Exosome-derived microRNA NGS library preparation

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Exosomes are lipid vesicles (30-150 nm in diameter) released by cells into the blood, urine, breast milk and many other body fluids. Various miRNA species were proposed to play an important role in the distant cell-to-cell signalling and regulation of gene/cell function, including tumorigenesis. Therefore, analysis of exosome-derived miRNA species constitutes a quickly evolving field with huge implications in research and diagnostics (Huang T. et al. 2019). Next-generation sequencing (NGS) proved to play a crucial role in the discovery of both known and novel small RNA species, however, the specifics of small RNA NGS library preparation present obstacles usually associated with limited RNA amount, so typical for the isolates from exosomes or body fluids. Since the miRNA represents a tiny fraction of total RNA, the limited amount primarily leads to the abundance of high adaptor-dimer content in the resulting library. Due to its size (18-34 nt), removing dimer sequences from the small RNA library might be limited or impossible. The presence of unwanted dimer molecules leads to a partial or complete loss of library during additional clean-ups and lower or even ruin the descriptive potential of sequencing data. Also, the limited input is more prone to bias the abundance of different miRNA species as more amplification steps in the library preparation protocol are needed. Here we present our experience with the QIAseq miRNA library kit (QIAGEN) applied on isolates from body fluid-derived exosomes.

Material and methods

Six samples of rabbit seminal plasma-derived exosomes were used to this study. Samples were separated and collected by ultra-centrifugation and size-exclusion chromatography (Institute of Animal Physiology and Genetics CAS, IAPG CAS) and stored in -80°C until the miRNA extraction.

miRNA extraction & quality control: miRNA was extracted using the miRNeasy Mini Kit (QIAGEN) according to the specialized protocol (Appendix A, miRNeasy Mini Handbook, QIAGEN) in combination with RNeasy MinElute kit (QIAGEN), which was used for enrichment of miRNAs and other small RNAs molecules (less than ~200 nt) in a separate fraction (IAPG CAS). Extracted miRNA samples were analysed on capillary gel electrophoresis (Bioanalyzer 2100, Agilent RNA Pico Chip (Agilent)). The concentrations of small RNA fractions were evaluated using the 2100 Expert software (version B.02.10.SI764, Agilent). Table 1 and Figure 1 shows the concentrations and traces of extracted small RNA isolates, respectively.

Sample ID	Concentration [ng/µl]	Max. miRNA input [ng]
1	0.61	4.89
2	0.73	5.86
3	0.08	0.64
4	1.08	8.61
5	0.76	6.10
6	0.82	6.56

Table 1. The metrics of miRNA samples recorded using the Bioanalyzer 2100 using Agilent RNA Pico Chip Assay

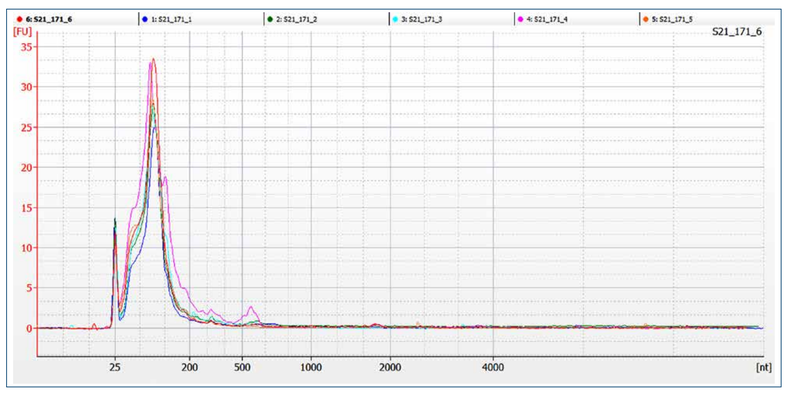

Figure 1. Traces of miRNA after analyses at Bioanalyzer 2100

The NGS miRNA libraries were prepared using the QIAseq miRNA library kit (QIAGEN, QIAseq® miRNA Library Kit Handbook). The QIAseq solution takes advantage of the presence of hydroxyl group at the 3' end and phosphate group at the 5' end of mature miRNA, which enables specific ligation of adapters. This allows universal reverse transcription and library preparation with minimal presence of other RNA species. The kit utilizes Universal Molecular Identifiers (UMIs), which enables unbiased analysis of the abundance of miRNA molecules.

Results

NGS library preparation

The manufacturer's protocol defines the input of 100ng of total RNA. However, the miRNA typically represents 0,01 % fraction of the total RNA. Given the limited nature of isolates, 5µl of each sample was used, resulting in input ranging from 0,64 to 8,61 ng miRNA (see Table 1). According to this miRNA input, the QIAseq miRNA NGS 3 'and QIAseq miRNA NGS 5' adapters were diluted to 1:10 (10x dilution). QIAseq miRNA NGS ILM Library Forward Primer and QIAseq miRNA NGS ILM IPD index containing 6 nt long index sequence were used for PCR amplification. Amplification was performed according to the protocol with 22x cycles to ensure the sufficient yield of NGS libraries for the sequencing

NGS library quality control

The prepared NGS miRNA libraries concentration was evaluated using the Qubit dsDNA High sensitivity Assay (Table 2). Size distribution was analyzed using the High Sensitivity DNA Assay Chip (Bioanalyzer 2100, Agilent) (Figure 2). Preparation of all miRNA NGS libraries resulted in sufficient concentration and typical size distribution (targeted ~180 bp), while no contamination of adaptor-dimers (~157 bp) was recorded.

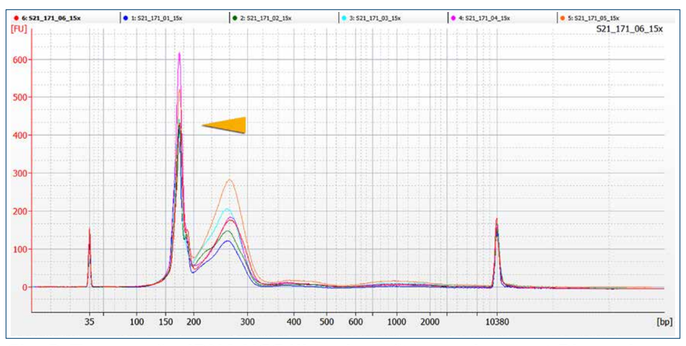

Fig. 2. Traces of miRNA NGS libraries after analyses at Bioanalyzer 2100. The peak in ~180 bp corresponds to miRNA library

Sample ID	c [ng/µl]	c [nM]	Ø library size [bp]
1	41,80	368.22	172
2	44,00	385.36	173
3	43,00	378.79	172
4	50,40	441.41	173
5	48,30	420.59	174
6	41,40	360.50	174

Table 2. The concentrations of miRNA NGS libraries (C (ng/µl) and C (nM)) measured using the Qubit HS DNA Assay. Average library size (Ø library size (bp)) estimated using the High Sensitivity DNA Chip on Bioanalyzer 2100 (Agilent)

Sequencing

miRNA libraries were sequenced on the shared 2x151 cycles experiment on the NovaSeq 6000 sequencer (Illumina), using NovaSeq S4 Reagent kit v1.5 (300 cycles), with aim of 60 M pair-end reads (30 M clusters) per sample (Table 3).

Sample ID	Milion PE Reads
1	90.56
2	97.21
3	96.17
4	102.03
5	101.07
6	126.06

Table 3. Results of sequencing experiment at NovaSeq 6000, yield of milions of pair reads/miRNA NGS library

Conclusion

Library preparation was done with modifications addressing the limited input of small RNA isolates from exosomes derived from seminal fluid. All six NGS libraries were successfully prepared without the presence of any adaptor dimers, although the input was as low as 0,64 ng. Besides the expected peak typical for miRNA libraries (~180 nt), a small proportion of non-small RNA libraries at larger sizes were also observed within all six libraries. Data evaluation (data not presented) showed more than 240 known miRNA in each group of samples. This number is particularly surprising, especially in the context of the miRNA origin: the miRNA contained in the exosomes from seminal plasma of the rabbit. Present results demonstrate the usability of the QIAseq miRNA library kit for NGS analysis of miRNA species even from limited input materials and without the need for laborious size-selection.