Detection of MicroRNAs in Dried Serum Blots

Santosh Kumar Patnaik, Reema Mallick and Sai Yendamuri

SUPPLEMENTARY MATERIAL

Material and Methods

Human serum samples

This study was approved by an institutional review board at the Roswell Park Cancer Institute. Frozen sera from ten individuals were obtained from the institute's Data Bank and BioRepository. Fresh serum from a healthy, young, male individual was prepared from venous blood collected without any additive using a 22 G needle, clotted at 37 ºC for 30-45 minutes in a polypropylene tube, and centrifuged twice for ten minutes at 1,500 g at room temperature (RT[a]). Frozen sera were kept at -80 ºC and were not thawed more than three times.

Preparation and storage of dried serum spots

Approximately 0.8 mm-thick GB003 blotting paper made of pure cellulose (Whatman®, Florham Park, NJ; www.whatman.com) was manually cut into 1x1 cm square pieces with a sterile steel blade. Forty-five µl of serum was blotted per piece, and the serum spots were dried on a clean, perforated, plastic surface for 1-2 hours under laminar airflow at RT (21 ºC to 24 ºC) under ambient humidity. Dried serum spots were stored in tightly capped, translucent, 2 ml or 15 ml polypropylene tubes in semi-darkness. Drying or storage at higher temperatures was done in air ovens without any humidity control. For storage under 95% humidity, a Steri-Cult™ cell culture incubator (Thermo®, Waltham, MA; www.thermo.com) was used. Samples of every sample-type were processed at least in duplicate in each experiment.

Extraction of RNA

RNA was extracted at the same time in an identical fashion from all samples being examined in an experiment. Four-hundred µl Trizol™ (Invitrogen®, Carlsbad, CA; www.invitrogen.com) was added to a 2 ml tube containing a 1x1 cm serum blot, and the tube was vortexed at 2000 rotations per minute at 4 ºC for 30 minutes on a Finemixer™ SH2000 vortexer (FinePCR®, Seoul, South Korea; www.finepcr.com). In some experiments, the tubes were kept at -80 ºC for up to a week before processing was resumed. Following further addition of 1.2 ml Trizol™ and then 0.24 ml chloroform, the tube was vortexed at RT for five minutes, and after another 2 minutes, centrifuged at 14,000 g at 4 ºC for 15 minutes. The top, aqueous phase was collected, mixed with an equal volume of ethanol, and processed on PureLink™ RNA spin-columns (Invitrogen®) on a QIAvac™ 24 Plus apparatus (Qiagen®, Valencia, CA; www.qiagen.com) as per manufacturers' suggested protocols. RNA was eluted from spin-columns using 100 µl water, and stored at -80 ºC. RNA from liquid serum was prepared similarly but with vortexing for only 2 minutes after adding Trizol™.

RNA quantification using Ribogreen dye

QuantIt™ Ribogreen RNA reagent (Invitrogen®) was used to quantify nucleic acid in less than 30 day-old RNA preparations in duplicate as per the method suggested by the manufacturer. Briefly, yeast tRNA (Ambion®, Austin, TX; www.ambion.com) was used to prepare standards

[a] C_q, quantification cycle; RT, room temperature; RT-qPCR, reverse transcription-quantitative polymerase chain reaction

of known concentration. RNA samples were diluted to 100 µl using 10 mM tris hydrochloride with 1 mM ethylenediaminetetraacetic acid at pH 7.5, and mixed with 100 µl of the same containing 2000x diluted Ribogreen. Fluorescence at 535 nm following excitation at 485 nm was measured for 0.1 s on a Victor Wallac™ 1420 plate reader (Perkin Elmer®, Waltham, MA; www.perkinelmer.com).

Semi-quantification of microRNAs by reverse transcription (RT)-quantitative PCR (qPCR)
Levels of mature microRNAs *miR-16*, *-21*, and *-223* were measured in less than ten day-old RNA preparations using TaqMan™ microRNA assays (Applied Biosystems®, Foster City, CA; www.appliedbiosystems.com). RNA preparations from the same time-point and/or experiment were assayed together. TaqMan™ microRNA reverse transcription kit (Applied Biosystems®) was used for reverse transcribing 9.9 µl RNA in 15 µl at 42 ºC for 30 minutes using a microRNA-specific oligonucleotide. MicroRNA-specific primers and 1.33 µl of RT reactions were used in triplicate 40- or 42-cycle quantitative PCR reactions of 20 µl volume in a 7900HT thermocycler (Applied Biosystems®); the denaturation step at 95 ºC was for 15 seconds, and the combined annealing and extension step at 60 ºC was for a minute. Fluorescence from the binding of microRNA-specific, carboxy-fluorescein dye-conjugated probes to amplification products was measured during PCR, and SDS™ software (version 2.3; Applied Biosystems®) was used to identify quantification cycle (C_q) values as the average of values from the triplicate PCR reactions. When an experiment was duplicated, all C_q values obtained in the second experiment were identically adjusted by addition of a constant such that the average C_q values for the reference sample-types (frozen serum, or serum blot dried and stored at RT) was the same in the two experiments.

Other
Statistical analyses and graphical plotting were done using Prism™ (version 5.0b; GraphPad Software®, La Jolla, CA; www.graphpad.com) and Excel™ software (version 2008 for Mac; Microsoft®, Redmond, WA; www.microsoft.com). All t and Mann Whitney U tests were two-tailed tests. The t tests assumed equal variances as suggested by P values of ≥ 0.05 in F tests.

Figure Legends
Supplementary figure 1
Effect of re-hydrating reagents on the recovery of microRNAs from dried serum blots.
MicroRNA *miR-16* was semi-quantified in RNA prepared from 30 hour-old dried serum spots prepared and stored at room temperature that were re-hydrated using indicated reagents (*PBS*, phosphate buffered saline; *GnHCl*, 6 M guanidine hydrochloride). Mean and standard error of mean of RT-qPCR C_q values for quadruplicate samples, and significant t test P values in comparisons with the *Trizol* group are shown.

Supplementary figure 2
Effect of the duration of the Trizol™-based re-hydration step on the recovery of microRNAs from dried serum blots. MicroRNA *miR-16* was semi-quantified in RNA prepared from identical dried serum blots with a Trizol™-using re-hydration step of either five or 30 minutes. Mean and range of RT-qPCR C_q values for duplicate samples are shown. The t test P value was 0.34.

Supplementary figure 3

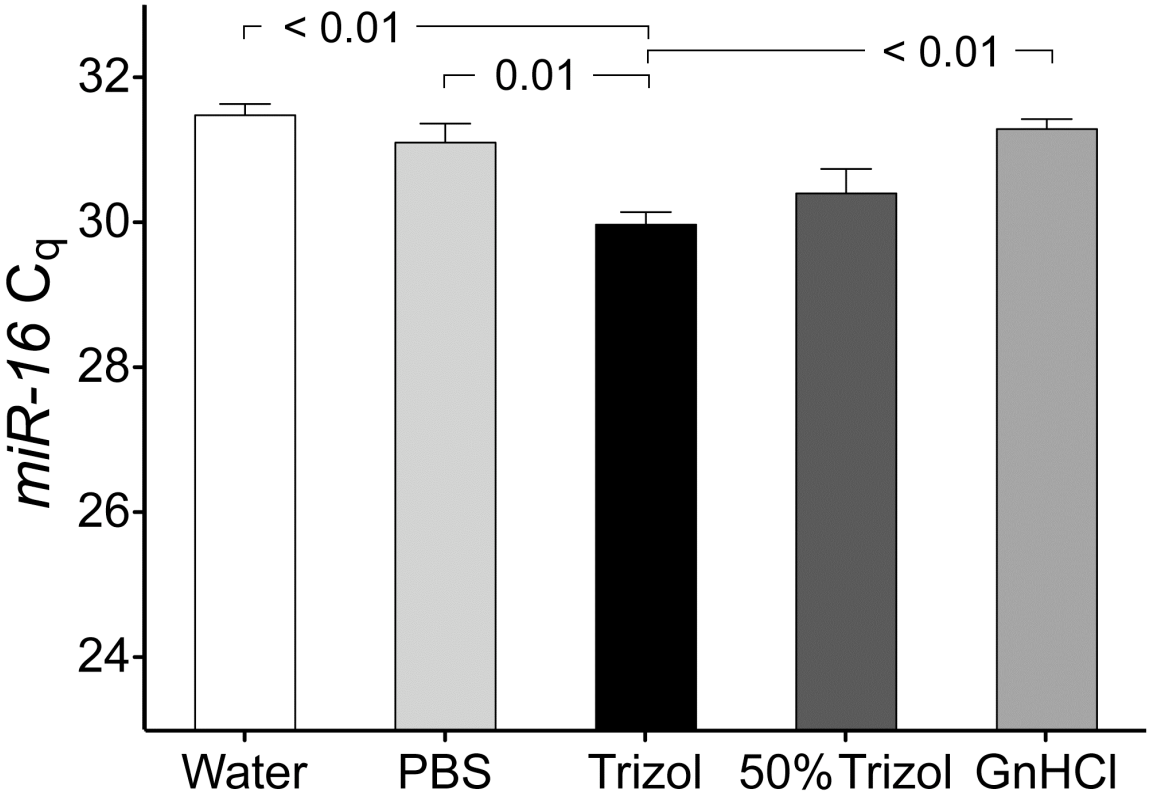
Equivalence of microRNA preservation in frozen and dried serum over time in a second time-series experiment. MicroRNA *miR-16* was semi-quantified in RNA prepared from serum that was kept frozen at -80 ºC or dried at room temperature for storage at indicated temperatures for 7, 14, 21 or 28 days in a second time-series experiment. Samples from the same time-point were processed for RT-qPCR together but separately from those from other time-points. Mean and range of RT-qPCR C_q values for duplicate samples are shown. Comparisons of the average C_q value observed for each sample-type at the four time-points along the time-series using paired t tests showed that the performance of blots over time was not different from that of frozen liquid serum (P values of 0.69, 0.43 and 0.48, respectively, for blots stored at -80 ºC, room temperature, and 37 ºC). P values in non-parametric Wilcoxon matched-pairs signed rank tests were 0.88, 0.63 and 0.38, respectively.

Supplementary figure 4
Effect of storage of blots at 45 ºC. MicroRNA *miR-16* was semi-quantified in RNA prepared from week-old sera either stored frozen at -80 ºC or as dried spots prepared at room temperature and stored at 45 ºC. Mean and range of RT-qPCR C_q values for duplicate samples are shown. The t test P value was 0.10.

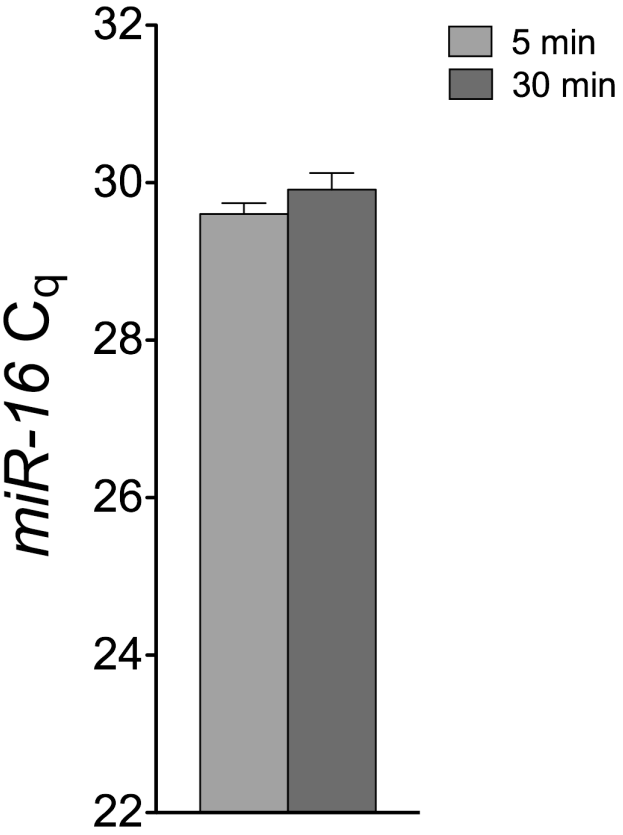
Supplementary figure 5
Effect of cellulose blot paper on the extractability of microRNAs from liquid sera. MicroRNA *miR-16* was semi-quantified in RNA prepared from equal volumes of liquid sera in presence (+) or absence (–) of a blank piece of 1 cm x 1 cm blot paper. The paper piece was added to sera in a micro-centrifuge tube just before RNA was extracted. Mean and range of RT-qPCR C_q values for duplicate samples are shown. The t test P value was 0.30.

Supplementary figure 6
Correlation of microRNA measurements in frozen and dried sera. MicroRNAs *miR-21* (*black*) and *miR-22*3 (*grey*) were semi-quantified in RNA prepared from sera from ten individuals that were kept frozen at -80 ºC, or dried and stored for 18 days at RT. Mean and range of RT-qPCR C_q values for duplicate samples, and best-fitting linear regression lines are shown.

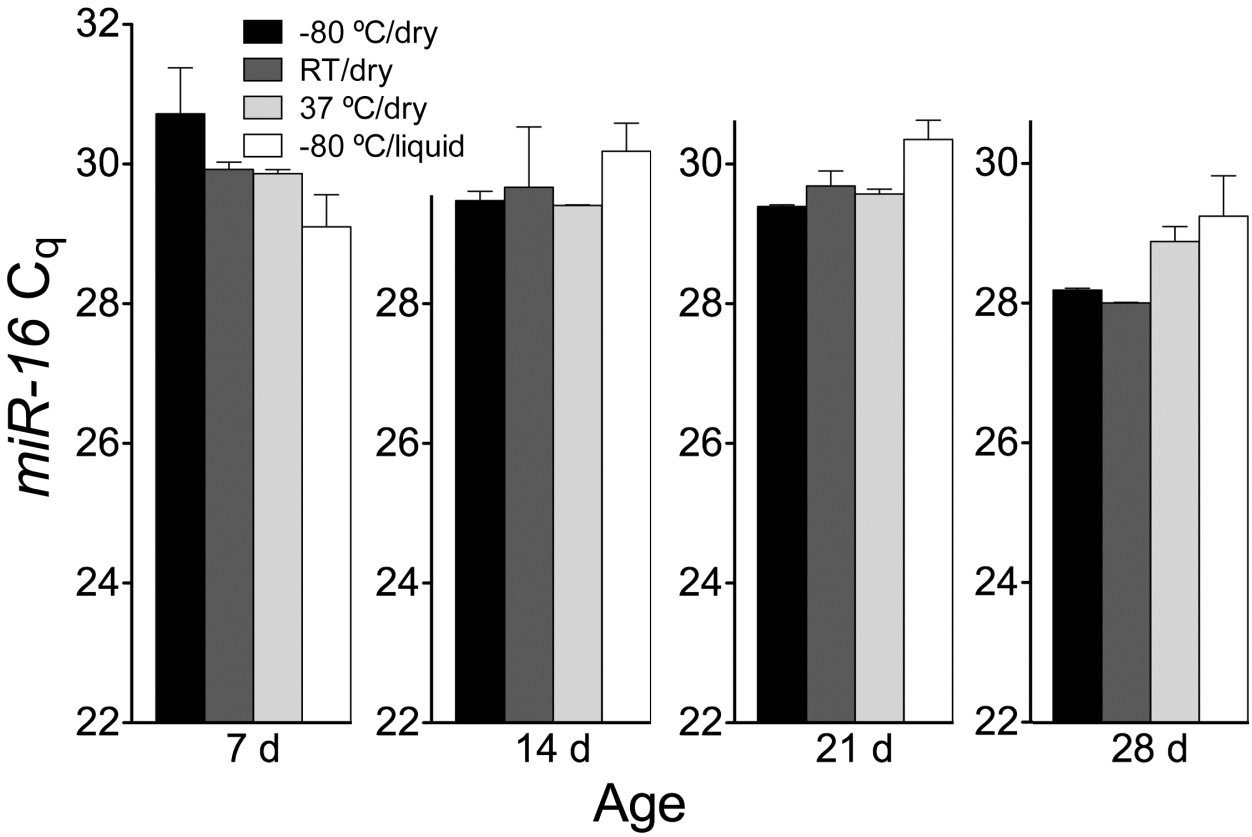
Supplementary figure 1



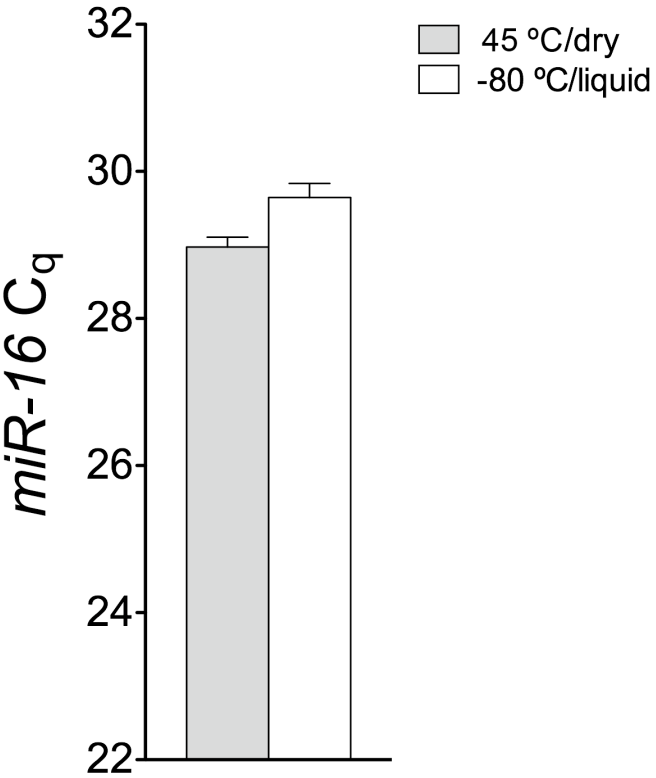
Supplementary figure 2



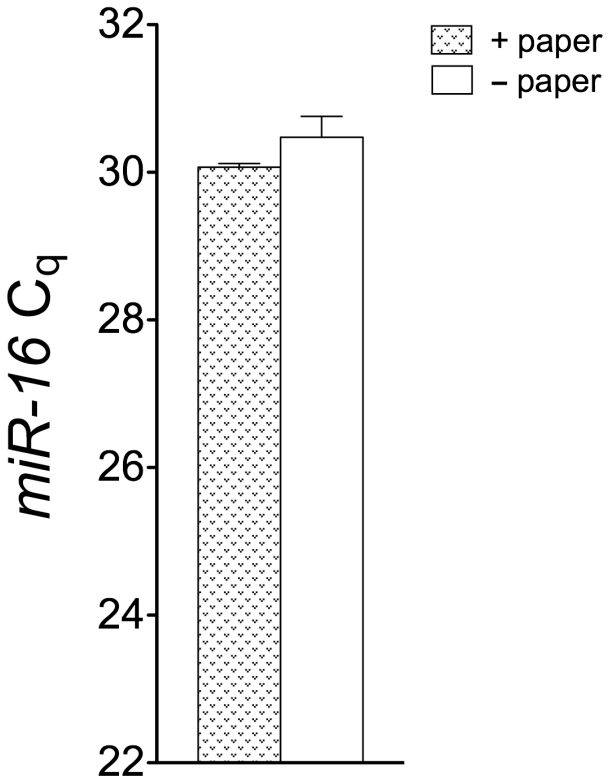
Supplementary figure 3



Supplementary figure 4



Supplementary figure 5



Supplementary figure 6