Analysis of circulating microRNAs and their post-transcriptional modifications in cancer serum by on-line solid-phase extraction capillary electrophoresis-mass spectrometry

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ABSTRACT

In this paper, an on-line solid-phase extraction capillary electrophoresis-mass spectrometry (SPE-CE-MS) method is described for the purification, preconcentration, separation, and characterization of endogenous microRNA (miRNA) and their post-transcriptional modifications in serum. First, analysis by CE-MS was optimized using a standard mixture of hsa-miR-21-5p (miR-21-5p) and hsa-let-7g-5p (let-7g-5p). For SPE-CE-MS, a commercial silicon carbide (SiC) resin was used to prepare the microcartridges. Under the optimized conditions with standards, the microcartridge lifetime (>25 analyses) and repeatability (2.8% RSD for the migration times; 4.4 and 6.4% RSD for the miR-21-5p and let-7g-5p peak areas, respectively) were good, the method was linear between 25 and 100 $nmol \cdot L^{-1}$, and the limit of detection (LOD) was around 10 $nmol \cdot L^{-1}$ (50 times lower than by CE-MS).

In order to analyze human serum samples, an off-line sample pretreatment based on phenol/chloroform/isoamyl alcohol (PCA) extraction was necessary prior to SPE-CE-MS. The potential of the SPE-CE-MS method to screen for B-cell chronic lymphocytic leukemia (CLL) was demonstrated by an analysis of serum samples from healthy controls and patients. MicroRNAs, specifically miR-21-5p and a 23 nucleotide long 5'-phosphorylated miRNA with 3'-uridylation (iso-miR-16-5p), were only detected in the CLL patients.

MicroRNAs (miRNAs) are a class of single-stranded, non-protein-coding RNAs that are19–23 nucleotides long. The function of a miRNA is to control gene expression post-transcriptionally, regulating messenger RNA (mRNA) by binding to the 3′ untranslated region (3′ UTR) of the target mRNA[1]. miRNAs play a major role in a wide range of normal cellular processes, including cell proliferation, development, and apoptosis[2]. Circulating miRNAs have been found in the extracellular environment, including in various biological fluids, such as the blood, saliva, and urine of both diseased and healthy people. These extracellular miRNAs are packaged in extracellular vesicles[3] or associated with RNA-binding proteins[4,5], leading to high stability and resistance to endogenous RNase activity, extreme temperature, and pH[6].

Deregulation of miRNAs has been associated with different diseases, such as cardiovascular diseases, diabetes, aberrant immune function, and especially cancer[7]. In comparison with longer biomarker molecules, miRNAs present several advantages: they have relatively smaller numbers of candidate sequences and higher stability due to their smaller size, which leads to an improved robustness of detection. Furthermore, they are detectable in accessible biofluids, such as serum. For these reasons, miRNAs are great candidates as minimally invasive biomarkers for early disease diagnosis, prognosis, and treatment outcome for different diseases[8]. In this sense, miR-21-5p is one of the most frequently upregulated miRNAs in solid tumors, and its levels have been associated with relapse-free survival[9,10].

Several analytical methods have been developed for the detection of miRNAs and the most commonly used are Northern blotting[11], microarrays[12], and a two-step procedure of modified reverse transcription polymerase chain reaction followed by quantitative PCR (RT-qPCR)[13]. The last method is very sensitive, but it is based on the indirect detection of complementary DNA. Furthermore, the above-mentioned methods are not capable of detecting the post-transcriptional modifications of miRNAs, such as 5’-end

phosphorylation and dephosphorylation, or unexpected isoforms with differing ends due to trimming or nucleotide additions[14]. These modifications have been reported to affect the stability of miRNA and be a mechanism for the regulation of miRNA activity[15]. Thus, the development of direct analytical methods able to obtain information regarding post-transcriptional modifications of miRNA is of great interest. In this respect, capillary electrophoresis-mass spectrometry (CE-MS) is a highly efficient technique for the separation and characterization of biomolecules; it is generally applied in the field of proteomics and metabolomics and less in genomics because of the emergence of next-generation sequencing techniques (NGS)[16–20]. CE-MS has rarely been used for the analysis of small RNAs, although it allows direct, label-free, multiplex analysis and precise mass identification of post-transcriptional modifications of miRNAs[21]. Small RNAs can be isolated from biological fluids using commercially available kits and then analyzed by CE-MS. However, the small injection volumes typical of CE compromise the concentration sensitivity. The use of highly selective and sensitive mass spectrometers is often not enough for robust detection; consequently, CE-MS has often been combined with different electrophoretic and chromatographic techniques for the on-line preconcentration of the target analytes, such as sample stacking, isotachophoresis, or on-line solid-phase extraction (SPE-CE)[22–27]. In SPE-CE, a microcartridge with an appropriate sorbent is inserted near the inlet of the separation capillary to purify and preconcentrate the target analytes from a large volume of sample. SPE-CE-MS has been extensively applied for the analysis of small molecules (drugs, metabolites, etc.), peptides, and proteins using the silica or polymeric sorbents typically used in off-line SPE (e.g., C18)[28–30] because of the versatility of these sorbents and their commercial availability. The use of other sorbents for improved selectivity, such as immunoaffinity sorbents, has also been studied with good results, although the very

specific conditions required for the extraction and compatibility with on-line MS detection[31–33].

In recent years, different commercially available kits, based on SPE spin microcolumns used in combination with liquid-liquid extraction and precipitation, have been developed for miRNA isolation and purification from biological fluids. These multistep sample pretreatments are widely used prior to PCR-based analytical methods due to their excellent recoveries and simplicity of usage[34–36]. However, to the best of our knowledge, the on-line coupling of these SPE methods to a high-performance separation technique with MS detection has not yet been explored, although it may reduce manual handling and increase analysis throughput while obtaining valuable structural information.

In this paper, for the first time, an SPE-CE-MS method for the purification, preconcentration, separation, and untargeted multiplex analysis of miRNAs from serum samples is described. The SPE-CE-MS analysis of miRNA was optimized using standards, and the figures of merit were compared with those of a previously developed sample stacking CE-MS method[21]. Subsequently, the applicability of the developed SPE-CE-MS method was demonstrated by the analysis of serum samples from healthy controls and patients with chronic lymphocytic leukemia (CLL), the most common type of leukemia in adults[37].

Experimental section

Materials and reagents

All the chemicals used in the preparation of background electrolytes (BGEs) and solutions were of analytical-reagent grade or better. Acetic acid (glacial), ammonium hydroxide (25%), guanidinium chloride (≥99.0%), ortho-phosphoric acid (85%), and sodium hydroxide (≥99.0%) were purchased from Merck (Darmstadt, Germany). Acetonitrile (LC-MS), 96% ethanol and methanol (LC-MS) were supplied by Panreac AppliChem (Barcelona, Spain). Propan-2-ol (LC-MS) was purchased from Scharlau (Barcelona, Spain). Ammonium acetate (NH_4Ac, ≥99.999%), ammonium bicarbonate (for LC-MS), chloroform (≥99.0%), and phenol/chloroform/isoamyl alcohol mixture (PCA, 25:24:1) were supplied by Sigma-Aldrich (Madrid, Spain). The TRIzol Reagent was purchased from Thermo Fisher Scientific (Barcelona, Spain). Synthetic miRNA hsa-miR-21-5p (miR-21-5p), hsa-let-7g-5p (let-7g-5p), and hsa-miR-16-5p with 3'-uridylation (iso-miR-16-5p) were purchased from IDT (Coralville, IA, USA).

Electrolyte solutions, sheath liquid, miRNA standards and serum samples.

All the solutions were stored in plastic bottles to minimize cation-adduct formation due to the glass-bottle Na^+ and K^+ leaching from and were degassed for 10 min by sonication before use. The optimized BGE for the CE-MS and SPE-CE-MS separation contained 25 mM NH_4Ac and was adjusted to pH 8.0 with ammonium hydroxide. The BGE was passed through a 0.20 µm nylon filter (Macherey-Nagel, Düren, Germany). The sheath liquid solution consisted of a mixture of 80:20 (v/v)

propan-2-ol/water with 2 mM NH_4Ac and was delivered at a flow rate of 3.3 $\mu L \cdot min^{-1}$ by a KD Scientific 100 series infusion pump (Holliston, MA, USA).

Stock solutions (100 $\mu mol \cdot L^{-1}$) of each miRNA were stored in a freezer at -20ºC. The concentrations of these standard solutions were confirmed by measuring the absorbance at 260 nm (10 mm path length) using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Rockford, IL, USA). Working standard solutions were prepared from the stock solutions by dilution in water and were stored in the fridge at 5ºC when not in use.

Human serum samples and data from donors were provided by the INCLIVA BioBank (PT13/0010/0004; B.0000768 ISCIII), integrated in the Valencian Biobanking Network and the Spanish National Biobanks Network. They were processed following standard operating procedures with the appropriate approval of the Ethical and Scientific Committees. Three healthy donor serum samples and three CLL serum samples from patients with stage IV of the disease were analyzed. All the samples corresponded to males and females aged between 75 and 85 years. Serum aliquots were stored in a freezer at -80ºC. Healthy and CLL samples were thawed and separately pooled before the analysis.

Apparatus

pH measurements were made with a Crison 2002 potentiometer and 52-03 electrode (Crison Instruments, Barcelona, Spain).

Centrifugal filtration was carried out at 25ºC in a cooled Rotanta 460 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany).

CE-MS

Fused silica capillaries were supplied by Polymicro Technologies (Phoenix, AZ, USA). All CE-MS experiments were performed in a 7100 CE coupled with an orthogonal G1603A sheath-flow interface to a 6220 oa-TOF LC/MS spectrometer (Agilent Technologies, Waldbronn, Germany).

ChemStation and MassHunter softwares (Agilent Technologies) were used for the CE and TOF mass spectrometer control, data acquisition and integration, and mass spectrum deconvolution. The TOF mass spectrometer was operated in negative electrospray ionization mode (ESI–) and the optimized parameters are presented in the Supporting Information.

Separations were performed at 15°C in a 72 cm long (L_T) × 75 µm i.d. × 365 µm o.d. capillary. All capillary rinses were performed at high pressure (930 mbar). For new ones and between workdays, the capillaries were flushed with 1 M NaOH (15 or 5 min), water (15 or 10 min), and BGE (30 or 15 min) off-line to avoid the unnecessary contamination of the MS system. The samples were hydrodynamically injected at 50 mbar for 10 s, and a separation voltage of +20 kV (normal polarity, cathode in the outlet) was applied. The autosampler was kept at 10°C using an external water bath (Minichiller 300, Peter Huber Kältemaschinenbau AG, Offenburg, Germany). Between consecutive runs, the capillary was conditioned by flushing with water (2 min) and BGE (2 min).

Sample stacking CE-MS

An electrophoretic preconcentration CE-MS method for the analysis of miRNA, described elsewhere[21], was adapted to our specific CE-MS setup. Separations were performed at 15°C in a 158 cm long (L_T) × 50 µm i.d. × 365 µm o.d. capillary with a

BGE of 25 mM NH_4Ac, adjusted to pH 6.0 with acetic acid. All capillary rinses were performed at high pressure (930 mbar). Before each injection, the capillary was rinsed with water (10 min) and BGE (3 min). Samples were injected at 930 mbar for 4 min (≈ 2.2 µL, according to the Hagen–Poiseuille equation[38]), and then the long plug of sample was pushed with BGE for 50 s (0.5 µL). Separation was performed at +20 kV.

SPE-CE-MS

The silicon carbide (SiC) resin was obtained from commercial microRNA purification kit spin columns (Norgen Biotek Corporation, Thorold, Canada).

Construction of the microcartridge or analyte concentrator was carried out as described elsewhere with little modifications[31,39,40]. The inlet end was prepared by connecting the microcartridge (0.7 cm L_T × 250 µm i.d. × 365 µm o.d. capillary) with a plastic sleeve to a previously conditioned inlet capillary (7.5 cm L_T × 75 µm i.d. × 365 µm o.d.). The microcartridge was completely filled by vacuum with the SiC particles. Another plastic sleeve was connected to the microcartridge, and a small piece of cotton (approximately 1 mm) was introduced in the plastic tube before connecting the separation capillary (64.5 cm L_T × 75 µm i.d. × 365 µm o.d.). This cotton frit prevented the SiC particles from leaking, which had promoted current instability or breakdown and poor reproducibility. Samples were hydrodynamically introduced at 930 mbar for 5 min (60 µL). A final flush for 40 s with BGE eliminated nonretained molecules and equilibrated the capillary before the electrophoretic separation. All these steps were performed by switching off the nebulizer gas and the ESI capillary voltage to prevent the entrance of contaminants into the MS. Then, both were switched on, a small volume of eluent with 60% (v/v) acetonitrile (ACN) was injected at 50 mbar for 10 s (50 nL); separation was conducted at +20 kV at 15°C, and 25 mbar of pressure was applied to

compensate for the microcartridge counter-pressure. Between consecutive runs, the capillary was rinsed for 4 min with water.

Before SPE-CE-MS, the serum samples were extracted with PCA or the TRIzol Reagent, both of which were followed by chloroform extraction. The detailed procedure is presented in the Supporting Information.

Quality parameters

The details regarding the estimation of the limit of detection (LOD), limit of quantification (LOQ), repeatability of migration time and peak area, linearity, and microcartridge lifetime in CE-MS ands SPE-CE-MS are given in the Supporting Information.

Results and discussion

CE-MS

CE-MS was optimized by analyzing standard solutions of the synthetic miR-21-5p and let-7g-5p miRNAs (Table S-1A).

Most MS studies on nucleic acids use ESI-, because it yields a better signal for such acidic compounds[21,41,42]. However, because the phosphate groups of the miRNAs have cation-binding sites, clusters corresponding to Na^+ and K^+ adducts are typically detected in the mass spectrum, decreasing the signal of the expected $[M\text{-}nH]^{n-}$ molecular ions. In order to increase the sensitivity of the detection of miRNA in ESI– and to decrease cation adduct formation, several BGE and sheath liquid compositions were tested. In general, an effective method to minimize alkali adduct formation is the addition of organic bases or ammonium salts[43]. In our case, appropriate results were

obtained with BGEs of ammonium acetate. Different BGE concentrations and acidities were tested at 10, 25, and 50 mM NH_4Ac and at pHs 6.0 and 8.0, adjusted with acetic acid and ammonium hydroxide, respectively. The lowest adduct formation and highest sensitivity were obtained with a BGE of 25 mM NH_4Ac at pH 8.0, in contrast to a previous study that reported a BGE of 25 mM NH_4Ac at pH 6.0 for the analysis of miRNA by sample stacking CE-MS[21]. This BGE was always freshly prepared in plastic tubes to minimize cation adduct formation due to the leaching of Na^+ and K^+ from glass bottles.

Different hydroorganic mixtures were also tested as sheath liquids, such as propan-2-ol, methanol, and acetonitrile at 60 and 80% (v/v). The highest sensitivity for miRNA detection was obtained with 80% (v/v) propan-2-ol (Figure S-1A). NH_4OH (0.5%, v/v) was also added to this mixture to improve miRNA ionization, but the sensitivity was lower than with ammonium salts as additives. The optimized sheath liquid contained 80% (v/v) propan-2-ol and 2 mM NH_4Ac, as in the Sample Stacking CE-MS method indicated before[21], which showed better results than those containing 1, 5, and 10 mM NH_4Ac.

As an example, Figure 1 shows the extracted ion electropherogram (EIE) and mass spectrum of a 5000 $nmol \cdot L^{-1}$ standard mixture of miR-21-5p and let-7g-5p in the optimized conditions. Both miRNA comigrated but showed molecular ions with different m/z values because of the molecular-mass differences. The most abundant ion was the $[M-5H]^{5-}$, and some Na^+ and K^+ adducts were still detected ($[M-6H+Na]^{5-}$ and $[M-6H+K]^{5-}$). Under these conditions, the LOD of the CE-MS method was 500 $nmol \cdot L^{-1}$ for both miRNAs.

Sample stacking CE-MS adaptation

Before exploring SPE-CE-MS, we adapted the sample stacking CE-MS method previously described for the analysis of miRNAs to the specific instrumental setup used in this study[21]. Sample stacking preconcentration during CE-MS was achieved by dissolving the sample in water, which had a lower conductivity than the BGE. A large volume of sample was injected hydrodynamically into the capillary between two plugs of BGE. When the separation voltage was applied, focusing and preconcentration of the ions happened at the narrow zone of the interface between the sample solution and the BGE because of a change in electrophoretic velocity due to a difference in the conductivities and, hence, the electric field.

As an example, Figure 2 shows the analysis of a 250 $nmol \cdot L^{-1}$ standard mixture of miR-21-5p and let-7g-5p in the adapted conditions. As expected from the results of the previous study[21], migration times were 5 times higher using sample stacking than by conventional CE-MS (Figure 2A), and the mass spectrum showed the same ion clusters as by CE-MS (compare Figures 2B and 1B). The LOD of the sample stacking CE-MS method adapted to our instrumental setup was 50 $nmol \cdot L^{-1}$, 10 times lower than that by CE-MS, 500 $nmol \cdot L^{-1}$. A slightly lower LOD (20 $nmol \cdot L^{-1}$) was obtained by sample stacking CE-MS in the original study[21], probably because of the better sensitivity of the mass spectrometer used in that case. Sample stacking allows a remarkable preconcentration factor, and in that study, it was demonstrated that it was possible to analyze miRNA from cancer serum samples[21]. As an alternative, in this study we investigated SPE-CE-MS to further enhance sensitivity, while reducing manual handling and increasing analysis throughput.

SPE-CE-MS optimization

The SiC resin from the spin microcolumns of the Norgen microRNA isolation kit was used to prepare the on-line SPE-CE microcartridges. The literature provided by the manufacturer cited better recoveries and linearities on miRNA purification than with silica-based or traditional phase-separation methods[44]. Another important aspect of the SiC resin was the small particle size, which facilitated packing the on-line microcartridges. The starting point for the optimization of the SPE-CE-MS methodology was the procedure recommended by the manufacturer, as well as on other procedures applied for the extraction of hydrophilic analytes, such as protein glycans, with graphitic carbon sorbents[45,46].

In SPE-CE-MS, after sample loading, the microcartridge located at the capillary inlet needed to be washed to eliminate nonspecifically retained molecules and also filled with BGE before the elution, separation, and detection. In preliminary studies using the optimized BGE for CE-MS (25 mM NH_4Ac, pH=8.0), some miRNA elution was detected during the washing and filling step with BGE (930 mbar, 2 min). This miRNA leakage was detrimental to achieving optimal sensitivity. Different strategies were explored, but it was found through those studies that changes in NH_4Ac concentration or pH and also ammonium phosphate and bicarbonate BGEs resulted in poorer performance.

Several modifications in the pressure and duration of the washing and filling step were, therefore, evaluated while the total volume of the flushed solution was minimized (4.0 μL, approximately 1.3 capillary volumes). This step was performed at low pressure (100 mbar, 360 s)[38], but lower sensitivities than those at high pressure (930 mbar, 40 s)[38] were obtained. Several other alternatives were explored, including the use of guanidinium chloride (<1 M) or alcohols, but the results were not better than those obtained with this fast and short washing and filling step. In the first case, the remaining guanidinium chloride caused later ion suppression during the analysis. In the second

case, despite the manufacturer's recommendation of the use of alcohols to increase miRNA retention by the SiC sorbent, methanol, ethanol, and propan-2-ol at concentrations between 10% and 70% (v/v) in the miRNA standard solutions or in the washing step promoted lower repeatability. This was probably because of the difficulty in rehydrating the sorbent or miRNA precipitation. In general, protocols for off-line miRNA extraction using spin columns include a drying step after sample loading and washing to evaporate the organic solvents. In SPE-CE-MS, we attempted to dry the microcartridge with air after sample loading and washing, prior to filling it with BGE. However, this procedural modification was discarded because bubbles were generated within the microcartridge, and electrophoretic separation was compromised.

Regarding the elution, ACN and MeOH hydroorganic mixtures at 40, 60 and 80% (v/v) were tested. The best sensitivity and repeatability of peak areas and migration times were obtained with a 60% (v/v) ACN (Figure S-1B) and no carry-over was observed between consecutive analyses.

The sample loading time was studied by loading a 35 $nmol \cdot L^{-1}$ standard mixture of miR-21-5p and let-7g-5p at 930 mbar from 3 to 20 min. As can be seen in Figure 3, the maximum amount of miRNA was detected between 5 and 10 min. Between 15 and 20 min of sample loading, analyte breakthrough caused a significant decrease of peak area. Hence, to reduce the total analysis time and to obtain the highest recoveries, a sample loading time of 5 min was selected as the best compromise for the rest of the experiments.

Under the optimized conditions, consecutive analyses of the standard mixture were repeatable in terms of migration times and peak areas: (at 50 $nmol \cdot L^{-1}$, the percent RSDs (n=3) were 2.8 and 4.4% for miR-21-5p and 2.8 and 6.4% for let-7g-5p. As can be seen in Figure S-2, the method was satisfactorily linear (r^2>0.97) between 25 and 100 $nmol \cdot L^{-1}$ for both miRNAs. When loading a concentration of 150 mM, the sorbent was

saturated, and there was not the expected increase in the peak areas. The LOD was 10 $nmol \cdot L^{-1}$, which was an improvement of 50 and 5 times compared with those of the CE-MS and sample stacking CE-MS methods, respectively. The LOQ was 25 $nmol \cdot L^{-1}$. The lifetimes of the microcartridges were around 25 analyses. As an example, Figure 4 shows the SPE-CE-MS analysis of a 50 $nmol \cdot L^{-1}$ miR-21-5p and let-7g-5p standard mixture in the optimized conditions. In comparison with CE-MS (Figure 1), the migration times were similar, the number of theoretical plates (N) was slightly higher, and the same ions were detected in the mass spectrum.

MicroRNA detection in cancer serum

The SPE-CE-MS method optimized with standards was applied to the analysis of biological fluids. Considering that miRNA levels in serum and plasma samples are similar[6] and that serum samples are more abundant in many clinical-sample repositories, serum samples were selected for miRNA profiling. Extracellular circulating miRNAs are found in the sera of both diseased and healthy people but at differing levels of concentration. miRNAs are resistant to endogenous RNase activity as well as extreme pHs and temperatures[6,8] because they are contained within microvesicles or in protein and lipoprotein complexes[9]. For this reason, no miRNAs were detected when serum samples were loaded without any off-line pretreatment (e.g., addition of a lysis buffer or phenol solution). In addition, current instability and breakdown during the electrophoretic separation was observed because of saturation of the sorbent with the sample matrix components due to nonspecific retention. Therefore, an off-line sample pretreatment was required to inhibit RNase activity, dissociate the nucleocomplexes, precipitate proteins, and extract the miRNAs. Two extraction procedures based on TRIzol and PCA were tested and followed by chloroform

extraction in both cases. In addition, it was necessary to desalt the extracts because the retention of the miRNAs was compromised as a result of the high abundance of salts. Drop dialysis was performed because it was a simple, rapid, and highly effective method to remove the salts from the small volume samples[47,48].

The sample pretreatments were optimized with healthy control serum samples spiked with the standard miRNAs, miR-21-5p and let-7g-5p. The PCA-extraction procedure was selected because of the limited compatibility of the TRIzol Reagent with MS, due to the presence of high concentrations of chaotropic salts (i.e., guanidinium thiocyanate). In the optimized conditions, the sample pretreatment recoveries were high, approximately 85%, calculated by a comparison of the SPE-CE-MS analyses for a 50 $nmol \cdot L^{-1}$ standard solution and a healthy control serum sample spiked at the same concentration. Consecutive analyses were repeatable in terms of migration times and peak areas (the percent RSDs (n=3) were 3.9 and 7.6% for miR-21-5p and 3.9 and 4.1% for let-7g-5p for the healthy control serum sample spiked at 50 $nmol \cdot L^{-1}$). Furthermore, the lifetimes of the microcartridges and LOD were similar to those with the standards (around 20 analyses and 10 $nmol \cdot L^{-1}$, respectively).

With the optimized sample pretreatment and SPE-CE-MS method, in concordance with the previous study using the sample stacking CE-MS method[21], no endogenous miRNAs were detected in the (unspiked) healthy control serum samples (Figure 5A,B), because of the extremely low abundance of miRNA in this type of samples. It is well-known that some miRNAs are up-regulated in sera from patients with different types of cancer[9,49–52]. CLL is a cancer of B-lymphocytes and is the most common type of leukemia in adults[37]. CLL derives from a combination of genetic (chromosomal abnormalities and gene mutations) as well as epigenetic (altered microRNA expression and DNA methylation) modifications[53]. Specific miRNA profiles have been associated with CLL progression, prognosis, and drug resistance[54]. With

SPE-CE-MS, in the analyzed CLL serum samples, two miRNAs were clearly detected at concentrations near the LODs (the EIEs and mass spectrum are shown in Figure 5C, D, respectively). The two miRNAs were tentatively identified using miRBase, which is a comprehensive database of miRNA sequences and expression (http://www.mirbase.org)[55]. The current version (miRBase 21) indexes 28645 miRNA entries and about 2000 sequences for humans. A screening of the most abundant miRNA species in CLL plasma and cell samples reported in the literature (Table S-2)[56–59] was performed, taking into account possible post-transcriptional modifications. The most likely possible candidates were miR-21-5p and miR-16-5p with 3′-uridylation (iso-miR-16-5p, Table S-1B). The analysis of a spiked CLL sample allowed the confirmation of the identities of the two miRNAs (Figure 5E,F). The detection of these two miRNA was in good agreement with the results reported for similar samples by sample stacking CE-MS and RT-qPCR[21,60]. miR-21-5p was the first serum miRNA biomarker discovered in the sera of CLL patients[10]; it is a representative oncogenic miRNA, and the overexpression of miR-21-5p as well as miR-16-5p in CLL has been previously reported[21,60]. In the future, the identities of the detected miRNAs could be unambiguously confirmed by high resolution tandem MS analysis with a hybrid mass spectrometer.

Concluding remarks

We have developed an on-line SPE-CE-MS method for the purification, preconcentration, separation, and characterization of endogenous microRNAs and their post-transcriptional modifications in serum. Under the optimized conditions with standards, the microcartridge lifetime (around 25 analyses) and repeatability (2.8 and <6.4% RSD for migration times and peak areas) were good, the method was linear

between 25 and 100 nmol·L^{-1}, and the LOD was 10 nmol·L^{-1} (i.e., 50 and 5 times lower than by CE-MS and sample stacking CE-MS, 500 and 50 nmol·L^{-1}, respectively). Regarding the analysis of the serum samples, it was necessary to apply an off-line sample pretreatment based on PCA extraction, but the recoveries for the miRNAs were >85%, and the performance of the SPE-CE-MS method was similar to that when the standards were used. Under the optimized conditions, it was possible to distinguish the healthy controls and CLL patients, because two endogenous miRNAs were detected at very low concentrations only in the diseased group. The results achieved show that SPE-CE-MS can be regarded as a promising technique for direct, high-throughput, sensitive, multiplex analyses capable of the preconcentration, quantification, and characterization of miRNA. The method also provides the unique structural information necessary for understanding the post-transcriptional regulation of miRNA function, and can be used in the discovery of new miRNA biomarkers for cancer and other diseases in noninvasive biological fluids, such as serum or plasma.

Supporting Information

Optimized MS parameters, serum-sample extraction, quality parameters, characteristics and molecular masses of the analyzed miRNAs, study of the sheath liquid in CE-MS and the eluent and linearity in SPE-CE-MS, and the list of miRNAs used for the screening of the CLL serum sample.

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The authors declare no competing financial interest.

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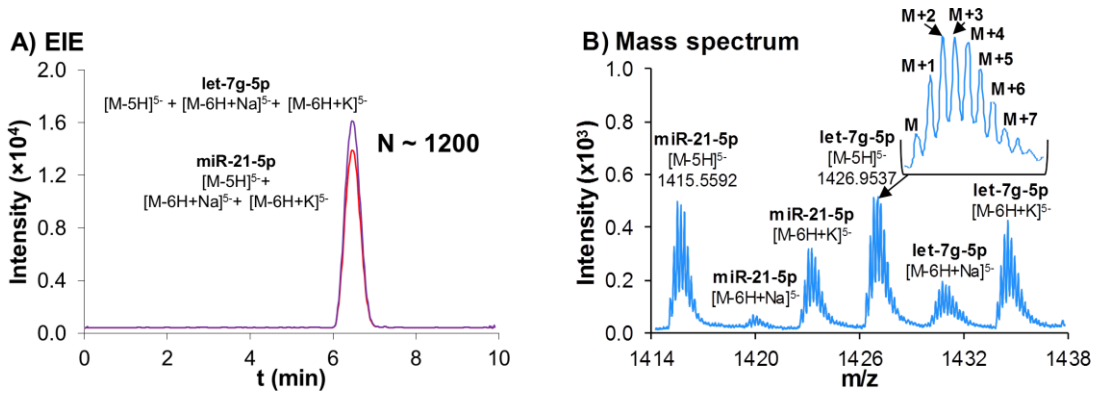


Figure 1. CE-MS for a 5000 nmol·L^{-1} miR-21-5p and let-7g-5p standard mixture. **(A)** Extracted ion electropherogram (EIE) and **(B)** mass spectrum. The number of theoretical plates (N) was calculated as $N = 5.54 \times (t_m/w_{1/2})^2$ where t_m is the migration time, and $w_{1/2}$ is the width at half the peak height.

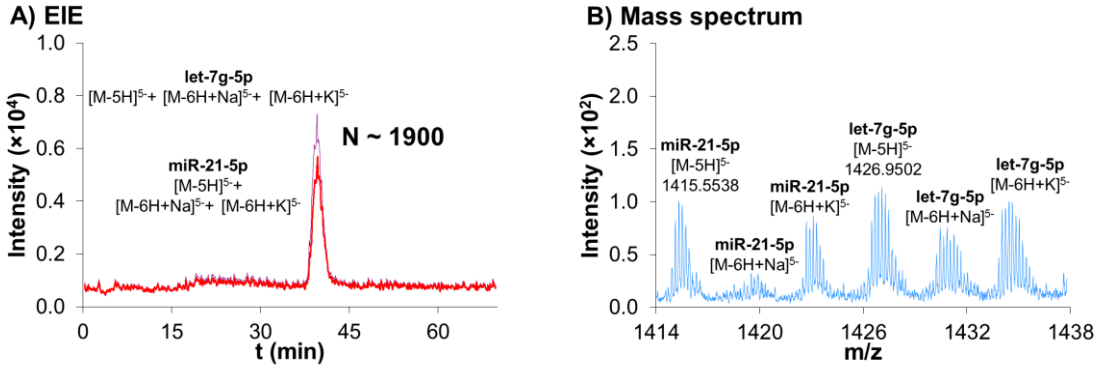


Figure 2. Sample stacking CE-MS for a 250 nmol·L^{-1} miR-21-5p and let-7g-5p standard mixture. **(A)** Extracted ion electropherogram (EIE) and **(B)** mass spectrum. The number of theoretical plates (N) was calculated as $N = 5.54 \times (t_m/w_{1/2})^2$ where t_m is the migration time, and $w_{1/2}$ is the width at half the peak height.

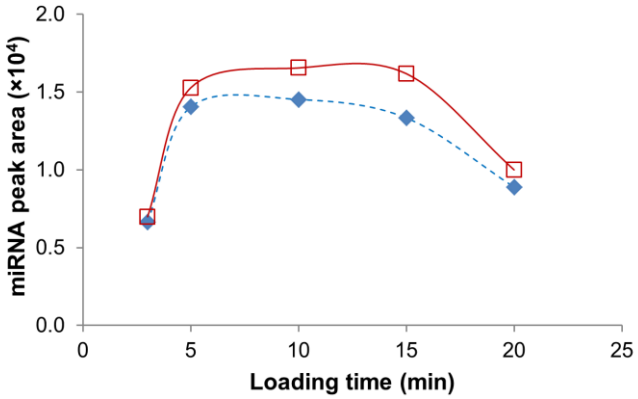


Figure 3. Plot of peak area of the eluted miR-21-5p (♦) and let-7g-5p (□) vs loading time at 930 mbar (35 nmol·L^{-1} standard mixture).

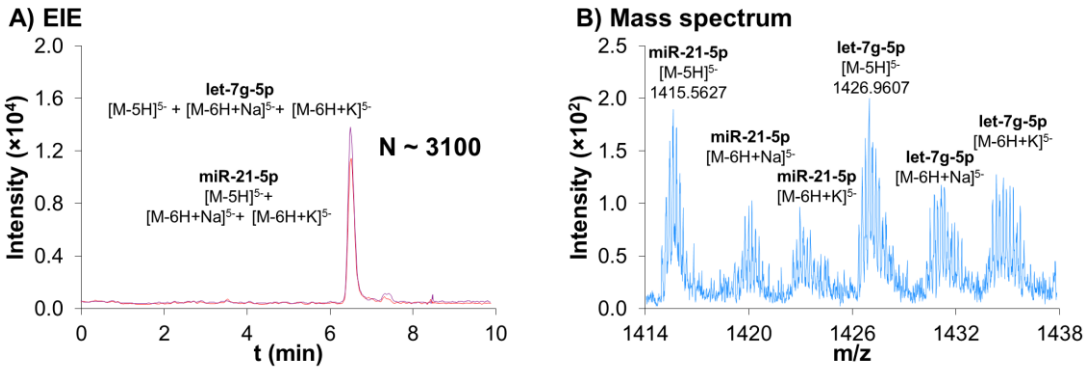


Figure 4. SPE-CE-MS for a 50 nmol·L^{-1} miR-21-5p and let-7g-5p standard mixture. **(A)** Extracted ion electropherogram (EIE) and **(B)** mass spectrum. The number of theoretical plates (N) was calculated as $N = 5.54 \times (t_m/w_{1/2})^2$ where t_m is the migration time, and $w_{1/2}$ is the width at half the peak height.

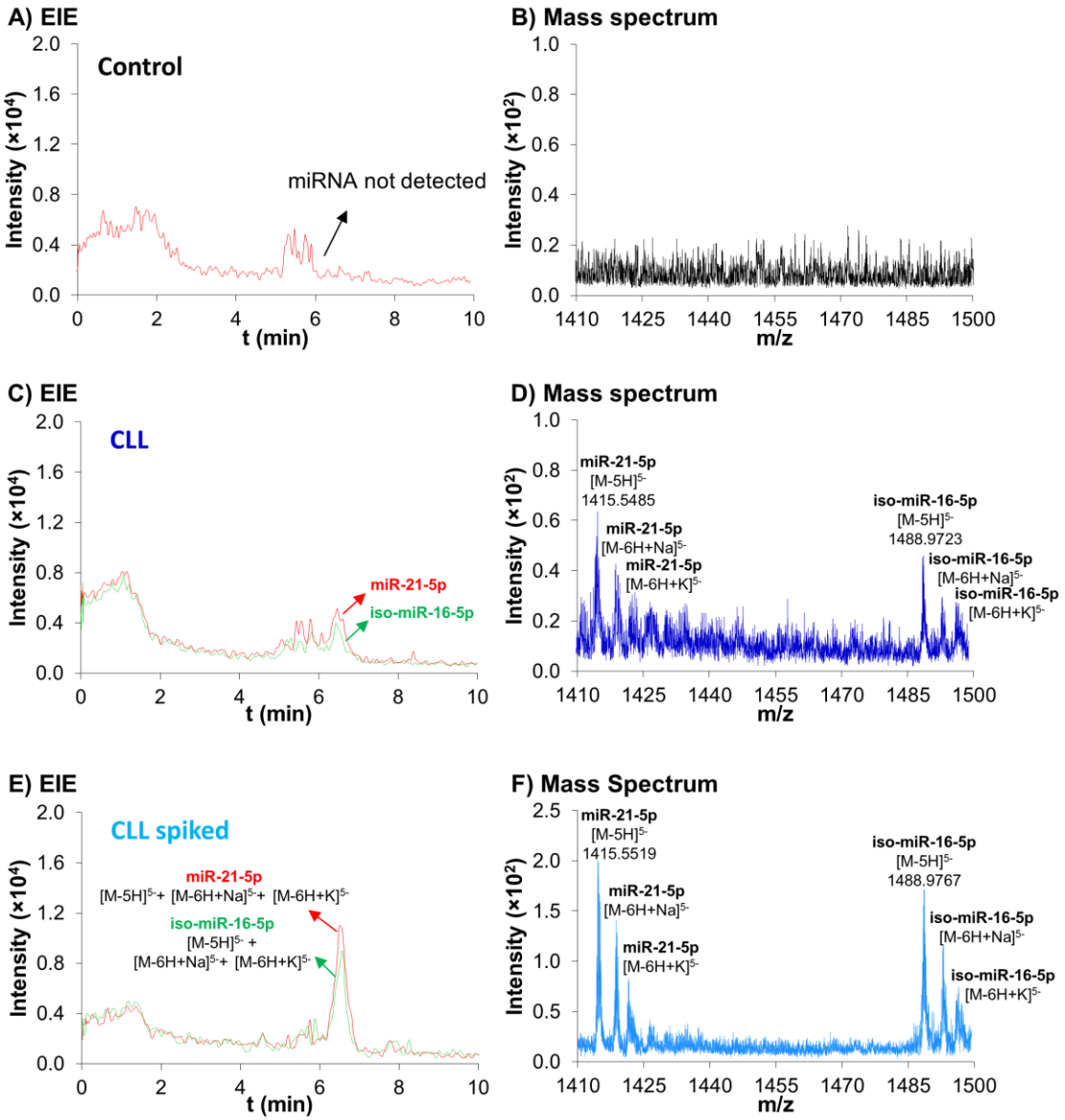
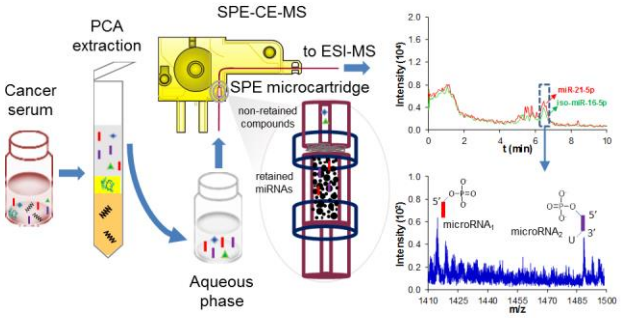


Figure 5. SPE-CE-MS for human serum samples. **(A,C,E)** Extracted ion electropherograms (EIEs) and **(B,D,F)** mass spectra of **(A,B)** a healthy control sample, **(C,D)** a CLL-patient sample, and **(E,F)** a CLL-patient sample spiked with 50 nM miR-21-5p and 50 nM iso-miR-16-5p. No endogenous miRNAs were detected in the control.



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Supporting Information

Analysis of circulating microRNAs and their post-transcriptional modifications in cancer serum by on-line solid-phase extraction capillary electrophoresis-mass spectrometry

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Optimized MS parameters

The MS parameters, optimized by infusion experiments with a 5000 $nmol \cdot L^{-1}$ let-7g-5p standard solution, were the following: capillary voltage 3500 V, drying gas temperature 350ºC, drying gas flow rate 6 $L \cdot min^{-1}$, nebulizer gas 7 psig, fragmentor voltage 225 V, skimmer voltage 70 V, OCT 1 RF Vpp voltage 300 V. Data were collected in profile at 1 spectrum/s between 100 and 3,200 *m/z*, with the mass range set to high resolution mode (4 GHz).

Serum samples extraction

According to the manufacturer's instructions, 0.3 mL of serum was mixed with 0.3 mL of PCA solution, centrifuged at 12,000 g and the aqueous phase (upper) was transferred to a new tube. To evaluate the extraction methodology, healthy control serum samples were spiked with 50 $nmol \cdot L^{-1}$ of the standard miRNAs, immediately after the first extraction to avoid degradation by endogenous serum RNases. Extraction with PCA was repeated twice more, until no protein was visible in the interface. Then, the aqueous phase was extracted with 0.2 mL chloroform to remove any residual phenol. Alternatively, for the extraction with the TRIzol Reagent, 0.3 mL serum was mixed with 3 mL of the TRIzol Reagent and 0.6 mL chloroform. In accordance to the manufacturer's instructions, the extraction was repeated only once. In both cases, drop dialysis of the obtained aqueous phases (upper) was performed with MF-Millipore membrane filters of mixed cellulose esters, with average pore size diameter of 25 nm and 25 mm of diameter (Millipore, Molsheim, France). The filter was floated with the glossy side up on a beaker with 50 mL of water. After 5 min for allowing the floating filter to wet completely, 100 µL of the sample extract was carefully placed on the center of the membrane and dialyzed for 1 hour at room temperature.

Quality parameters

All quality parameters were calculated from data obtained by measuring peak area and migration time (t_m) from the extracted ion electropherogram (EIE) considering the *m/z* of the most abundant miR-21-5p and let-7g-5p ions from the cluster resolved for the $[M-5H]^{5-}$, and the sodium and potassium adducts $[M-6H+Na]^{5-}$ and $[M-6H+K]^{5-}$.

An estimation of the LODs was obtained by analyzing low-concentration standard mixtures (close to the LOD level, as determined from the approach based on S/N=3). LOQ was determined from the approach based on S/N=10. Reproducibility in SPE-CE-MS was evaluated as the relative standard deviation (percent RSD) of peak areas and t_m. Linearity range was studied between 10 and 150 $nmol \cdot L^{-1}$. The lifetimes of the microcartridges was investigated by iteratively analyzing a standard mixture of 50 $nmol \cdot L^{-1}$.

Table S-1. Characteristics and molecular masses of the **(A)** standard miRNAs, **(B)** miRNA tentatively identified by SPE-CE-MS in a CLL-patient serum sample.

miRNA ID	Sequence	Modification	m/z $[M+2-5H]^{5-}$ a)		Calculated M_r ([M+2]) a)		
			Theoretical	Observed	Theoretical	Observed	Error (ppm)
(A) Standard miRNAs							
hsa-miR-21-5p (miR-21-5p)	UAGCUUAUCAGACUGAUGUUGA	5' phos	1415.5707	1415.5592	7082.89	7082.84	8
hsa-let-7g-5p (let-7g-5p)	UGAGGUAGUAGUUUGUACAGUU	5' phos	1426.9677	1427.9537	7139.88	7139.81	10
(B) miRNA tentatively identified in a CLL-patient serum sample							
hsa-miR-21-5p (miR-21-5p)	UAGCUUAUCAGACUGAUGUUGA	5' phos	1415.5707	1415.5485	7082.89	7082.78	15
hsa-iso-miR16-5p	UAGCAGCACGUAAAUAUUGGCGU	5' phos, 3'-U	1488.9890	1488.9723	7449.98	7449.90	11

a) M+2 corresponds to the most abundant ion of the isotopic distribution cluster resolved for the -5 molecular ion.

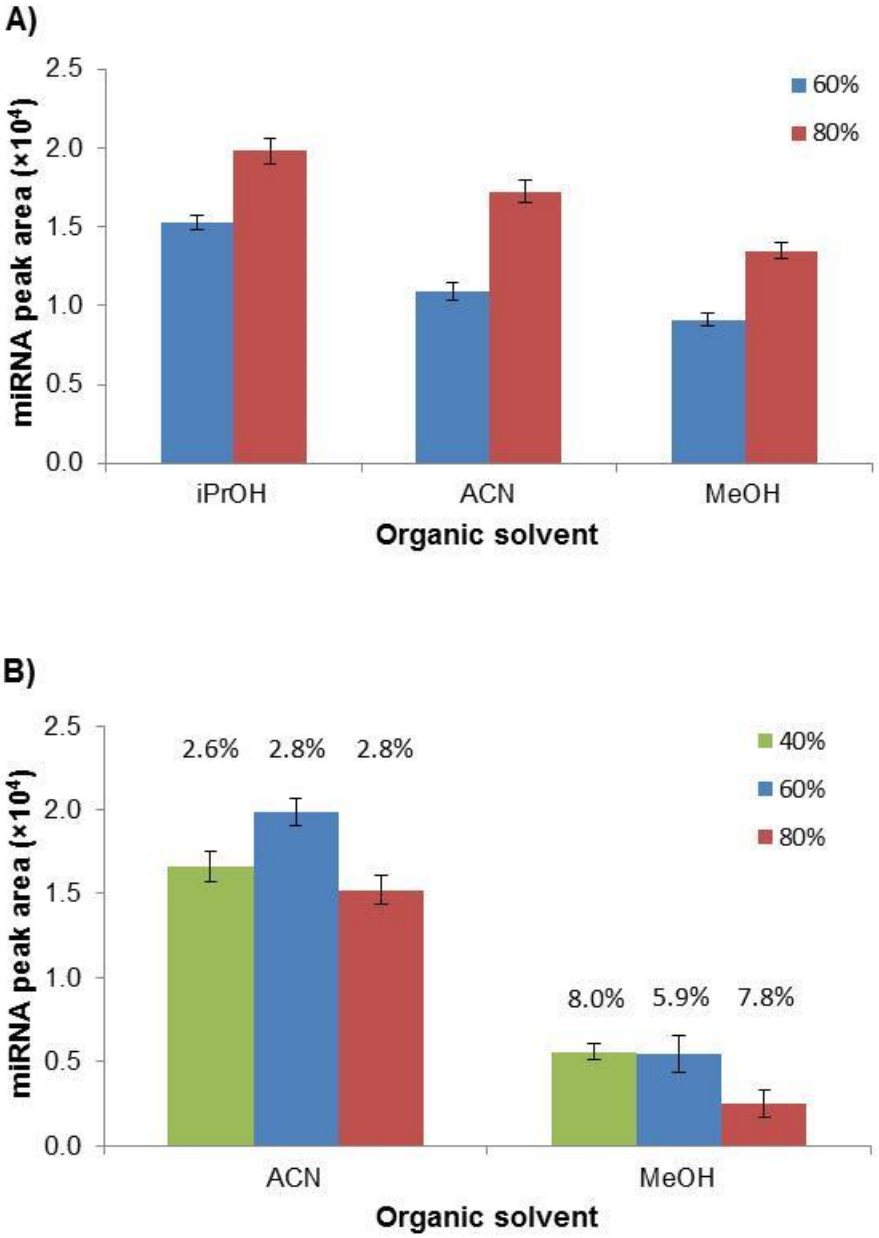

Figure S-1. Effect of the organic solvent **(A)** in the sheath liquid (CE-MS, 5,000 nmol·L^{-1} miR-21-5p standard) and **(B)** in the eluent (SPE-CE-MS, 50 nmol·L^{-1} miR-21-5p standard). Peak area of the injected (CE-MS) or eluted miRNA (SPE-CE-MS). All measurements were performed in triplicate (standard deviation is given as error bars, percentage RSD for migration times is given in numbers).

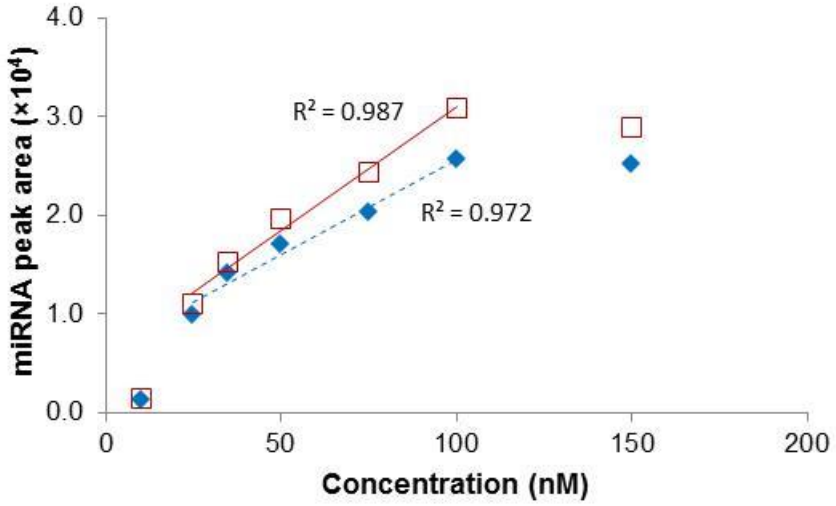

Figure S-2. Plot of peak area of the eluted miR-21-5p (♦) and let-7g-5p (□) vs concentration of the loaded standard mixture (930 mbar, 5 min). Regression line and R^2 value in the linearity range.

Table S-2. miRNAs reported in human B-cell chronic lymphocytic leukemia (CLL) plasma and cell samples used for the screening of endogenous miRNA in a CLL-patient serum sample by SPE-CE-MS (miRBase 21, http://www.mirbase.org [49]).

miRNA ID	Sequence	Length, nt
hsa-miR-15a-5p	UAGCAGCACAUAAUGGUUUGUG	22
hsa-miR-16-5p	UAGCAGCACGUAAAUAUUGGCG	22
hsa-miR-16-2-3p	CCAAUAUUACUGUGCUGCUUUA	22
hsa-miR-20a-5p	UAAAGUGCUUAUAGUGCAGGUAG	23
hsa-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA	22
hsa-miR-23b-5p	UGGGUUCCUGGCAUGCUGAUUU	22
hsa-miR-24-3p	UGGCUCAGUUCAGCAGGAACAG	22
hsa-miR-29a-5p	ACUGAUUUCUUUUGGUGUUCAG	22
hsa-miR-93-5p	CAAAGUGCUGUUCGUGCAGGUAG	23
hsa-miR-101-5p	CAGUUAUCACAGUGCUGAUGCU	22
hsa-miR-106a-5p	AAAAGUGCUUACAGUGCAGGUAG	23
hsa-miR-146a-5p	UGAGAACUGAAUUCCAUGGGUU	22
hsa-miR-150-5p	UCUCCCAACCCUUGUACCAGUG	22
hsa-miR-155-5p	UUAAUGCUAAUCGUGAUAGGGGU	23
hsa-miR-195-5p	UAGCAGCACAGAAAUAUUGGC	21
hsa-miR-221-5p	ACCUGGCAUACAAUGUAGAUUU	22
hsa-miR-221-3p	AGCUACAUUGUCUGCUGGGUUUC	23
hsa-miR-222-5p	CUCAGUAGCCAGUGUAGAUCCU	22
hsa-miR-483-5p	AAGACGGGAGGAAAGAAGGGAG	22
hsa-miR-486-5p	UCCUGUACUGAGCUGCCCCGAG	22