Research use only

Mus musculus Transcriptome Sequencing

Report

March 2019

Humanizing Genomics
macrogen

ver. MGTR3.4_HS_mm10

Project Information

Client Name	Macrogen Japan
Company/Institution	Macrogen Corp. Japan
Order Number	HN00101712
Species	*Mus musculus*
Reference	UCSC mm10
Annotation	RefSeq_2017_06_12
Read Length	101
Number of Samples	2
Library Kit	TruSeq Stranded mRNA LT Sample Prep Kit
Library Protocol	TruSeq Stranded mRNA Sample Preparation Guide, Part # 15031047 Rev. E
Reagent	NovaSeq 6000 S4 Reagent Kit
Sequencing Protocol	NovaSeq 6000 System User Guide Document # 1000000019358 v02
Type of Sequencer	NovaSeq
Sequencing Control Software	1000000019358 v02

Project Results Summary

In this study, *Mus musculus* whole transcriptome sequencing was performed in order to examine the different gene expression profiles, and to perform gene annotation on set of useful genes based on gene ontology pathway information.

Analyses were successfully performed on all 2 paired-ends samples. Figure 1 shows the throughput of raw data and trimmed data. Figure 2 shows the Q30 percentage (% of bases with quality over phred score 30) of each sample's raw and trimmed data.

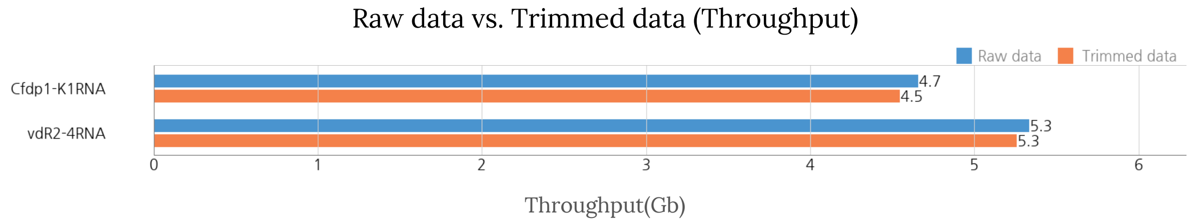

Figure 1. Throughput output of Raw and Trimmed data

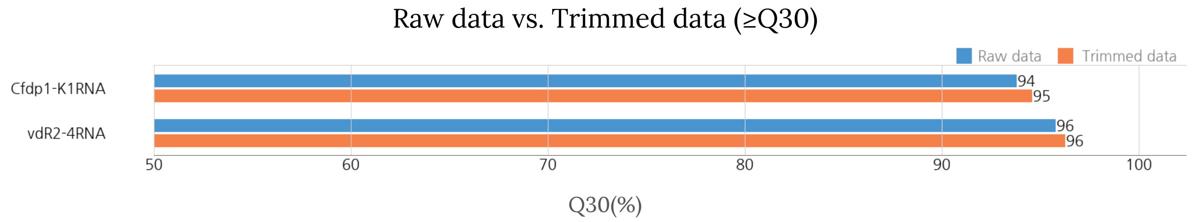

Figure 2. Q30 score of Raw and Trimmed data

Trimmed reads are mapped to reference genome with HISAT2. Figure 3 shows the overall read mapping ratio, the ratio of mapped reads to trimmed reads.

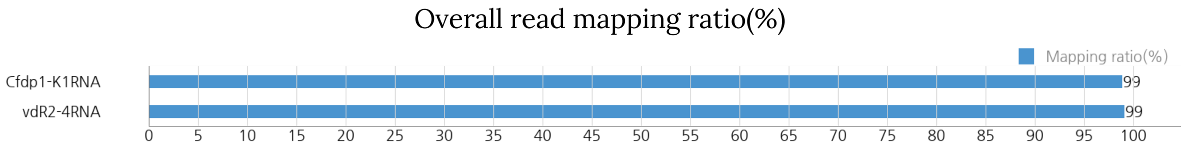

Figure 3. Overall read mapping ratio(%)

After the read mapping, Stringtie was used for transcript assembly. Expression profile was calculated for each sample and transcript/gene as read count and FPKM (Fragment per Kilobase of transcript per Million mapped reads).

DEG (Differentially Expressed Genes) analysis was performed on a comparison pair (Cfdp1-K1RNA_vs_vdR2-4RNA) as requested using FPKM. The results showed 694 genes which satisfied |fc|>=2 conditions in comparison pair.

Figure 4 shows the result of hierarchical clustering (distance metric= Euclidean distance, linkage method= complete) analysis. It graphically represents the similarity of expression patterns between samples and genes.

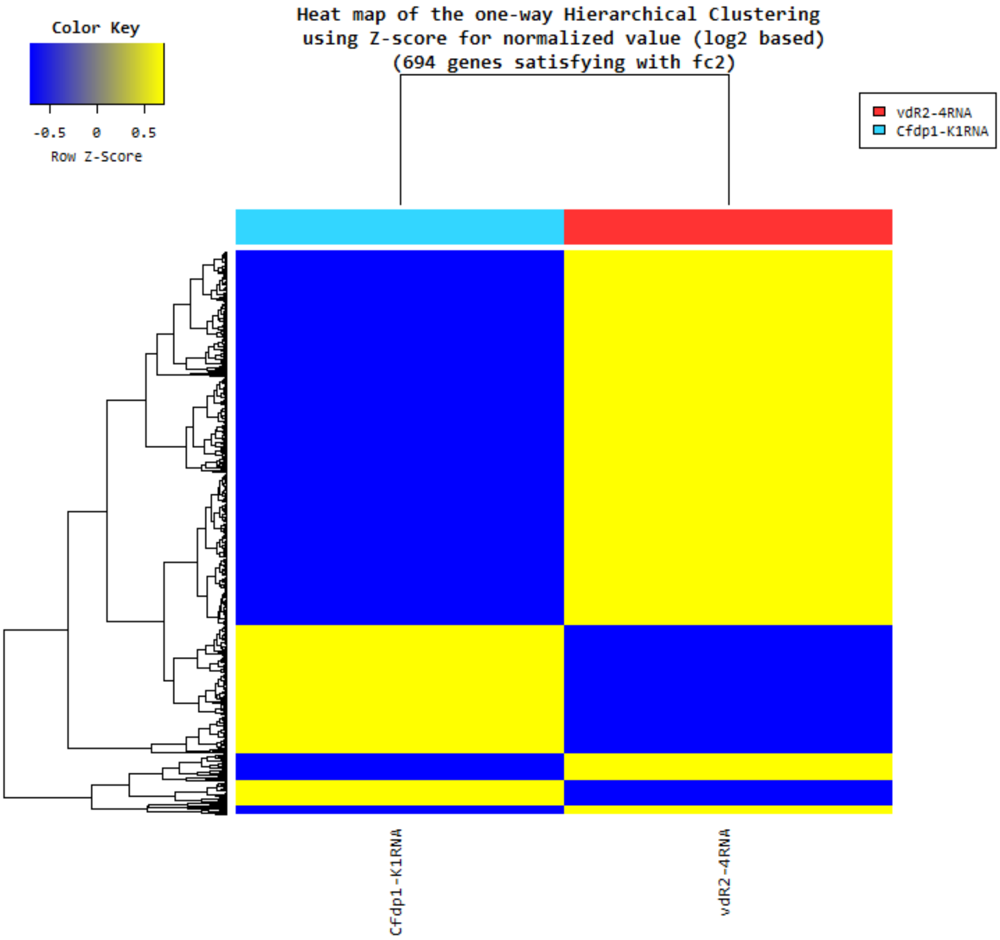

Figure 4. Heatmap for DEG list

DEG list was further analyzed with Gene Ontology (http://geneontology.org/) for gene set enrichment analysis per biological process (BP), cellular component (CC) and molecular function (MF). The Figure 5, 6 and 7 show the significant gene set by each category.

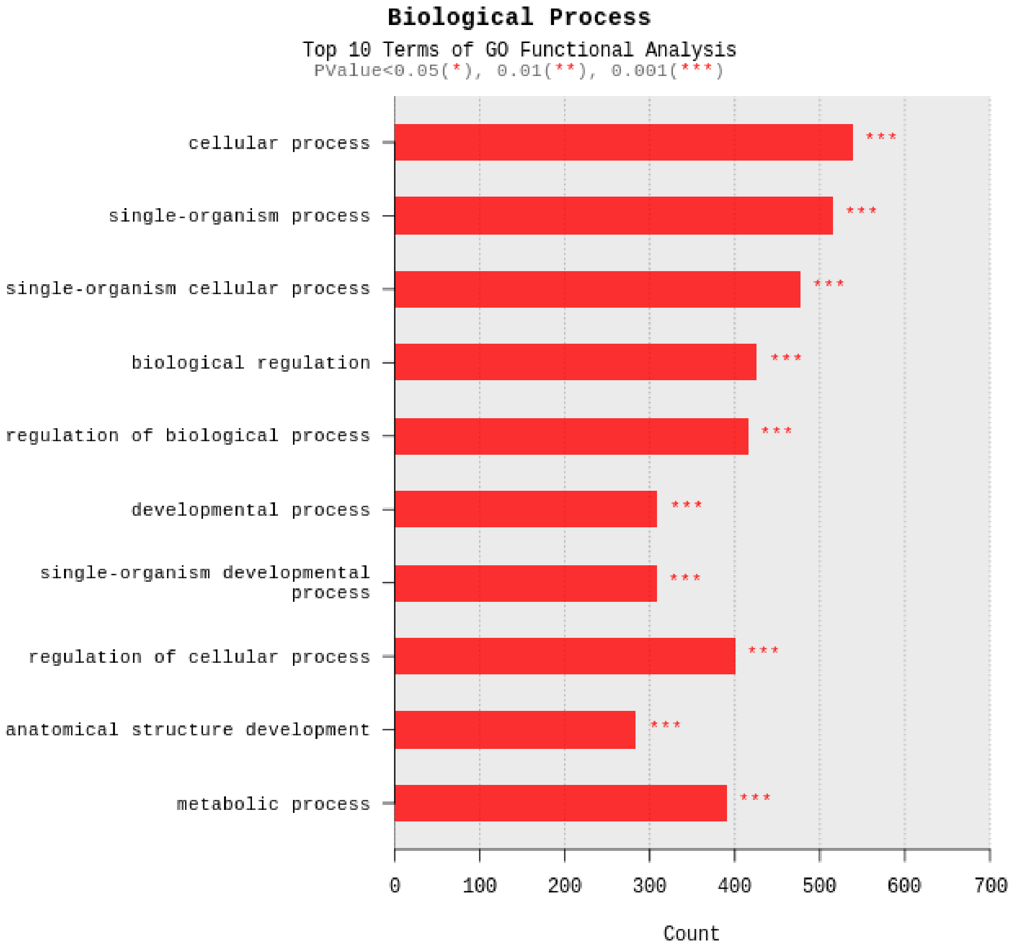

Figure 5. Gene Ontology terms related to Biological Process

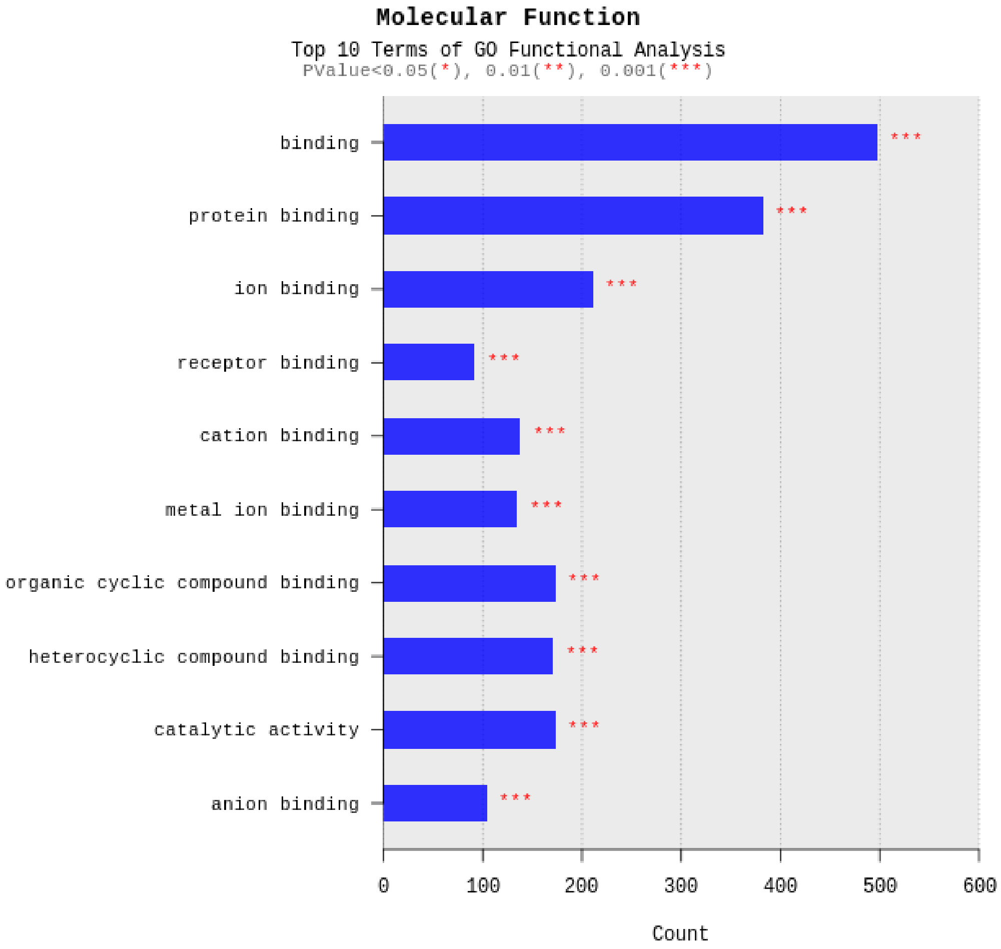

Figure 6. Gene Ontology Terms related to Molecular Function

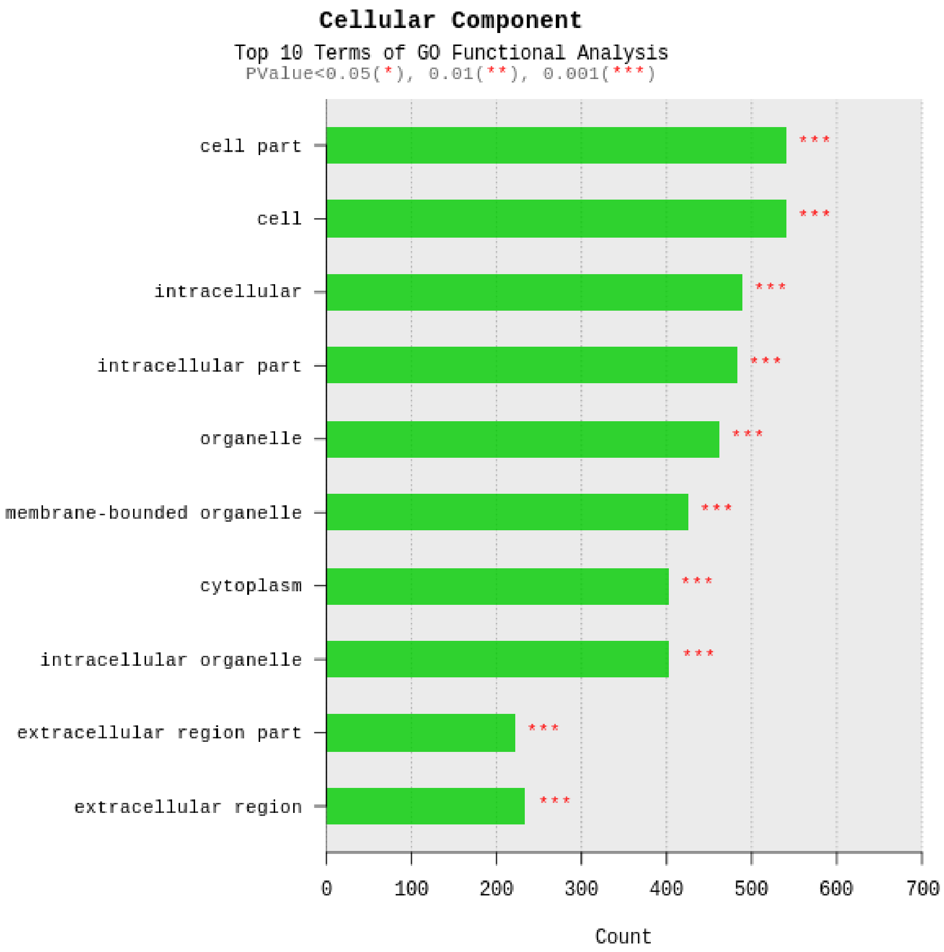

Figure 7. Gene Ontology Terms related to Cellular Component

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1. Experimental Methods and Workflow

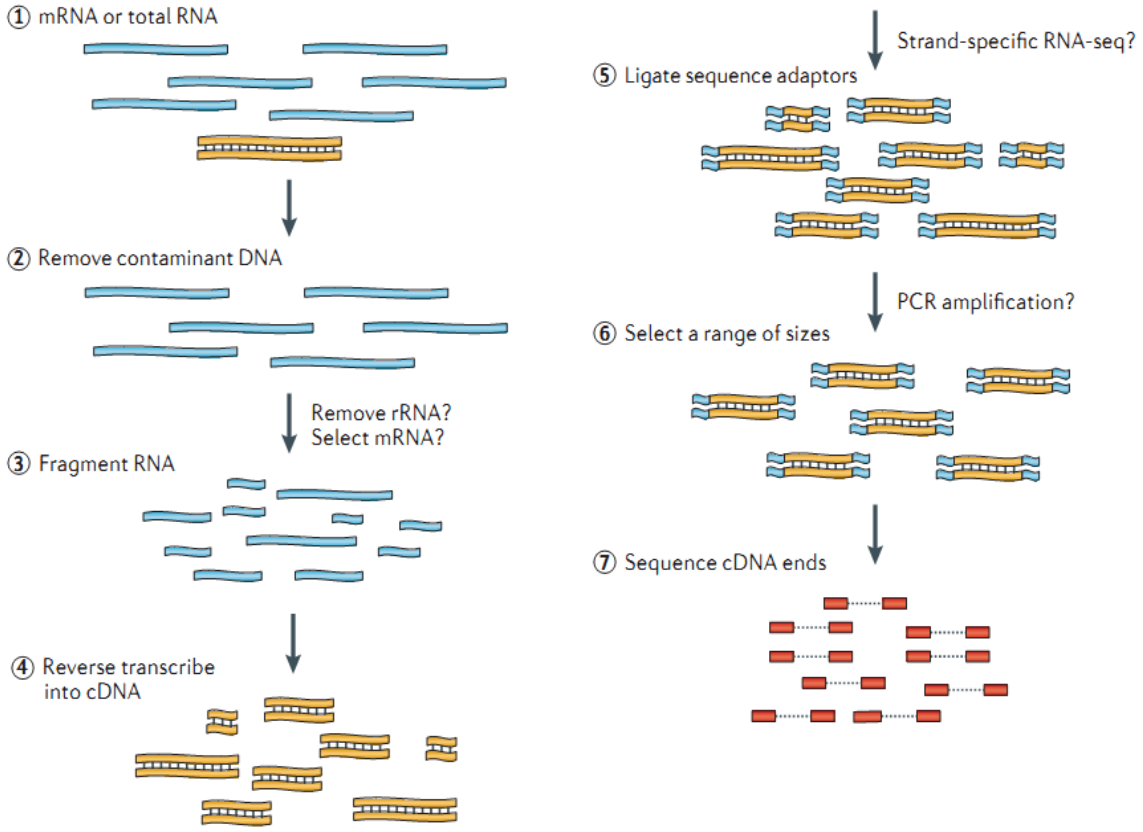


Figure 8. RNA Sequencing Experiment Workflow

REFERENCE Nat Rev Genet. 2011 Sep 7;12(10):671-82

1) Isolate the Total RNA from Sample of interest (Cell or Tissue).

2) Eliminate DNA contamination using DNase.

3) Choose an appropriate kit for library prep process depending on the types of RNA. For mRNA with poly-A tail, use mRNA purification kit; for non-coding RNAs, such as lincRNA, use ribo-zero RNA removal Kit to purify RNA of interest.

4) Randomly fragment purified RNA for short read sequencing.

5) Reverse transcribe fragmented RNA into cDNA.

6) Ligate adapters onto both ends of the cDNA fragments.

7) After amplifying fragments using PCR, select fragments with insert sizes between 200-400 bp. For paired-end sequencing, both ends of the cDNA is sequenced by the read length.

2. Analysis Methods and Workflow

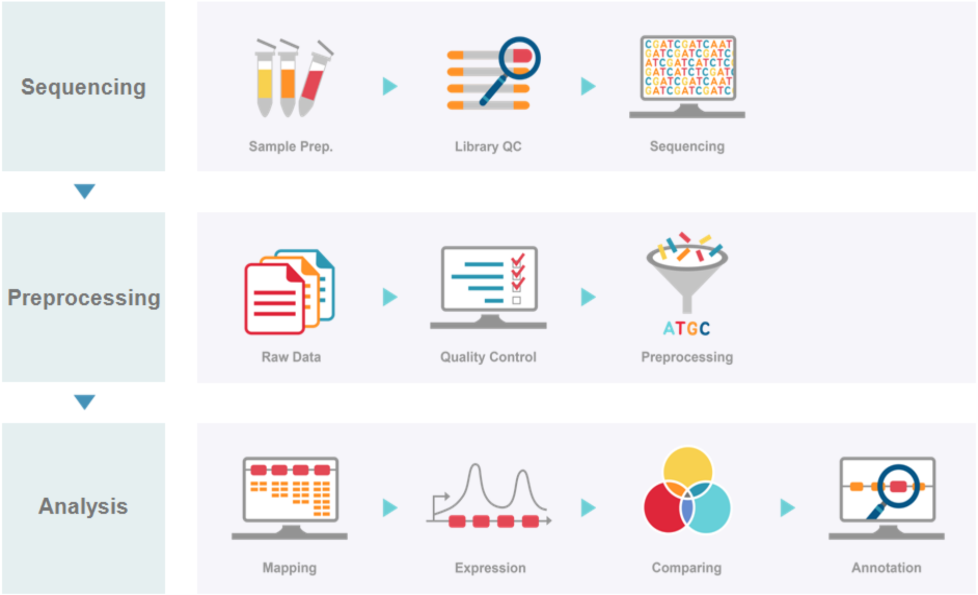


Figure 9. Analysis Workflow

1) Analyze the quality control of the sequenced raw reads. Overall reads' quality, total bases, total reads, GC (%) and basic statistics are calculated.

2) In order to reduce biases in analysis, artifacts such as low quality reads, adaptor sequence, contaminant DNA, or PCR duplicates are removed.

3) Trimmed reads are mapped to reference genome with HISAT2, splice-aware aligner.

4) Transcript is assembled by StringTie with aligned reads.

5) Expression profiles are represented as read count and normalization value which is based on transcript length and depth of coverage. The FPKM (Fragments Per Kilobase of transcript per Million Mapped reads) value or the RPKM (Reads Per Kilobase of transcript per Million mapped reads) is used as a normalization value.

6) In groups with different conditions, genes or transcripts that express differentially are filtered out through statistical hypothesis testing.

7) In case of known gene annotation, functional annotation and gene-set enrichment analysis are performed using GO and KEGG database on differentially expressed genes.

3. Summary of Data Production

3. 1. Raw Data Statistics

(Refer to Path: result_RNAseq/Analysis_statistics/rawData/raw_throughput.stats)

The total number of bases, reads, GC (%), Q20 (%), Q30 (%) are calculated for 2 samples. For example, in Cfdp1-K1RNA, 46,100,372 reads are produced, and total read bases are 4.7Gbp. The GC content (%) is 49.57% and Q30 is 93.77%.

Table 1. Raw data stats

Index	Sample id	Total read bases*	Total reads	GC (%)	Q20 (%)	Q30 (%)
1	Cfdp1-K1RNA	4,656,137,572	46,100,372	49.57	97.87	93.77
2	vdR2-4RNA	5,331,793,636	52,790,036	50.52	98.66	95.75

(* Total read bases = Total reads x Read length)

- Total read bases: Total number of bases sequenced
- Total reads: Total number of reads
- GC (%): GC content
- Q20 (%): Ratio of bases that have phred quality score greater than or equal to 20
- Q30 (%): Ratio of bases that have phred quality score greater than or equal to 30

3. 2. Average Base Quality at Each Cycle

(Refer to Path: Analysis_statistics/rawData/A_fastqc/)

The quality of produced data is determined by the phred quality score at each cycle. Box plot containing the average quality at each cycle is created with FastQC.

The x-axis shows number of cycles and y-axis shows phred quality score. Phred quality score 20 means 99% accuracy and reads over score of 20 are accepted as good quality.

LINK http://www.bioinformatics.babraham.ac.uk/projects/fastqc

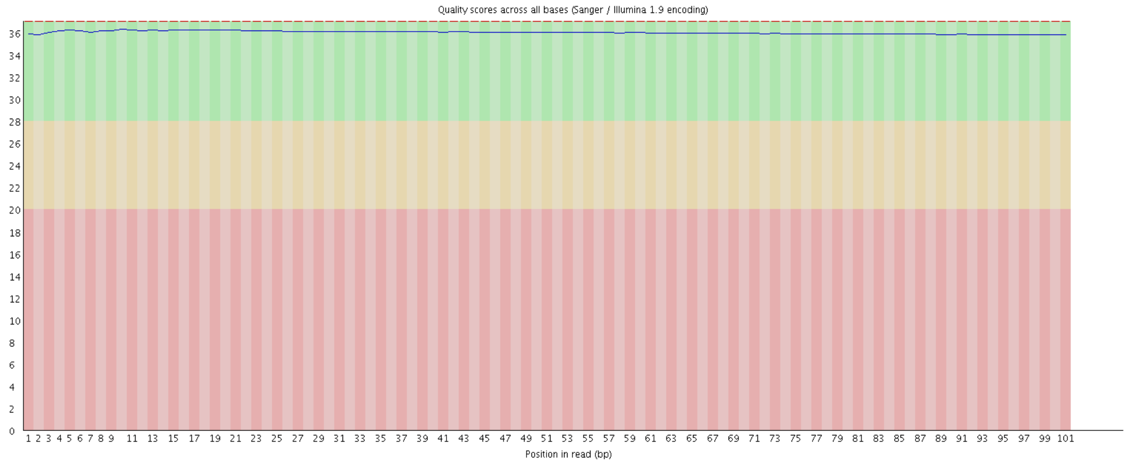


Figure 10. Read quality at each cycle of Cfdp1-K1RNA (read1)

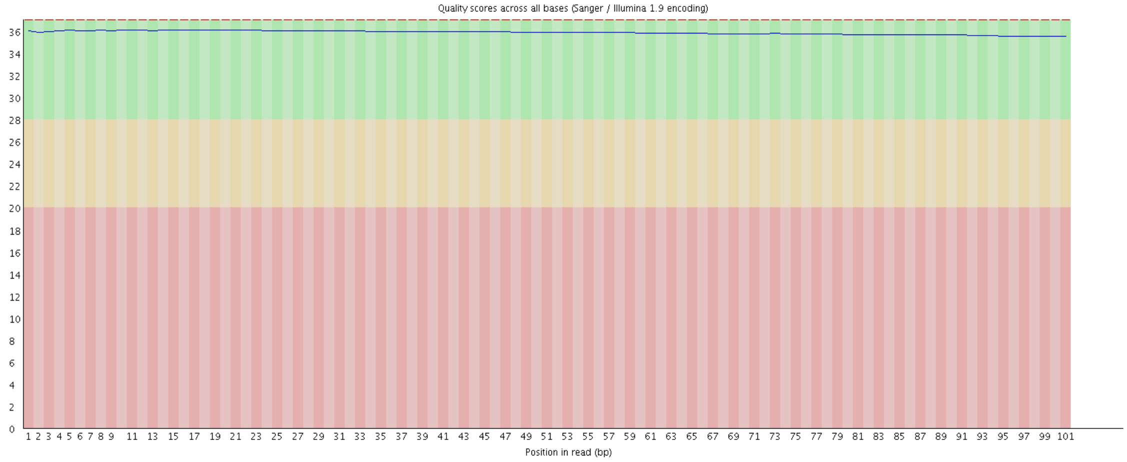


Figure 11. Read quality at each cycle of Cfdp1-K1RNA (read2)

- Yellow box: Interquartile range (25-75%) of phred score at each cycle
- Red line: Median of phred score at each cycle
- Blue line: Average of phred score at each cycle
- Green background: Good quality
- Orange background: Acceptable quality
- Red background: Bad quality

3. 3. Trimming Data Statistics

(Refer to Path: result_RNAseq/Analysis_statistics/trimmedData/trim_throughput.stats)

Trimmomatic program is used to remove adapter sequences and bases with base quality lower than three from the ends. Also using sliding window method, bases of reads that does not qualify for window size 4, and mean quality 15 are trimmed. Afterwards, reads with length shorter than 36bp are dropped to produce trimmed data.

Table 2. Trimming Data Stats

Index	Sample id	Total read bases	Total reads	GC(%)	Q20(%)	Q30(%)
1	Cfdp1-K1RNA	4,543,927,517	45,262,214	49.59	98.39	94.57
2	vdR2-4RNA	5,253,493,756	52,245,252	50.53	98.99	96.25

- Total read bases: Total number of read bases after trimming
- Total reads: Total number of reads after trimming
- GC (%): GC Content
- Q20 (%): Ratio of bases that have phred quality score greater than or equal to 20
- Q30 (%): Ratio of bases that have phred quality score greater than or equal to 30

3. 4. Average Base Quality at Each Cycle after Trimming

(Refer to Path: result_RNAseq/Analysis_statistics/trimmedData/A_fastqc/)

Figure 12 and 13 show average base quality at each cycle after trimming.

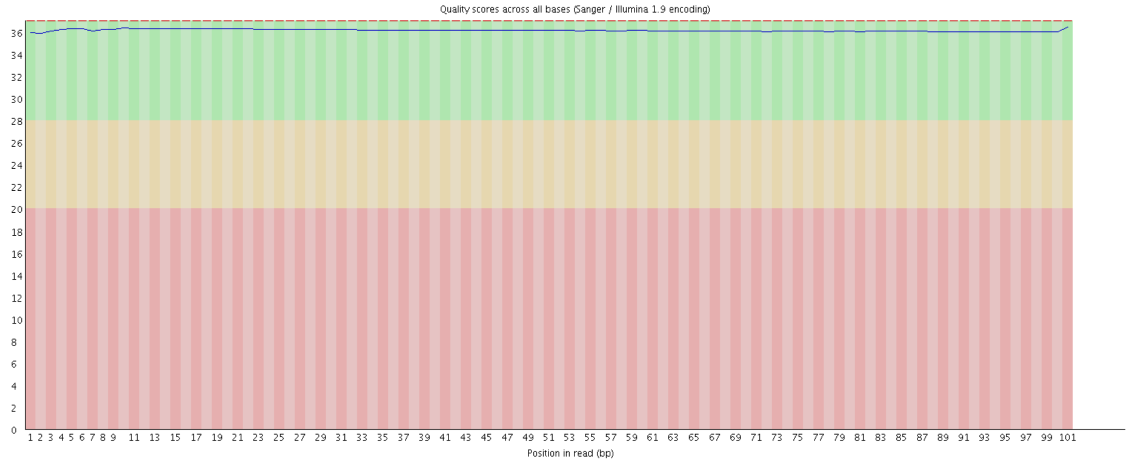


Figure 12. Average base quality of Cfdp1-K1RNA (read1) at each cycle after trimming

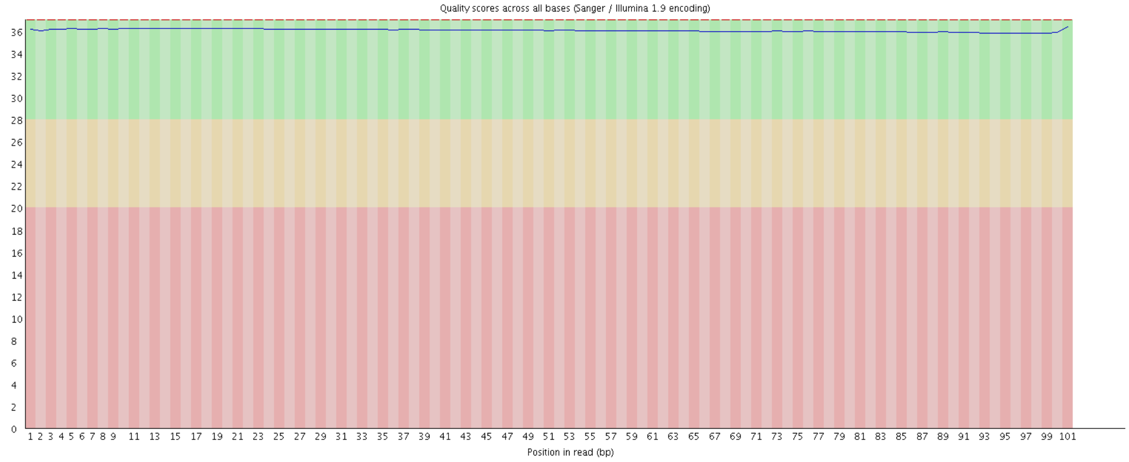


Figure 13. Average base quality of Cfdp1-K1RNA (read2) at each cycle after trimming

- Yellow box: Interquartile range (25-75%) of phred score at each cycle
- Red line: Median of phred score at each cycle
- Blue line: Average of phred score at each cycle
- Green background: Good quality
- Orange background: Acceptable quality
- Red background: Bad quality

4. Reference Mapping and Assembly Results

4. 1. Mapping Data Statistics

(Refer to Path: result_RNAseq/Analysis_statistics/mapping.hisat.stats)

In order to map cDNA fragments obtained from RNA sequencing, UCSC mm10 was used as a reference genome. Table 3 shows the statistic obtained from HISAT2, which is known to handle spliced read mapping through Bowtie2 aligner. You can check number of processed reads, mapped reads.

Table 3. Mapped Data Stats

Sample ID	# of processed reads	# of mapped reads (%)	# of unmapped reads (%)
Cfdp1-K1RNA	45,262,214	44,725,812 (98.81%)	536,402 (1.19%)
vdR2-4RNA	52,245,252	51,767,696 (99.09%)	477,556 (0.91%)

- Processed reads: Number of cleaned reads after trimming
- Mapped reads: Number of reads mapped to reference
- Unmapped reads: Number of reads that failed to align

4. 2. Expression Profiling

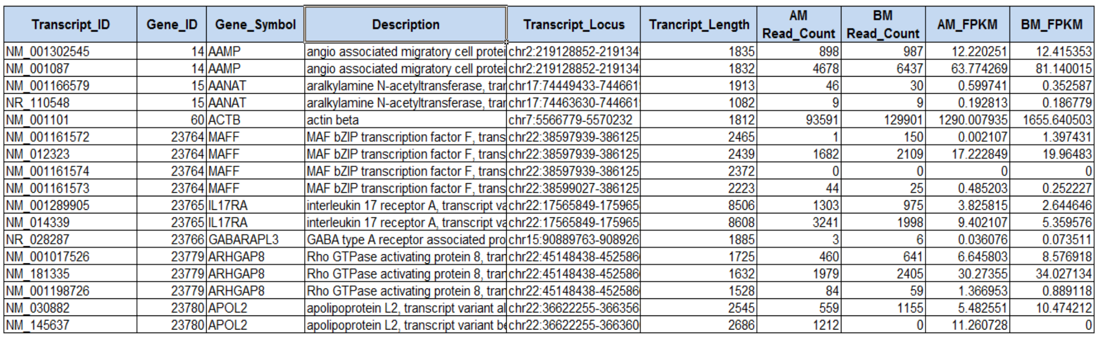
Known genes and transcripts are assembled with StringTie based on reference genome model. After assembly, the abundance of gene/transcript is calculated in the read count and normalized value as FPKM (Fragments Per Kilobase of transcript per Million mapped reads) for a sample.

4. 2. 1. Known Transcripts Expression Level

(Refer to Path: result_RNAseq_excel/Expression_profile/StringTie/ Expression_Profile.mm10.transcript.xlsx)

Table 4 is an example of known transcript expression level per sample in expression value. This result is obtained by -e option of StringTie does not consider novel transcript assembly.

Table 4. Known transcripts Expression Level (example)



Transcript_ID	Gene_ID	Gene_Symbol	Description	Transcript_Locus	Trancript_Length	AM Read_Count	BM Read_Count	AM_FPKM	BM_FPKM
NM_001302545	14	AAMP	angio associated migratory cell protei	chr2:219128852-219134	1835	898	987	12.220251	12.415353
NM_001087	14	AAMP	angio associated migratory cell protei	chr2:219128852-219134	1832	4678	6437	63.774269	81.140015
NM_001166579	15	AANAT	aralkylamine N-acetyltransferase, trar	chr17:74449433-744661	1913	46	30	0.599741	0.352587
NR_110548	15	AANAT	aralkylamine N-acetyltransferase, trar	chr17:74463630-744661	1082	9	9	0.192813	0.186779
NM_001101	60	ACTB	actin beta	chr7:5566779-5570232	1812	93591	129901	1290.007935	1655.640503
NM_001161572	23764	MAFF	MAF bZIP transcription factor F, trans	chr22:38597939-386125	2465	1	150	0.002107	1.397431
NM_012323	23764	MAFF	MAF bZIP transcription factor F, trans	chr22:38597939-386125	2439	1682	2109	17.222849	19.96483
NM_001161574	23764	MAFF	MAF bZIP transcription factor F, trans	chr22:38597939-386125	2372	0	0	0	0
NM_001161573	23764	MAFF	MAF bZIP transcription factor F, trans	chr22:38599027-386125	2223	44	25	0.485203	0.252227
NM_001289905	23765	IL17RA	interleukin 17 receptor A, transcript va	chr22:17565849-175965	8506	1303	975	3.825815	2.644646
NM_014339	23765	IL17RA	interleukin 17 receptor A, transcript va	chr22:17565849-175965	8608	3241	1998	9.402107	5.359576
NR_028287	23766	GABARAPL3	GABA type A receptor associated pro	chr15:90889763-908926	1885	3	6	0.036076	0.073511
NM_001017526	23779	ARHGAP8	Rho GTPase activating protein 8, trar	chr22:45148438-452586	1725	460	641	6.645803	8.576918
NM_181335	23779	ARHGAP8	Rho GTPase activating protein 8, trar	chr22:45148438-452586	1632	1979	2405	30.27355	34.027134
NM_001198726	23779	ARHGAP8	Rho GTPase activating protein 8, trar	chr22:45148438-452586	1528	84	59	1.366953	0.889118
NM_030882	23780	APOL2	apolipoprotein L2, transcript variant al	chr22:36622255-366356	2545	559	1155	5.482551	10.474212
NM_145637	23780	APOL2	apolipoprotein L2, transcript variant be	chr22:36622255-366360	2686	1212	0	11.260728	0

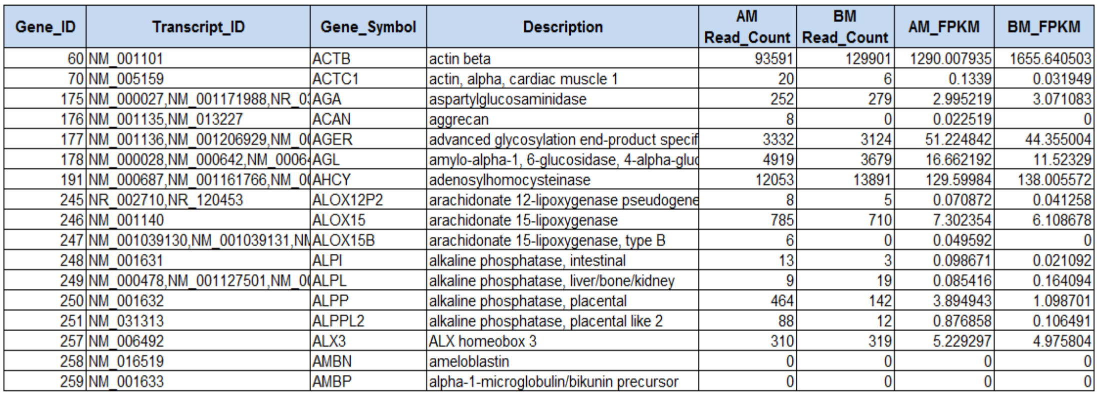
- Transcript_ID: Splicing variant (isoform/transcript)
- Gene_ID: Gene ID
- Gene_Symbol: Symbol of gene
- Gene_Description: Description of gene
- Transcript_Locus: Transript locus
- Transcript_Length: Transcript length
- [Sample Name]_Read_Count: Read count of a sample
- [Sample Name]_FPKM: FPKM normalized value of a sample

4. 2. 2. Known Genes Expression Level

(Refer to Path: result_RNAseq_excel/Expression_profile/StringTie/
Expression_Profile.mm10.gene.xlsx)

Table 5 is an example of known gene expression level per sample in expression value. This result is obtained by -e option of StringTie does not consider novel transcript assembly.

Table 5. Known genes Expression Level (example)



Gene_ID	Transcript_ID	Gene_Symbol	Description	AM Read_Count	BM Read_Count	AM_FPKM	BM_FPKM
60	NM_001101	ACTB	actin beta	93591	129901	1290.007935	1655.640503
70	NM_005159	ACTC1	actin, alpha, cardiac muscle 1	20	6	0.1339	0.031949
175	NM_000027,NM_001171988,NR_0	AGA	aspartylglucosaminidase	252	279	2.995219	3.071083
176	NM_001135,NM_013227	ACAN	aggrecan	8	0	0.022519	0
177	NM_001136,NM_001206929,NM_0	AGER	advanced glycosylation end-product specif	3332	3124	51.224842	44.355004
178	NM_000028,NM_000642,NM_0006	AGL	amylo-alpha-1, 6-glucosidase, 4-alpha-glu	4919	3679	16.662192	11.52329
191	NM_000687,NM_001161766,NM_0	AHCY	adenosylhomocysteinase	12053	13891	129.59984	138.005572
245	NR_002710,NR_120453	ALOX12P2	arachidonate 12-lipoxygenase pseudogene	8	5	0.070872	0.041258
246	NM_001140	ALOX15	arachidonate 15-lipoxygenase	785	710	7.302354	6.108678
247	NM_001039130,NM_001039131,NM	ALOX15B	arachidonate 15-lipoxygenase, type B	6	0	0.049592	0
248	NM_001631	ALPI	alkaline phosphatase, intestinal	13	3	0.098671	0.021092
249	NM_000478,NM_001127501,NM_0	ALPL	alkaline phosphatase, liver/bone/kidney	9	19	0.085416	0.164094
250	NM_001632	ALPP	alkaline phosphatase, placental	464	142	3.894943	1.098701
251	NM_031313	ALPPL2	alkaline phosphatase, placental like 2	88	12	0.876858	0.106491
257	NM_006492	ALX3	ALX homeobox 3	310	319	5.229297	4.975804
258	NM_016519	AMBN	ameloblastin	0	0	0	0
259	NM_001633	AMBP	alpha-1-microglobulin/bikunin precursor	0	0	0	0

- Gene_ID: Gene ID
- Transcript_ID: Splicing variant (isoform/transcript)
- Gene_Symbol: Symbol of gene
- Gene_Description: Description of gene
- [Sample Name]_Read_Count: Read count of a sample
- [Sample Name]_FPKM: FPKM normalized value of a sample

5. Differentially Expressed Gene Analysis Results

5. 1. Data Analysis Quality Check and Preprocessing

There is a process that sorts differentially expressed gene among samples by FPKM value of known genes. In preprocessing, there are data quality and similarity checks among samples in case of biological replicates exist.

(Refer to Path: result_RNAseq_excel/DEG_result/Analysis_Result.html)

5. 1. 1. Sample Information and Analysis Design

Total of 2 samples was used for analysis. For more information of samples and comparison pair, please refer to Sample.Info.txt file.

Index	Sample.ID	Sample.Group
1	vdR2-4RNA	vdR2-4RNA
2	Cfdp1-K1RNA	Cfdp1-K1RNA

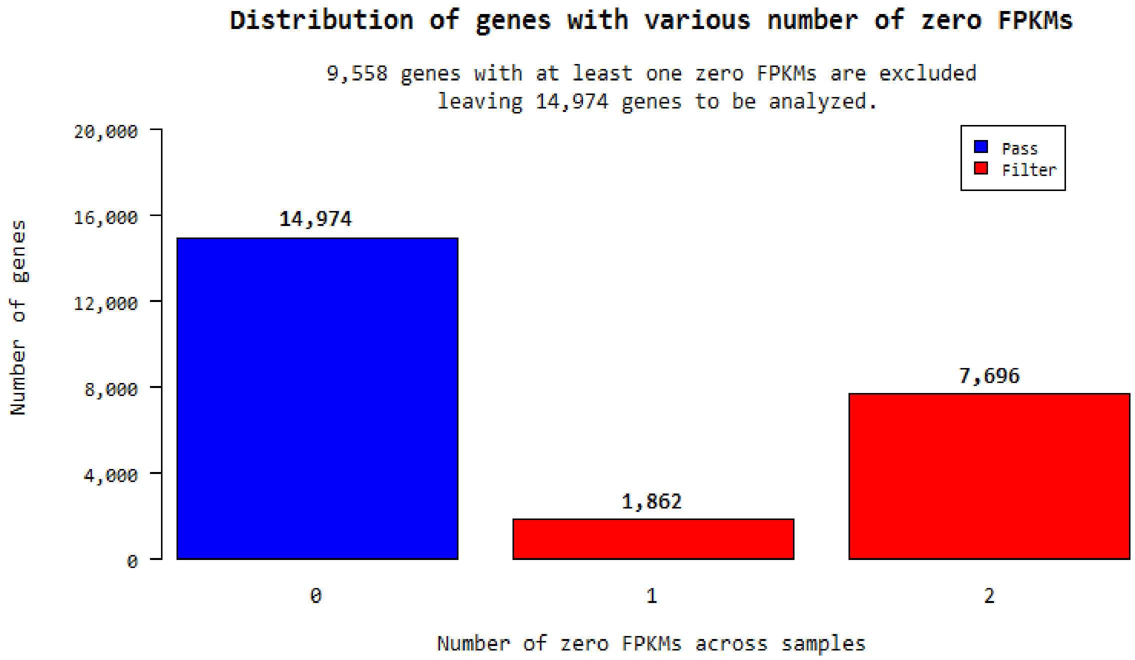
Comparison pair and statistical method for each pair are shown below.

Index	Test vs. Control	Statistical Method
1	Cfdp1-K1RNA vs. vdR2-4RNA	Fold Change, Hierarchical Clustering

5. 1. 2. DATA Quality Check

(Refer to Path: result_RNAseq_excel/DEG_result/Data Quality Check/)

For 2 samples, if more than one FPKM value was 0, it was not included in the analysis. Therefore, from total of 24,532 genes, 9,558 were excluded and only 14,974 genes were used for statistic analysis.

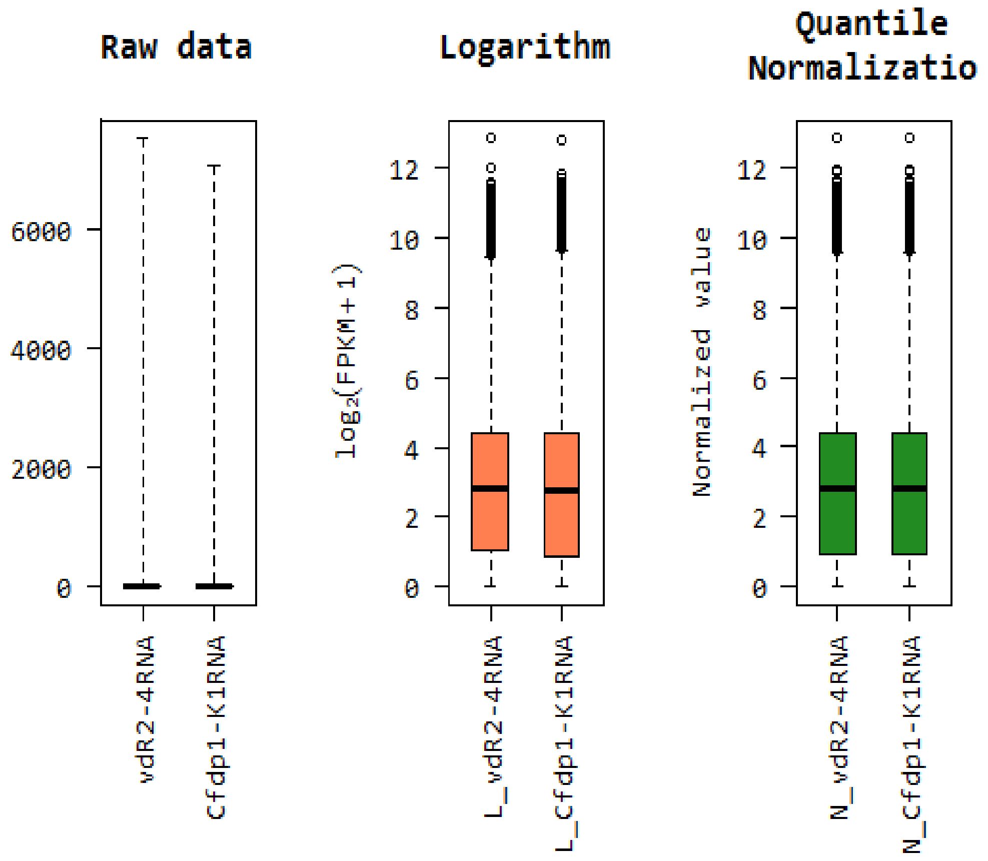


5. 1. 3. Data Transformation and Normalization

To facilitate log2 transformation, 1 was added to the raw signal (FPKM). This process is performed because raw signals are scattered along wide range and most signals are concentrated on the low signal value, so log transformation reduces the range of the signals and produces more even data distribution. After log transformation, in order to reduce systematic bias, quantile normalization is used with preprocessCore' R library.

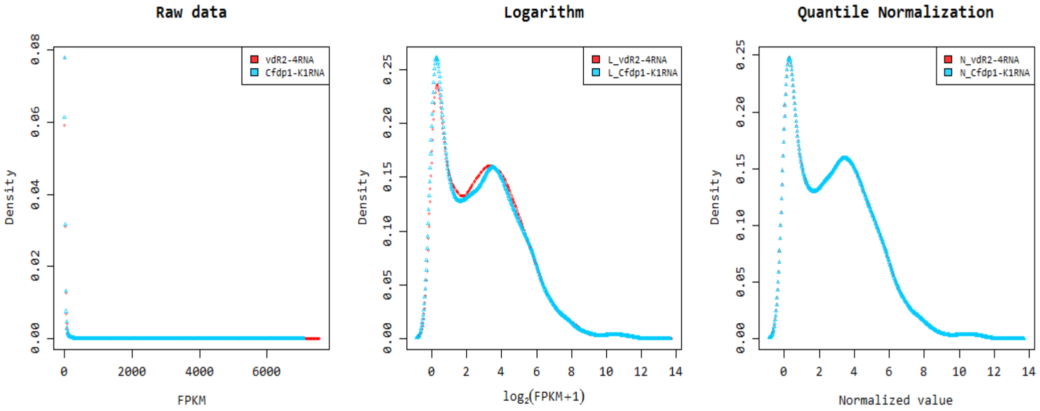
5. 1. 3. 1. Boxplot of Expression Difference between samples.

Below boxplots show the corresponding sample's expression distribution based on percentile (median, 50 percentile, 75 percentile, maximum and minimum) based on raw signal (FPKM), Log2 transformation of FPKM+1 and Quantile Normalization.



5. 1. 3. 2. Expression Density Plot per sample

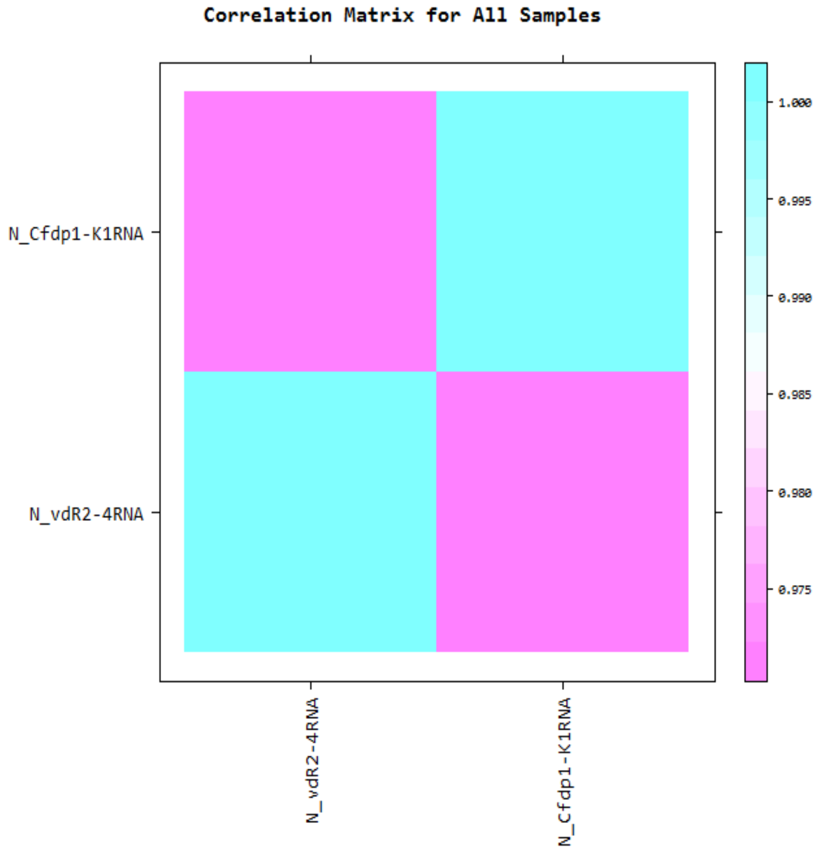
Below density plots show the corresponding samples expression distribution before and after of raw signal (FPKM), Log2 transformation of FPKM+1 and Quantile Normalization.



5. 1. 4. Correlation Analysis between samples

The similarity between samples are obtained through Pearson’s coefficient of the normalized value. For range: -1≤ r ≤ 1,the closer the value is to 1, the more similar the samples are.

Correlation matrix of all samples is as follows.



5. 2. Differentially Expressed Gene Analysis Workflow

Below shows the orders of DEG (Differentially Expressed Genes) analysis.

1) the FPKM value of known genes obtained through -e option of the StringTie were used as the original raw data.

- Raw data

(Refer to Path: result_RNAseq_excel/Expression_profile/StringTie/Expression_Profile.mm10.gene.xlsx)

: 24,532 genes, 2 samples

2) During data preprocessing, low quality transcripts are filtered. Afterwards, log2 transformation of FPKM+1 and quantile normalization are performed.

- Processed data

(Refer to Path: result_RNAseq_excel/DEG_result/data2.xlsx)

: 14,974 genes, 2 samples

3) Statistical analysis is performed using Fold Change per comparison pair.
The significant results are selected on conditions of |fc|>=2.

- Significant data

(Refer to Path: result_RNAseq_excel/DEG_result/data3_fc2.xlsx)

: 694 genes

4) For significant lists, hierarchical clustering analysis is performed to group the similar samples and genes. These results are graphically depicted using heatmap and dendogram.

- Hierarchical Clustering (Euclidean Distance, Complete Linkage)

(Refer to Path: result_RNAseq_excel/DEG_result/Cluster image/)

5) For significant lists, gene-set enrichment analysis was performed based on gene ontology(
http://geneontology.org/).
Please refer to the gomap_stat sheet and the gomap_genes sheet of data3 file.

Following result are provided.

- gomap_stat
- gomap_genes

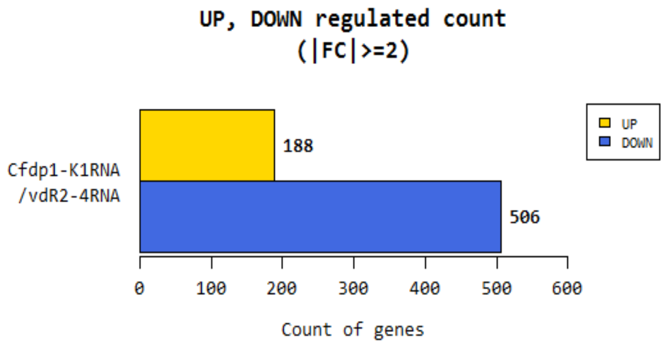
5. 3. Significant Gene Results

(Refer to Path: result_RNAseq_excel/DEG_result/Plots/)

These are DEG result of Cfdp1-K1RNA_vs_vdR2-4RNA meeting fc2 by example.

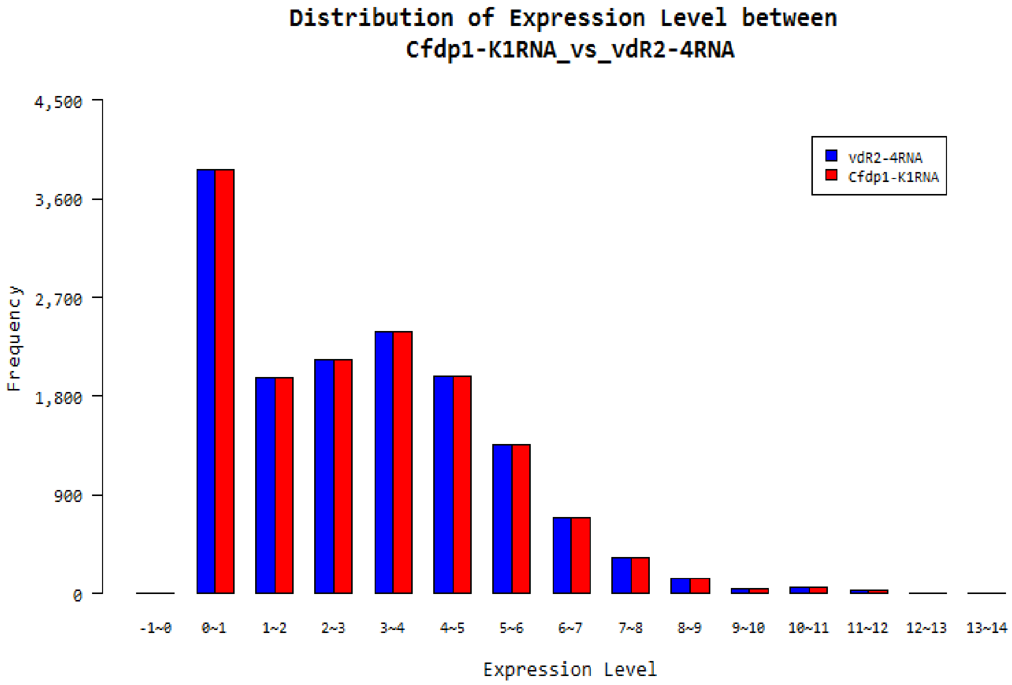
5. 3. 1. Up, Down Regulated Count by Fold Change

Shows number of up and down regulated genes based on fold change of comparison pair.



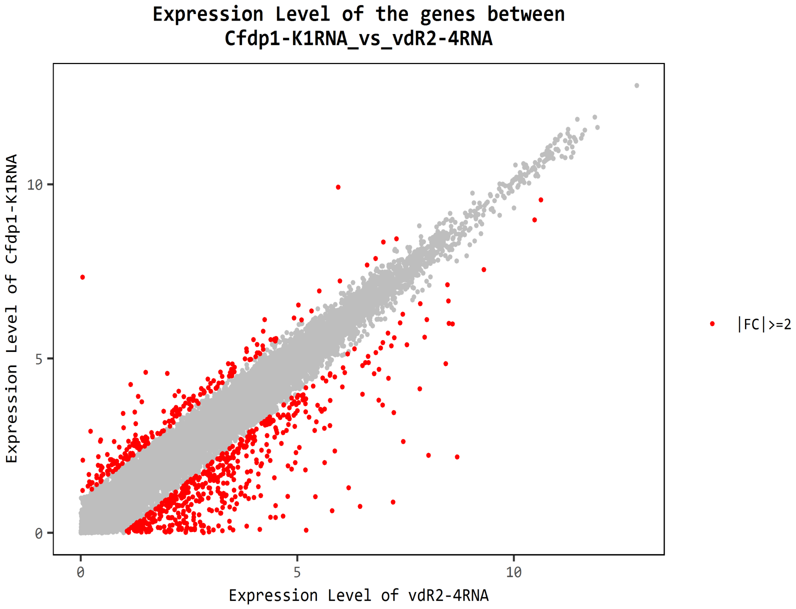
5. 3. 2. Distribution of Expression Level between two groups

Shows distribution of normalized value of each group for comparison pair.



5. 3. 3. Scatter Plot of Expression Level between two groups

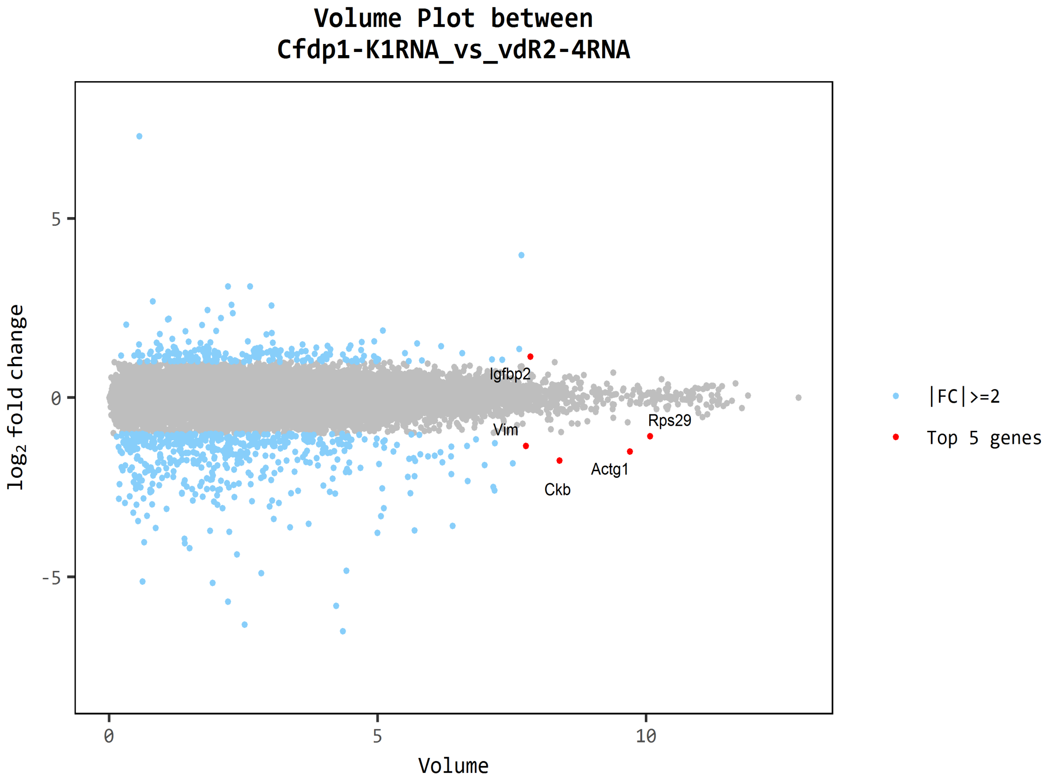
Shows expression levels between comparison pair as a scatter plot. X-axis is control and Y-axis is average normalized value of the group.



5. 3. 4. Volume Plot

Expression volume is defined as the geometric mean of two group's expression level. In order to confirm the genes that show higher expression difference compared to the control according to expression volume, volume plot is drawn. (X-axis: Volume, Y-axis: log2 Fold Change).

For example, even though fold change might be different by two-fold, the genes with higher volume may be more credible.

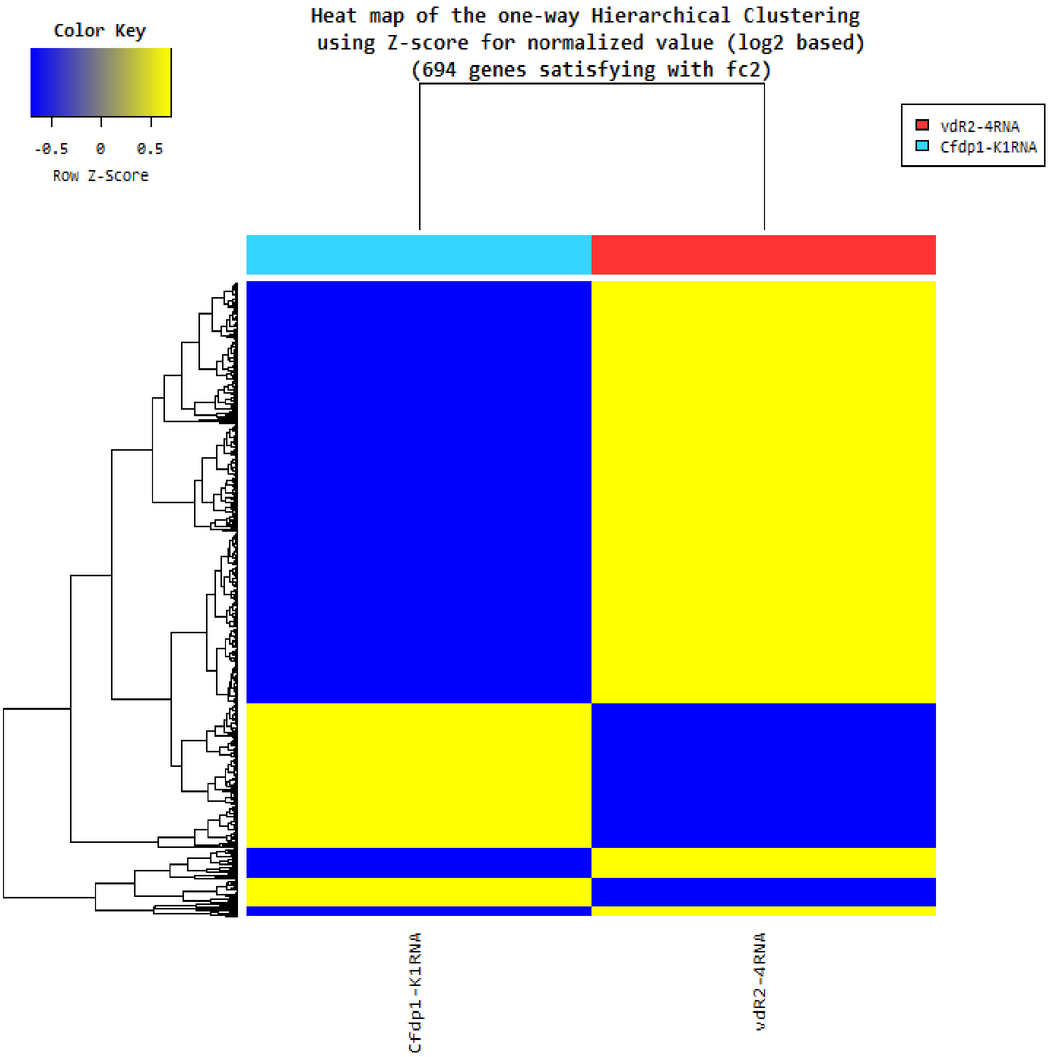


- Red dot: Top five genes by volume which satisfies, |fc|>=2

5. 3. 5. Hierarchical Clustering Analysis

(Refer to Path: result_RNAseq_excel/DEG_result/Cluster image/)

Heatmap shows result of hierarchical clustering analysis (Euclidean Method, Complete Linkage) which clusters the similarity of genes and samples by expression level (normalized value) from significant list.



5. 4. GO Enrichment Analysis

(Refer to Path: result_RNAseq_excel/DEG_result/GO)

For Gene-Enrichment test which based on Gene Ontology (http://geneontology.org/) DB was conducted with significant gene list.

Progressing about 3 categories of GO. The gene or gene product, molecule associated with GO ID was summarized by parsing the ontology file and the annotation file (multispecies annotation provided by Uniprot, or the annotation provided by each type reference DB for the GO consortium) for the GO graph structure.

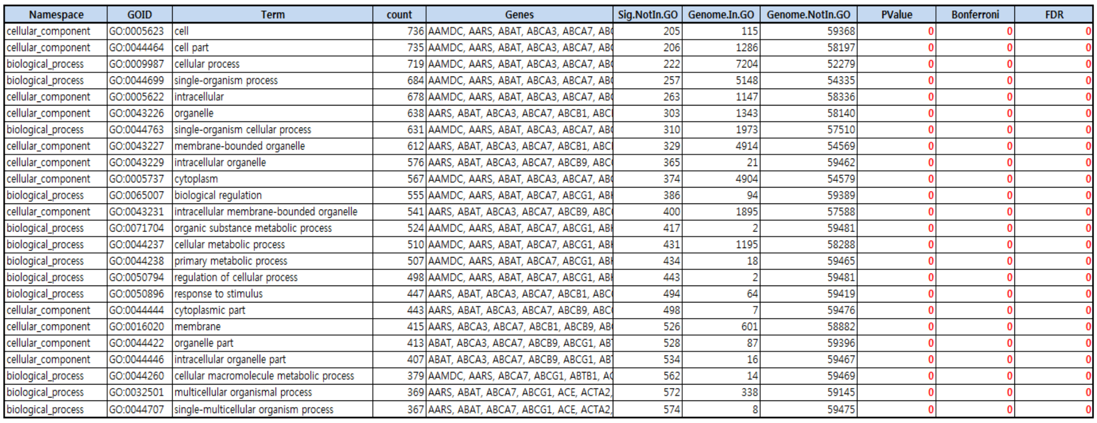
- Link for the ontology documentation: http://geneontology.org/page/ontology-documentation
- Link for the ontology files: http://geneontology.org/page/download-ontology
- Link for the annotation files: http://geneontology.org/page/download-annotations

The two results are provided for enrichment analysis.

- gomap_stat
- gomap_genes

5. 4. 1. gomap_stat Sheet

The result of associated gene and enrichment test was summarized by GO ID. The significance of specific GO ID in enrichment test with DEG set was calculated by modified fisher's exact test.

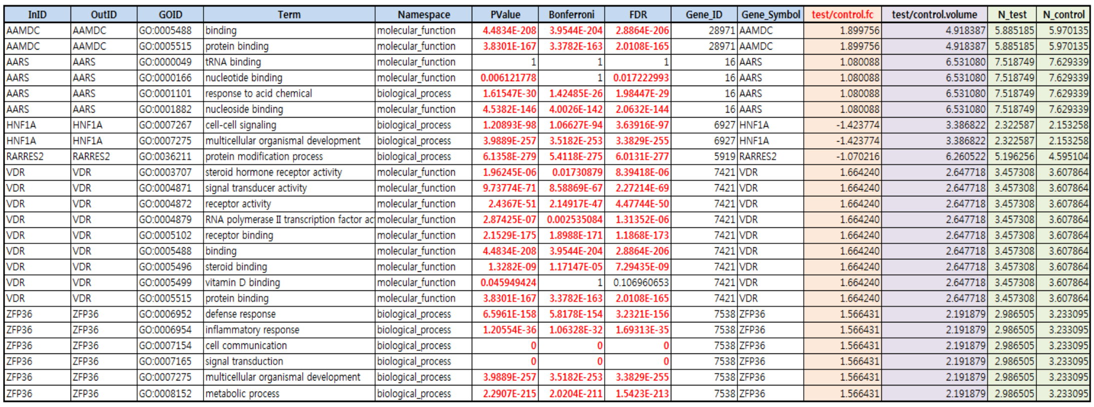


Namespace	GOID	Term	count	Genes	Sig.NotIn.GO	Genome.In.GO	Genome.NotIn.GO	PValue	Bonferroni	FDR
cellular_component	GO:0005623	cell	736	AAMDC, AARS, ABAT, ABCA3, ABCA7, AB	205	115	59368	0	0	0
cellular_component	GO:0044464	cell part	735	AAMDC, AARS, ABAT, ABCA3, ABCA7, AB	206	1286	58197	0	0	0
biological_process	GO:0009987	cellular process	719	AAMDC, AARS, ABAT, ABCA3, ABCA7, AB	222	7204	52279	0	0	0
biological_process	GO:0044699	single-organism process	684	AAMDC, AARS, ABAT, ABCA3, ABCA7, AB	257	5148	54335	0	0	0
cellular_component	GO:0005622	intracellular	678	AAMDC, AARS, ABAT, ABCA3, ABCA7, AB	263	1147	58336	0	0	0
cellular_component	GO:0043226	organelle	638	AARS, ABAT, ABCA3, ABCA7, ABCB1, ABC	303	1343	58140	0	0	0
biological_process	GO:0044763	single-organism cellular process	631	AAMDC, AARS, ABAT, ABCA3, ABCA7, AB	310	1973	57510	0	0	0
cellular_component	GO:0043227	membrane-bounded organelle	612	AARS, ABAT, ABCA3, ABCA7, ABCB1, ABC	329	4914	54569	0	0	0
cellular_component	GO:0043229	intracellular organelle	576	AARS, ABAT, ABCA3, ABCA7, ABCB9, ABC	365	21	59462	0	0	0
cellular_component	GO:0005737	cytoplasm	567	AAMDC, AARS, ABAT, ABCA3, ABCA7, AB	374	4904	54579	0	0	0
biological_process	GO:0065007	biological regulation	555	AAMDC, AARS, ABAT, ABCA7, ABCG1, AB	386	94	59389	0	0	0
cellular_component	GO:0043231	intracellular membrane-bounded organelle	541	AARS, ABAT, ABCA3, ABCA7, ABCB9, ABC	400	1895	57588	0	0	0
biological_process	GO:0071704	organic substance metabolic process	524	AAMDC, AARS, ABAT, ABCA7, ABCG1, AB	417	2	59481	0	0	0
biological_process	GO:0044237	cellular metabolic process	510	AAMDC, AARS, ABAT, ABCA7, ABCG1, AB	431	1195	58288	0	0	0
biological_process	GO:0044238	primary metabolic process	507	AAMDC, AARS, ABAT, ABCA7, ABCG1, AB	434	18	59465	0	0	0
biological_process	GO:0050794	regulation of cellular process	498	AAMDC, AARS, ABAT, ABCA7, ABCG1, AB	443	2	59481	0	0	0
biological_process	GO:0050896	response to stimulus	447	AARS, ABAT, ABCA3, ABCA7, ABCB1, ABC	494	64	59419	0	0	0
cellular_component	GO:0044444	cytoplasmic part	443	AARS, ABAT, ABCA3, ABCA7, ABCB9, ABC	498	7	59476	0	0	0
cellular_component	GO:0016020	membrane	415	AARS, ABCA3, ABCA7, ABCB1, ABCB9, AB	526	601	58882	0	0	0
cellular_component	GO:0044422	organelle part	413	ABAT, ABCA3, ABCA7, ABCB9, ABCG1, AB	528	87	59396	0	0	0
cellular_component	GO:0044446	intracellular organelle part	407	ABAT, ABCA3, ABCA7, ABCB9, ABCG1, AB	534	16	59467	0	0	0
biological_process	GO:0044260	cellular macromolecule metabolic process	379	AAMDC, AARS, ABCA7, ABCG1, ABTB1, A	562	14	59469	0	0	0
biological_process	GO:0032501	multicellular organismal process	369	AARS, ABAT, ABCA7, ABCG1, ACE, ACTA2,	572	338	59145	0	0	0
biological_process	GO:0044707	single-multicellular organism process	367	AARS, ABAT, ABCA7, ABCG1, ACE, ACTA2,	574	8	59475	0	0	0

- Namespace: 3 categories of Gene ontology (Cellular Component, Molecular Function, Biological Process)
- GOID: Gene ontology ID
- Term: Gene ontology term
- count: The number of unique genes associated with GO ID
- Genes: Associated genes with GO ID (connect by comma)
- Sig.NotIn.GO: The number of genes which are not associated GO ID
- Genome.In.GO: The number of genes associated with GO ID of total sample of gene species
- Genome.NotIn.GO: The number of genes which are not associated with GO ID of total sample of gene species
- PValue: Raw p-value was calculated by modified fisher's exact test
- Bonferroni: Adjusted p-value by Bonferroni
- FDR: Adjusted p-value by FDR

5. 4. 2. gomap_genes Sheet

The result of associated GO ID and DEG analysis was summarized based on gene. The GO ID which associated with specific gene was summarized with statistic such as fold change, p-value, volume, and normalized value.

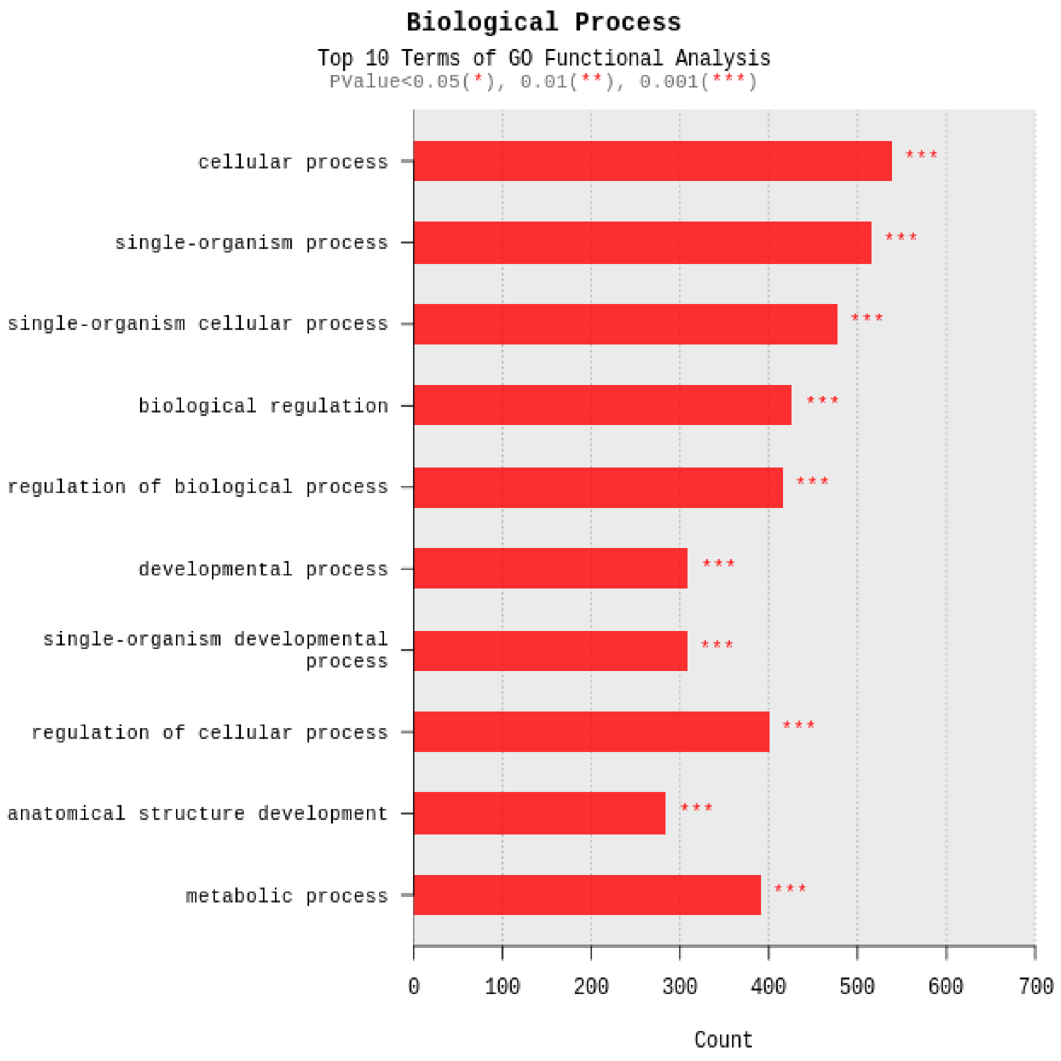


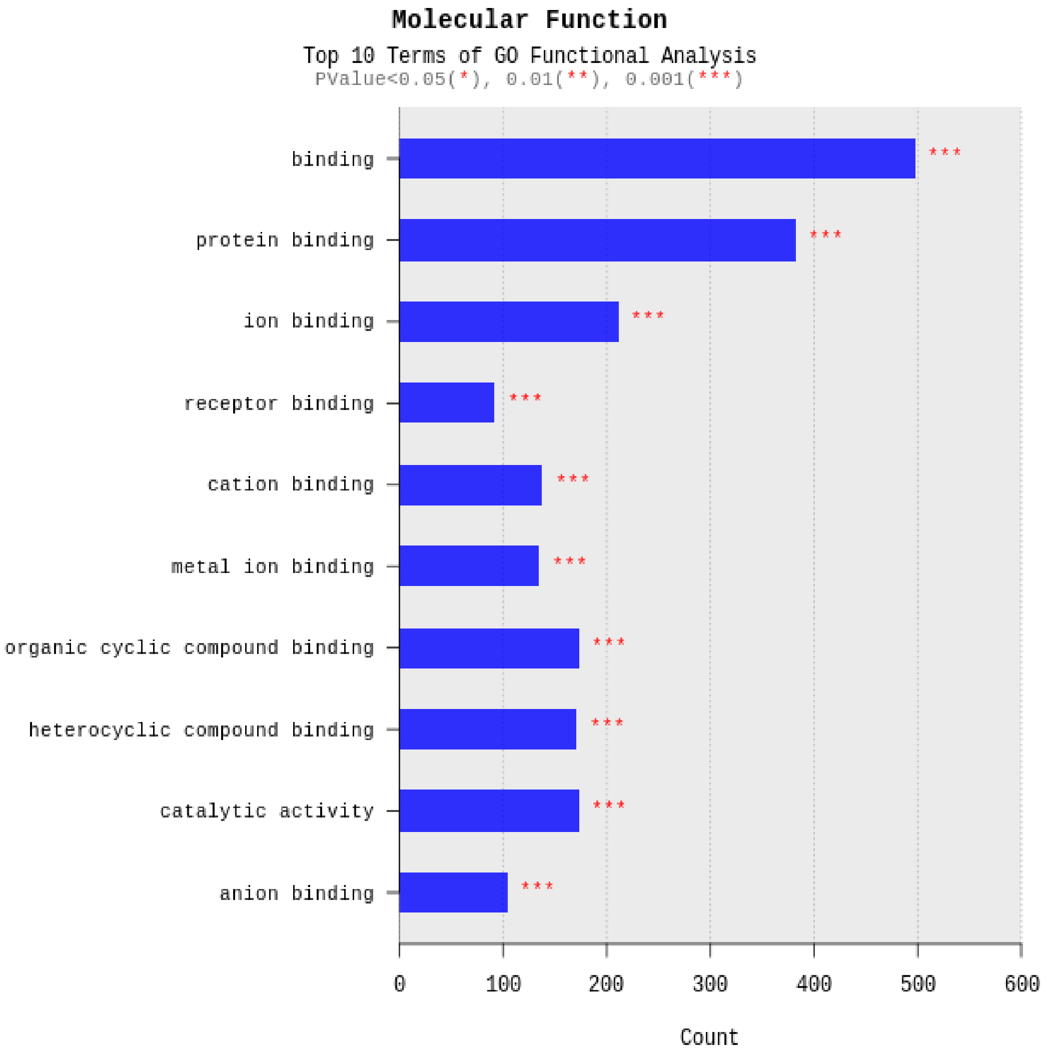
InID	OutID	GOID	Term	Namespace	PValue	Bonferroni	FDR	Gene_ID	Gene_Symbol	test/control.fc	test/control.volume	N_test	N_control
AAMDC	AAMDC	GO:0005488	binding	molecular_function	4.4834E-208	3.9544E-204	2.8864E-206	28971	AAMDC	1.899756	4.918387	5.885185	5.970135
AAMDC	AAMDC	GO:0005515	protein binding	molecular_function	3.8301E-167	3.3782E-163	2.0108E-165	28971	AAMDC	1.899756	4.918387	5.885185	5.970135
AARS	AARS	GO:0000049	tRNA binding	molecular_function	1	1	1	16	AARS	1.080088	6.531080	7.518749	7.629339
AARS	AARS	GO:0000166	nucleotide binding	molecular_function	0.006121778	1	0.017222993	16	AARS	1.080088	6.531080	7.518749	7.629339
AARS	AARS	GO:0001101	response to acid chemical	biological_process	1.61547E-30	1.42485E-26	1.98447E-29	16	AARS	1.080088	6.531080	7.518749	7.629339
AARS	AARS	GO:0001882	nucleoside binding	molecular_function	4.5382E-146	4.0026E-142	2.0632E-144	16	AARS	1.080088	6.531080	7.518749	7.629339
HNF1A	HNF1A	GO:0007267	cell-cell signaling	biological_process	1.20893E-98	1.06627E-94	3.63916E-97	6927	HNF1A	-1.423774	3.386822	2.322587	2.153258
HNF1A	HNF1A	GO:0007275	multicellular organismal development	biological_process	3.9889E-257	3.5182E-253	3.3829E-255	6927	HNF1A	-1.423774	3.386822	2.322587	2.153258
RARRES2	RARRES2	GO:0036211	protein modification process	biological_process	6.1358E-279	5.4118E-275	6.0131E-277	5919	RARRES2	-1.070216	6.260522	5.196256	4.595104
VDR	VDR	GO:0003707	steroid hormone receptor activity	molecular_function	1.96245E-06	0.01730879	8.39418E-06	7421	VDR	1.664240	2.647718	3.457308	3.607864
VDR	VDR	GO:0004871	signal transducer activity	molecular_function	9.73774E-71	8.58869E-67	2.27214E-69	7421	VDR	1.664240	2.647718	3.457308	3.607864
VDR	VDR	GO:0004872	receptor activity	molecular_function	2.4367E-51	2.14917E-47	4.47744E-50	7421	VDR	1.664240	2.647718	3.457308	3.607864
VDR	VDR	GO:0004879	RNA polymerase II transcription factor ac	molecular_function	2.87425E-07	0.002535084	1.31352E-06	7421	VDR	1.664240	2.647718	3.457308	3.607864
VDR	VDR	GO:0005102	receptor binding	molecular_function	2.1529E-175	1.8988E-171	1.1868E-173	7421	VDR	1.664240	2.647718	3.457308	3.607864
VDR	VDR	GO:0005488	binding	molecular_function	4.4834E-208	3.9544E-204	2.8864E-206	7421	VDR	1.664240	2.647718	3.457308	3.607864
VDR	VDR	GO:0005496	steroid binding	molecular_function	1.3282E-09	1.17147E-05	7.29435E-09	7421	VDR	1.664240	2.647718	3.457308	3.607864
VDR	VDR	GO:0005499	vitamin D binding	molecular_function	0.045949424	1	0.106960653	7421	VDR	1.664240	2.647718	3.457308	3.607864
VDR	VDR	GO:0005515	protein binding	molecular_function	3.8301E-167	3.3782E-163	2.0108E-165	7421	VDR	1.664240	2.647718	3.457308	3.607864
ZFP36	ZFP36	GO:0006952	defense response	biological_process	6.5961E-158	5.8178E-154	3.2321E-156	7538	ZFP36	1.566431	2.191879	2.986505	3.233095
ZFP36	ZFP36	GO:0006954	inflammatory response	biological_process	1.20554E-36	1.06328E-32	1.69313E-35	7538	ZFP36	1.566431	2.191879	2.986505	3.233095
ZFP36	ZFP36	GO:0007154	cell communication	biological_process	0	0	0	7538	ZFP36	1.566431	2.191879	2.986505	3.233095
ZFP36	ZFP36	GO:0007165	signal transduction	biological_process	0	0	0	7538	ZFP36	1.566431	2.191879	2.986505	3.233095
ZFP36	ZFP36	GO:0007275	multicellular organismal development	biological_process	3.9889E-257	3.5182E-253	3.3829E-255	7538	ZFP36	1.566431	2.191879	2.986505	3.233095
ZFP36	ZFP36	GO:0008152	metabolic process	biological_process	2.2907E-215	2.0204E-211	1.5423E-213	7538	ZFP36	1.566431	2.191879	2.986505	3.233095

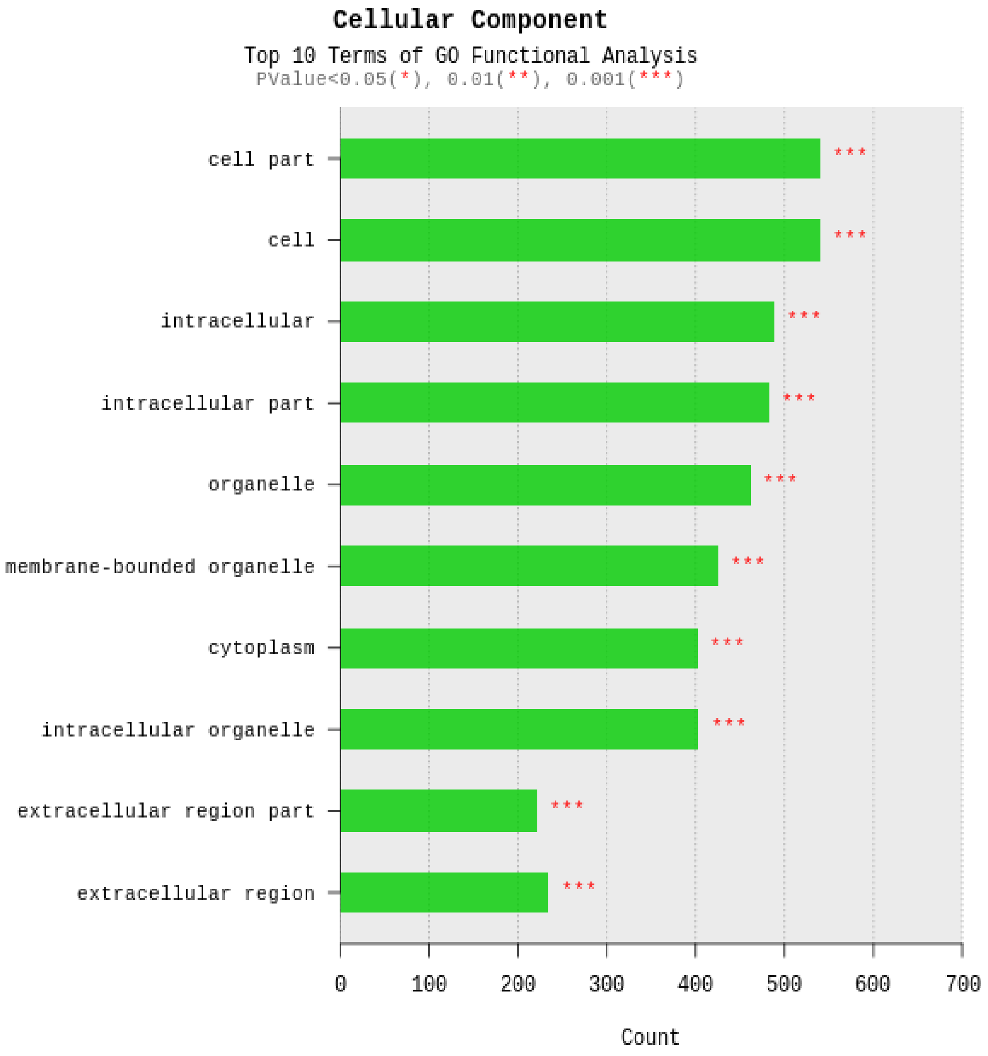
- InID: Iuput ID for GO enrichment analysis
- OutID: Mapping ID as gene symbol from input ID through GO enrichment analysis
- GOID: Gene ontology ID
- Term: Gene ontology term
- Namespace: 3 categories of gene ontology (Cellular Component, Molecular Function, Biological Process)
- PValue: Raw p-value was calculated by modified fisher's exact test
- Bonferroni: Adjusted p-value by Bonferroni
- FDR: Adjusted p-value by FDR

The bar plot below shows the results of the enrichment analysis based on Gene Ontology DB for significant genes.

(These plots were made based on gomap_stat result.)




Molecular Function
Top 10 Terms of GO Functional Analysis
PValue<0.05(*), 0.01(**), 0.001(***)
binding
protein binding
ion binding
receptor binding
cation binding
metal ion binding
organic cyclic compound binding
heterocyclic compound binding
catalytic activity
anion binding
***
0
100
200
300
400
500
600
Count


Cellular Component
Top 10 Terms of GO Functional Analysis
PValue<0.05(*), 0.01(**), 0.001(***)
cell part
cell
intracellular
intracellular part
organelle
membrane-bounded organelle
cytoplasm
intracellular organelle
extracellular region part
extracellular region
***
0
100
200
300
400
500
600
700
Count

6. Data Download Information

6. 1. Raw Data

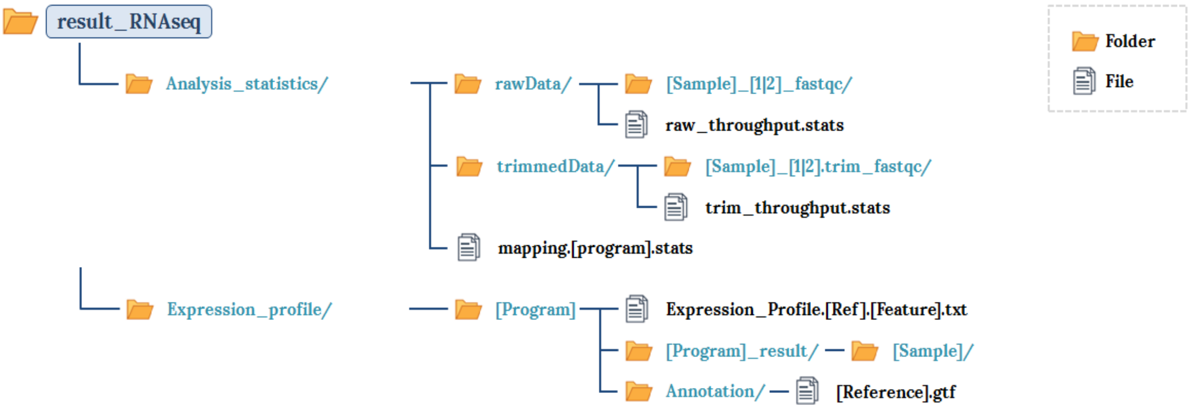
Raw data is the FASTQ file that isn't trimmed adapter sequence.

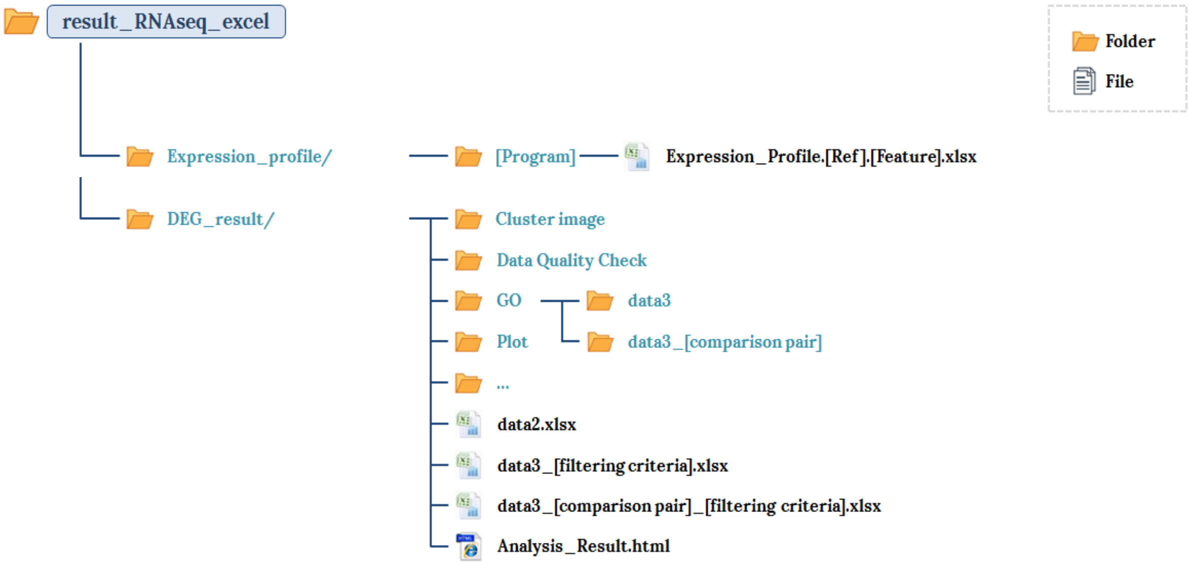
Download link	File size	md5sum
Cfdp1-K1RNA_1.fastq.gz	1.2G	34d313aab0592c10d9a8c1181e14dfb5
Cfdp1-K1RNA_2.fastq.gz	1.23G	68288067dba6036df06abc1a1fef372e
vdR2-4RNA_1.fastq.gz	1.31G	a793383ab0387867c2d5edaae7627c38
vdR2-4RNA_2.fastq.gz	1.35G	ed193bf6585ada34d4a1d450d52971f1

- fastq.gz : This is a zip file of raw data used in analysis.
- md5sum : In order to verify the integrity of files, md5sum is used. If the values of md5sum are the same, there is no forgery, modification or omission.

6. 2. Analysis Results

Download link	File size
HN00101712_result_RNAseq.zip (md5sum: 7f33ecd78a315ed837bf635cddbec896)	30.45M
HN00101712_result_RNAseq_excel.zip (md5sum: 621bddc7040d0df14b635ddd4db9ce28)	14.35M


result_RNAseq
Analysis_statistics/
rawData/
[Sample]_[1|2]_fastqc/
raw_throughput.stats
trimmedData/
[Sample]_[1|2].trim_fastqc/
trim_throughput.stats
mapping.[program].stats
Expression_profile/
[Program]
Expression_Profile.[Ref].[Feature].txt
[Program]_result/
[Sample]/
Annotation/
[Reference].gtf
Folder
File



- result_RNAseq_excel
  - Expression_profile/
    - [Program]
      - Expression_Profile.[Ref].[Feature].xlsx
  - DEG_result/
    - Cluster image
    - Data Quality Check
    - GO
      - data3
      - data3_[comparison pair]
    - Plot
    - ...
    - data2.xlsx
    - data3_[filtering criteria].xlsx
    - data3_[comparison pair]_[filtering criteria].xlsx
    - Analysis_Result.html

⚠ The data retention period is three months,
please send an e-mail (ngs@macrogen.com)
or contact representative if you want longer retention period.

7. Appendix

7. 1. Phred Quality Score Chart

Phred quality score numerically express the accuracy of each nucleotide. Higher Q number signifies higher accuracy. For example, if Phred assigns a quality score of 30 to a base, the chances of having base call error are 1 in 1000.

Quality of phred score	Probability of incorrect base call	Base call accuracy
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1000	99.9%
40	1 in 10000	99.99%

Phred Quality Score Q is calculated with $-10\log_{10}P$, where P is probability of erroneous base call.

Q-Score Binning

Illumina NovaSeq sequencer groups quality scores into specific ranges, or bins, and assigns a value to each range. Q-scores is typically updated when significant characteristics of the sequencing platform changes, such as new hardware, software, or chemistry versions.

7. 2. Programs used in Analysis

7. 2. 1. FastQC v0.11.7

LINK http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

FastQC is a program that performs quality check on the raw sequences before analysis to make sure data integrity. The main function is importing BAM, SAM, FastQ files and providing quick overview on which section has problems. It provides such results as graphs and tables in html files.

7. 2. 2. Trimmomatic 0.38

LINK http://www.usadellab.org/cms/?page=trimmomatic

Trimmomatic is a program that performs trimming depending on various parameters on illumina paired-end or single-end.

- ILLUMINACLIP: Cut adapter and other illumina-specific sequences from the read.
- SLIDINGWINDOW: Perform a sliding window trimming, cutting once the average quality within the window falls below a threshold.
- LEADING: Cut bases off the start of a read, if below a threshold quality.
- TRAILING: Cut bases off the end of a read, if below a threshold quality.
- CROP: Cut the read to a specified length.
- HEADCROP: Cut the specified number of bases from the start of the read.
- MINLEN: Drop the read if it is below a specified length.
- TOPHRED33: Change quality score to phred33.
- TOPHRED64: Change quality score to phred64.

7. 2. 3. HISAT2 version 2.1.0, Bowtie2 2.3.4.1

LINK https://ccb.jhu.edu/software/hisat2/index.shtml

HISAT2 is a fast and sensitive alignment program for mapping next-generation sequencing reads to genomes. Its first implementation based on an extension of BWT for graphs, designed a graph FM index (GFM). In addition to using one global GFM index, HISAT2 uses a large set of small GFM indexes that collectively cover the whole genome (each index representing a genomic region of 56 Kbp, with 55,000 indexes needed to cover the human population). These small indexes (called local indexes), combined with several alignment strategies, enable rapid and accurate alignment of sequencing reads. This new indexing scheme is called a Hierarchical Graph FM index (HGFM).

7. 2. 4. StringTie version 1.3.4d

LINK https://ccb.jhu.edu/software/stringtie/

StringTie is a fast and highly efficient assembler of RNA-Seq alignments into potential transcripts. It uses a novel network flow algorithm as well as an optional de novo assembly step to assemble and quantitate full-length transcripts representing multiple splice variants for each gene locus.

7. 3. References

1. BOLGER, Anthony M.; LOHSE, Marc; USADEL, Bjoern. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics, 2014, btu170.
2. KIM, Daehwan; LANGMEAD, Ben; SALZBERG, Steven L. HISAT: a fast spliced aligner with low memory requirements. Nature methods, 2015, 12.4: 357-360.
3. LI, Heng, et al. The sequence alignment/map format and SAMtools. Bioinformatics, 2009, 25.16: 2078-2079.
4. PERTEA, Mihaela, et al. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nature biotechnology, 2015, 33.3: 290-295.
5. PERTEA, Mihaela, et al. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. Nature Protocols, 2016, 11.9: 1650-1667.

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