## Title

Optimizing preservation of extracellular vesicular miRNAs derived from clinical cerebrospinal fluid



## Journal

Cancer Biomarkers, 17(2)

## ISSN

1574-0153

## Authors

Akers, Johnny C
Ramakrishnan, Valya
Yang, Isaac
et al.

## Publication Date

2016-03-25

## DOI

10.3233/cbm-160609

Peer reviewed

HHS Public Access

Author manuscript

*Cancer Biomark*. Author manuscript; available in PMC 2016 August 20.

Published in final edited form as:

*Cancer Biomark*. 2016 March 25; 17(2): 125–132. doi:10.3233/CBM-160609.

# Optimizing Preservation of Extracellular Vesicular miRNAs Derived from Clinical Cerebrospinal Fluid

**Johnny C. Akers**[1], **Valya Ramakrishnan**[1], **Isaac Yang**[2], **Wei Hua**[3], **Ying Mao**[3], **Bob S. Carter**[1,†], and **Clark C. Chen**[1,†]

[1]Center for Theoretical and Applied Neuro-Oncology, University of California, San Diego, CA

[2]Department of Neurosurgery, University of California, Los Angeles, CA

[3]Department of Neurosurgery, Huashan Hospital, Fudan University, Shanghai, China

## Abstract

**BACKGROUND—**Tumor specific genetic material can be detected in extracellular vesicles (EVs) isolated from blood, cerebrospinal fluid (CSF), and other biofluids of glioblastoma patients. As such, EVs have emerged as a promising platform for biomarker discovery. However, the optimal procedure to transport clinical EV samples remains poorly characterized.

**METHODS—**We examined the stability of EVs isolated from CSF of glioblastoma patients that were stored under different conditions. EV recovery was determined by Nanoparticle tracking analysis, and qRT-PCR was performed to determine the levels of miRNAs.

**RESULTS—**CSF EVs that were lyophilized and stored at room temperature (RT) for seven days exhibited a 37–43% reduction in EV number. This reduction was further associated with decreased abundance of representative miRNAs. In contrast, the EV number and morphology remained largely unchanged if CSF were stored at RT. Total RNA and representative miRNA levels were well-preserved under this condition for up to seven days. A single cycle of freezing and thawing did not significantly alter EV number, morphology, RNA content, or miRNA levels. However, incremental decreases in these parameters were observed after two cycles of freezing and thawing.

**CONCLUSIONS—**These results suggest that EVs in CSF are stable at RT for at least seven days. Repeated cycles of freezing/thawing should be avoided to minimize experimental artifacts.

## INTRODUCTION

Development of “liquid biopsy” platforms for monitoring response to cancer therapeutics has the potential to fundamentally reshape the treatment paradigm for oncologic care [1,2]. The discovery that neoplastic cells secrete Extracellular Vesicles (EVs) that contain cancer

Corresponding author: Clark C. Chen, M.D., Ph.D., Center for Theoretical and Applied Neuro-Oncology, University of California, San Diego, Phone: 858 246 09674, FAX: 858 534 8899, clarkchen@ucsd.edu.

†These authors shared responsibility as senior authors

**COMPLIANCE WITH ETHICAL RESEARCH STANDARDS**

The authors declare no conflicts of interest. All research on human subjects presented in this paper was conducted in accordance with the ethical research standards prescribed by the responsible national/institutional committee on human experimentation and with the WMA Declaration of Helsinki as of its 7th revision in 2013. Informed consent was obtained from all human subjects participating in the study.

specific or enriched genetic material has led to mounting interest in EVs as a platform for liquid biopsy [2,3,4]. Fueling this interest are studies documenting that cancer specific EVs can be detected in the bio-fluid of cancer patients [5,6,7].

We previously demonstrated that the biologic and clinical behavior of glioblastomas [8,9], the most aggressive form of brain cancer is regulated by key microRNAs (miRNAs) [10,11,12,13]. Moreover, we showed that profiles of cerebrospinal fluid (CSF) derived from glioblastoma patients can serve as a biomarker for tumor burden and, by extension, therapeutic response [6,14]. Clinical translation of this and other observations will undoubtedly require trials that necessitate transport of bio-specimens to centralized sites for molecular characterization. However, the optimal procedure to transport clinical EV samples remains poorly characterized. Here we explored the effect of lyophilization and freeze thawing on the stability of EVs and their associated miRNAs, with the goal of developing the optimal condition for transport.

## METHODS

### Clinical Specimen Collection

All research performed were approved by IRB boards at University of California, San Diego Human Research Protections Program under IRB 120345X and were in accordance with the principles expressed at the declaration at Helsinki. Each patient was consented by a dedicated clinical research specialist prior to collection. Written consent was obtained for each patient. The consent process was approved by the ethics committee, and all records were documented in our electronic record system. The written consent from patients was also scanned into our electronic filing system. CSF was collected by ventricular/lumbar drain placement or cisternal aspiration at the time of craniotomy. Collected CSF specimens were 0.8μm filtered, snapped frozen and stored at −80°C.

### EV Isolation

The EV fraction was isolated by differential centrifugation as previously described [6,15]. CSFs were diluted 1:1 with 1× PBS (Mediatech) prior to centrifugation. Samples were centrifuged at 2,000×g for 20 min to remove cellular debris. The supernatant was further centrifuged at 120,000×g for 2 h in a Type 70 Ti rotor (Beckman) to pellet the EVs. All centrifugation steps were performed at 4°C. EV pellets were resuspended in PBS and stored at −80°C.

### EV Lyophilization

Equal volume of EVs was aliquoted into 1.5ml tubes and frozen at −80°C. Frozen EVs were than lyophilized using a FreeZone Benchtop Freeze Dry system for 24 hours (Labconco). Lyophilized samples were stored at room temperature for seven days. To rehydrate, the lyophilized EVs were resuspended with distilled water to the same volume as the pre-lyophilized samples and incubated on ice for 15 min prior to any further analyses.

### EV Quantification and Assessment

The number of vesicles recovered was determined by Nanoparticle Tracking Analysis (NTA) on a Nanosight LM-10HS equipped with a 405nm laser (Malvern) that was calibrated with polystyrene latex microbeads at 100 nm and 200 nm prior to analysis. Resuspended vesicles were diluted with PBS to achieve between 20–100 objects per frame. EVs were manually injected into the sample chamber at ambient temperature. Each sample was measured in triplicate at camera setting 13 with acquisition time of 30 s and detection threshold setting of 7. At least 200 completed tracks were analyzed per video. The NTA analytical software version 2.3 was used for capturing and analyzing the data.

The morphology of the EV isolated were assessed using transmission electron microscopy (TEM) as previously described [15].

### Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

RNA from the EV fraction was extracted using the Qiagen miRNeasy Mini Kit. RNA concentration and quality were determined using the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific) or Ribogreen™ assay. 20 ng of RNA was used for cDNA synthesized using the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon). The resultant cDNA were diluted 20× and four microliter was used for qRT-PCR in triplicate wells as previously described [6].

### Determination of miRNA Copy Number

To determine copy number of miRNA (miR-21, miR-103, miR-24, miR-125), standard curves were generated by serial dilution of known quantities of miRNA mimic (Qiagen), followed by cDNA synthesis using the miRCURY LNA™ Universal RT microRNA PCR system [16]. The cDNAs were then used for the generation of standard curve.

## RESULTS

### Clinical CSF EVs stored under Room Temperature (RT) and −80°C

We wished to determine the general stability of EVs in clinical cerebrospinal fluids derived from glioblastoma patients when stored at room temperature. To this end, CSF samples that were stored at room temperature for 1 and 7 days after collection from patient and before EV extraction. The EV yield was compared to reference samples that were immediately stored at −80°C after collection. When analyzed by Nanoparticle Tracking Analysis (NTA) [17], the total number of EVs recovered (Figure 1A) or the size profile of EVs (Figure 1C) did not significantly differ. Transmission Electron Micrograph (TEM) revealed no detectable change in morphology of EVs isolated from CSF stored in RT for 1 day, RT for 7 days or −80°C for 7 days (Figure 1B). We further extracted total RNA from EVs isolated after CSF was stored in various conditions. We previously demonstrated the abundance of miR-21, miR-24, miR-103, and miR-125 in clinical CSF EV [14]. Here we use changes in the abundance of these representative miRNAs as a gauge of the effects of storage condition on EV miRNA stability. We observed no significant difference in total RNA extracted (Figure 1D) as determined by UV absorbance reading. Since material other than RNA can also contribute to the absorbance readings, we also quantified the RNA recovered using a

fluorescent nucleic acid stain, Ribogreen. While total RNA yield as determined by RiboGreen assay was lower than NanoDrop, the relative abundance of RNA between samples was largely unaffected (Supp Table 1). Similarly, the levels of the various representative miRNA transcripts were found to be stable between CSF stored at −80°C or CSF stored at room temperature (Figure 1E). We thus conclude that EV miRNAs appeared to be stable in CSF at room temperature for up to 7 days.

### Effects of lyophilization on clinical CSF EV stability and EV associated miRNAs

Lyophilization is a commonly used method for long-term preservation of DNA and RNA. We wish to test the effect of lyophilization on EV stability and the stability of EV associated miRNAs. EVs were isolated from CSFs, resuspended in PBS, aliquoted, and lyophilized using a commercial benchtop freeze-dryer. Lyophilized samples were stored at room temperature for 7 days prior to rehydration with distilled water and then subjected to further analysis. Compared to non-lyophilized EVs stored at −80°C, we observed a significant reduction (37 to 43%) of EV numbers in two out of three CSF specimens processed in this manner (Figure 2A). However, this reduction in EV number was not associated with significantly changes in EV sizes (Figure 2C) or in EV morphology on TEM (Figure 2B). These results suggest that lyophilization induced EV disintegration. Although we did not noticed a significant difference in total RNA yield when compared to non-lyophilized controls (Figure 2D and Supp Table 2). When representative miRNAs (miR-21, miR-24, miR-103, and miR-125) were quantitated by qRT-PCR, we observed a significant reduction (up to 80%) in miRNA levels in the lyophilized samples (Figure 2E). These results suggest that lyophilization of EVs isolated from clinical CSF should be avoided if miRNAs is the analytic platform of choice.

### Effects of multiple freeze-thaw cycles on clinical CSF EV

Clinical bio-fluid such as CSF is often stored at −80°C after collection. We wished to determine the effect of freeze-thaw cycles [18] on the stability of EVs and their miRNA cargo. EVs were isolated by differential centrifugation after the clinical CSFs were subjected to 1, 2 or 3 freeze thaw cycles. A single cycle of freezing and thawing did not significant alter EV number (Figure 3A), EV size (Figure 3B), or EV morphology (Figure 3C). However, additional freeze-thaw cycles lead to incremental decreases in the number of EV recovered (Figure 3A). We observed an approximate 30% reduction in EV number after 3 consecutive freeze-thaw cycles. Both total RNA yield and the abundance of the representative miRNAs (miR-21, miR-24, miR-103, and miR-125) were stable after 1 freeze-thaw cycle (Figure 3D and 3E, Supp Table 3). However, additional freeze thaw cycles (>2 cycles) lead to incremental decreases in the total RNA and the abundance of the representative miRNAs (miR-21, miR-24, miR-103, and miR-125).

## DISCUSSION

While CSF EV miRNAs have been proposed as biomarkers for brain tumors [5,6] and other neurodegenerative diseases [19], it remains unclear as to the optimal conditions for storage and transport for maximizing the preservation of these samples. Undoubtedly, validation of any biomarker platform and clinical translation of such platform will require the transfer of

bio-fluids between clinical centers. Our study demonstrates several key considerations for maximizing the EV associated miRNA derived from clinical CSF samples. While lyophilization is generally associated with increased stability of DNA and RNAs [20,21], our results show that this process disrupts EVs isolated from clinical CSF, which in turn compromises the recovery of miRNAs from these specimens. The findings may harbor implications in the utilization of commercially available, lyophilized EVs. In contrast, the CSF EVs and their associated miRNAs appear remarkably stable when stored at room temperature for up to 7 days of storage.

Another key consideration involve serial degradation of CSF EV and EV associated miRNAs upon repeat freeze thawing. While one single freeze-thaw cycle did not significantly affect the content of CSF EVs and EV associated miRNAs, additional freeze thaw cycles (>2 cycles) lead to incremental decreases in EV number, total RNA, and the abundance of the representative miRNAs (miR-21, miR-24, miR-103, and miR-125).

Based on our results, we propose the following considerations for the storage and processing of CSF EVs with intent for miRNA based analytics. First, clinical CSF should be aliquoted to appropriate volume required for the miRNA based analytic prior to storage at −80°C as to avoid unnecessary freeze thawing. Second, CSF can be safely stored and transported at room temperature for up to seven days. Finally, lyophilization of CSF EVs should be avoided in general.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

The work is supported by NIH UH2 TR000931-0, NIH PO1 2P30CA023100-28. Additional funding also provided by the Doris Duke Charitable Foundation Clinical Scientist Development Award, Sontag Foundation Distinguished Scientist Award, Burroughs Wellcome Fund Career Awards for Medical Scientists, the Kimmel Scholar Award, a Grant from Accelerated Brain Cancer Cure, the William Guy Forbeck Research Foundation, and Program of International Science & Technology Cooperation of China (2014DFA31470).

## REFERENCES

1. Crowley E, Di Nicolantonio F, Loupakis F, Bardelli A. Liquid biopsy: monitoring cancer-genetics in the blood. Nat Rev Clin Oncol. 2013; 10:472–484. [PubMed: 23836314]
2. Santiago-Dieppa DR, Steinberg J, Gonda D, Cheung VJ, Carter BS, et al. Extracellular vesicles as a platform for 'liquid biopsy' in glioblastoma patients. Expert Rev Mol Diagn. 2014; 14:819–825. [PubMed: 25136839]
3. Rabinowits G, Gercel-Taylor C, Day JM, Taylor DD, Kloecker GH. Exosomal microRNA: a diagnostic marker for lung cancer. Clin Lung Cancer. 2009; 10:42–46. [PubMed: 19289371]
4. Taylor DD, Gercel-Taylor C. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecol Oncol. 2008; 110:13–21. [PubMed: 18589210]
5. Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol. 2008; 10:1470–1476. [PubMed: 19011622]
6. Akers JC, Ramakrishnan V, Kim R, Skog J, Nakano I, et al. MiR-21 in the extracellular vesicles (EVs) of cerebrospinal fluid (CSF): a platform for glioblastoma biomarker development. PLoS One. 2013; 8:e78115. [PubMed: 24205116]

7. Resnick KE, Alder H, Hagan JP, Richardson DL, Croce CM, et al. The detection of differentially expressed microRNAs from the serum of ovarian cancer patients using a novel real-time PCR platform. Gynecol Oncol. 2009; 112:55–59. [PubMed: 18954897]
8. Bartek J Jr, Ng K, Bartek J, Fischer W, Carter B, et al. Key concepts in glioblastoma therapy. J Neurol Neurosurg Psychiatry. 2012; 83:753–760. [PubMed: 22396442]
9. Ng K, Kim R, Kesari S, Carter B, Chen CC. Genomic profiling of glioblastoma: convergence of fundamental biologic tenets and novel insights. J Neurooncol. 2012; 107:1–12. [PubMed: 22002595]
10. Kushwaha D, Ramakrishnan V, Ng K, Steed T, Nguyen T, et al. A genome-wide miRNA screen revealed miR-603 as a MGMT-regulating miRNA in glioblastomas. Oncotarget. 2014; 5:4026–4039. [PubMed: 24994119]
11. Ramakrishnan V, Kushwaha D, Koay DC, Reddy H, Mao Y, et al. Post-transcriptional regulation of O(6)-methylguanine-DNA methyltransferase MGMT in glioblastomas. Cancer Biomark. 2011; 10:185–193. [PubMed: 22674304]
12. Zhang W, Zhang J, Hoadley K, Kushwaha D, Ramakrishnan V, et al. miR-181d: a predictive glioblastoma biomarker that downregulates MGMT expression. Neuro Oncol. 2012; 14:712–719. [PubMed: 22570426]
13. Moskwa P, Zinn PO, Choi YE, Shukla SA, Fendler W, et al. A functional screen identifies miRs that induce radioresistance in glioblastomas. Mol Cancer Res. 2014; 12:1767–1778. [PubMed: 25256711]
14. Akers JC, Ramakrishnan V, Kim R, Phillips S, Kaimal V, et al. miRNA contents of cerebrospinal fluid extracellular vesicles in glioblastoma patients. J Neurooncol. 2015; 123:205–216. [PubMed: 25903655]
15. Thery C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol. 2006 Chapter 3: Unit 3 22.
16. Trang P, Wiggins JF, Daige CL, Cho C, Omotola M, et al. Systemic delivery of tumor suppressor microRNA mimics using a neutral lipid emulsion inhibits lung tumors in mice. Mol Ther. 2011; 19:1116–1122. [PubMed: 21427705]
17. Filipe V, Hawe A, Jiskoot W. Critical evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates. Pharm Res. 2010; 27:796–810. [PubMed: 20204471]
18. Bass DM, Baylor M, Chen C, Upadhyayula U. Dansylcadaverine and cytochalasin D enhance rotavirus infection of murine L cells. Virology. 1995; 212:429–437. [PubMed: 7571412]
19. Liu CG, Song J, Zhang YQ, Wang PC. MicroRNA-193b is a regulator of amyloid precursor protein in the blood and cerebrospinal fluid derived exosomal microRNA-193b is a biomarker of Alzheimer's disease. Mol Med Rep. 2014; 10:2395–2400. [PubMed: 25119742]
20. Mareninov S, De Jesus J, Sanchez DE, Kay AB, Wilson RW, et al. Lyophilized brain tumor specimens can be used for histologic, nucleic acid, and protein analyses after 1 year of room temperature storage. Journal of Neuro-Oncology. 2013; 113 10.1007/s11060-11013-11135-11061.
21. Wu Y, Wu M, Zhang Y, Li W, Gao Y, et al. Lyophilization is suitable for storage and shipment of fresh tissue samples without altering RNA and protein levels stored at room temperature. Amino Acids. 2012; 43:1383–1388. [PubMed: 22215254]

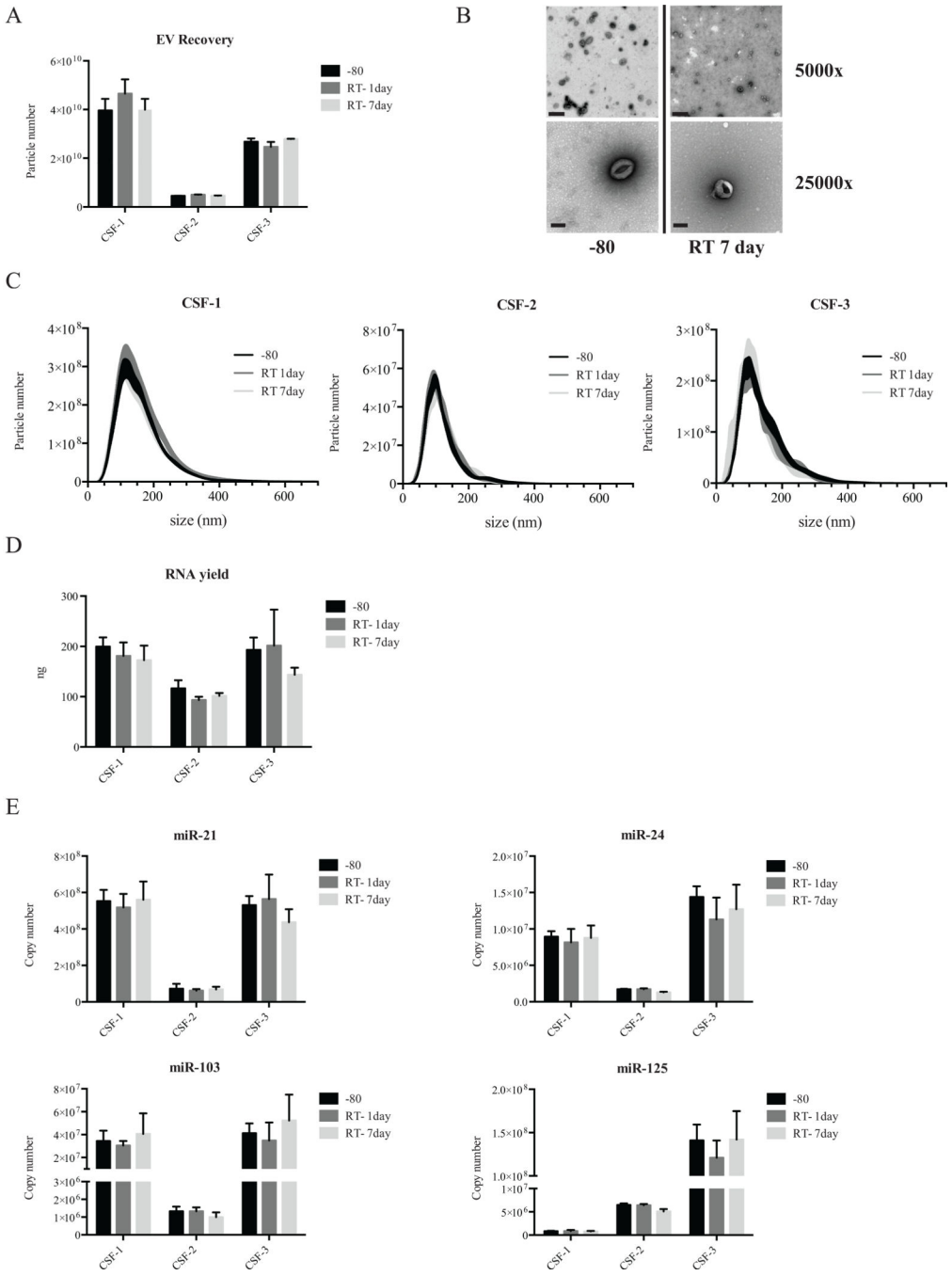


**Fig. 1. Clinical CSF EVs stored under Room Temperature (RT) and −80°C**

EVs isolated from CSF that were stored at room temperature for 1 and 7 days were comparable to EVs isolated from CSF stored at −80°C. (A) Total number of EVs recovered as determined by Nanoparticle tracking analysis (NTA). (B) Representative TEM images of EVs isolated from CSF stored under different conditions. Scale bar represents 500nm in 5000× magnification images, and 200nm in 25000× magnification images. (C) EV size profile as determined by NTA. (D) Comparison of total EV RNA yield extracted using Qiagen miRNeasy mini kit. (E) Detection of miR-21, miR-24, miR-103 and miR-125

transcripts in EVs by qRT-PCR. EV miRNAs appeared to be stable in CSF at room temperature for up to 7 days. Experiments were performed in triplicate and data are expressed as mean ± SEM.

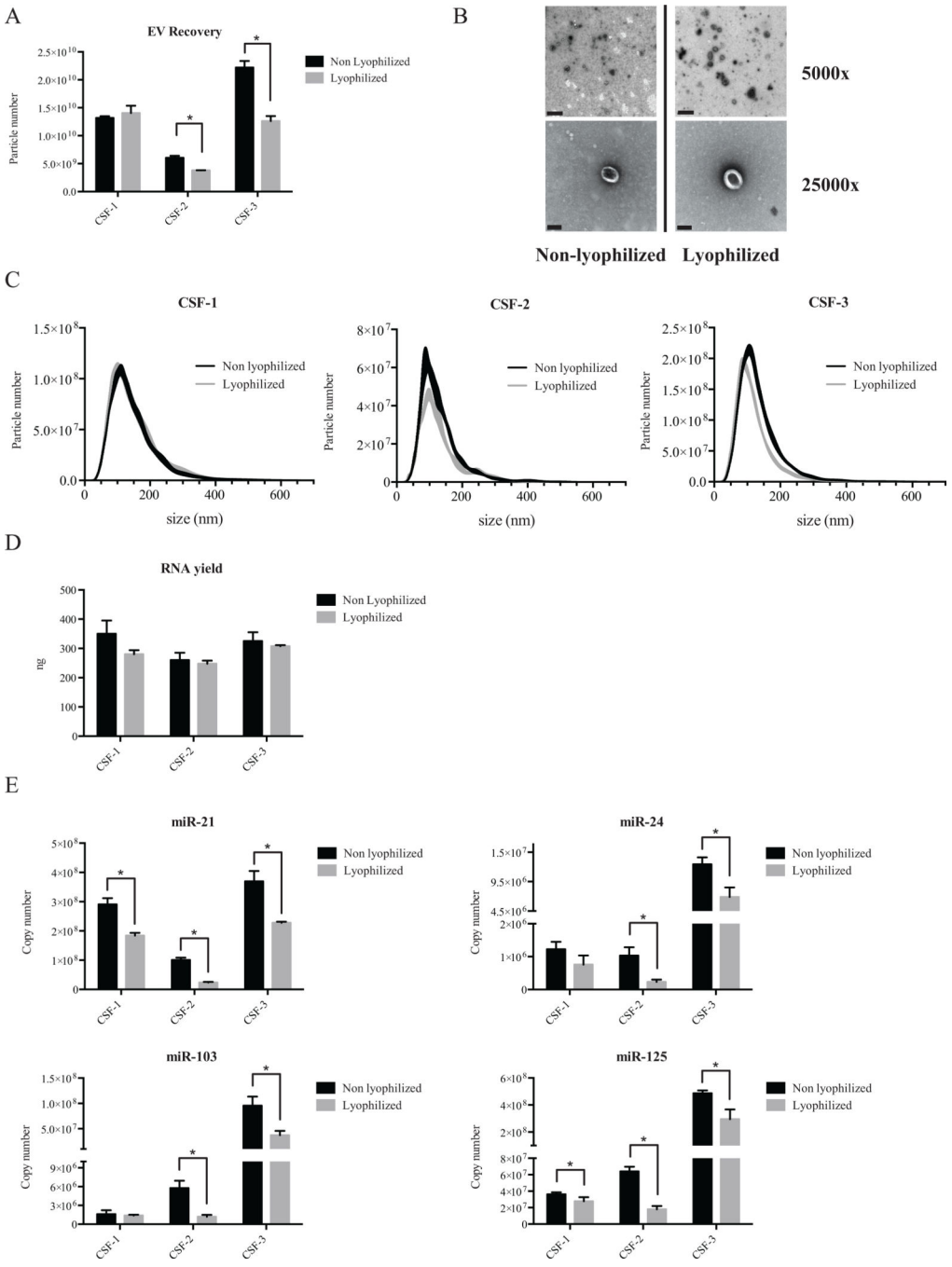


**Fig. 2. Effects of lyophilization on clinical CSF EV stability and EV associated miRNAs**

Lyophilized CSF EV samples were stored at room temperature for 7 days prior to rehydration with distilled water and then subjected to parallel analysis against non-lyophilized EVs. (A) Total number of EVs recovered as determined by NTA. (B) Representative TEM images of non-lyophilized EVs and EVs that were lyophilized and rehydrated. Scale bar represents 500nm in 5000× magnification images, and 200nm in 25000× magnification images. (C) EV size profile as determined by NTA. (D) Comparison of total EV RNA yield extracted using Qiagen miRNeasy mini kit. (E) Detection of miR-21,

miR-24, miR-103 and miR-125 transcripts in EVs by qRT-PCR. Significant reductions in miRNA levels were observed in lyophilized EVs. Lyophilization was performed in duplicate and data are expressed as mean ± SEM. * indicates $p<0.05$ as determined by Student's t-test.

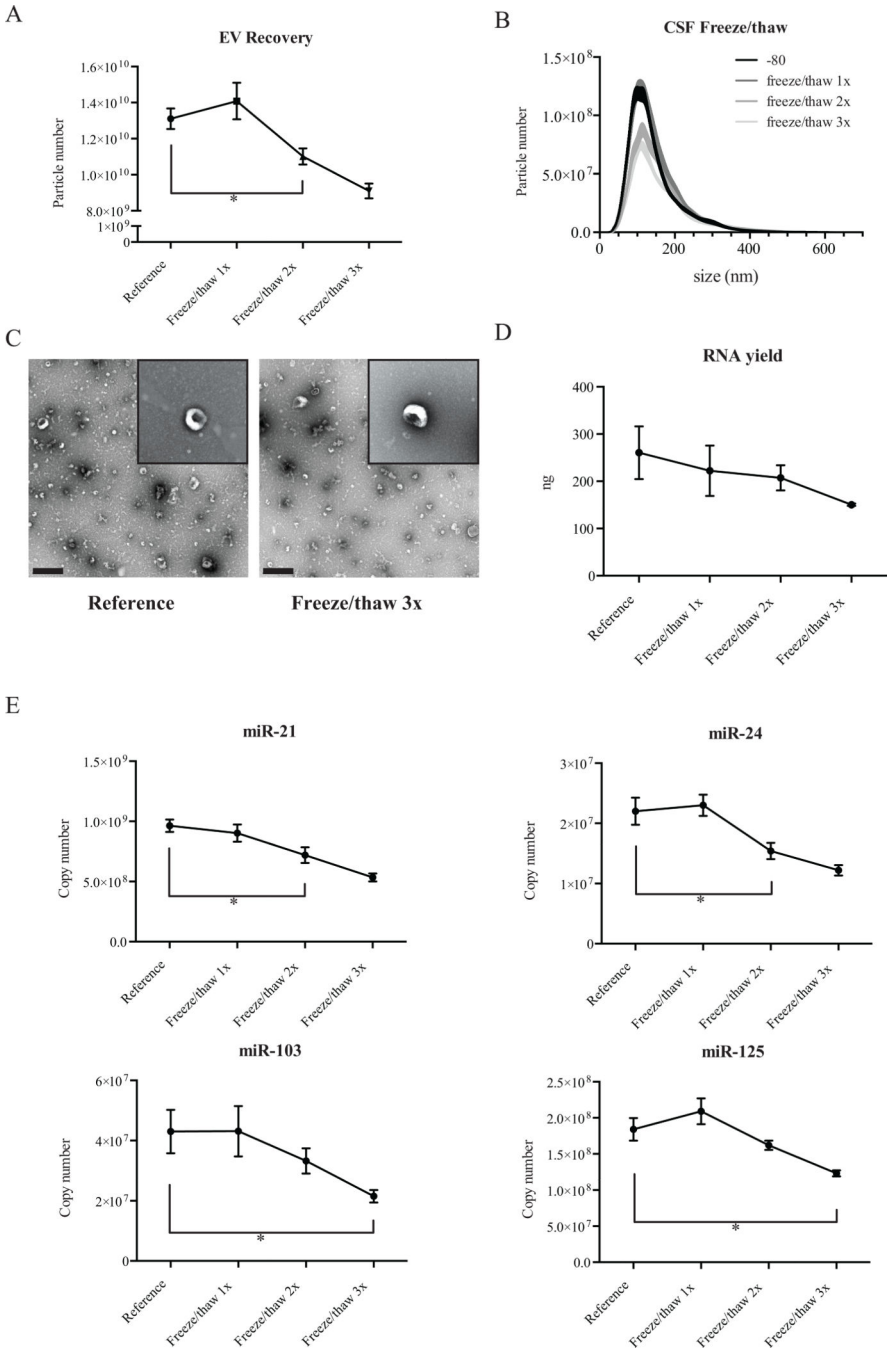


**Fig. 3. Effects of multiple freeze-thaw cycles on clinical CSF EV**

CSFs were subjected to multiple rounds of freezing and thawing prior to EV isolation. Recovered EVs were characterized by **(A–B)** NTA, and **(C)** TEM. Scale bar represents 500nm. Inset shows a magnified view of the EV. **(D)** EV RNA was then extracted and **(E)** miRNA levels were quantitated by qRT-PCR. A single cycle of freezing and thawing did not significantly alter EV number, morphology, RNA content, or miRNA levels. However, incremental decreases in these parameters were observed after two cycles of freezing and

thawing. Experiments were performed in triplicate and data are expressed as mean ± SEM. * indicates $p<0.05$ as determined by Student's t-test.