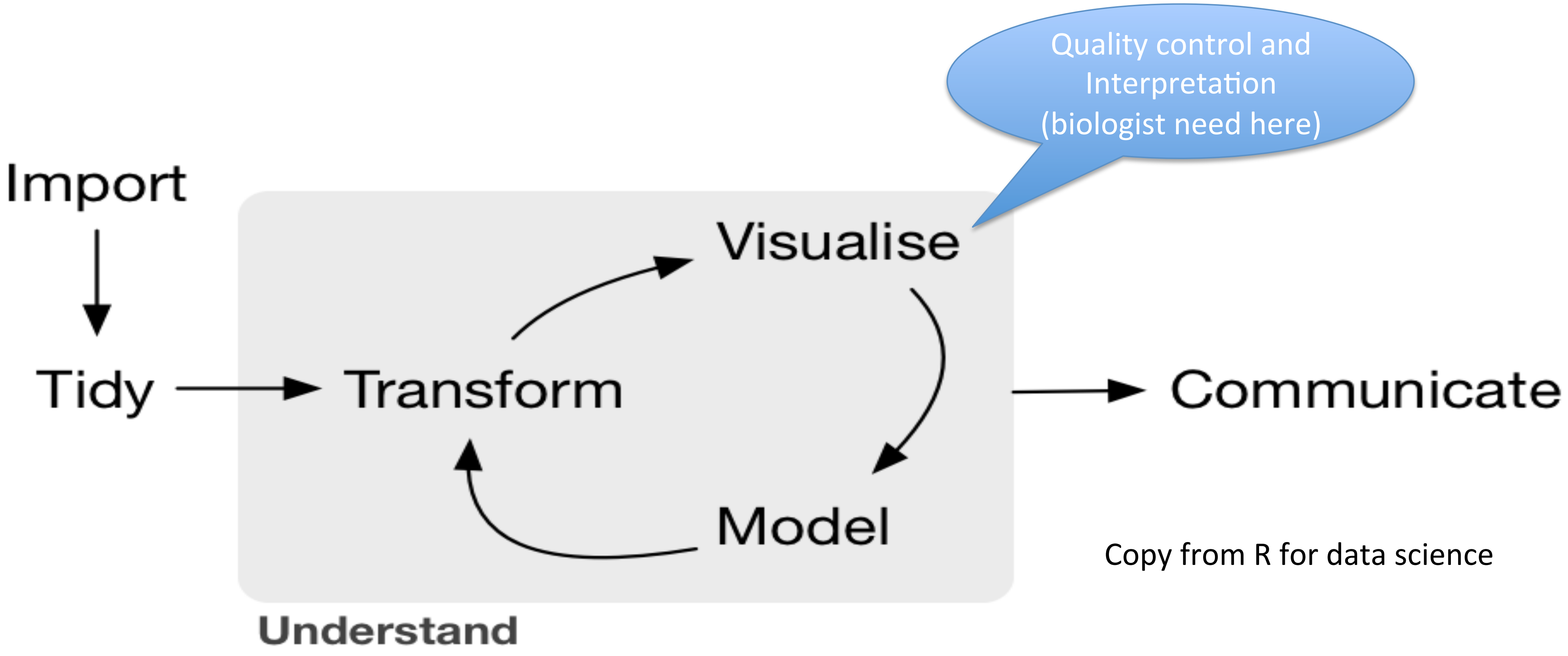
- Usually, we will do many modification on the raw data to get the final data we need, which will make the code very complicate.

- Linux has a pipe which facilitate complicate operation. R also has a pipe command, and here it is:

  %>%

Check the magnittr package for more details.

Summary: a daily life for data scientist



Copy from R for data science

A few advice to newbie when do science

Actually I also am a newbie, but I also some advice to the more newer ones.

1. Tidy up the workspace of your project. (like make some folders: result, code, figures, reference, document, discussion, tables, annotation, data, et.al.)
2. Record every command ever used. (So if result go wrong, we know where we do mistake, thus can avoid make same mistake twice)
3. Double check every step!!

Further reading

http://hadley.nz

Advance R. http://adv-r.had.co.nz/

R for data science. http://r4ds.had.co.nz/pipes.html

ggplot2: elegant graphics for data analysis. (This one is not free)

ggplot2:http://docs.ggplot2.org/0.9.3.1/geom_bar.html

Bimedical data science: http://genomicsclass.github.io/book/

Bioconductor tutorial:https://www.bioconductor.org/help/course-materials/

I recommend read the "R for data science" first.

Acknowledgement

冯伟兴教授和其它实验室的老师和同学们。