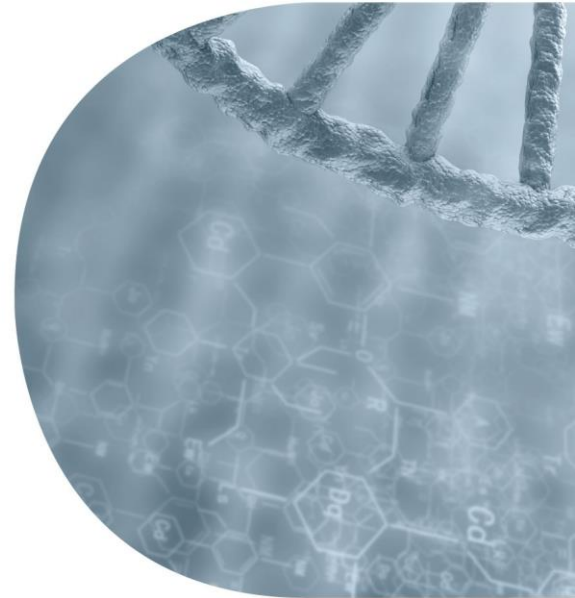


Homo sapiens Transcriptome Sequencing Report

December 2023



Project Information

Client Name	Customer
Company/Institution	Institution
Order Number	HN000000000
Species	<i>Homo sapiens</i>
Reference	GRCh38
Annotation	NCBI_109.20200522
Type of Read	Paired-ends
Read Length	101
Number of Samples	15
Library Kit	TruSeq Stranded Total RNA with Ribo-Zero H/M/R
Type of Sequencer	Illumina platform

Project Results Summary

In this study, *Homo sapiens* 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 15 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.

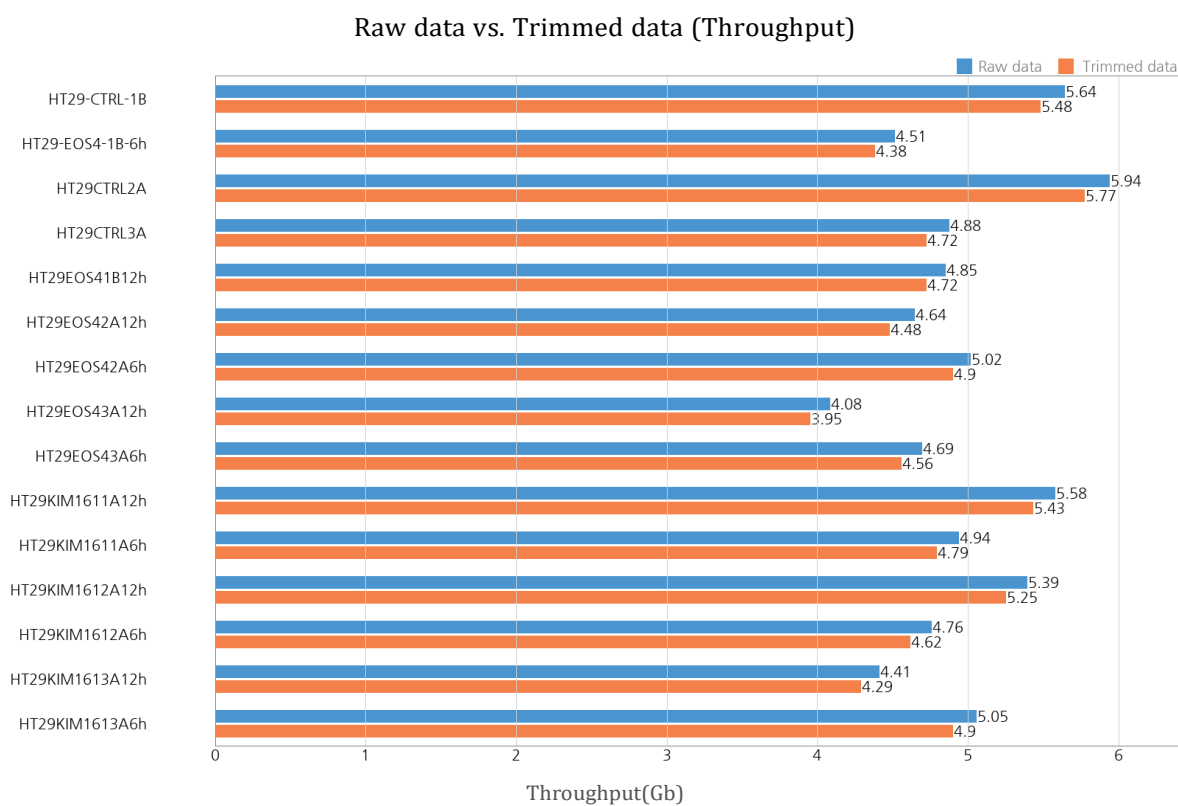


Figure 1. Throughput output of Raw and Trimmed data

Raw data vs. Trimmed data ($\geq Q30$)

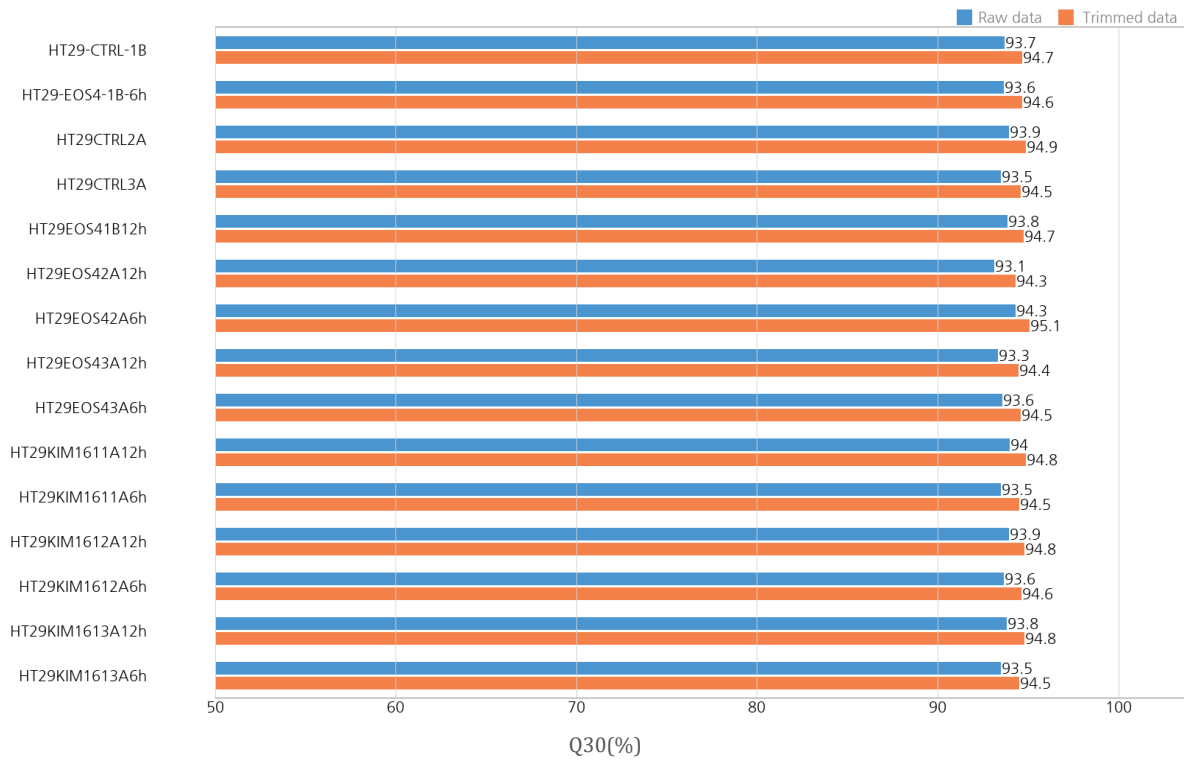


Figure 2. Q30 score of Raw and Trimmed data

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.

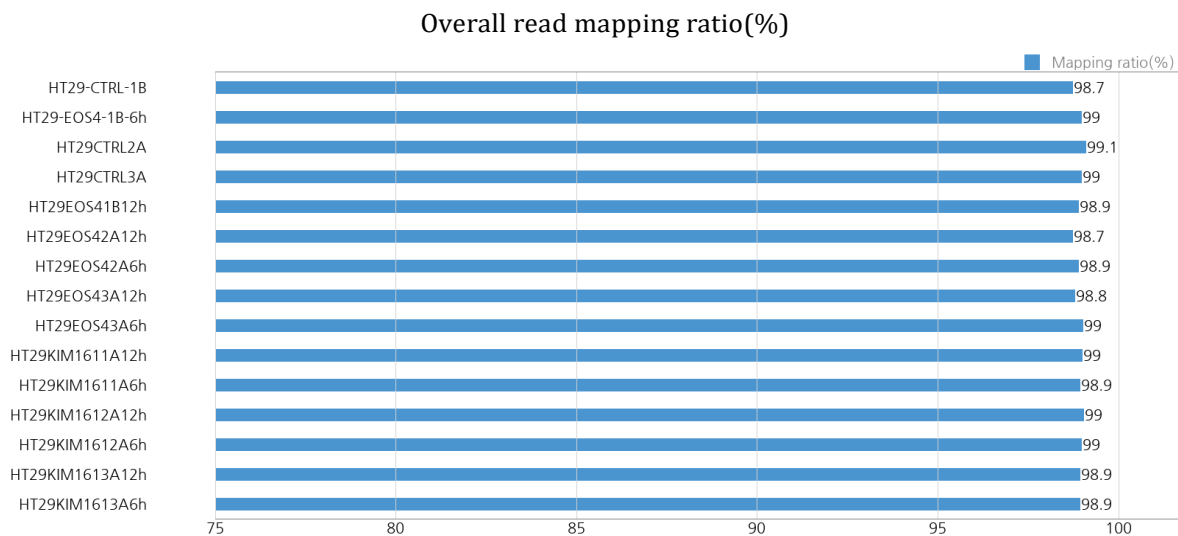


Figure 3. Overall read mapping ratio(%)

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

DEG (Differentially Expressed Genes) analysis was performed on 4 comparisons pairs as requested using DESeq2. The results showed 548 genes which satisfied $|fc| \geq 2$ & $nbinomWaldTest$ raw p-value < 0.05 conditions in at least one of comparison pairs.

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.

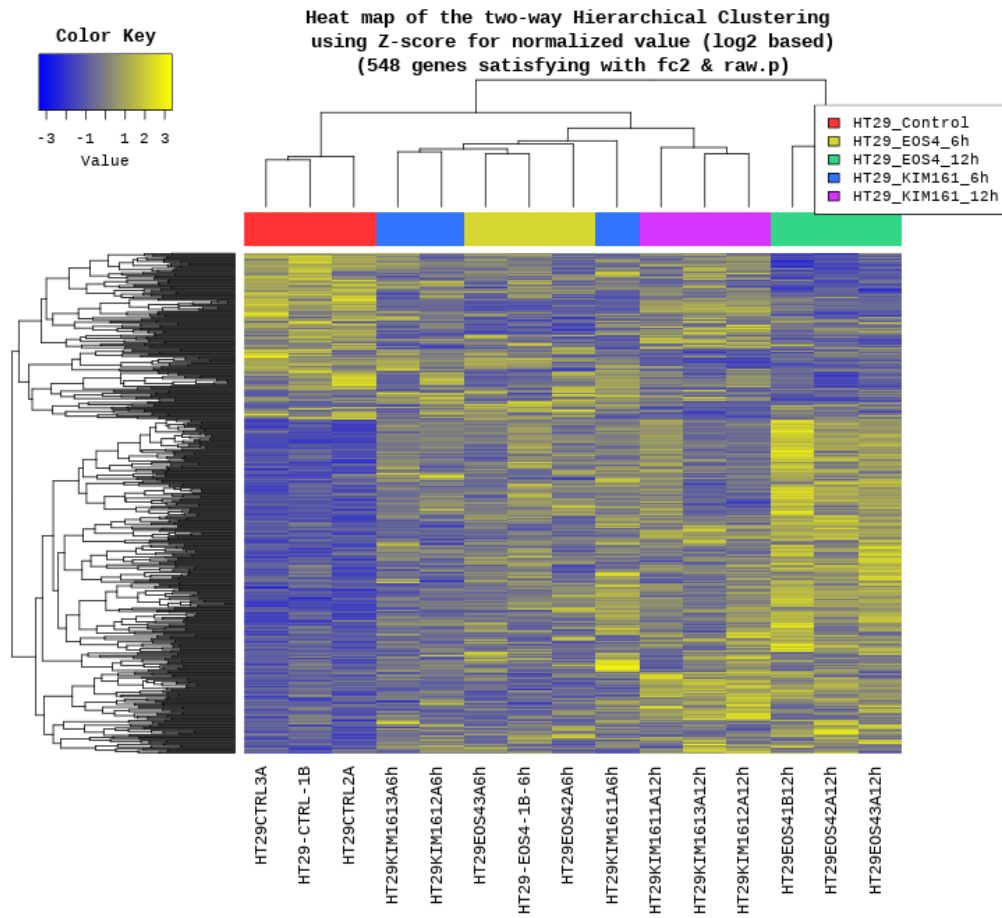


Figure 4. Heatmap for DEG list

DEG list was further analyzed with gProfiler (<https://biit.cs.ut.ee/gprofiler/orth>) 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.

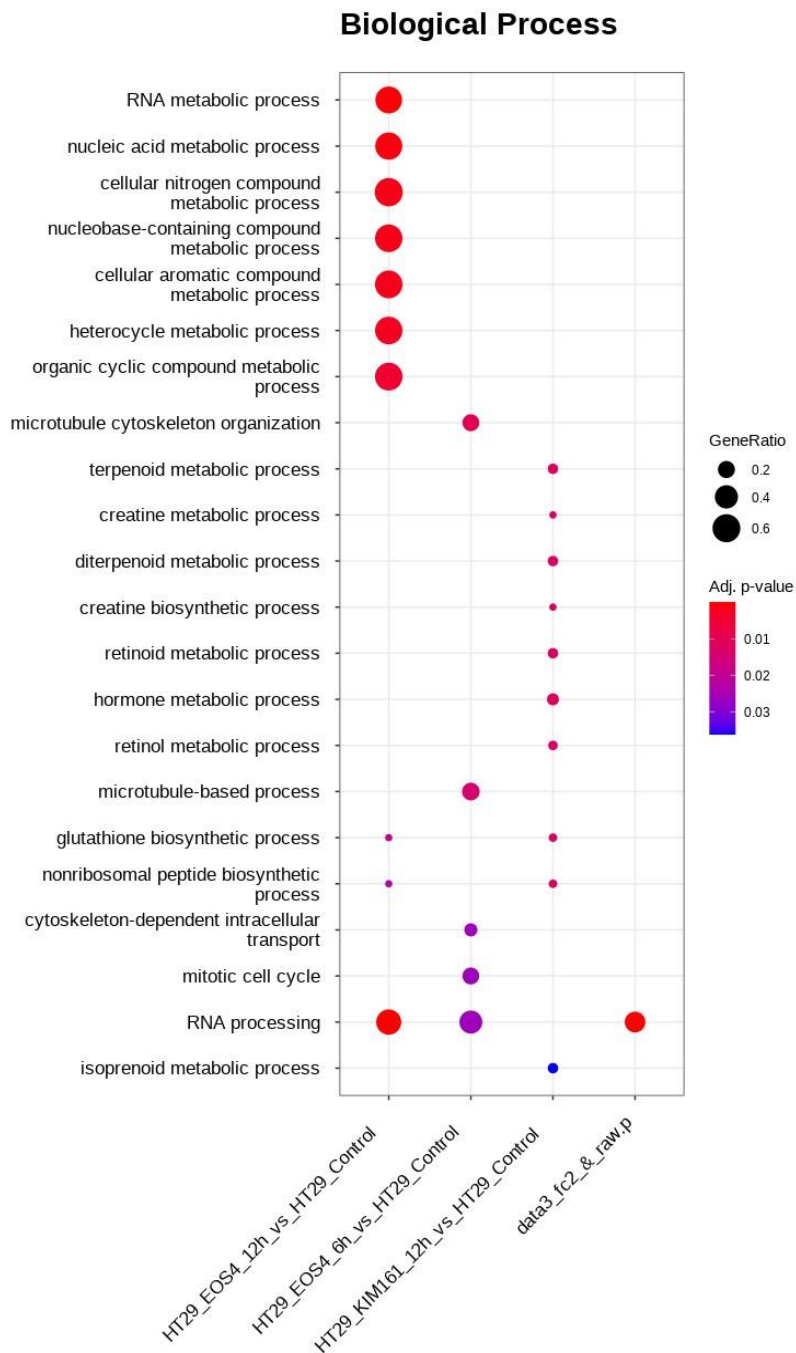


Figure 5. Gene Ontology terms related to Biological Process

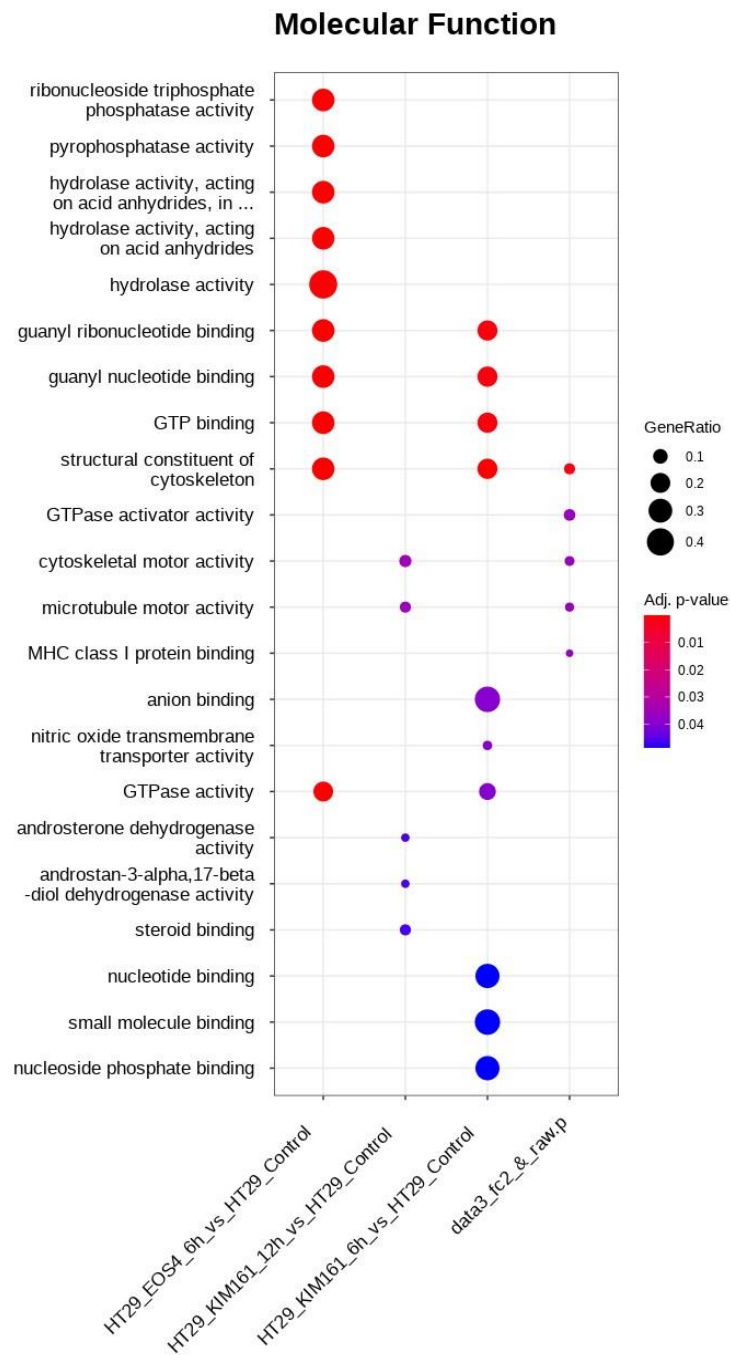


Figure 6. Gene Ontology Terms related to Molecular Function

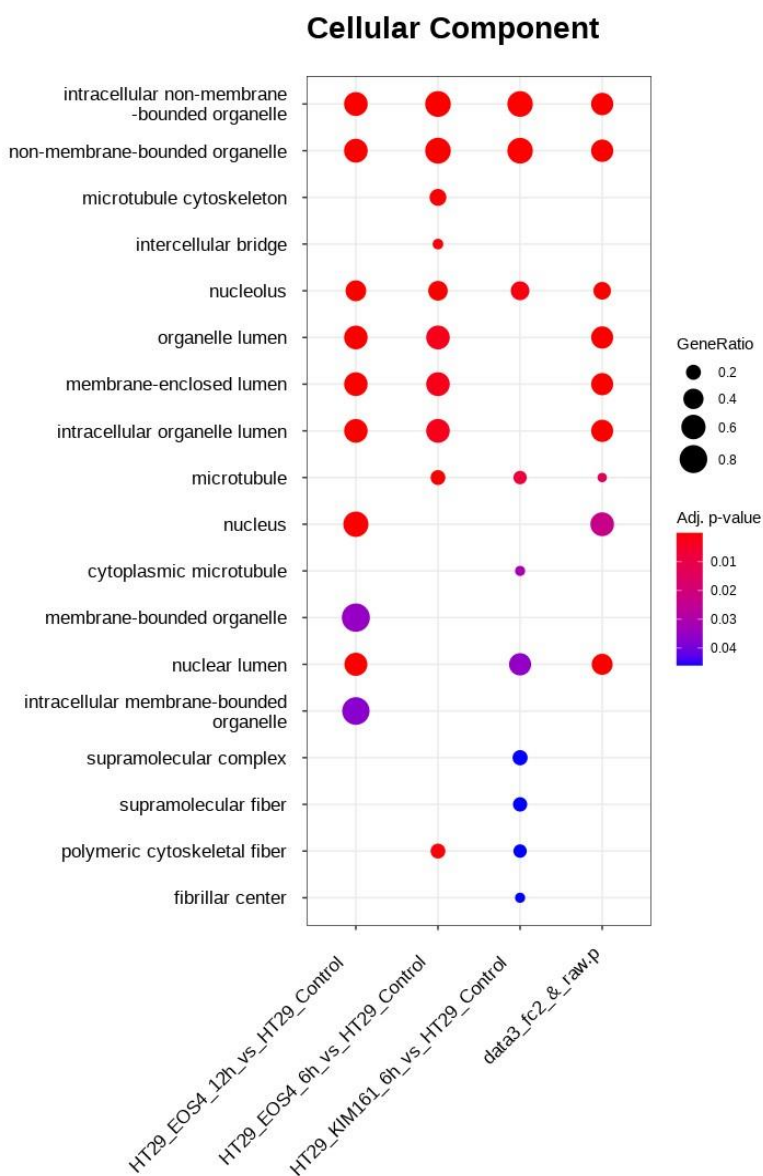


Figure 7. Gene Ontology Terms related to Cellular Component

Table of Contents

Project Information	02
Project Results Summary	03
1. Experimental Methods and Workflow	11
2. Analysis Methods and Workflow	12
3. Summary of Data Production	13
3. 1. Raw Data Statistics	13
3. 2. Average Base Quality at Each Cycle	14
3. 3. Trimming Data Statistics	15
3. 4. Average Base Quality at Each Cycle after Trimming	16
4. Reference Mapping and Assembly Results	17
4. 1. Mapping Data Statistics	17
4. 2. Expression Profiling	19
5. Differentially Expressed Gene Analysis Results	21
5. 1. Data Analysis Quality Check and Preprocessing	21
5. 2. Differentially Expressed Gene Analysis Workflow	27
5. 3. Significant Gene Results	29
5. 4. GO Enrichment Analysis	35
6. Data Download Information	42
6. 1. Raw Data	42
7. Appendix	45
7. 1. Phred Quality Score Chart	45
7. 2. Programs used in Analysis	46
7. 3. References	47

1. Experimental Methods and Workflow

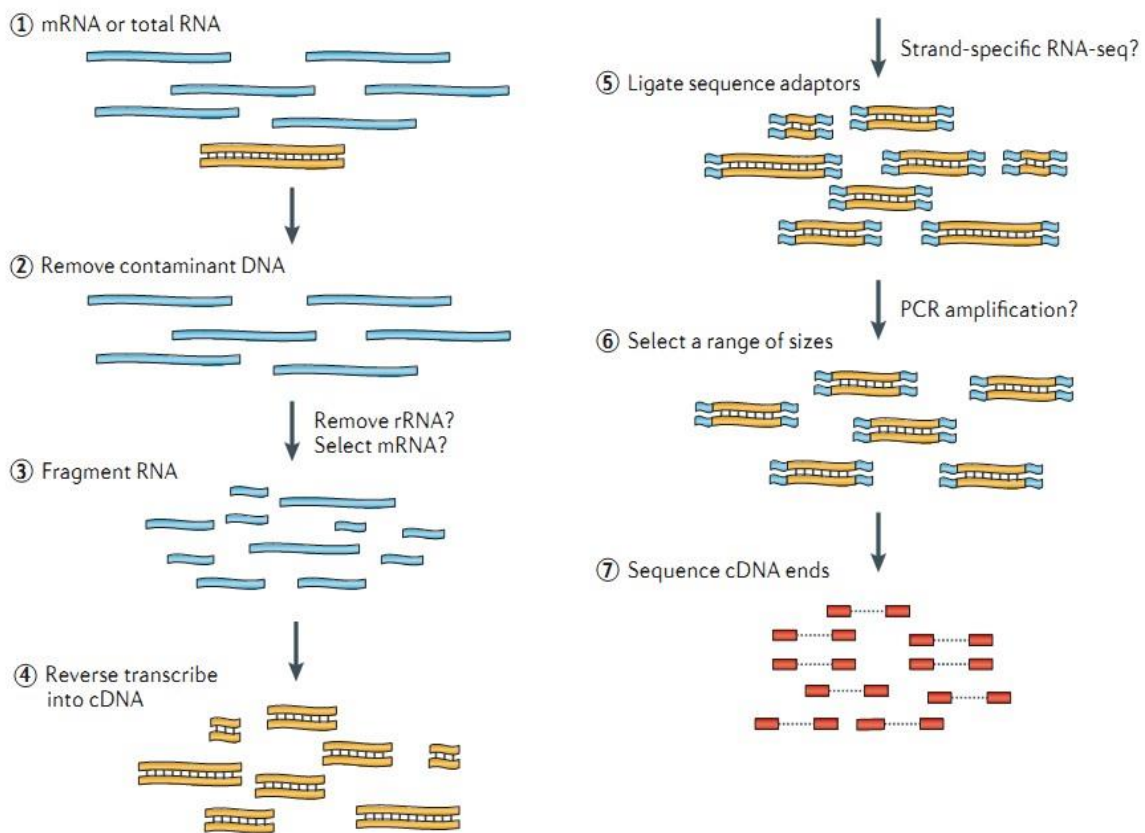


Figure 8. RNA Sequencing Experiment Workflow

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 adaptors 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 values which are calculated based on transcript length and depth of coverage. Normalization values are provided as FPKM (Fragments Per Kilobase of transcript per Million Mapped reads) / RPKM (Reads Per Kilobase of transcript per Million mapped reads) and TPM (Transcripts Per Kilobase Million).
- 6) In groups with different conditions, genes or transcripts that express differentially are filtered out through statistical hypothesis testing.

3. Summary of Data Production

3. 1. Raw Data Statistics

(Refer to Path: result_RNAseq/Analysis_statistics/raw_throughput.txt)

The total number of bases, reads, GC (%), Q20 (%), Q30 (%) are calculated for 15 samples. For example, in HT29-CTRL-1B, 55,866,450 reads are produced, and total read bases are 5.6Gbp. The GC content (%) is 48.33% and Q30 is 93.65%.

Table 1. Raw data stats

Sample id	Total read bases*	Total reads	GC (%)	Q20 (%)	Q30 (%)
HT29-CTRL-1B	5,642,511,450	55,866,450	48.33	97.66	93.65
HT29-EOS4-1B-6h	4,513,338,924	44,686,524	48.02	97.64	93.61
HT29CTRL2A	5,940,781,014	58,819,614	48.98	97.77	93.9
HT29CTRL3A	4,876,214,552	48,279,352	48.1	97.56	93.47
HT29EOS41B12h	4,848,909,606	48,009,006	48.2	97.78	93.84
HT29EOS42A12h	4,641,703,460	45,957,460	48.04	97.35	93.08
HT29EOS42A6h	5,017,224,288	49,675,488	48.71	98.0	94.29
HT29EOS43A12h	4,081,033,472	40,406,272	47.18	97.48	93.32
HT29EOS43A6h	4,691,329,406	46,448,806	47.44	97.63	93.56
HT29KIM1611A12h	5,577,591,074	55,223,674	48.42	97.82	93.97
HT29KIM1611A6h	4,937,782,536	48,888,936	48.16	97.59	93.46
HT29KIM1612A12h	5,389,584,826	53,362,226	48.39	97.81	93.9
HT29KIM1612A6h	4,758,873,762	47,117,562	48.34	97.66	93.62
HT29KIM1613A12h	4,411,208,734	43,675,334	48.41	97.72	93.79
HT29KIM1613A6h	5,054,461,170	50,044,170	47.33	97.56	93.45

(* 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>

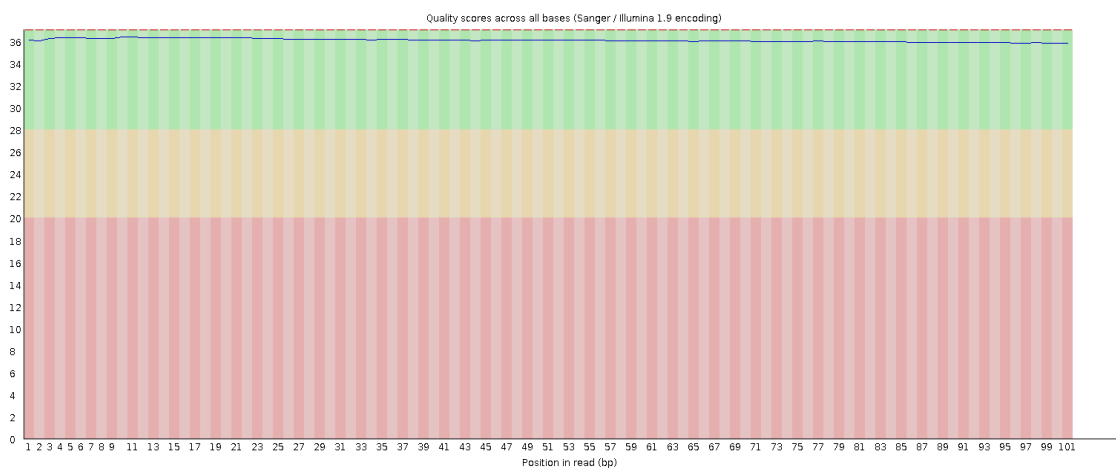


Figure 10. Read quality at each cycle of HT29-CTRL-1B (read1)

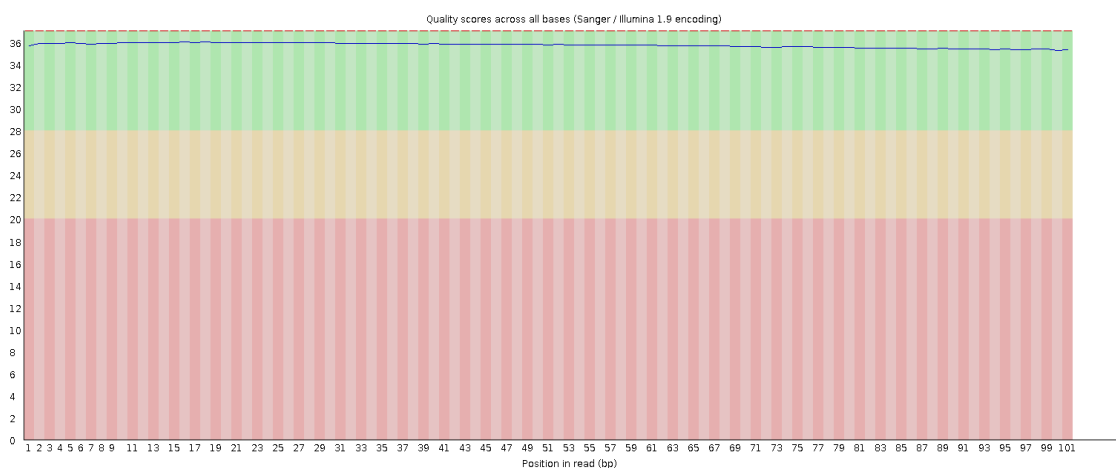


Figure 11. Read quality at each cycle of HT29-CTRL-1B (read2)

- 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/trim_throughput.txt)

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

Sample id	Total read bases	Total reads	GC(%)	Q20(%)	Q30(%)
HT29-CTRL-1B	5,477,489,013	54,606,140	48.33	98.35	94.65
HT29-EOS4-1B-6h	4,380,659,754	43,672,618	48.03	98.34	94.63
HT29CTRL2A	5,774,085,669	57,563,728	48.98	98.42	94.86
HT29CTRL3A	4,722,656,570	47,065,938	48.11	98.31	94.55
HT29EOS41B12h	4,722,140,137	47,054,082	48.2	98.39	94.73
HT29EOS42A12h	4,478,948,586	44,670,268	48.03	98.19	94.28
HT29EOS42A6h	4,900,435,767	48,832,120	48.71	98.52	95.06
HT29EOS43A12h	3,948,126,138	39,373,554	47.18	98.26	94.44
HT29EOS43A6h	4,555,175,632	45,407,576	47.44	98.31	94.55
HT29KIM1611A12h	5,432,480,723	54,131,512	48.43	98.43	94.85
HT29KIM1611A6h	4,788,804,049	47,769,896	48.15	98.29	94.48
HT29KIM1612A12h	5,249,615,481	52,315,168	48.38	98.41	94.78
HT29KIM1612A6h	4,615,028,960	46,062,354	48.35	98.33	94.61
HT29KIM1613A12h	4,286,870,669	42,724,514	48.41	98.4	94.76
HT29KIM1613A6h	4,898,353,314	48,832,032	47.34	98.29	94.5

- 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.

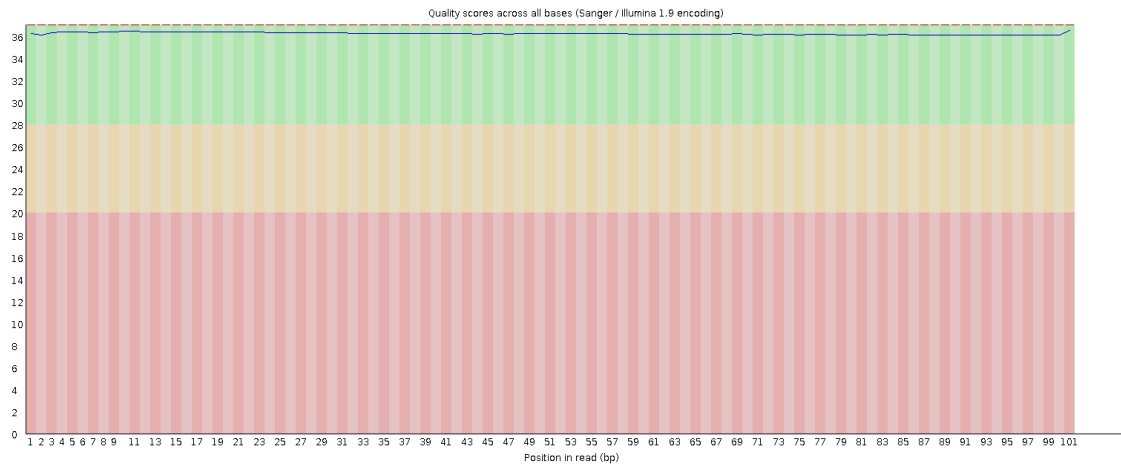


Figure 12. Average base quality of HT29-CTRL-1B (read1) at each cycle after trimming

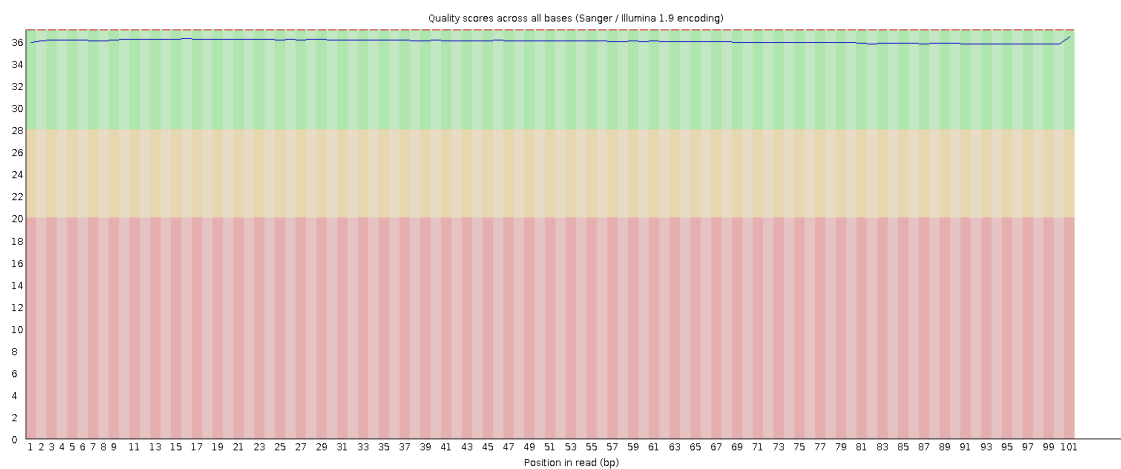


Figure 13. Average base quality of HT29-CTRL-1B (read2) at each cycle after trimming

- 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.txt)

In order to map cDNA fragments obtained from RNA sequencing, GRCh38 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 (%)
HT29-CTRL-1B	54,606,140	53,904,560 (98.72%)	701,580 (1.28%)
HT29-EOS4-1B-6h	43,672,618	43,220,628 (98.97%)	451,990 (1.03%)
HT29CTRL2A	57,563,728	57,038,464 (99.09%)	525,264 (0.91%)
HT29CTRL3A	47,065,938	46,582,710 (98.97%)	483,228 (1.03%)
HT29EOS41B12h	47,054,082	46,531,814 (98.89%)	522,268 (1.11%)
HT29EOS42A12h	44,670,268	44,101,877 (98.73%)	568,391 (1.27%)
HT29EOS42A6h	48,832,120	48,292,036 (98.89%)	540,084 (1.11%)
HT29EOS43A12h	39,373,554	38,891,297 (98.78%)	482,257 (1.22%)
HT29EOS43A6h	45,407,576	44,956,205 (99.01%)	451,371 (0.99%)
HT29KIM1611A12h	54,131,512	53,582,555 (98.99%)	548,957 (1.01%)
HT29KIM1611A6h	47,769,896	47,258,850 (98.93%)	511,046 (1.07%)
HT29KIM1612A12h	52,315,168	51,805,380 (99.03%)	509,788 (0.97%)
HT29KIM1612A6h	46,062,354	45,586,445 (98.97%)	475,909 (1.03%)

HT29KIM1613A12h	42,724,514	42,269,093 (98.93%)	455,421 (1.07%)
HT29KIM1613A6h	48,832,032	48,308,182 (98.93%)	523,850 (1.07%)

- **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 values as FPKM (Fragments Per Kilobase of transcript per Million mapped reads) and TPM (Transcripts Per Kilobase Million) for a sample.

4. 2. 1. Known Transcripts Expression Level

(Refer to Path: result_RNAseq/Expression_profile/StringTie/Expression_Profile.GRCh38.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	Transcript Length	AM		BM		AM_FPKM	BM_FPKM	AM_TPM	BM_TPM
						Read_Count	Read_Count	Read_Count	Read_Count				
NM_130786		1A1BG	alpha-1-B glycoprotein	chr19:58345183-58353492	3382	88	163	0.432396	0.678319	0.947053	1.504474		
NR_040112		3A2MP1	alpha-2-macroglobulin pseudog	chr12:9228533-9234207	1201	0	0	0	0	0	0	0	0
XM_017013947		9NAT1	N-acetyltransferase 1, transcrip	chr8:18170419-18223689	2704	0	21	0	0.108737	0	0.241173	0	0
NM_001291962		9NAT1	N-acetyltransferase 1, transcrip	chr8:18170467-18223689	2122	0	0	0	0	0	0	0	0
NM_000015		10NAT2	N-acetyltransferase 2	chr8:18391282-18401218	1285	0	0	0	0	0	0	0	0
NM_001085		12SERPINA3	serpin family A member 3	chr14:94612377-94624053	1590	8	75	0.084216	0.664787	0.184454	1.474461		
XM_005247104		13AADAC	arylacetamide deacetylase, tra	chr3:151814008-151828488	1620	0	12	0	0.102866	0	0.228152	0	0
NM_001086		13AADAC	arylacetamide deacetylase	chr3:151814116-151828488	1563	109	108	1.152579	0.971041	2.524427	2.153715		
NM_024452712		14AAMP	angio associated migratory cell	chr2:218264127-218270181	2002	106	101	0.879142	0.710738	1.925533	1.576378		
NM_001302945		14AAMP	angio associated migratory cell	chr2:218264129-218270137	1763	1621	1797	15.408498	14.424821	33.74635	31.99344		
NM_001087		14AAMP	angio associated migratory cell	chr2:218264129-218270137	1760	9332	10212	88.854179	82.119453	194.6122	182.1363		
NM_001166579		15AANAT	aralkylamine N-acetyltransferas	chr17:764653351-76470117	1913	2	8	0.010678	0.052728	0.023387	0.116948		
XM_017024259		15AANAT	aralkylamine N-acetyltransferas	chr17:76465946-76470797	4252	4	11	0.013221	0.03452	0.028958	0.076564		
NR_110548		15AANAT	aralkylamine N-acetyltransferas	chr17:76467548-76470117	1082	0	0	0	0	0	0	0	0
NM_001088		15AANAT	aralkylamine N-acetyltransferas	chr17:76467603-76470117	971	0	0	0	0	0	0	0	0
XR_933220		16AARS	alanyl-tRNA synthetase, transc	chr16:70252295-70289509	3258	90	160	0.461517	0.694592	1.010834	1.540566		
NM_001605		16AARS	alanyl-tRNA synthetase	chr16:70252394-70289509	3344	22367	68204	112.089745	288.669189	245.5037	640.2521		

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

4. 2. 2. Known Genes Expression Level

(Refer to Path: result_RNAseq/Expression_profile/StringTie/Expression_Profile.GRCh38.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	AM_TPM	BM_TPM
1	NM_130786	A1BG	alpha-1-B glycoprotein	88	163	0.432396	0.678319	0.947053	1.504474
2	NM_000014.NM_001347423	A2M	alpha-2-macroglobulin	0	0	0	0	0	0
3	NR_040112	A2MP1	alpha-2-macroglobulin pseudogene	0	0	0	0	0	0
9	NM_000662.NM_001160170	NAT1	N-acetyltransferase 1	288	217	2.411185	1.490984	5.281078	3.306918
10	NM_000015.XM_017012938	NAT2	N-acetyltransferase 2	10	6	0.097138	0.050729	0.212756	0.112513
12	NM_001085	SERPINA3	serpin family A member 3	8	75	0.084216	0.664787	0.184454	1.474461
13	NM_001086.XM_005247104	AADAC	arylacetamide deacetylase	108	120	1.152579	1.073907	2.524427	2.381867
14	NM_001087.NM_001302545	AAMP	angio associated migratory cell prot	11059	12110	105.141819	97.255012	230.2861	215.7062
15	NM_001088.NM_001166579	AANAT	aralkylamine N-acetyltransferase	6	19	0.023899	0.087248	0.052345	0.193512
16	NM_001605.XR_933220	AARS	alanyl-tRNA synthetase	22457	68364	112.551262	289.363781	246.5145	641.7927
18	NM_000663.NM_001127448	ABAT	4-aminobutyrate aminotransferase	327	175	1.143824	0.441216	2.505251	0.978593
19	NM_005502.XM_005251773	ABCA1	ATP binding cassette subfamily A n	1496	2718	2.403716	3.695532	5.264719	8.196482
20	NM_001606.NM_212533.XM	ABCA2	ATP binding cassette subfamily A n	2500	3986	5.218521	6.986245	11.42982	15.4951
21	NM_001089	ABCA3	ATP binding cassette subfamily A n	2214	4876	5.619098	10.452255	12.30719	23.18251
22	NM_001271696.NM_0012714	ABCB7	ATP binding cassette subfamily B n	2618	1974	9.550061	6.097788	20.91695	13.52455
23	NM_001025091.NM_001090	ABCF1	ATP binding cassette subfamily F n	11449	11921	56.366045	49.563715	123.4553	109.9295
24	NM_000350	ABCA4	ATP binding cassette subfamily A n	62	139	0.140036	0.267738	0.306712	0.593827

- 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 read count 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/DEG_result/[DataSet]/Analysis_Result.html)

5. 1. 1. Sample Information and Analysis Design

Total of 15 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	HT29-CTRL-1B	HT29_Control
2	HT29CTRL2A	HT29_Control
3	HT29CTRL3A	HT29_Control
4	HT29EOS42A6h	HT29_EOS4_6h
5	HT29EOS43A6h	HT29_EOS4_6h
6	HT29-EOS4-1B-6h	HT29_EOS4_6h
7	HT29EOS41B12h	HT29_EOS4_12h
8	HT29EOS42A12h	HT29_EOS4_12h
9	HT29EOS43A12h	HT29_EOS4_12h
10	HT29KIM1611A6h	HT29_KIM161_6h
11	HT29KIM1612A6h	HT29_KIM161_6h
12	HT29KIM1613A6h	HT29_KIM161_6h
13	HT29KIM1611A12h	HT29_KIM161_12h
14	HT29KIM1612A12h	HT29_KIM161_12h
15	HT29KIM1613A12h	HT29_KIM161_12h

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

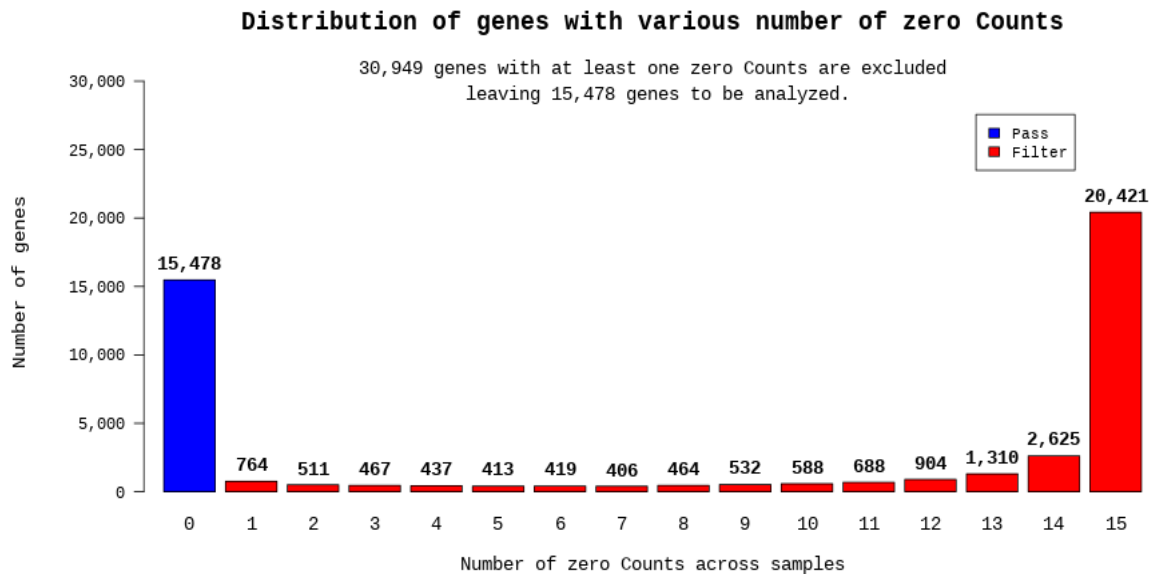
Index	Test vs. Control	Statistical Method
1	HT29_EOS4_6h vs. HT29_Control	Fold Change, nbinomWaldTest using DESeq2, Hierarchical Clustering

2	HT29_EOS4_12h vs. HT29_Control	Fold Change, nbinomWaldTest using DESeq2, Hierarchical Clustering
3	HT29_KIM161_6h vs. HT29_Control	Fold Change, nbinomWaldTest using DESeq2, Hierarchical Clustering
4	HT29_KIM161_12h vs. HT29_Control	Fold Change, nbinomWaldTest using DESeq2, Hierarchical Clustering

5. 1. 2. DATA Quality Check

(Refer to Path: result_RNAseq/DEG_result/[DataSet]/Data Quality Check/)

For 15 samples, if more than one read count value was 0, it was not included in the analysis. Therefore, from total of 46,427 genes, 30,949 were excluded and only 15,478 genes were used for statistic analysis.



5. 1. 3. Data Transformation and Normalization

In order to reduce systematic bias, size factors were estimated from the read count data (estimateSizeFactors method).

Using them, the read count data was normalized with Relative Log Expression (RLE) method in DESeq2 R library.

Then, statistical test was performed with the normalized data.

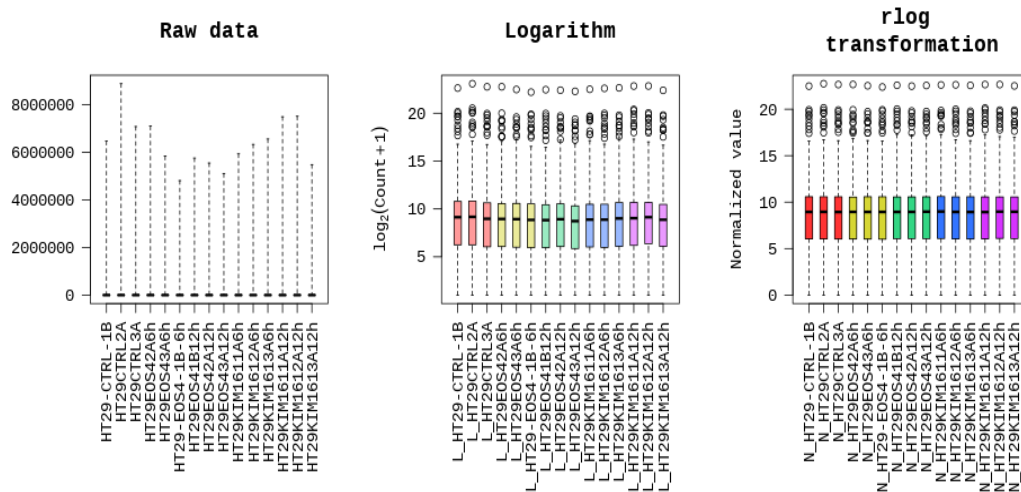
$\log_2(\text{read count}+1)$ and regularized log (rlog) transformed values were used for data visualization. rlog transformation is a method to minimize differences between samples for genes/transcripts in low expression. It transforms count data into \log_2 scale and normalizes them with a library size factor. rlog is robust in the case when the size factors vary widely.

These logarithm figures were used only for visualization.

To proceed a statistical test, RLE normalized count was adopted for negative binomial Wald Test(nbinomWaldTest) in DESeq2.

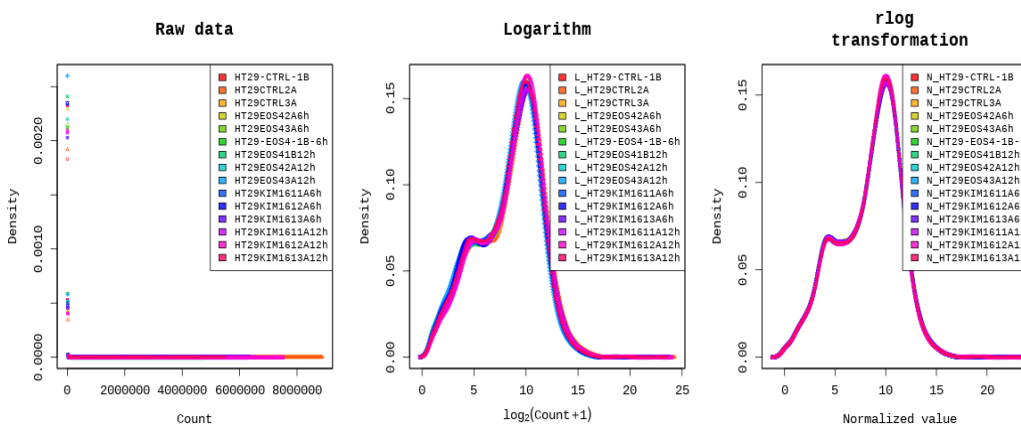
5. 1. 3. 1. Boxplot of Expression Difference between samples.

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



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

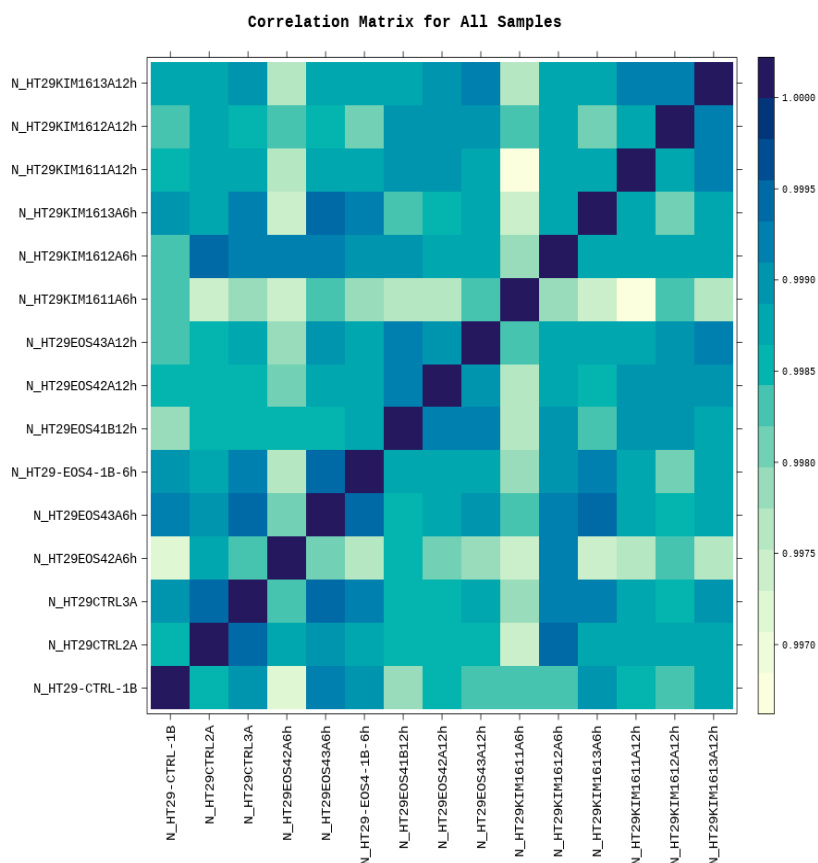
Below density plots show the corresponding samples expression distribution before and after of raw signal (read count), Log₂ transformation of read count+1 and RLE Normalization.



5. 1. 4. Correlation Analysis between samples

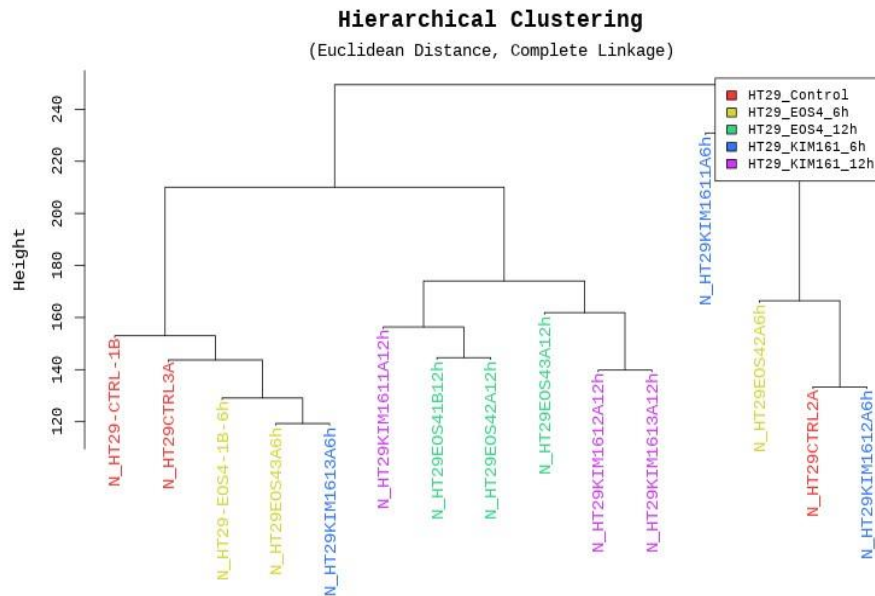
The similarity between samples are obtained through Pearson's coefficient of the rlog transformed value. For range: $-1 \leq r \leq 1$, the closer the value is to 1, the more similar the samples are.

Correlation matrix of all samples is as follows.



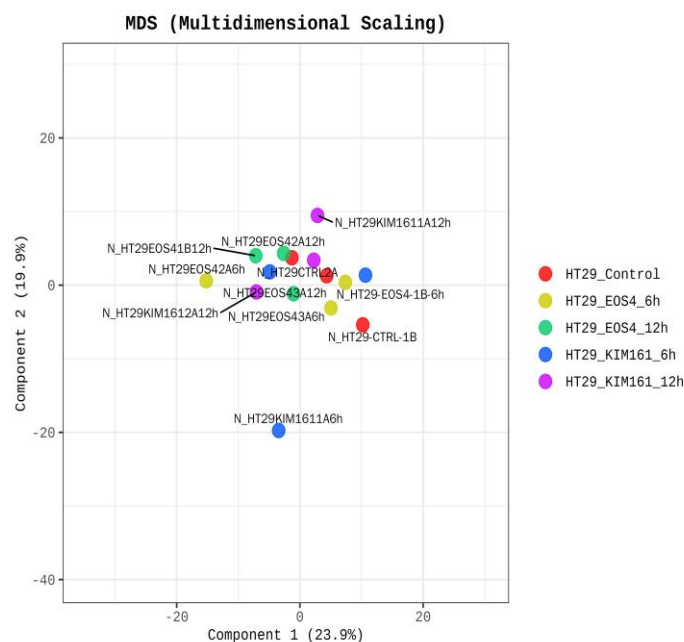
5. 1. 5. Hierarchical Clustering Analysis

Using each sample's rlog transformed value, the high expression similarities were grouped together. (Distance metric = Euclidean distance, Linkage method= Complete Linkage)



5. 1. 6. Multidimensional Scaling Analysis

Using each sample's rlog transformed value, the similarity between samples is graphically shown in a 2D plot. It employs two components that well preserve the degree of similarity between samples. This allows identification any outlier samples, or similar expression patterns between sample groups.



5. 2. Differentially Expressed Gene Analysis Workflow

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

- 1) the read count 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/Expression_profile/StringTie/
Expression_Profile.GRCh38.gene.xlsx)

: 46,427 genes, 15 samples

- 2) During data preprocessing, low quality transcripts are filtered. Afterwards, RLE Normalization are performed.

- Processed data

(Refer to Path: result_RNAseq/DEG_result/[DataSet]/data2.xlsx)

: 15,478 genes, 15 samples

- 3) Statistical analysis is performed using Fold Change, nbinomWaldTest using DESeq2 per comparison pair.

The significant results are selected on conditions of $|fc| \geq 2$ & nbinomWaldTest raw p-value < 0.05 .
(data3_*.xlsx contains significant genes which satisfied $|fc| \geq 2$ & nbinomWaldTest raw p-value < 0.05 conditions in more than at least one of total comparison pairs.)

(Refer to Path: result_RNAseq/DEG_result/[DataSet]/)

- Significant data (data3_fc2_&_raw.p.xlsx)

: 548 genes

- Significant data (data3_HT29_EOS4_6h_vs_HT29_Control_fc2_&_raw.p.xlsx)

: 128 genes

- Significant data (data3_HT29_EOS4_12h_vs_HT29_Control_fc2_&_raw.p.xlsx)

: 376 genes

- Significant data (data3_HT29_KIM161_6h_vs_HT29_Control_fc2_&_raw.p.xlsx)

: 110 genes

- Significant data (data3_HT29_KIM161_12h_vs_HT29_Control_fc2_&_raw.p.xlsx)

: 173 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 dendrogram.

- Hierarchical Clustering (Euclidean Distance, Complete Linkage)

(Refer to Path: result_RNAseq/DEG_result/[DataSet]/Cluster image/)

- 5) For significant lists, gene-set enrichment analysis was performed based on gene ontology(
<https://biit.cs.ut.ee/gprofiler/>).

Please refer to the GO_stat sheet and the GO_genes sheet of data3 file.

Following result are provided.

- GO_stat
- GO_genes

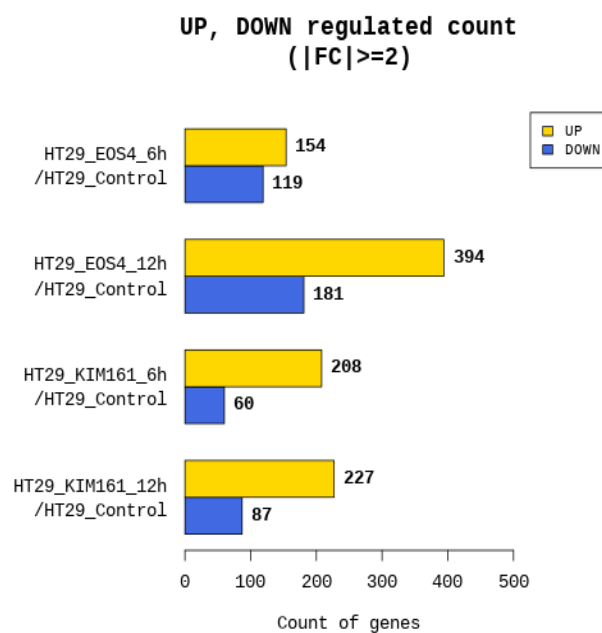
5. 3. Significant Gene Results

(Refer to Path: result_RNAseq/DEG_result/[DataSet]/Plots/)

These are DEG result of HT29_EOS4_6h_vs_HT29_Control meeting fc2_&_raw.p by example.

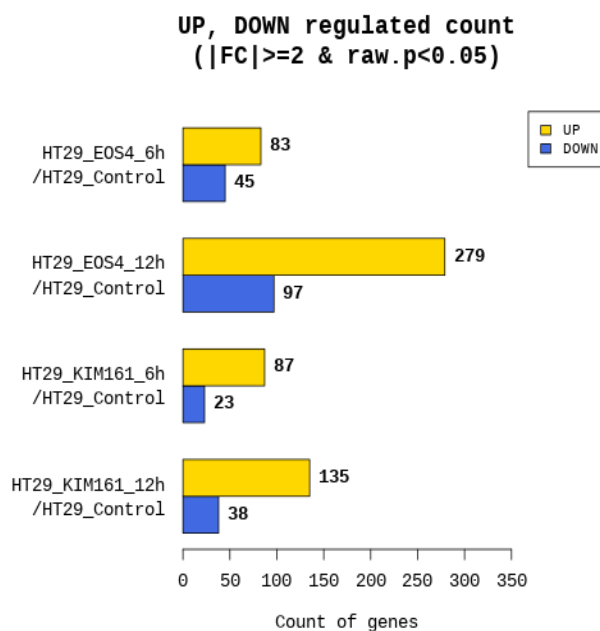
5. 3. 1. Up, Down Regulated Count by Fold Change

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



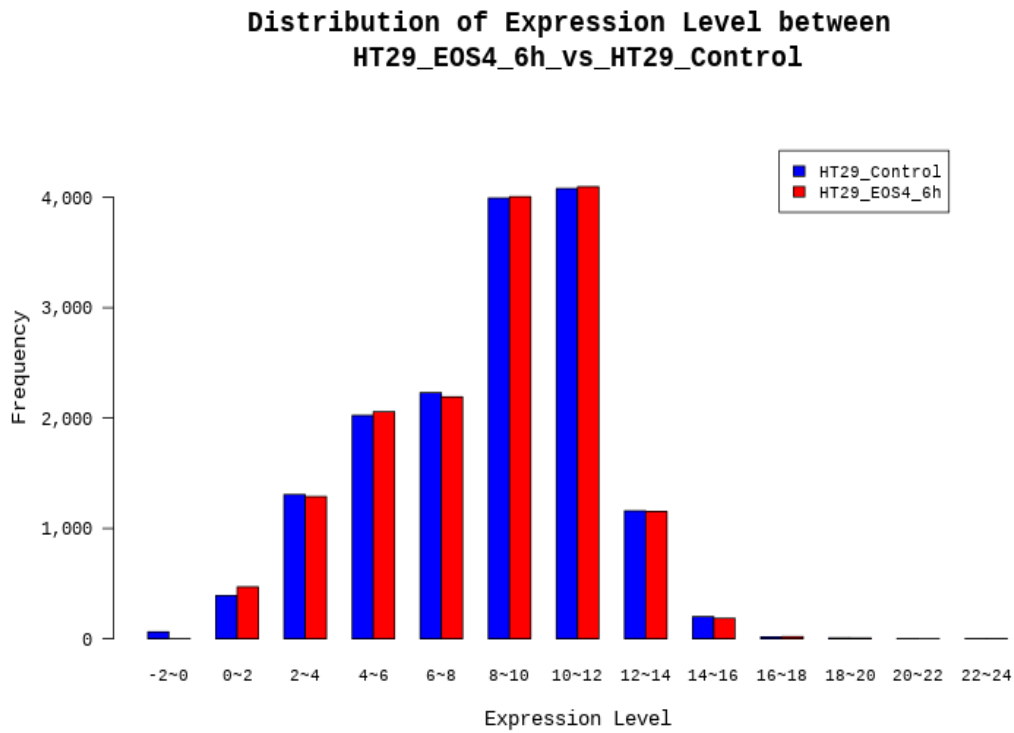
5. 3. 2. Up, Down Regulated Count by Fold Change and p-value

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



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

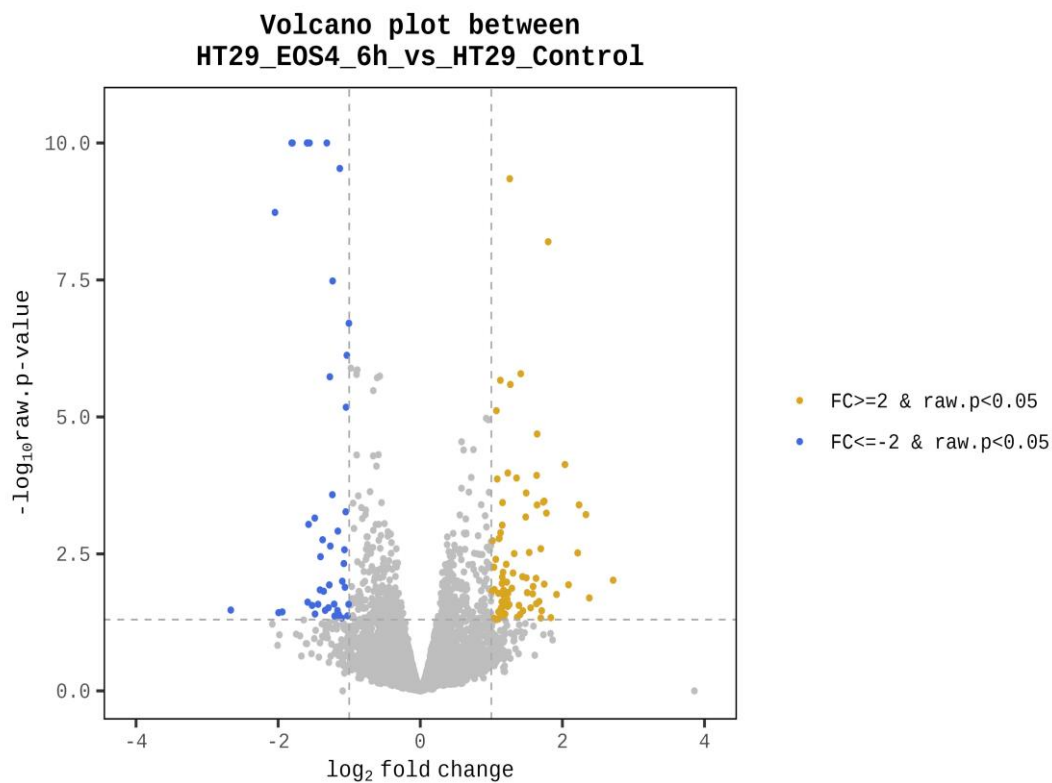
Shows distribution of normalized value of each group for comparison pair.



5. 3. 4. Volcano Plot of Expression Level of two groups.

Log2 fold change and p-value obtained from the comparison between two groups plotted as volcano plot.

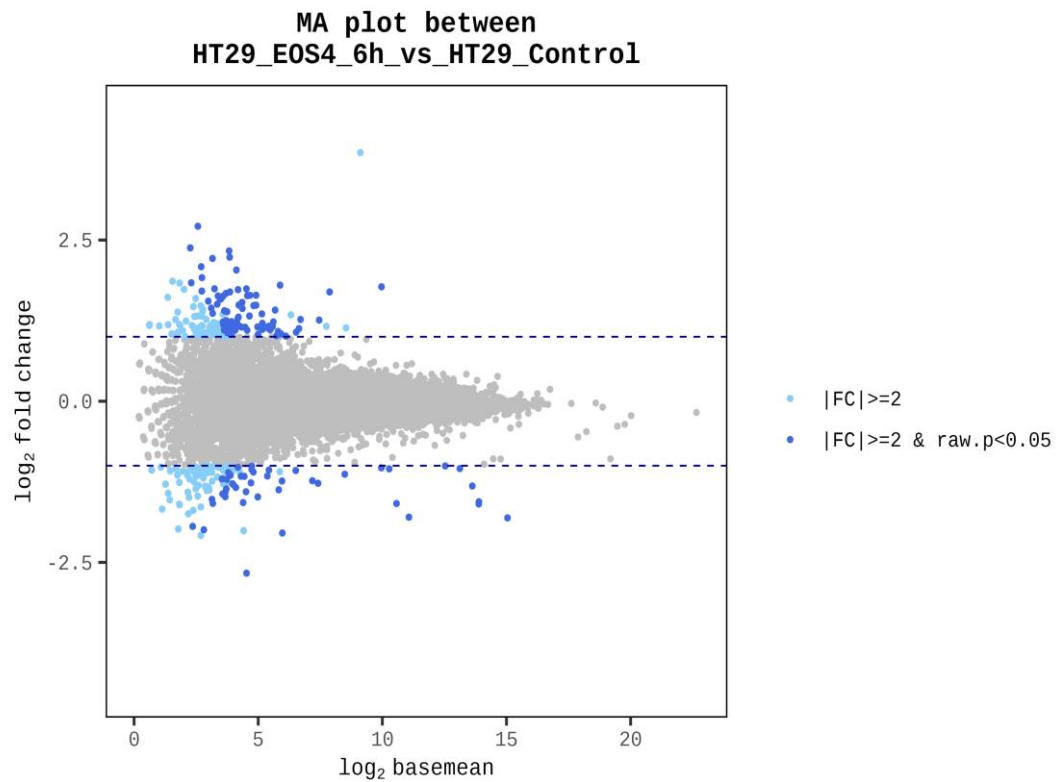
(X-axis: log₂ Fold Change, Y-axis: -log₁₀ p-value)



5. 3. 5. MA Plot

In order to confirm the transcripts that show higher expression difference compared to the control according to overall average expression level, MA plot is drawn. (X-axis: mean of normalized counts, Y-axis: log₂ Fold Change).

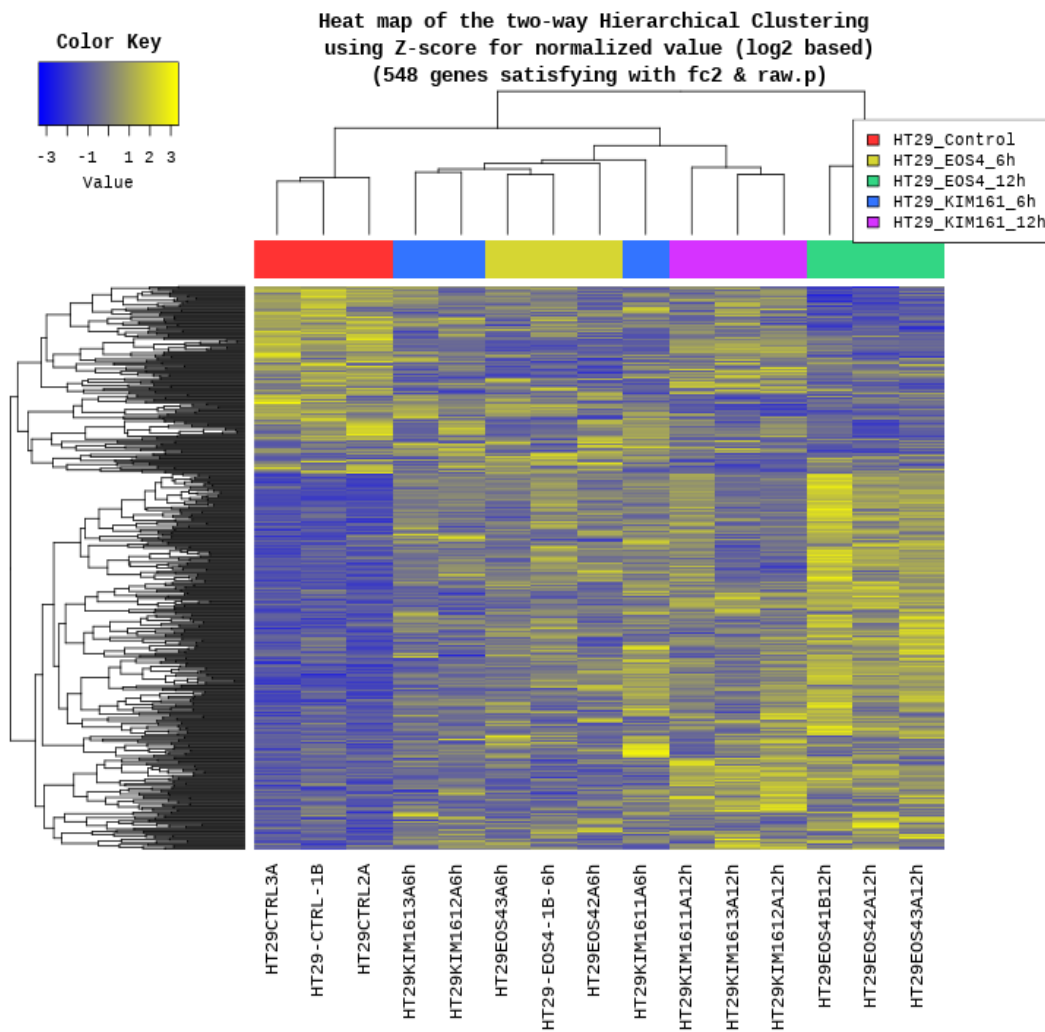
For example, even though fold change might be different by two-fold, the gene with higher mean of normalized counts may be more credible.



5. 3. 6. Hierarchical Clustering Analysis

(Refer to Path: result_RNAseq/DEG_result/[DataSet]/Cluster image/)

Heatmap shows result of hierarchical clustering analysis (Euclidean Method, Complete Linkage) which clusters the similarity of genes and samples by expression level (rlog transformed value) from significant list more than at least one of total comparison pairs.



5. 4. GO Enrichment Analysis

(Refer to Path: result_RNAseq/DEG_result/[DataSet]/gprofiler)

For Enrichment test which based on Gene Ontology (<http://geneontology.org/>) DB was conducted with significant gene list using g:Profiler tool (<https://biit.cs.ut.ee/gprofiler/>).

The g:Profiler tool performs statistical enrichment analysis to find over-representation of information from Gene Ontology terms, biological pathways, regulatory DNA elements, human disease gene annotations, and protein-protein interaction networks.

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>

Enrichment test result was summarized at each sheet of DEG result(data3-*.xlsx file) by 2 forms below.

- GO_stat
- GO_genes

5. 4. 1. GO_stat Sheet

The result of associated gene and test stat was summarized by term_id. The significance of specific term_id in enrichment test with DEG set was summarized.

source	term_id	term_name	adjusted_p_value	term_size	query_size	intersection_size	effective_domain_size	intersections
GO:CC	GO:0022626	cytosolic ribosome	2.72198E-17	115	1921	50	18797	6134, 6206, 6155, 6204, 6168, 200916,
GO:BP	GO:0006614	SRP-dependent cotranslational protein targeting to membrane	3.60328E-15	96	1824	44	17816	6134, 6206, 6155, 6204, 6168, 6747, 61
GO:MF	GO:0003735	structural constituent of ribosome	1.32911E-14	170	1860	59	18098	6134, 6206, 6155, 6204, 6168, 200916,
GO:BP	GO:0006613	cotranslational protein targeting to membrane	2.03613E-14	101	1824	44	17816	6134, 6206, 6155, 6204, 6168, 6747, 61
GO:MF	GO:0005198	structural molecule activity	4.45523E-14	739	1860	151	18098	6134, 6206, 127294, 4586, 301, 3887, 6
GO:BP	GO:0045047	protein targeting to ER	7.18306E-14	109	1824	45	17816	6134, 6206, 6155, 6204, 6168, 6747, 61
GO:CC	GO:0044391	ribosomal subunit	2.36014E-13	195	1921	61	18797	6134, 6206, 6155, 6204, 6168, 200916,
GO:BP	GO:0072599	establishment of protein localization to endoplasmic reticulum	2.82077E-13	113	1824	45	17816	6134, 6206, 6155, 6204, 6168, 6747, 61
GO:BP	GO:0070972	protein localization to endoplasmic reticulum	4.06119E-11	137	1824	47	17816	6134, 6206, 6155, 6204, 6168, 6747, 61
GO:CC	GO:0005840	ribosome	1.34069E-10	246	1921	65	18797	6134, 6206, 6155, 6204, 6168, 200916,
GO:CC	GO:0022625	cytosolic large ribosomal subunit	1.69728E-10	64	1921	29	18797	6134, 6155, 6168, 200916, 6167, 6161,
GO:BP	GO:000184	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	7.11348E-10	122	1824	42	17816	6134, 6206, 6155, 6204, 6168, 6167, 61
GO:CC	GO:0044459	plasma membrane part	1.34094E-09	2879	1921	400	18797	165829, 10326, 6405, 4283, 8322, 5743,
GO:CC	GO:0071944	cell periphery	1.8891E-09	5662	1921	709	18797	829, 165829, 10326, 23256, 6405, 4283,
GO:CC	GO:0005886	plasma membrane	5.37824E-09	5539	1921	692	18797	165829, 10326, 23256, 6405, 4283, 505
GO:CC	GO:0044444	cytoplasmic part	5.37824E-09	9685	1921	1125	18797	6134, 829, 84532, 10326, 5332, 23256,
GO:CC	GO:0005737	cytoplasm	5.47219E-09	11534	1921	1309	18797	6134, 829, 84532, 10326, 5332, 23256,
GO:BP	GO:0009888	tissue development	5.79564E-09	2068	1824	305	17816	6405, 5054, 8322, 5743, 144165, 12729
GO:BP	GO:0006612	protein targeting to membrane	5.95069E-09	195	1824	54	17816	6134, 6206, 6155, 6204, 6168, 6747, 51
GO:BP	GO:0051179	localization	1.23607E-08	6751	1824	824	17816	6134, 829, 10326, 10734, 23256, 6405,
GO:CC	GO:1903561	extracellular vesicle	1.65132E-08	2165	1921	309	18797	829, 5054, 10103, 2098, 9518, 4151, 41
GO:CC	GO:0043230	extracellular organelle	1.66899E-08	2167	1921	309	18797	829, 5054, 10103, 2098, 9518, 4151, 41
GO:CC	GO:0044445	cytosolic part	2.71585E-08	252	1921	60	18797	6134, 6206, 6155, 6204, 6168, 338321,
GO:BP	GO:0032501	multicellular organismal process	3.22915E-08	7718	1824	922	17816	6134, 829, 6405, 5670, 5054, 7079, 832

- source: Code for the data source. Ex> GO:BP | GO:CC | GO:MF ...
- term_id: ID for the enriched term/functional category
- term_name: Readable name for the enriched term
- adjusted_p_value: Adjusted p-value by FDR
- query_size: The number of unique DEG that are annotated to the data source (the functional category).
- intersection_size: The number of unique DEG that are annotated to the term_id
- term_size: The number of genes of species that are annotated to the term_id.
- effective_domain_size: The number of genes of species that are annotated to the data source (the functional category).
- intersections: list of unique DEG that are annotated to the term_id

5. 4. 2. GO_genes Sheet

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

source	term_id	term_name	adjusted_p_value	intersection_size	Gene_ID	Transcript_ID	Gene_Symbol	test/control.fc	test/control.logCPM	test/control.raw.pval	test/control.bh.pval	N_control_1	N_control_2	N_test_1	N_test_2
GO:CC	GO:0044444	cytoplasmic part	3.37824E-09	1125	9	NM_000662.NN	NAT1	2.593577	0.965259	1.66575E-06	1.40133E-05	1.167645	0.902212	1.879864	1.926688
GO:CC	GO:0005737	cytoplasm	5.47219E-09	1309	9	NM_000662.NN	NAT1	2.593577	0.965259	1.66575E-06	1.40133E-05	1.167645	0.902212	1.879864	1.926688
GO:BP	GO:0070887	cellular response to ch	6.97255E-05	417	9	NM_000662.NN	NAT1	2.593577	0.965259	1.66575E-06	1.40133E-05	1.167645	0.902212	1.879864	1.926688
GO:BP	GO:0050896	response to stimulus	0.000405905	1045	9	NM_000662.NN	NAT1	2.593577	0.965259	1.66575E-06	1.40133E-05	1.167645	0.902212	1.879864	1.926688
GO:CC	GO:0005829	cytosol	0.078450245	563	9	NM_000662.NN	NAT1	2.593577	0.965259	1.66575E-06	1.40133E-05	1.167645	0.902212	1.879864	1.926688
GO:CC	GO:0005622	intracellular	0.110987379	1522	9	NM_000662.NN	NAT1	2.593577	0.965259	1.66575E-06	1.40133E-05	1.167645	0.902212	1.879864	1.926688
GO:MF	GO:0004060	arylamine N-acetyltras	0.573292063	1	9	NM_000662.NN	NAT1	2.593577	0.965259	1.66575E-06	1.40133E-05	1.167645	0.902212	1.879864	1.926688
GO:CC	GO:0005575	cellular_component	1	1921	9	NM_000662.NN	NAT1	2.593577	0.965259	1.66575E-06	1.40133E-05	1.167645	0.902212	1.879864	1.926688
GO:BP	GO:0008150	biological_process	1	1824	9	NM_000662.NN	NAT1	2.593577	0.965259	1.66575E-06	1.40133E-05	1.167645	0.902212	1.879864	1.926688
GO:CC	GO:0044459	plasma membrane pa	1.34094E-09	400	24	NM_000350	ABCA4	-8.936138	3.797432	1.4729E-62	6.89976E-60	4.626902	4.764929	1.879864	1.961991
GO:CC	GO:0071944	cell periphery	1.8891E-09	709	24	NM_000350	ABCA4	-8.936138	3.797432	1.4729E-62	6.89976E-60	4.626902	4.764929	1.879864	1.961991
GO:CC	GO:0016020	membrane	0.000133938	1085	24	NM_000350	ABCA4	-8.936138	3.797432	1.4729E-62	6.89976E-60	4.626902	4.764929	1.879864	1.961991
GO:CC	GO:0097458	neuron part	0.000244106	234	24	NM_000350	ABCA4	-8.936138	3.797432	1.4729E-62	6.89976E-60	4.626902	4.764929	1.879864	1.961991
GO:CC	GO:0042995	cell projection	0.000353388	283	24	NM_000350	ABCA4	-8.936138	3.797432	1.4729E-62	6.89976E-60	4.626902	4.764929	1.879864	1.961991
GO:CC	GO:0044425	membrane part	0.000390902	813	24	NM_000350	ABCA4	-8.936138	3.797432	1.4729E-62	6.89976E-60	4.626902	4.764929	1.879864	1.961991
GO:BP	GO:0050896	response to stimulus	0.000405905	1045	24	NM_000350	ABCA4	-8.936138	3.797432	1.4729E-62	6.89976E-60	4.626902	4.764929	1.879864	1.961991
GO:BP	GO:0051606	detection of stimulus	1	35	24	NM_000350	ABCA4	-8.936138	3.797432	1.4729E-62	6.89976E-60	4.626902	4.764929	1.879864	1.961991
GO:BP	GO:0008150	biological_process	1	1824	24	NM_000350	ABCA4	-8.936138	3.797432	1.4729E-62	6.89976E-60	4.626902	4.764929	1.879864	1.961991
GO:CC	GO:0044444	cytoplasmic part	5.37824E-09	1125	34	NM_000016.NN	ACADM	2.326451	4.229202	9.93422E-14	2.78049E-12	3.715210	3.505224	4.754088	4.772040
GO:CC	GO:0005737	cytoplasm	5.47219E-09	1309	34	NM_000016.NN	ACADM	2.326451	4.229202	9.93422E-14	2.78049E-12	3.715210	3.505224	4.754088	4.772040
GO:BP	GO:0008888	tissue development	5.79564E-09	305	34	NM_000016.NN	ACADM	2.326451	4.229202	9.93422E-14	2.78049E-12	3.715210	3.505224	4.754088	4.772040
GO:BP	GO:0032501	multicellular organism	3.22915E-08	922	34	NM_000016.NN	ACADM	2.326451	4.229202	9.93422E-14	2.78049E-12	3.715210	3.505224	4.754088	4.772040
GO:BP	GO:0048731	system development	3.55854E-08	626	34	NM_000016.NN	ACADM	2.326451	4.229202	9.93422E-14	2.78049E-12	3.715210	3.505224	4.754088	4.772040
GO:BP	GO:0048513	animal organ develop	3.78565E-08	478	34	NM_000016.NN	ACADM	2.326451	4.229202	9.93422E-14	2.78049E-12	3.715210	3.505224	4.754088	4.772040

- source: Code for the data source. Ex> GO:BP | GO:CC | GO:MF ...
- term_id: ID for the enriched term/functional category
- term_name: Readable name for the enriched term
- adjusted_p_value: Adjusted p-value by FDR
- intersection_size: The number of unique DEG that are annotated to the term_id

data3.GO_*.gprofiler.png: Top 20 terms of Gene Ontology Enrichment Analysis result were described by dot plot.

(Plotting based on GO_stat)

data3.GO_*.gprofiler.sizefilt.png: After term_size filtering (min=10, max=500), top 20 terms of Gene Ontology Enrichment Analysis result were described by dot plot.

(Plotting based on GO_stat. Please refer to ./gprofiler/data3*.GO/folder.)

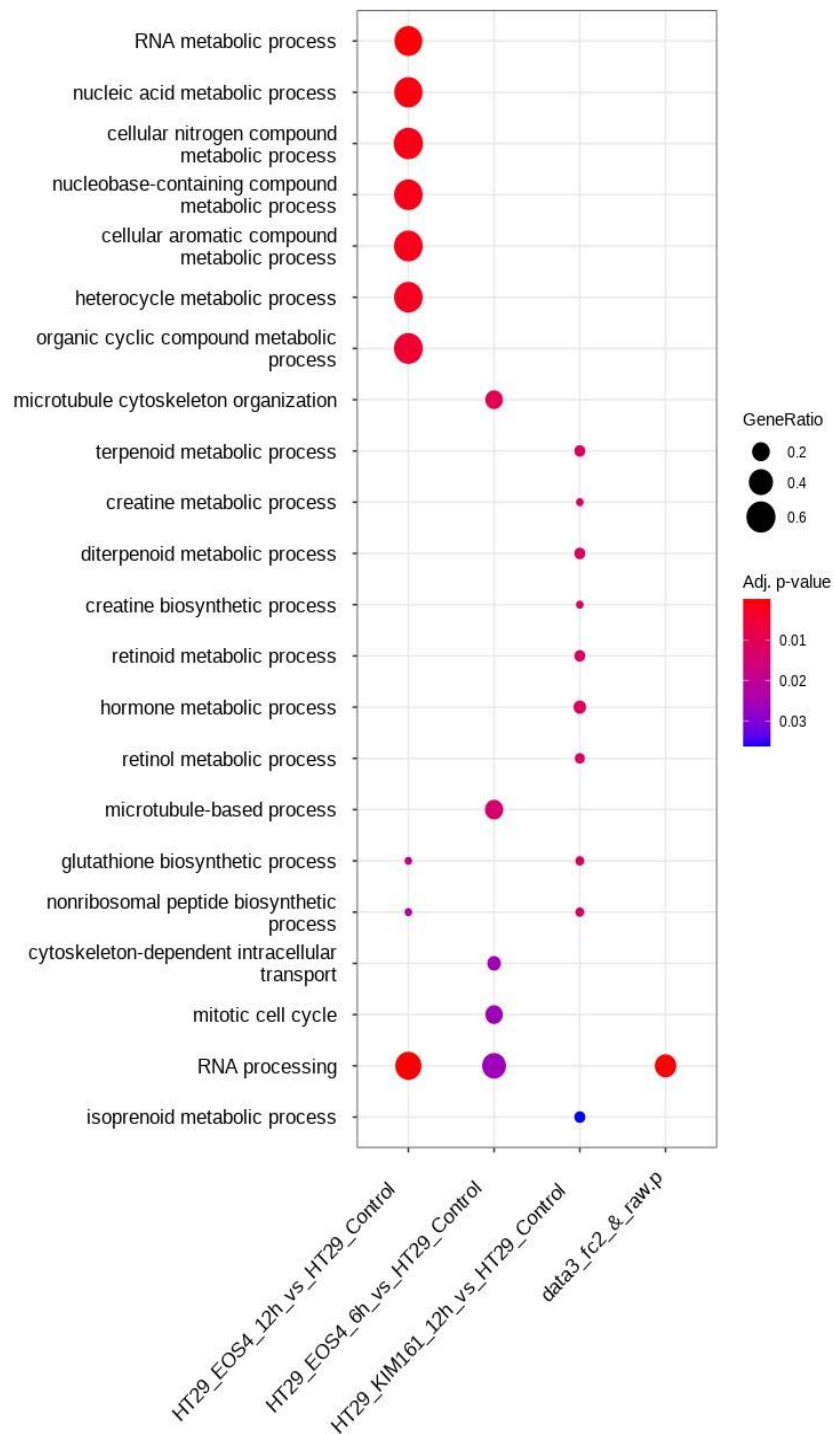
- term_size filtering: The GO Terms that are very large or small do not contribute to interpretability of results, and their statistical significance can be inflated when using certain statistical enrichment methods (e.g., Hypergeometric test).

- GeneRatio: GeneRatio is calculated as the ratio of intersection_size and query_size.

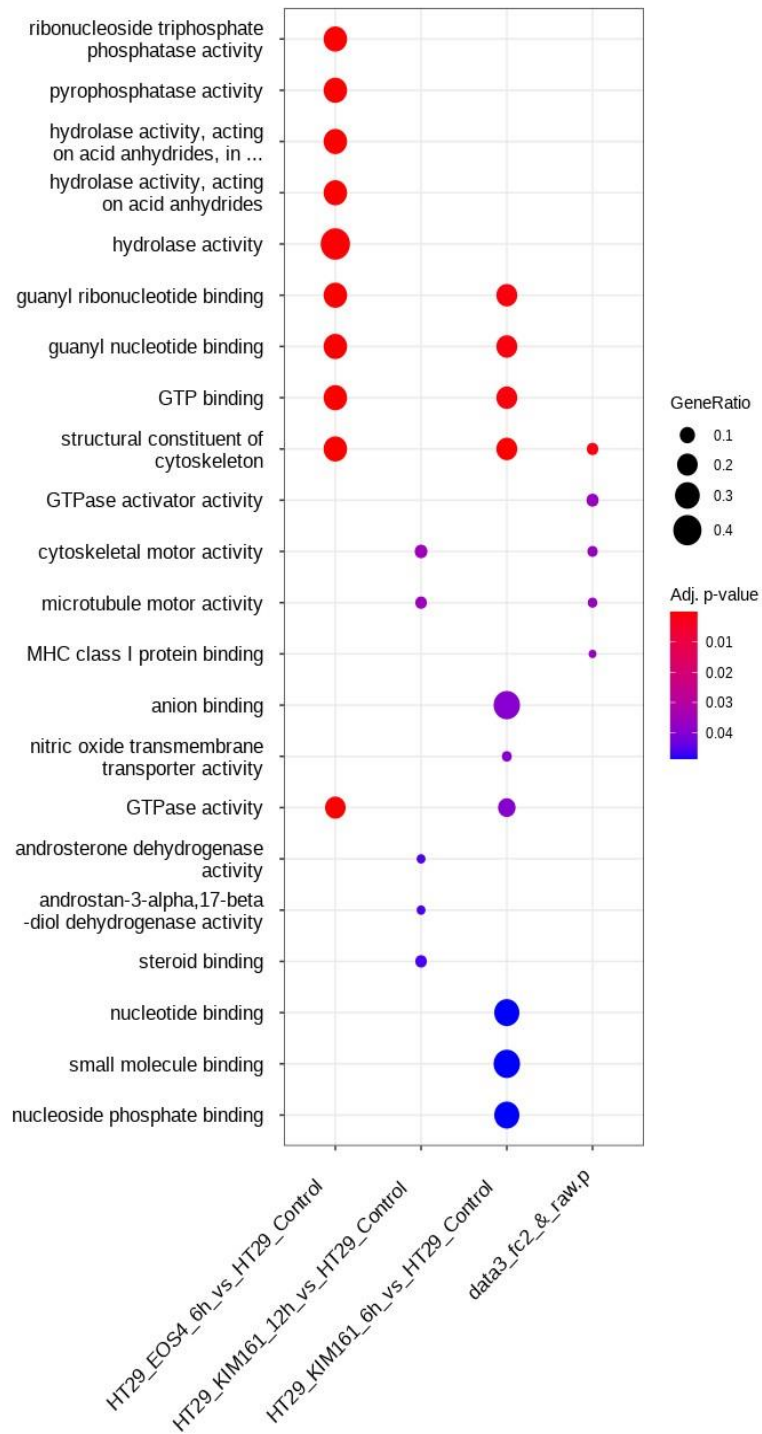
The dot plot below shows the results of the enrichment analysis based on Gene Ontology DB for significant genes.

These dot plots are examples for data3.GO_*.gprofiler.png (without term_size filtering).

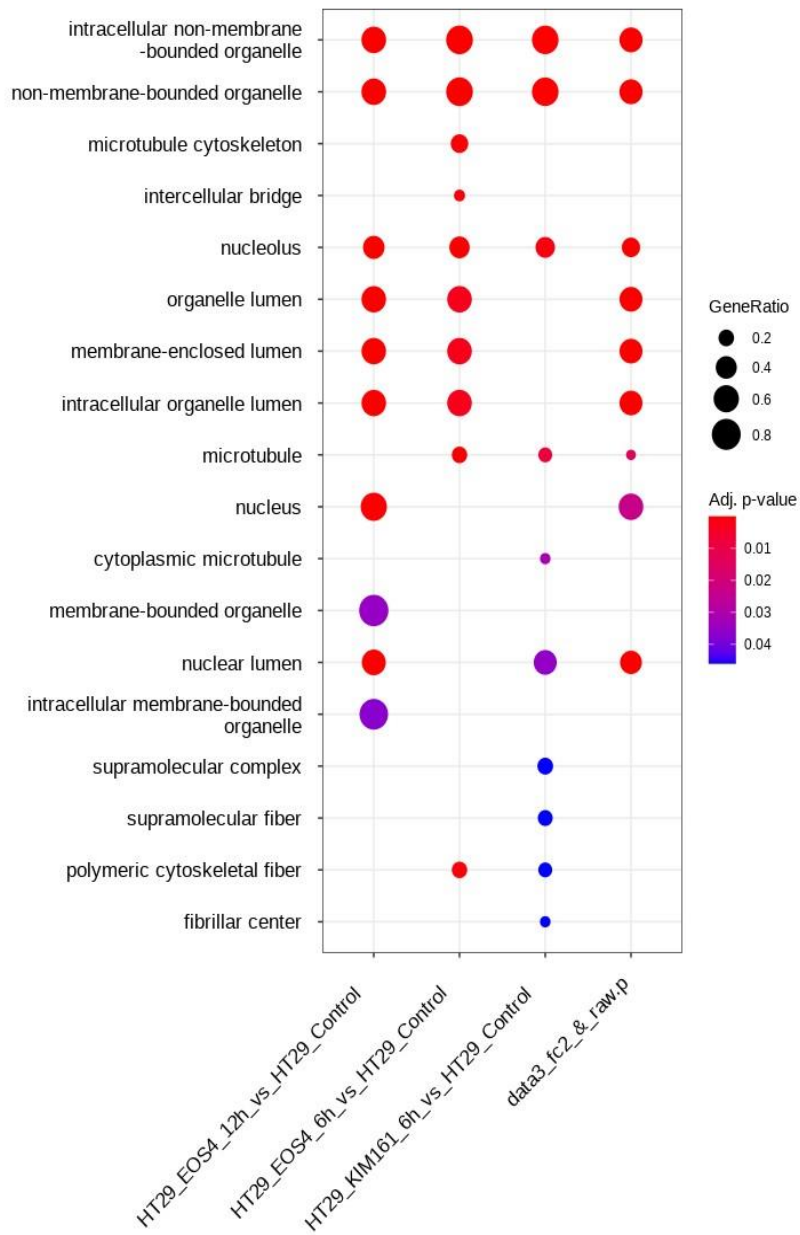
Biological Process



Molecular Function



Cellular Component



6. Data Download Information

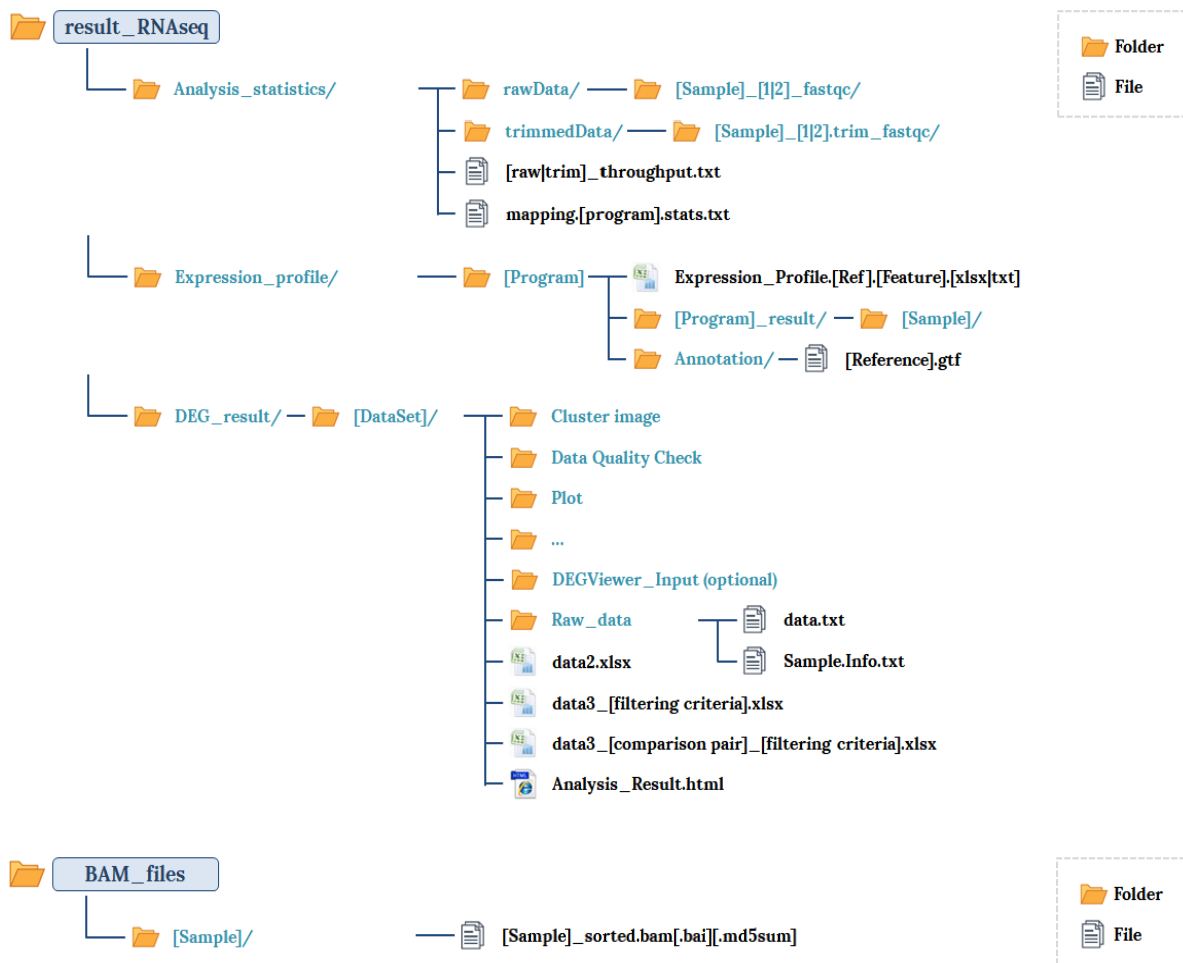
6. 1. Raw Data

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

File name	File size	md5sum
XXXXXXXXXXXXX.fastq.gz	1.41G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX.fastq.gz	1.46G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX_1.fastq.gz	1.13G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX_2.fastq.gz	1.16G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX.fastq.gz	1.47G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX.fastq.gz	1.5G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX.fastq.gz	1.21G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX.fastq.gz	1.26G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX_1.fastq.gz	1.21G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX 2.fastq.gz	1.25G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX 1.fastq.gz	1.16G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX_2.fastq.gz	1.22G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX_1.fastq.gz	1.25G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX.fastq.gz	1.27G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX.fastq.gz	1.02G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX 2.fastq.gz	1.07G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX.fastq.gz	1.18G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX fastq.gz	1.22G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX.fastq.gz	1.38G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX.fastq.gz	1.42G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX.fastq.gz	1.24G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX.fastq.gz	1.29G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX.fastq.gz	1.34G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX.fastq.gz	1.38G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX.fastq.gz	1.19G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX_2.fastq.gz	1.23G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX.fastq.gz	1.1G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX fastq.gz	1.13G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX.fastq.gz	1.26G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXX.fastq.gz	1.32G	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

- 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.



Your data will be retained in our server for 3 months.
 Should you wish to extend the retention period, please contact us.

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	Characters
10	1 in 10	90%	!"#\$%&'()*+,-./0123456789:;h=i?
20	1 in 100	99%	@ABCDEFGHIJ
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.

7. 2. Programs used in Analysis

7. 2. 1. FastQC v0.11.7

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

[LINK https://ccb.jhu.edu/software/hisat2/index.shtml](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 2.1.3b

[LINK https://ccb.jhu.edu/software/stringtie/](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

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