Exosomal CircRNA 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 CircRNA Identification

Find circ (v1.2) (Memczak et al, 2013) and CIRI2 (v2.0.6) (Gao et al, 2015) are used to detect and identify circRNA to improve the accuracy of circRNA identification.

2.1.5 Quantitative Analysis of CircRNA

The expression levels of known and new circRNAs in each sample are statistically analyzed, and the expression levels are normalized by TPM (Zhou et al, 2010). Normalized expression level = (read count*1,000,000) / libsize (libsize: sum of sample circRNA read count).

2.1.6 CircRNA Difference Analysis

For samples with biological duplications, differential expression analysis between the two comparison combinations is performed by DESeq2 (v1.42.0) (Love MI et al., 2014), which provided a statistical procedure to determine differential expression in digital gene expression data using a model based on negative binomial distribution. For samples without biological duplications, edgeR (Robinson MD et al., 2010) TMM algorithm is used to standardize read count data for analysis.

2.1.7 Enrichment Analysis of Different CircRNA Source Genes

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 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 padj<0.05 as the threshold of significant enrichment. The differentially expressed genes in the KEGG pathway is analyzed by clusterProfiler.

2.1.8 Prediction of miRNA binding sites

circRNA can inhibit the function of miRNA by binding to miRNA (Hansen TB et al, 2013). Target miRNA sites of circRNA are predicted by miRanda software.

2.1.9 Prediction of CircRNA Coding Potential

IRESfinder software (Zhao J et al, 2018) is used to predict IRES scores, and then CPC, CNCI, and PAFM are used to identify whether circRNA has coding potential.

3. References

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