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Detection of MicroRNAs in Dried Serum Blots

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Abstract

We observed the preservation of microRNAs in unrefrigerated dried serum blots. Preservation was not adversely affected by drying or storing at 37, 45 or 60 °C instead of room temperature, but was harmed when blots were dried incompletely before storage. Preservation of microRNAs in serum was not diminished if instead of being kept frozen at –80 °C it was stored as dried blots at room temperature for five months or at 37 °C for four weeks. Dried blots can thus be a convenient and safer way to save, transport, and store serum for microRNA assays.

Keywords

blotting; dried serum; exosome; microRNA; RNA preservation; RNA stability; serum storage

Recent studies have shown the presence of extracellular microRNAs in blood, and this finding can enhance the utility of microRNAs as biomarkers to diagnose, prognosticate, and monitor disease status via non-invasive methods. Alterations in circulating microRNAs have been observed in diverse conditions such as breast cancer [1] and myocardial injury [2]. As naked RNA is rapidly degraded by the strong ribonuclease activity of plasma [3;4], it seems likely that microRNAs released upon cellular lysis do not survive in the circulation. Instead, microRNAs detected in the plasma are likely to be those within membrane-enveloped, 50-100 nm-sized secretory microvesicles termed exosomes that are released by cells into the extracellular space and that eventually enter the vascular compartment. Dried serum spots (DSS) have been used to assay different analytes, like anti-rubella antibodies [5] and cholesterol [6], but their suitability for analyzing microRNAs is unknown. Though the stability of RNAs is temperature-sensitive, often requiring storage of RNA or RNA-containing specimens in a frozen state, viral RNAs such as those of HIV-1 [7] can be detected in unrefrigerated DSS even after months of storage. We speculated that microRNAs too may be detectable in dried sera as, akin to viral RNAs, they are protected from ribonucleases by encapsulation and, possibly, from chemical hydrolysis by being in the macromolecule-rich interior of the exosome. To test this hypothesis, 45 µl fresh serum was spotted per 1x1 cm piece of 0.8 mm-thick, Whatman® GB003 pure cellulose paper (mean weight, 32 mg; range, 29-36; standard deviation, 3; 2.2% moisture-content as suggested by weighing before and after drying at 60 °C for three hours) and dried at room temperature (RT²; 21-24 °C). Weighing of the blots showed that drying was

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complete within an hour. DSS were stored at RT for 30 hours before being re-hydrated for 30 minutes at 4 °C for RNA extraction using Trizol™ for semi-quantification of *miR-16*, a microRNA abundant in serum [3], by reverse transcription-quantitative PCR (RT-qPCR; material and methods are detailed in supplementary material). The microRNA could be detected in RNA extracted from the dried blots (supplementary fig. 1), whereas no RT-qPCR signal was detected from blank blots that were not spotted with serum (data not shown). MicroRNA yield was highest when Trizol™ was used for re-hydration; water, phosphate-buffered saline, 50% Trizol™, and 6 M guanidine hydrochloride all reduced yields, with average C_q value increases of 1.5, 1.1, 0.4 and 1.3, with U test P values of 0.03, 0.03, 0.34, and 0.03, respectively (supplementary fig. 1). RNA yield was similar when the Trizol™-using re-hydration step was reduced from 30 to five minutes (supplementary fig. 2).

To test the effect of higher temperatures, DSS were dried or stored at 37, 45 or 60 °C, and microRNA yield was assessed by *miR-16* RT-qPCR after storage for three days. Drying at either 37 or 45 °C instead of RT did not reduce microRNA preservation (U test P values of 0.11 and 0.34, respectively), while drying at 60 °C improved it as indicated by an average increase of 1.3 in the C_q value (U test P = 0.03; fig. 1A). Storage of blots dried at RT at 37, 45 or 60 °C instead of RT for three days too did not reduce microRNA preservation (U test P values of 0.11, 0.20 and 0.89, respectively; fig. 1A). DSS prepared on glass fiber paper (Whatman® GF/F) instead of cellulose paper had poorer preservation of microRNAs as indicated by an average increase of 1.1 in the C_q value (U test P = 0.03; fig. 1B).

To test the effect of humidity, blots dried at RT were stored at 37 °C for 30 hours with or without exposure to 95% humidity. *miR-16* RT-qPCR assays suggested that the exposure to humidity during storage reduced microRNA preservation, with an average increase in C_q value of 0.6 which, however, was not statistically significant (U test P = 0.69; fig. 1C). The effect of humidity was also tested by comparing microRNA yield from blots that were dried fully or incompletely to 50% and stored at RT for 30 hours. As shown in fig. 1C, incompletely dried blots had a lower yield as suggested by an average increase in C_q value of 1.6 (U test P = 0.03).

To compare microRNA preservation in DSS to that in -80 °C -frozen liquid serum, blots prepared at RT were stored at -80 °C, RT or 37 °C for 7-28 days. RNA yield was assessed by both *miR-16* RT-qPCR and Ribogreen dye-based RNA assays (fig. 2A and 2B). Because the RNA preparations and assays were performed separately for each time-point, a cross-time-point comparison could not be made. However, comparisons of measurements for each sample-type at the four time-points using Wilcoxon matched-pairs signed rank tests showed that the performance of blots over time was not different from that of frozen liquid. For the *miR-16* RT-qPCR and the Ribogreen dye-based assays, respectively, P values were 0.63, 0.63 and 0.13, and 0.88, 0.88 and 0.25 for blots stored, respectively, at -80 °C, RT and 37 °C. A similar result was obtained in a second experiment (supplementary fig. 3). Even after five months of storage, microRNA yield from DSS kept at RT was not reduced compared to that from -80 °C-frozen liquid serum (fig. 2C; U test P = 0.06). Similar equivalence between frozen and dried sera was also observed when blots were stored at 45 °C for a week (supplementary fig. 4). To rule out an effect of the blot paper on the extractability of RNA, blank blots were added to some tubes of liquid serum immediately before RNA extraction. No significant effect of the cellulose paper-piece on RNA yield was seen (supplementary fig. 5).

To assess equivalent detectability of inter-individual variations in microRNA levels using frozen or dried sera, DSS were prepared with sera from ten individuals and stored at RT for 18 days. RNA was extracted from the blots as well as from -80 °C-frozen liquid sera, and

²CI, confidence interval; C_q , quantification cycle; DSS, dried serum spot; RT, room temperature; RT-qPCR, reverse transcription-quantitative polymerase chain reaction

levels of *miR-21* and *miR-223* were quantified by RT-qPCR (supplementary fig. 6). For *miR-21*, the Pearson correlation coefficient for C_q values for dried and liquid sera was 0.82 (95% confidence interval [CI] = 0.39-0.96; t test P value = 0.0037). For *miR-223*, the coefficient was 0.89 (95% CI = 0.60-0.97; P = 0.0005). For comparison, the coefficients for the duplicate sets of dried sera, for *miR-223*, and of the liquid sera, for *miR-21*, were 0.83 (95% CI = 0.42-0.96; P = 0.0030) and 0.85 (95% CI = 0.47-0.96; P = 0.0019), respectively.

With microRNAs emerging as biomarkers for diverse diseases, the ability to detect them in DSS can facilitate the development and use of microRNA-based assays by simplifying sample storage and transportation. We found that microRNAs can indeed be detected in DSS. Cellulose was a better matrix than glass-fiber, and Trizol™ was better than water or saline for re-hydrating the blots for RNA isolation (fig. 1B, supplementary fig. 1). Preservation of microRNAs was as good in dried serum spots as in -80 °C-frozen liquid sera even after storage of the blots at 45 °C for a week, at 37 °C for four weeks, or at RT for five months (fig. 2, and supplementary figs. 3-4). A high correlation was observed between measurements of specific microRNAs in RNA prepared from liquid and dried sera of ten individuals (supplementary fig. 6). The observation that drying or storage at 37, 45 or 60 °C did not reduce RNA yield from DSS (fig. 1A) suggests that microRNAs in DSS may remain preserved in spite of the high temperatures that are prevalent in certain geographical regions, or those which the blots may get exposed to during transportation. Incomplete drying of blots decreased microRNA preservation. Thus, as has been shown for viral RNAs [8], a detrimental effect of humidity on microRNA preservation was observed (fig. 1C). In this study humidity was not specifically controlled, e.g., through the use of desiccants, and it is possible that even better microRNA preservation may be achieved through the use of desiccants to ensure complete dryness during storage.

This exploratory study shows the feasibility of using dried serum spots for quantification of microRNAs and suggests that blotting and drying as paper spots can be a convenient and less hazardous way to save, transport, and store biological fluids for such purpose. Using material like SampleTanker™ [9], it may be feasible to handle volumes of serum larger than the 45 µl used in this study. Dried blots may also be usable for profiling microRNAs in other body fluids such as saliva [10] and urine [11]. It is also possible that non-microRNA RNA such as mRNAs that are present in exosomes might be similarly preserved. Because exosomes are generated as vesicles within a cell, microRNAs might be detectable in dried cellular material. Such findings can also have implications for fields such as forensic science and archaeobiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Effect of temperature, paper-type, and humidity on microRNA preservation in dried serum blots

miR-16 was semi-quantified in RNA from (A) three day-old blots prepared and stored at indicated temperatures (°C; *RT*, room temperature); (B) 30 hour-old blots prepared on glass fiber or cellulose; and, (C) 30 hour-old blots dried completely (+) or not (-) at *RT*, and stored at *RT* or 37 °C in presence (+) or absence (-) of 95% humidity. Mean and standard error of mean of *RT*-qPCR C_q values for quadruplicate samples are shown.

Figure 2. Equivalence of microRNA preservation in frozen and dried serum over time

A. *miR-16* was semi-quantified in RNA from sera kept frozen at -80°C or dried at RT for storage at indicated temperatures for 7-28 days. Samples from the same time-point were processed for RT-qPCR together but separately from those from other time-points. **B.** RNA yields (ng) from the samples as suggested by Ribogreen assay. **C.** Like A, but after storage for five months, with blots stored at RT. Mean and range of RT-qPCR C_q values (A, C) or RNA yields (B) for duplicate (A, B) or quadruplicate (C) samples are shown.