APPLICATION NOTE

Automated microRNA purification with the chemagic 360 Instrument.

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Abstract

The purification of small RNA species is challenging because of the low concentrations and the small fragment sizes. Nevertheless, the information it delivers is highly valuable for cancer research and research of conditions connected to the metabolic syndrome. The chemagic™ miRNA kits enable the automated and efficient extraction of small RNA species from 1.0 and 0.2 ml plasma in 24-well or 96-well format in maximal 2 hours with minimal hands-on time. The chemagic miRNA kits show equivalent yields and increased qPCR performance compared to a representative manual miRNA extraction kit. Fragment analysis confirmed the isolation of the miRNA fragments of interest and a sequencing analysis revealed high diversity and reliable performance. Furthermore, the optional addition of glycogen to the small RNA isolation further boosted the yield, reduced C_T values for selected miRNA targets and increased diversity by helping to capture more low-level miRNA transcripts.

Chemagic 360 instrument used for the miRNA extraction with chemagic miRNA kits.

Introduction

Liquid biopsies have shown to be important sources for markers to help detect cancer in its initial stages. Markers such as circulating cell-free DNA and RNA (cfDNA and cfRNA), circulating tumor cells (CTCs) and exosomes have shown to be useful. The importance of cfDNA has been well established, while cfRNA supplies a complementary molecular profile to give a clearer picture of a patient's oncology[1, 2]. Out of the cfRNA population, most of these ribonucleic acids are unstable, yet microRNA (miRNA) molecules have greater longevity and have been essential tools for the study of tumor and metabolic markers[3].

Analysis of microRNA (miRNA) in plasma samples opens a wide range of interesting information for many research applications. They play roles in both homeostasis in healthy individuals and diseases such as cancer, diabetes, in addition to cardiovascular and kidney pathologies. MicroRNA biogenesis involves the movement of pre-miRNA molecules from the nucleus to the cytoplasm and release into the extracellular environment via exosomes. Extraction of high-quality miRNA is a crucial factor as the miRNA profile can be easily altered upon haemolysis[4].

The isolation of miRNA represents a significant challenge due to low concentrations, small fragment sizes of about 19-22 nucleotides (nt) and furthermore, miRNAs are often bound in protein-complexes contaminating proteins or inhibitors. Thus, a high demand for precise purification workflows is required[5].

In this application note, we demonstrate the efficient automated isolation of high-quality miRNA from plasma using our chemagic miRNA 200 Kit H96 and chemagic miRNA 1k Kit H24 on the chemagic 360 instrument.

Product overview

Revvity developed two chemagic miRNA kits for the isolation of miRNA from 1.0 ml and 0.2 ml plasma with the chemagic 360 instrument.

The chemagic miRNA kits are based on chemagen technology using M-PVA Magnetic Beads for the isolation of miRNA. The miRNA binds to paramagnetic beads, which are magnetically separated from the sample material. During subsequent steps, contaminants are removed, and the purified miRNA is transferred into an elution medium. The automated sample processing by the chemagic 360 instrument excludes cross contamination and ensures safe handling of infectious sample material.

	chemagic™ miRNA 1k Kit H24 (CMG-1223)	chemagic™ miRNA 200 Kit H96 (CMG-1224)
chemagic Rod Head	24	96
Format	24-well	96-well
Preps/Kits	240	960
Sample Volume	1.0 ml	0.2 ml
Processing Time	90 min (including 10 min hands-on time)	90 min (including 10 min hands-on time)

Materials and methods

The sample preparation with the chemagic miRNA 1k Kit H24 and chemagic miRNA 200 Kit H96 does not involve any centrifugation or filtration, which leads to a total hands-on time of only 10 min. Nucleic acids were extracted from 1.0 ml and 0.2 ml plasma from three healthy donors with the chemagic miRNA kits CMG-1223 and CMG-1224, respectively. Plasma from Donor 1 and Donor 2 stabilized with citrate was purchased from UKA Blutspendedienst, University Hospital Aachen; blood from Donor 3 was collected in Streck - Cell-Free DNA BCT® tubes (218962, Streck, Inc., La Vista, NE, USA), plasma was prepared by double centrifugation (2.000 x g, 20 min, plasma aspirated above the buffy coat layer followed by a centrifugation at 3.300 x g, 30 min). All plasma samples were stored at -20°C. Prior to the nucleic acid extraction, the samples were thawed in a water bath at 30°C for 10 min. A synthetic RNA spike-in mix (339390, Qiagen, Hilden, Germany) composed of three RNA templates (UniSp2: 2 fmol/µl, UniSp4: 0.02 fmol/µl, UniSp5: 0.00002 fmol/µl) was added to the plasma samples according to the manufacturer's instructions to serve as quality control for miRNA isolation. The automated nucleic acid extraction on a chemagic 360 instrument was compared to a manual, silica spin-based competitor extraction kit.

In addition, the miRNA extraction on the chemagic 360 instrument was performed with the addition of 5 µg glycogen (R0551, Thermo Fisher Scientific™, Waltham, MA, USA) per sample to further increase the efficiency of miRNA isolation from plasma.

After miRNA isolation, the concentration of the purified eluates was determined with the Qubit™ microRNA Assay Kit (Q32880, Thermo Fisher Scientific™, Waltham, MA, USA), the assay is highly selective for small RNA over rRNA or large mRNA.

Reverse transcription (RT) of the purified miRNA into cDNA was performed with the miRCURY LNA RT Kit (339340, Qiagen, Hilden, Germany) using UniSp6 and cel-miR-39-3p RNA Spike-ins as cDNA synthesis controls. After cDNA synthesis, quantitative real-time PCR (qPCR) was performed with the miRCURY LNA SYBR® Green PCR Kit (339345, Qiagen, Hilden, Germany) on a QuantStudio™ 5 (A34322, Thermo Fisher Scientific™, Waltham, MA, USA). In total, three assays targeting human miRNAs (YP00204001: hsa-miR-486-5p miRCURY LNA miRNA PCR Assay, YP00206010: hsa-miR-126-5p miRCURY LNA miRNA PCR Assay, YP00204606: hsa-miR-22-3p miRCURY LNA miRNA PCR Assay) and three assays targeting the spike-in RNAs (YP00203950: UniSp2 miCURY LNA miRNA PCR Assay, YP00203953: UniSp4 miCURY LNA miRNA PCR Assay, YP00203955: UniSp5 miCURY LNA miRNA PCR Assay) were performed as well as one assay used for the detection of the cDNA synthesis control (YP00203954: UniSp6 miCURY LNA miRNA PCR Assay). All PCR Assays were purchased from Qiagen (Hilden, Germany). The preparation of the spike-ins, cDNA and qPCR were performed as instructed by the manufacturer.

Results and discussions

Small RNA species such as miRNAs are highly specific and informative disease biomarkers, which represent a strong tool for future applications[6]. miRNA was isolated from 0.2 ml and 1.0 ml from three different donors with the chemagic kits on the chemagic 360 instrument and manually from 0.2 ml plasma with the competitor Q. Figure 2 shows the yields per ml plasma determined with the Qubit™ microRNA Assay Kit. The concentration and yield are highly donor-dependent and therefore can vary strongly. In all cases, the yields achieved with the chemagic miRNA extraction kits are comparable to the corresponding competitor extraction from 0.2 ml (Figure 2 A and B). The miRNA yields per ml of plasma were ranging from 261 – 339 ng/ml with CMG-1224 (%CV of replicates 7.4 to 24.6 %), 232 to 329 ng/ml (%CV of replicates 7.4 to 24.6 %) with CMG-1223 and 251 to 259 ng/ml (%CV of replicates 7.1 – 11.6 %) with %CV values as described in literature[7]. Furthermore, the scalability of the miRNA isolation with both chemagic kits confirmed that the quantity of miRNA recovered was not volume dependent.

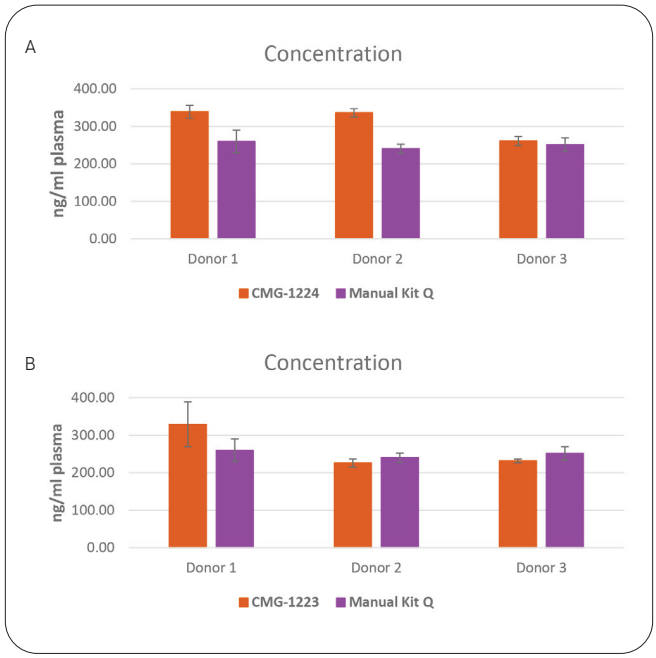

Figure 2: Comparable yields of miRNA extracted with the chemagic miRNA kits compared to a competitor kit. miRNA was isolated from 0.2 ml (A) and 1.0 ml (B) from three different donors with the chemagic kits on the chemagic 360 instrument and manually with competitor Q.

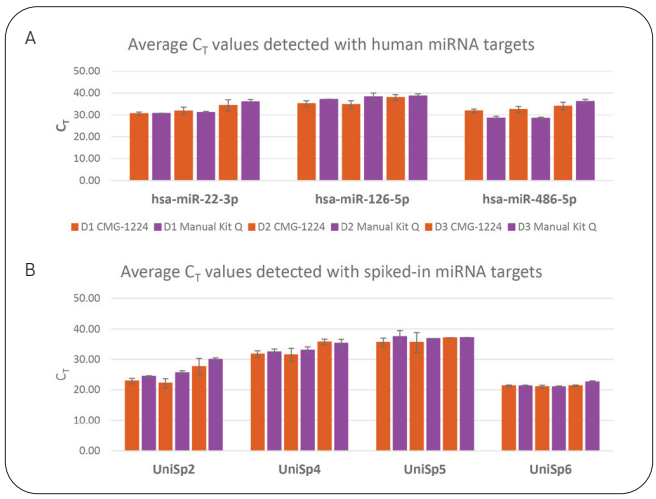

Figure 3: Comparable C_T values detected for miRNA extracted from 0.2 ml plasma with the chemagic miRNA kit CMG-1224 compared to a manual competitor kit. A) Detection of human miRNA targets, B) detection of spiked controls UniSP2, UniSP4, and UniSP5 as well as the cDNA synthesis control UniSP6.

The eluates obtained from the automated extraction on the chemagic 360 instrument with CMG-1224 and CMG-1223 and the manual competitor kit were used for a cDNA synthesis with subsequent qPCR. The C_Ts for the UniSP6 cDNA control showed a very uniform amplification among all tested samples (Figure 3B and Figure 4B) indicating that the cDNA synthesis efficiency was equally efficient in all extracted samples. Therefore, the detected C_T differences can be directly related to the performance of the miRNA extraction procedure. The C_T values obtained for three human miRNA targets are very comparable between the chemagic miRNA kits and the competitor kit as shown in Figure 3A and Figure 4A. CMG-1223 even shows decreased C_T values with the human targets in comparison with manual Kit Q (Figure 4A). Using the miRNA spike-ins with defined concentrations not only confirms the C_T results obtained with the human targets but also proves that the miRNAs of very high to only weak abundancy are successfully extracted with the chemagic miRNA extraction kits. In addition, the expected C_T shifts of 6.6 cycles (100-fold increment) between UniSP2 and UniSP4 and between UniSP4 and UniSP5 (1000-fold increment) were confirmed (Figure 3A and Figure 4A)[8].

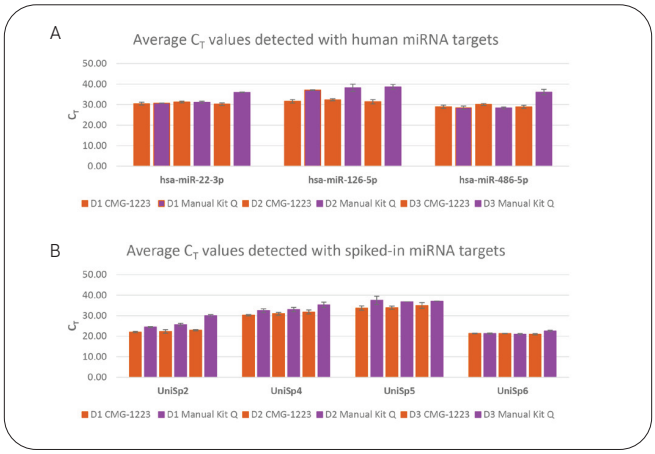

Figure 4: Comparable C_T values detected for miRNA extracted from 1.0 ml plasma with the chemagic miRNA kit CMG-1223 compared to a manual competitor kit. A) Detection of human miRNA targets, B) detection of spiked controls UniSP2, UniSP4, and UniSP5 as well as the cDNA synthesis control UniSP6.

In a further experimental setup, the use of glycogen in the miRNA isolation was evaluated with the chemagic miRNA kits. Figures 5 and 6 clearly show that the addition of only 5 µg glycogen to the chemagic miRNA extraction setup is beneficial to decrease C_T values obtained with human miRNA targets as well as with the spike-ins, which corresponds to earlier findings[9]. The control UniSP6 proves that the cDNA synthesis was performed with equal efficiency among all samples. Thus, the variations observed in the C_T values can be directly related to the miRNA isolation procedure. In conclusion, the addition of glycogen to the lysates is beneficial but not mandatory to yield excellent miRNA C_T results.

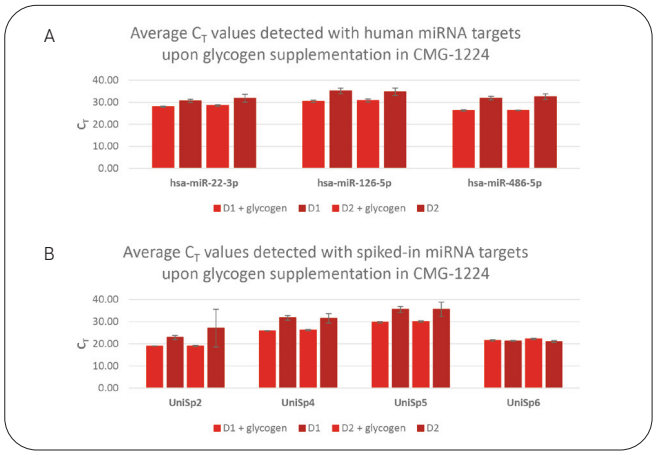

Figure 5: Decreased C_T values detected for miRNA extracted from 1.0 ml plasma with the addition of 5 µg glycogen to the chemagic miRNA kit CMG-1224. A) Detection of human miRNA targets, B) detection of spiked controls UniSP2, UniSP4, and UniSP5 as well as the cDNA synthesis control UniSP6.

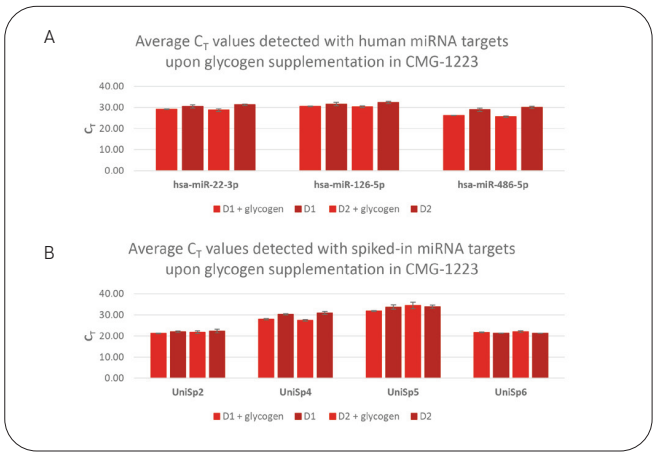

Figure 6: Decreased C_T values detected for miRNA extracted from 1.0 ml plasma with the addition of 5 µg glycogen to the chemagic miRNA kit CMG-1223. A) Detection of human miRNA targets, B) detection of spiked controls UniSP2, UniSP4, and UniSP5 as well as the cDNA synthesis control UniSP6.

To determine the abundance and diversity of the miRNA extracted pool, a workflow was developed by TGen (Phoenix, AZ, USA) to sequence these molecules so that investigators can analyze the population of these miRNA molecules in individuals. Using the CMG-1224 kit for 0.2 ml plasma, the resulting eluates were analyzed to determine their size population (Figure 7). Fragments analyzed showed a peak around 20-40 nucleotides (nt), in addition to other RNA species from these plasma samples. To show that this preparation included the miRNA fragments of interest and to prepare for sequencing, libraries were constructed using the Revvity NEXTFlex Small RNA 3.0 kit (Figure 8). This result indicates the presence of libraries generated from miRNA and shows a uniform peak of approximately over 150 base pairs (bp). Based on sequencing experience based on other extraction methods, libraries that are approximately 150-160 bp sequence well. In this case, the extraction using the CMG-1224 kit showed high diversity and performed reliably, based on sequencing analysis. As we saw with the RT-PCR results and overall yield, the addition of glycogen not only boosted yield, reduced C_T values for selected miRNA targets, but it also showed by sequencing analysis that it increased diversity by helping to capture more low-level miRNA transcripts, while not significantly increasing more abundant miRNA species.

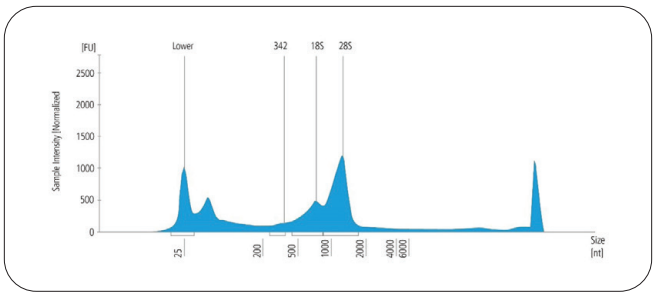

Figure 7: Fragment analysis of miRNA extracted from 0.2 ml plasma. Note the markers indicating the sizes in nucleotides (nt) and the 28S and 18S rRNA sizes.

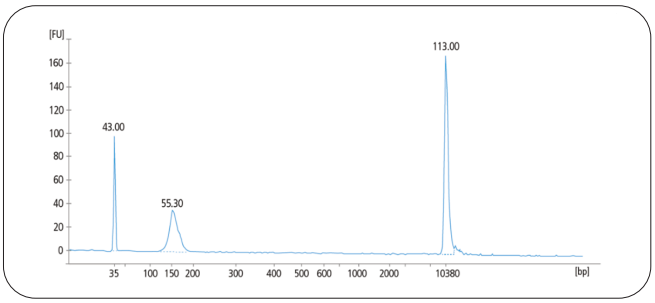

Figure 8: Fragment analysis of miRNA libraries (cDNA) constructed using the miRNA extracted from 0.2 ml plasma with the Revvity NEXTFLEX Small RNA-Seq kit. Note the markers indicating the sizes in base pairs (bp).

Conclusion

This application note shows that the newly developed chemagic miRNA extraction kits perform excellently with regards to miRNA yields and detection in qPCR. The kits are a time-efficient solution for the automated extraction of small RNA species with a hands-on-time of only 10 min. Both kits were compared to miRNA isolation with a representative manual miRNA extraction kit and showed at least equal or better performance. qPCR with three human miRNA targets as well as synthetic miRNA spike-ins proved that miRNAs in high as well as low abundancies are isolated and amplified optimally. Fragment analysis confirmed the successful isolation of miRNA and further sequencing analysis showed high diversity and a reliable performance with miRNA eluates derived from the extraction with CMG-1224.

Acknowledgement

Fragment analysis of miRNA isolated with CMG-1224 as well as miRNA libraries preparation was performed by the Translational Genomics Research Institute, Neurogenomics DIvision, (Phoenix, AZ USA); Revvity chemagen sincerely thanks Dr. Joanna Palade, Dr. Eric Alsop and Kendall Jensen for their significant contributions to this application note.

References

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