APPLICATION NOTE

Optimizing the extraction of small RNA from plasma for NGS applications.

Key features

- Consistent and scalable small RNA extraction with comparable yields to manual methods
- Fast, automated workflow (10 min hands-on time) with on-board lysis and no heating required
- Suitable for plasma, serum or extracellular vesicles at input volumes of 200 µl or 1 ml
- Suitable for various downstream assays and proven compatibility with Revvity NEXTFLEX® Small RNA-Seq Kit v4

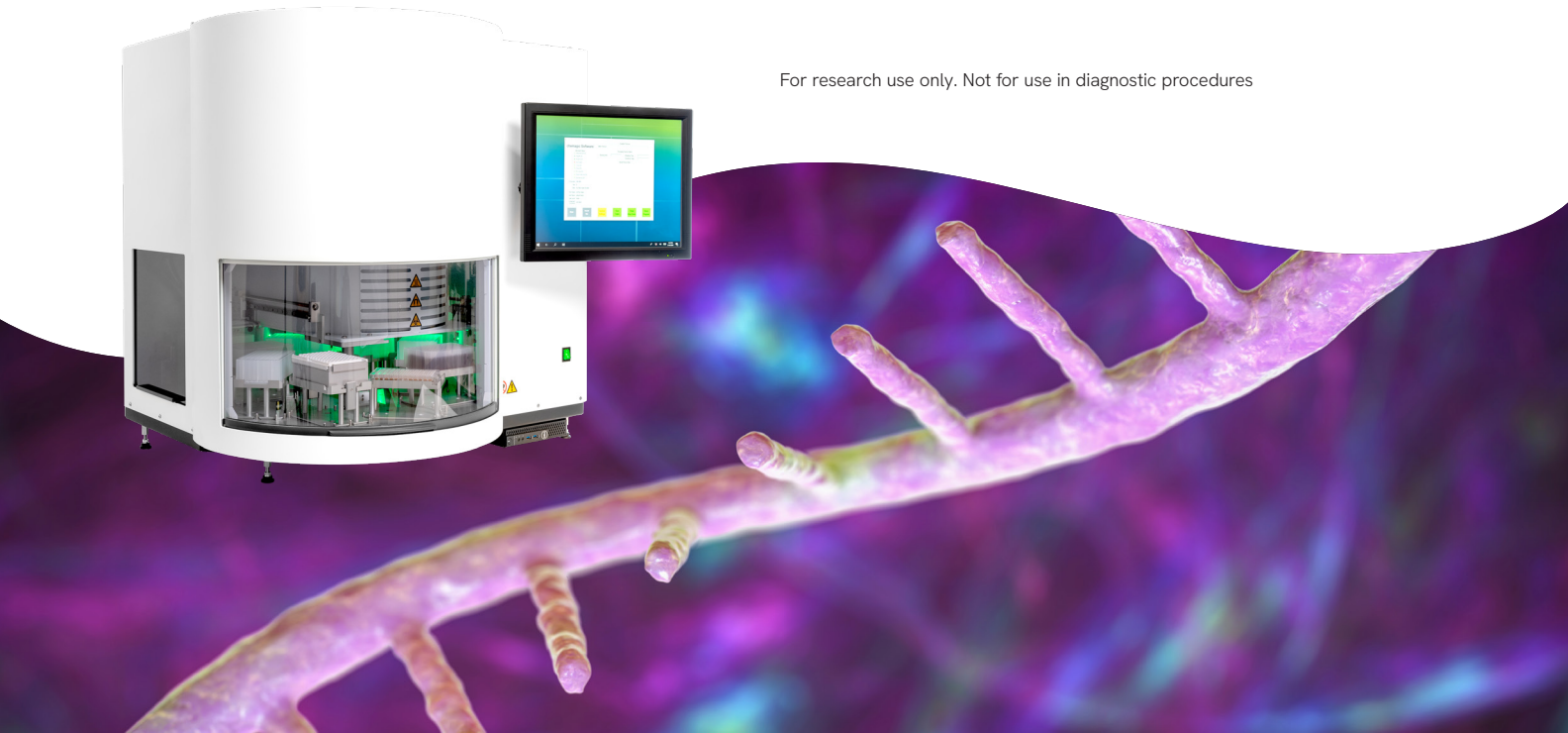
Introduction

MicroRNAs (miRNA) play an important role in the regulation of protein levels. They are tissue specific, stable in extracellular space and their expression profiles have been shown to correlate with health status in multiple studies. They can be reliably detected in tissues and diverse body fluids, which make them ideal biomarker molecules.

Researchers interested in miRNA are moving from studying tissues to plasma, as this is easy to obtain. However, this sample type poses many challenges, as the available amount of circulating cell free RNA in plasma is low, the amount of miRNA is very variable from sample to sample and there is high amount of fragmented RNA molecules (mRNA and rRNA) that can interfere in downstream analysis.

To realize the full potential of miRNA as biomarkers, there is a need for a workflow able to extract efficiently circulating cell free RNA at high yield and purity. Then this RNA is converted into libraries to allow massively parallel sequencing and analysis of miRNA.

Here we present a method for rapid high-throughput automated extraction of circulating free RNA from very small volumes of plasma (200 µL). We also show that the purified RNA obtained is suitable for the preparation of small RNA libraries with our gel-free Small RNA sequencing library preparation kit, opening the door to the possibility of a fully automated workflow.



Methods

PLASMA SAMPLE PREPARATION

Frozen, citrate-stabilized plasma from a healthy donor was purchased from the University Hospital Aachen Blood Donation Service. Plasma was thawed in a water bath at room temperature, aliquoted and frozen at -20°C. Prior to small RNA extraction, the plasma was thawed in a water bath at room temperature.

SMALL RNA EXTRACTION

96 samples of 200 µL of plasma was used as input on the chemagic™ 360 instrument equipped with a 96-rod head. Small RNA was purified using the chemagic™ miRNA 200 H96 Kit (CMG-1224). For 10 samples, 5 µl of eluate was quantified with the Thermo Scientific® Qubit® miRNA Assay. Samples were kept at -20°C immediately after quantification.

LIBRARY PREPARATION

4 µL of extracted RNA was used as input. Small RNA libraries were constructed manually using the NEXTFLEX® Small RNA-seq Kit v4 (NOVA-5132-41) according to the manufacturer's instructions. Libraries were quantified using the High Sensitivity assay on the 2100 Bioanalyzer® Instrument and the Thermo Scientific® Qubit® dsDNA HS Assay kit. Following this, 12 libraries were pooled and run on an Illumina® MiSeq® platform at 1x75.

Results

The isolation of small RNA using the chemagic™ miRNA 200 H96 Kit on the chemagic™ 360 instrument generates 35 – 40 µL eluate/sample with an average concentration of 0.93 ng/µL of RNA recovered and CV=6.3% in the samples tested (Figure 1).

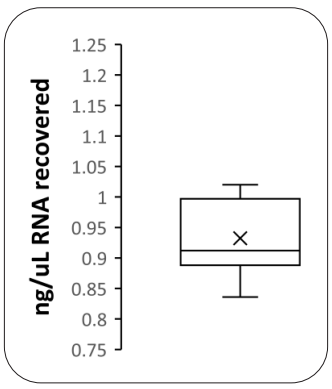


Figure 1: Highly reproducible small RNA extractions on chemagic™ 360 instrument. 200 µL of plasma from a healthy donor was used and small RNA was extracted in one automated run.

Analysis of the size of the libraries obtained showed a peak at 158 bp corresponding to the expected product, together with smaller peaks below 100 bp and at 190 bp that correspond to single stranded indexed primers that do not interfere with sequencing (Figure 2 shows two examples of library profiles obtained).

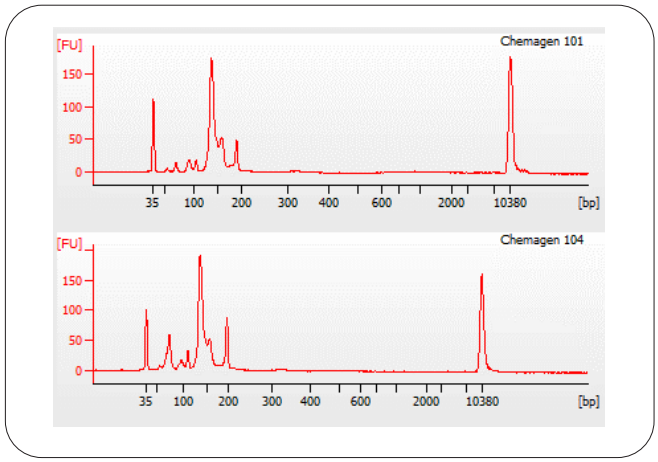


Figure 2: Representative profiles of libraries obtained using 4 µL of extracted RNA as input for NEXTFLEX® Small RNA-seq Kit v4.

After sequencing, Small RNA analysis was performed using a PerkinElmer custom script. Alignment reference was mature miRNA from mirBase v22.1. The results obtained for each of the 12 samples are listed in the table below.

The % <16 bp refers to the amount of inserts with size of 16 bp or less, which cannot be mapped to unique sites in the genome. With an average of 57.78%, this value is in agreement with values reported for this sample type. Similarly, the amount of miRNA detected averages 9.97%, in agreement with previous data published for plasma.

Sample #	% Dimer	% <16 bp	% miRNA	% tRNA	% rRNA
1	7.48	55.52	8.55	0.05	0.36
2	5.11	65.09	8.07	0.33	0.31
3	5.76	61.04	11.28	0.08	0.27
4	7.17	60.53	9.73	0.04	0.24
5	5.77	67.23	4.07	0.03	0.17
6	5.42	68.68	4.46	0.02	0.15
7	8.45	55.65	8.46	0.05	0.27
8	7.68	48.42	14.59	0.04	0.38
9	7.23	66.07	6.20	0.03	0.19
10	7.39	49.11	13.27	0.09	0.32
11	5.98	48.92	16.24	0.06	0.47
12	5.16	47.04	14.67	0.22	0.54

Conclusions

In this note, we show that the chemagic™ miRNA 200 Kit H96 (CMG-1224 for 0.2 mL of plasma) can be used to automate the extraction of small RNA from plasma, producing highly reproducible amounts of material suitable for multiple applications downstream, including NGS. This kit is also available in a different format as CMG-1223 chemagic™ miRNA 1k Kit H24 for 1 mL of plasma. The combination of chemagic™ miRNA method with the gel-free NEXTFLEX® Small RNA-Seq Kit v4 makes the implementation of a fully automated end-to-end workflow for high throughput biomarker discovery possible.

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