

## **Exosomal LncRNA Method (Illumina)**

# **1. Experimental Procedure**

## **1.1 Exosome enrichment, RNA extraction, RNA quantification**

Exosomes are obtained by exoRNeasy Maxi Kit (Qiagen, 77164) or ultracentrifugation. RNA is extracted by exoRNeasy Maxi Kit and RNA integrity is detected by RNA Nano 6000 Assay Kit of Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

## **1.2 Library Construction, Quality Control and Sequencing**

RNA libraries are prepared by Ovation SoLo RNA-seq system (NuGEN), and constructed by removing ribosomal RNA and micro amplification. Fragmentation of exosomal RNA, cDNA is synthesized by reverse transcription. After terminal repair, A-tail addition, splice and purification, the first round of amplification is performed. Removed ribosomal RNA and then performed the second round of amplification and purification of the library. cDNA fragments ranging from 250-300bp are selected by AMPure XP.

After library construction, Qubit® fluorometer measured the concentration of the library, diluted the library to 1ng/μl, detected the length of the insert size by Agilent 2100 bioanalyzer, and quantified the effective concentration by qPCR to ensure the quality of the library.

Following library preparation and pooling of different samples, sequencing is performed on Illumina platform.

# **2. Bioinformatics Analysis Pipeline**

## **2.1 Data Quality Control**

### **2.1.1 Raw Data**

The original fluorescence image files obtained from sequencing platform are transformed to short reads (Raw data) by base calling and these short reads are recorded in FASTQ format (Cock P. et al, 2010), which contains sequence information and corresponding sequencing quality information.

### **2.1.2 Evaluation of Data (Data Quality Control)**

Sequence artifacts, including reads containing adapter contamination, low-quality nucleotides and unrecognizable nucleotide (N), undoubtedly set the barrier for the subsequent reliable bioinformatics analysis. Hence quality control is an essential step and applied to guarantee the meaningful downstream analysis. We used Fastp (v0.23.1) (Chen S. et al, 2018) to perform basic statistics on the quality of the raw reads.

The steps of data processing were as follows:

- (1) Discard a paired reads if either one read contains adapter contamination;
- (2) Discard a paired reads if more than 10% of bases are uncertain in either one read;
- (3) Discard a paired reads if the proportion of low quality (Phred quality <5) bases is over 50% in either one read.

### **2.1.3 Mapping to Reference Genome**

We used Hisat2 (v2.2.1) to compare clean reads with the reference genome to obtain the location information of reads on the reference genome. The higher mapping rate, the higher accuracy rate of finding junction reads (Kim D et al., 2015).

As a comparison tool, Hisat2 can generate a concatenation database based on gene

model annotation files, quickly process a large number of sequencing data, provide high-precision comparison results, and efficiently detect junction reads, which has a better effect than other non-concatenation comparison tools.

#### **2.1.4 Transcript Assembly**

Based on the comparison results, Stringtie (v2.2.3) is used to concatenate reads into transcripts and quantify them (Pertea M et al., 2015). Stringtie has specific parameters for different libraries, which can concatenate transcripts more accurately and achieve transcription quantification.

#### **2.1.5 Transcript Identification**

Stringtie is used to merge the transcripts obtained from the concatenated samples, and the transcripts whose chain direction is uncertain and length does not exceed 200nt are removed. Gffcompare (v0.12.6) is used to compare with the known database, filter the known transcripts in the database, and finally predict the coding potential of the selected new transcripts.

#### **2.1.6 Quantification**

The expression levels of mapped, spliced, screened transcripts and predicted transcripts need to be quantitatively analyzed. FPKM is the number of fragments per kilobase of transcript sequence sequenced per million base pairs. It is a simple and commonly used expression level normalization method that can standardize sequencing depth and genome size.

#### **2.1.7 Differential Expression Analysis**

We used edgeR (v4.0.16) (Robinson MD et al., 2010) software to analyze the

significance of expression differences, and p value or padj was used to determine the significance level. It can be divided into 3 steps:

- (1) Standardize the original read count, mainly to correct the sequencing depth;
- (2) Calculate p value of the statistical model;
- (3) The FDR value is obtained by multiple hypothesis testing;

### **2.1.8 Enrichment Analysis**

We used clusterProfiler (v4.8.1) (Yu G et al, 2012) to achieve functional enrichment analysis of differentially expressed genes in GO (Gene Ontology). Differentially expressed genes are significantly enriched, and  $\text{padj} < 0.05$  as the threshold of significant enrichment. KEGG (Kyoto Encyclopedia of Genes and Genomes) is the main public database of pathway significant enrichment analysis, hypergeometric test is applied to identify the pathway of significant enrichment in candidate target genes, and  $\text{padj} < 0.05$  as the threshold of significant enrichment. The differentially expressed genes in the KEGG pathway is analyzed by clusterProfiler.

### **2.1.9 Prediction and Functional Analysis of LncRNA Target Genes**

LncRNA is rich in biological functions and widely participates in various important physiological processes of organisms. LncRNA can regulate the expression of target genes at the transcriptional and post-transcriptional levels, and identify the differential functional pathways generated by different treatments (Schmitt AM et al., 2016). Predict the target genes of lncRNAs, and functional enrichment analysis (GO/KEGG) is performed on target genes to predict the main functions of lncRNAs.

### **2.1.10 Alternative Splicing Analysis**

Alternative splicing means that the same precursor mRNA has multiple splicing procedures to form different mRNAs. It is an important mechanism to regulate gene expression and produce protein diversity. Alternative splicing analysis is performed by rMATS (Shen S et al., 2014). rMATS (v4.3.0) can analyze SE (Exon Skip), RI (Retained Intron), ME (Mutually Exclusive Exon), A5SS (Alternative 5 ' splicing site), A3SS (Alternative 3 ' splicing site).

### **2.1.11 Variant Sites Analysis**

SNP (Single Nucleotide Polymorphism) refers to a genetic marker formed by a single nucleotide mutation on the genome, which has a large number and abundant polymorphisms. InDel stands for insertion deletion, which refers to the insertion deletion of a small fragment in a sample relative to the reference genome, which may contain one or more bases. GATK (v4.6.0.0) (McKennaAetal.,2010) is used to analyze the variant sites of the sample, and SnpEff is used to annotate the variant sites.

## **3. References**

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