Comparison of serum exosome isolation methods on co-precipitated free microRNAs

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Background. Exosomes are nano-sized extracellular vesicles containing different biomolecules such as proteins and microRNAs (miRNAs) that mediate intercellular communication. Recently, numerous studies have reported the important functions of exosomal miRNAs in disease development and the potential clinical application as diagnostic biomarkers. Up to now, the most commonly used methods to extract exosomes are ultracentrifugation (UC) and precipitation-based commercial kit (e.g., ExoQuick). Generally, both UC and ExoQuick method could co-isolate contaminating proteins along with exosomes, with the UC method yielding even purer exosomes than ExoQuick. However, the comparison of these two methods on co-precipitated free miRNAs is still unknown.

Methods. In this study, we isolated exosomes from the human serum with exogenously added cel-miR-39 by UC and ExoQuick and compared the proportion of cel-miR-39 co-precipitated with exosomes extracted by these two methods.

Results. Using exogenous cel-miR-39 as free miRNAs in serum, we concluded that ExoQuick co-isolates a small proportion of free miRNAs while UC hardly precipitates any free miRNAs. We also found that incubation at 37 ℃ for 1 h could decrease the proportion of free miRNAs, and exosomal miRNAs like miR-126 and miR-152 also decreased when RNase A was used. In conclusion, our findings provide essential information about the details of serum exosome isolation methods for further research on exosomal miRNAs.

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Abstract

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Methods. In this study, we isolated exosomes from the human serum with exogenously added cel-miR-39 by UC and ExoQuick and compared the proportion of cel-miR-39 co-precipitated with exosomes extracted by these two methods.

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Key words: exosome, isolation method, ultracentrifugation, ExtraPEG, microRNAs, serum

40

Introduction

41
42   Exosomes are small secreted extracellular vesicles of 30-200 nm in diameter with the same
43   structure as cell membrane (Pegtel & Gould 2019; Thery et al. 2002; Tkach & Thery 2016).
44   They convey intercellular communications by delivery of biomolecules and affect multiple
45   physiological processes under normal or diseased conditions (He et al. 2018; Meckes et al. 2010;
46   Valadi et al. 2007). They originate from multivesicular bodies (MVBs) which contain many
47   small vesicles called intraluminal endosomal vesicles (ILVs) and the ILVs become exosomes
48   when the MVBs fuse with the plasma membrane, releasing the ILVs into the extracellular space
49   (Colombo et al. 2014). Moreover, exosomes have been found in diverse biological fluids, such as
50   serum, urine, saliva and breast milk (Thery et al. 2006).
51   MicroRNAs (miRNAs), a class of 19-23 nt non-coding RNAs are known as a mediator of
52   post-transcriptional regulation, which can negatively regulate the expression of target mRNAs
53   (Bartel 2004). While the majority of miRNAs are located within the cell, recently, significant
54   number of miRNAs have been found in extracellular environment, including various biological
55   fluids and cell culture media, commonly known as circulating miRNAs or extracellular miRNAs
56   (Turchinovich et al. 2011). Recently, an increasing number of extracellular miRNAs have been
57   detected in exosomes isolated from biological fluids and cell culture media (Valadi et al. 2007).
58   Moreover, some exosomal miRNAs have been shown to regulate disease development and
59   reported as biomarkers of different types of cancers, cardiovascular disease and brain injury
60   (Grimolizzi et al. 2017; Lugli et al. 2015; Zhang et al. 2017). In addition to exosomal miRNAs,
61   there are still some circulating miRNAs free in the serum (Mitchell et al. 2008). It remains
62   unclear whether these free miRNAs can be co-precipitated with exosomes and how to remove
63   these free miRNAs from exosome pellets if so.
64   The most commonly used methods to extract exosomes are ultracentrifugation (UC) and
65   commercially available kits, like ExoQuick from Systems Biosciences, exploiting sedimentation,
66   with pros and cons for each method (Alvarez et al. 2012; Umezu et al. 2013). Generally, UC is
67   the most reliable "gold standard" method but time-consuming; and precipitation methods such as
68   ExoQuick can obtain higher yields of exosomes but co-precipitate more impure proteins at the
69   same time (Shao et al. 2016). However, if and to what extent these two exosome isolation
70   methods can co-isolate serum free miRNAs remain to be investigated.
71   In this study, regarding exogenous cel-miR-39 as free miRNAs in serum, we concluded that
72   ExoQuick co-isolates a small proportion of free miRNAs while UC hardly precipitates any free
73   miRNAs. Further, we found that incubation at 37℃ for 1h can decrease the proportion of free
74   miRNAs in exosome pellets and exosomal miRNAs like miR-126 and miR-152 (Grimolizzi et al.
75   2017; Lugli et al. 2015; Ong et al. 2014; Zhang et al. 2017) also decreased when RNase A was
76   used. In conclusion, our findings provide essential information on the details of serum exosome
77   isolation methods for further studies on exosomal miRNAs.

78

Materials & Methods

79

80 ***Sample collection***

81   Human blood samples were collected into serum collection tubes from the Shanghai Chest

82 Hospital. Blood samples were centrifuged at 3000 rpm at 4 ℃ for 10 min for serum collection,

83 followed by an additional centrifugation step at 3000 g for 15 min. The supernatants of different

84 samples were mixed and then equally divided into several portions (500 µL) for subsequent

85 experiments.

86   This study was approved by the Ethical Committee of the School of Biomedical Engineering,

87 Shanghai Jiao Tong University (Registration No. 2019045) and carried out in accordance with

88 the Declaration of Helsinki. All participants provided written informed consent.

89

90 ***Exogenous miRNA addition***

91   The serum (500 µL) were added with 5 µL 7×10^9 copies/µL cel-miR-39 or 5 µL 7×10^6

92 copies/µL cel-miR-39 or 5 µL ddH$_2$O. The sequence of cel-miR-39 is: forward 5'-

93 UCACCGGGUGUAAAUCAGCUUG-3'.

94

95 ***Exosome isolation***

96 *ExoQuick^{TM} Kit*

97   Exosomes from 500 µL serum were isolated by ExoQuick^{TM} kit (System Biosciences Inc.,

98 CA, USA) according to the manufacturer's recommendations. Briefly, 120 µL EXOQ5A-1 was

99 added to serum and the mixture was incubated at 4 ℃ for 30 min. After incubation, the mixture

100 was centrifuged at 13000 rpm for 2 min. The supernatant was removed and the exosome pellet

101 was used for subsequent experiments.

102 *Ultracentrifugation (UC)*

103   Exosomes from 500 µL serum were also isolated by UC method. Briefly, the serum was

104 transferred into 12.5 mL ultracentrifuge tube (Beckman). The tube then was filled with PBS

105 (HyClone, SH30256.01) followed by ultracentrifugation twice for 70 min at 100,000 g, 4 ℃ in

106 an SW 40Ti swinging-bucket rotor (Beckman). The supernatant was discarded and the exosome

107 pellet was used for subsequent experiments.

108

109 ***Exosome identification***

110 *Transmission electron microscopy (TEM)*

111   The exosome pellet was resuspended in a small volume of PBS. According to Thery et al

112 (Thery et al. 2006), the exosomes (5 µL) was dripped onto a copper grid (Zhongjingkeyi, CHN,

113 BZ110223b) firstly. After one minute, the droplet was sucked out using the air-laid paper. And

114 then, the 2% uranyl acetate (Merck, 1005) solution (5 µL) was dripped onto the same copper grid

115 for negative-staining and sucked out again one minute later.

116 *Nanoparticle tracking analysis (NTA)*

117   The size distribution of the exosome pellet was measured by ZetaView (Particle Metrix). The

118 pellet was resuspended in a proper volume of PBS to achieve the optimal detectable

119   concentration (about 10^7 particles per mL) of its corresponding software (ZetaView 8.03.04.01).
120   For each measurement, 3-5 mL of the diluted sample was injected into the machine.
121   *Western blot*
122   Western blotting was performed according to the manufacturer's instruction manual. Briefly,
123   each pellet (30 μg) was mixed with loading buffer (5x) and heated at 95 ℃ for 5 min. Proteins
124   were loaded on 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE; EpiZyme, PG112)
125   and separated at constant 55 V for 30 min first and then 120 V until the loading buffer running
126   out of the SDS-PAGE. Then, proteins were transferred to a nitrocellulose membrane (GE
127   Healthcare, 10600002) at constant 300 mA for 1.5 h. Next, the membrane was blocked with 5%
128   nonfat milk powder suspended in tris-buffered saline and tween 20 (TBST) for 1 h at room
129   temperature. The blots were probed with TSG101 (Abcam, 133586) and subsequently probed
130   with horseradish peroxidase conjugated secondary anti-rabbit antibodies (Jackson). Finally, the
131   blots were visualized using the enhanced chemiluminescent (ECL; Thermo, 1856136) and
132   chemiluminescent imaging system (Tanon, 5200).
133
134   **RNase A and RNase Inhibitor treatment**
135   The exosome pellet was suspended in 500 μL PBS and a determined volume of RNase A
136   (Thermo Scientific, MAN0012003) was added in the RNase A treatment groups to reach a final
137   concentration of 10 μg/mL, 20 μg/mL or 30 μg/mL. And RNase inhibitor (Thermo Scientific,
138   EO0381) was added in the RNase inhibitor treatment groups to reach a final concentration of 1
139   U/μL. Then the mixture was incubated at 37℃ for 1 h.
140
141   **RNA isolation**
142   Exosomal RNA was extracted by miRNeasy Mini Kit (Qiagen, 217004) according to the
143   manufacturer's recommendations. The final elution volume was 35 μL.
144
145   **Reverse Transcription Polymerase Chain Reaction (RT-PCR)**
146   The cDNA of cel-miR-39, miR-126 and miR-152 were synthesized with the Revert Aid First
147   Strand cDNA Synthesis Kit (Thermo Scientific, K1622). Briefly, 5 μL total RNA from each
148   sample were mixed with 5 μL of RT-Primer, 2 μL of 10×T4 Buffer, 2 μL of 10mM dNTP Mix, 1
149   μL of RNase inhibitor, 1 μL of Transcriptase, 0.5 μL of T4 Polynucleotide Kinase, 0.25 μL of T4
150   Kinase, and 3.25 μL of nuclease-free water. Then the 20 μL of mixture was incubated at 16 ℃
151   for 30 min, 42 ℃ for 30 min, 85 ℃ for 5 min followed by cooling at 4 ℃ in the PCR instrument
152   (LongGene, A100).
153   Note that 5 μL of 35 μL extracted RNA were used in RT-PCR, so if all the exogenous cel-
154   miR-39 had been co-isolated with exosomes, the current concentration of cel-miR-39 in this
155   procedure would be 10^9 copies/μL or 10^6 copies/μL. Therefore, 10^9 copies/μL or 10^6 copies/μL
156   cel-miR-39 were used as a standard control.
157   Here, stemloop RT primers were used for cDNA synthesis. The sequence of miR-126 is: 5'-
158   UCGUACCGUGAGUAAUAAUGCG-3'. The RT primer of miR-126 is: 5'-

159 GATGAGGAGTGTCGTGGAGTCGGCAATTTCCTCATCACGCATTA-3'. The sequence of
160 miR-152 is: 5'-AGGUUCUGUGAUACACUCCGACU-3'. The RT primer of miR-152 is: 5'-
161 GATGAGGAGTGTCGTGGAGTCGGCAATTTCCTCATCAAGTCGGAG-3'. The RT primer
162 of cel-miR-39 is: 5'- GATGAGGAGTGTCGTGGAGTCGGCAATTTCCTCATCCAAGCTG -
163 3'.

164

165 ***Quantitative real-time PCR (qRT-PCR)***

166 To validate the quantity of cel-miR-39, miR-126 and miR-152 in the RNA samples, qPCR was
167 performed using Fluorescence Polymerase Chain Reaction (PCR) Detection Kit for the Analysis
168 of Human miRNA Gene Expression (Jiangsu MicroDiag Biomedicine Technology Co. Ltd.,
169 China) according to the manufacturer's recommendations. Briefly, the synthesized cDNA of cel-
170 miR-39, miR-126 and miR-152 from each sample were mixed with $10\times$ Buffer, 25 mM MgCl2,
171 25 mM dNTP Mix, F-primer, R-primer, miRNA-specific probe, HS Tag, UDG and ddH2O.
172 Meanwhile, 10^2 - 10^7 copies of cel-miR-39, miR-126 and miR-152 were amplificated in this
173 procedure to get a curve for calculating miRNA copy numbers in each sample.

174 The PCR reaction mixture was incubated at 37 ℃ for 5 min, and then 94 ℃ for 5 min, followed
175 by 50 cycles of 94 ℃ for 15 sec, 60 ℃ for 60 sec, and cooling at 50 ℃ for 30 sec, which was
176 performed using a real-time fluorescent quantitative PCR instrument (Roche, LightCycler 480).
177 Here, the miRNA-specific probes were used. The F-primer of miR-126 is: 5'-
178 CTCCGTCAGGGTCGTACCGTGAGTAA-3'. The R-primer of miR-126 is: 5'-
179 CTCAAGTGTCGTGGAGTCGGC-3'. The specific probe of miR-126 is: 5'-FAM-
180 TTTCCTCATCACGCATTA-MGB-3'. The F-primer of miR-152 is: 5'-
181 CTCCGTCAGGGAGGTTCTGTGATACA-3'. The R-primer of miR-152 is: 5'-
182 CTCAAGTGTCGTGGAGTCGGC-3'. The specific probe of miR-152 is: 5'-FAM-
183 TTTCCTCATCAAGTCGGAG-MGB-3'. The F-primer of cel-miR-39 is: 5'-
184 CGTATGAGCGTCACCGGGTGTAAATCA-3'. The R-primer of miR-152 is: 5'-
185 CTCAAGTGTCGTGGAGTCGGCAA-3'. The specific probe of miR-152 is: 5'-FAM-
186 TTTCCTCATCCAAGCTG-MGB-3'.

187

188 ***Statistical analysis***

189 Each experiment was repeated at least three times. Data were analyzed and all statistical
190 graphs were generated by GraphPad Prism 6.0 (GraphPad Software Inc, La Jolla, CA, USA).
191 Differences between two groups were analyzed using one-way or two-way ANOVA and the
192 probability value below 0.05 was considered significant.

193

194 # Results

195 ***Workflow of this research and the identification of exosomes***

196 The experimental procedure of this research is summarized (Fig. 1), with details given in
197 subsequent sections. First, 5 μL 7×10^9 copies/μL or 7×10^6 copies/μL exogenous cel-miR-39 was
198 added in 500 μL human serum while same volume of ddH$_2$O was added in another group as

199   blank control (step I). Next, the exosomes were isolated by ExoQuick or UC method (step II) and
200   characterized using TEM, NTA and western blot (Fig. 2(a-f)). TEM showed exosomes' "cup-
201   shaped" morphology and co-isolated impure proteins in both methods (Fig. 2(a, d)). NTA
202   analysis showed exosomes' similar particle size distribution mostly between 30-200 nm (Fig. 2(b,
203   d)), however, ExoQuick-isolated exosomes tended to be a little smaller than those from UC,
204   which may be caused by more co-precipitated impure proteins from ExoQuick method.
205   Furthermore, exosomes from both methods obtained the exosome-associated protein marker,
206   CD63, TSG101, CD9 (Fig. 2(e, f)) (Thery et al. 2018). Following, to clear up the co-isolation
207   free miRNA in exosome pellets, 10 μg/mL, 20 μg/mL or 30 μg/mL RNase A were added in
208   RNase A group and incubated with exosomes at 37 ℃ for 1 h. Moreover, 1 U/μL RNase
209   inhibitor were used to eliminate the effect of environmental RNase; the control group was a
210   blank control with no enzyme addition, which was also placed at 4 ℃ for 1 h (step III). Then,
211   total RNA including miRNA of exosomes was extracted by QIAGEN miRNeasy Mini Kit and
212   cel-miR-39, miR-126 or miR-152 in exosome samples were detected using RT-PCR followed by
213   qPCR. 5 μL 10^9 copies/μL or 10^6 copies/μL cel-miR-39 was used as standard control in PCR
214   procedure (step IV, V and VI).
215
216   ***ExoQuick co-isolates a small proportion of free miRNAs***
217   As described in **Figure 1**, higher concentration ($7×10^9$ copies/μL) of cel-miR-39 was added in
218   500 μL serum and then exosomes were extracted by ExoQuick method. The ratio of cel-miR-39
219   in exosome samples to standard cel-miR-39 sample (10^9 copies/μL) showed that about 2%
220   exosome-independent cel-miR-39 were co-isolated with exosomes extracted by ExoQuick
221   method (Fig. 3(a)). To measure the effect of RNase A to remove exosome-independent miRNAs,
222   10 μg/mL, 20 μg/mL, 30 μg/mL RNase A and 1 U/μL RNase inhibitor were respectively added
223   in the same exosome samples, and the results of PCR showed that RNase A can decrease the
224   quantity of co-isolated cel-miR-39 in exosomes but there are no significance difference between
225   different concentrations of RNase A and RNase inhibitor groups (Fig. 3(a)). Moreover, RNase A
226   decreased the quantity of exosomal miR-126 and miR-152 at the same time compared with
227   control and RNase inhibitor groups (Fig. 3(b)). Interestingly, RNase inhibitor reduced the
228   amount of exosomal miR-126 but not reduced miR-152 (Fig. 3(b)).
229   To verify if the co-isolation is due to the high concentration of cel-miR-39, low concentration
230   ($7×10^6$ copies/μL) of miR-39 were exogenously added in serum. Similar to the results of high
231   concentration cel-miR-39 experiment, nearly 2% cel-miR-39 were co-isolated with exosomes
232   and RNase A decreased the amount of exosomal miR-126 and miR-152 (Fig. 3(c-d)). And
233   RNase inhibitor reduced the amount of exosomal miR-126, not the miR-152 in low concentration
234   cel-miR-39 experiment as well (Fig. 3(d)). These results indicated that ExoQuick method can co-
235   isolate 2% free miRNA in serum regardless of the concentrations of miRNAs and RNase A not
236   only can reduce the number of exosome-independent miRNAs, but also reduce the miRNAs in
237   the exosomes.
238

239 ***Ultracentrifugation co-isolates almost no proportion of free miRNAs***
240   To investigate if UC can co-isolate free miRNAs in serum with exosomes as ExoQuick
241 method did, higher concentration (7×10^9 copies/μL) of cel-miR-39 was added in 500 μL serum
242 and then exosomes were extracted by UC method. The results showed that only 0.03%
243 exogenous cel-miR-39 were co-isolated with exosomes extracted by UC method (Fig. 4(a)).
244 Meanwhile, exosomal miR-126 and miR-152 were decreased by RNase A and miR-126 rather
245 than miR-152 was reduced by RNase inhibitor (Fig. 4(b)). These results indicated that UC
246 method hardly precipitate any exosome-independent miRNAs with exosomes and RNase A can
247 reduce the miRNAs in exosomes no matter which method is used.
248
249 ***Incubation at 37 ℃ for 1 h reduces the amount of co-isolated free miRNAs***
250   In the previous experiments, we noticed that RNase inhibitor and RNase A both reduced the
251 amount of exogenous cel-miR-39. These two reagents both require a 37 ℃ incubation with
252 exosomes for 1 h. We speculated if incubation at 37 ℃ for 1 h could cause the reduction of cel-
253 miR-39. Here, we isolated the exosomes by ExoQuick method, and the results showed that
254 incubation at 37 ℃ for 1 h reduced the amount of cel-miR-39 as much as the RNase A did (Fig.
255 5(a)). Consistent with the previous results in Fig. 3(b, d) and 4(b), RNase A reduced the amount
256 of exosomal miR-126 and miR-152, but incubation at 37 ℃ for 1 h reduced the amount of miR-
257 126 but not miR-152 like the RNase inhibitor did (Fig. 5(b)). These results indicated that
258 incubation at 37 ℃ for 1 h without RNase inhibitor and RNase A can remove the co-isolated free
259 miRNAs from exosome pellets, but RNase A can reduce the number of miRNAs in exosomes as
260 well.
261
262
263

264 **Discussion**
265   Circulating miRNAs, especially exosomal miRNAs, are strongly associated with the
266 development of different diseases, such as the manifestation, invasion and metastasis of cancers
267 (Melo et al. 2014; Zhou et al. 2014). Therefore, circulating miRNAs can serve as novel
268 biomarkers for diagnosis, prognosis of diseases and even drugs for diseases therapy (Cheng
269 2015; He et al. 2015; Huang et al. 2015). However, exosomal miRNAs are remarkably stable
270 compared to circulating miRNAs that are not contained in exosomes, and different fractions of
271 circulating miRNAs may play different roles in the progression of diseases, and serve as
272 diagnosis biomarkers (Grimolizzi et al. 2017). Therefore, if miRNAs existing in exosome-free
273 serum fractions contaminates exosomal miRNAs during laboratory studies and clinical
274 applications, it will have a great negative impact on the sensitivity and accuracy of research and
275 diagnostics. It has been commonly recognized that both UC and ExoQick methods co-precipitate
276 contaminating proteins, but exosomes extracted by UC are much purer than ExoQuick on
277 proteins (Shao et al. 2016; Tkach & Thery 2016). However, it is still unknown if UC is also

278   much more reliable than ExoQuick on miRNAs. Here we used exogenous cel-miR-39 as free
279   miRNA in serum and miR-126, miR-152 as exosomal miRNAs, to investigate if and to what
280   extent these two methods would co-precipitate free miRNAs in serum.
281   First, we found that compared with ExoQuick method, UC co-isolated much less exogenous
282   cel-miR-39 with exosomes, which suggests exosomes extracted by UC are much purer than
283   ExoQuick in exosomal miRNAs (Fig. 3(a, c) and 4(a)). Although 2% contaminating miRNAs
284   may be too small to be considered, they can really make difference in some studies about
285   exosomal miRNAs. Rekker et al (Rekker et al. 2014) compared UC with ExoQuick method for
286   miRNAs profiling, and found that 17 out of 375 miRNAs had a slightly different levels between
287   these two methods. Some of these 17 different miRNAs may be predominantly free in serum so
288   that their levels in exosomes extracted by ExoQucik method were higher than that by UC. Based
289   on our finding, when studying exosome-specific miRNAs, the standard UC method is preferred.
290   In previous studies, it was found that exosomal miRNAs could be protected from RNase by
291   the bilayer membrane of exosomes and hence RNase A was used to degrade exosome-
292   independent miRNAs (Muller et al. 2014; Quackenbush et al. 2014; Rekker et al. 2014).
293   However, our results showed that although RNase A reduced the ratio of co-precipitated
294   miRNAs (Fig. 3(a, c) and 5(a)), it reduced the levels of exosomal miRNAs at the same time no
295   matter which methods were used (Fig. 3(b, d), 4(b) and 5(b)). Furthermore, we have also found
296   that a simple incubation at 37 ℃ for 1 h can reduce the amount of exosome-independent
297   miRNAs as effectively as the addition of RNase A, but hardly reduce the amount of exosomal
298   miRNAs at the same time (Fig. 5(a-b)).
299   These results suggested that merely incubation at 37 ℃ could remove exosome-independent
300   miRNAs free in serum effectively, and RNase A was not recommended for degrading miRNAs
301   out of exosomes. Earlier reports have shown that the methods of incubation at room temperature
302   (RT) or 37 ℃ have been used to load drugs into exosomes (Haney et al. 2015; Ma et al. 2016). It
303   is possible that exosomal membranes, when incubated with RNase A at 37 ℃ for 1 h, could
304   display a certain level of permeability to the enzyme, and could not fully protect miRNAs from
305   RNase A to some degree.
306   It is worth noting that incubation at 37 ℃ for 1 h (with or without RNase inhibitor) slightly
307   reduced miR-126 instead of miR-152 (Fig. 3(b, d), 4(b) and 5(b)). So, there must be a difference
308   between miR-126 and miR-152. Turchinovich et al reported that a large part of extracellular
309   miRNAs are associated with Ago proteins and we found from their miRNA Array data that the
310   level of serum miR-126 associated with AGO1 and AGO2 is much higher than that of miR-152
311   (Turchinovich & Burwinkel 2012; Turchinovich et al. 2011). Therefore, perhaps it is because UC
312   and ExoQuick methods both can precipitate AGO-associated miR-126 and exosome-independent
313   miR-126 can be degraded by incubation as cel-miR-39 did.
314
315   **Conclusions**

316   Our study provides critical information on the effect of free miRNAs in the serum when using
317   the UC and ExoQuick methods to isolate serum exosomes and analyze exosomal miRNAs. We
318   have found that ExoQuick method can co-precipitate much more free miRNAs in serum than
319   UC. Furthermore, we have also found that a simple incubation at 37 ℃ for 1 h can reduce the
320   amount of exosome-independent miRNAs as effectively as the addition of RNase A, but the
321   latter would reduce the amount of exosomal miRNAs as well. Thus, for studies of exosomal
322   miRNA analysis, UC method is recommended, but not the use of RNase to remove exosome-
323   independent miRNAs. In conclusion, these findings on the details of serum exosome isolation
324   methods are essential for further studies on exosome-specific miRNAs.
325

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327   Not applicable.

328

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481

Figure 1

Figure 1. The experimental flowchart of this research.

(I) exogenously added cel-miR-39 in serum, blank control: ddH$_2$O, (II) isolation of exosomes by ExoQuick or UC method, (III) using 10 µg/mL, 20 µg/mL, 30 µg/mL RNase A or 1 U/µL RNase inhibitor or nothing with incubation at 37 ℃ for 1 h or nothing (control) to treat exosome pellets, (IV) extraction of total RNA including miRNA by miRNeasy Mini Kit, (V) detecting the quantity of cel-miR-39, miR-126 or miR-152 in exosome samples by qRT-PCR.
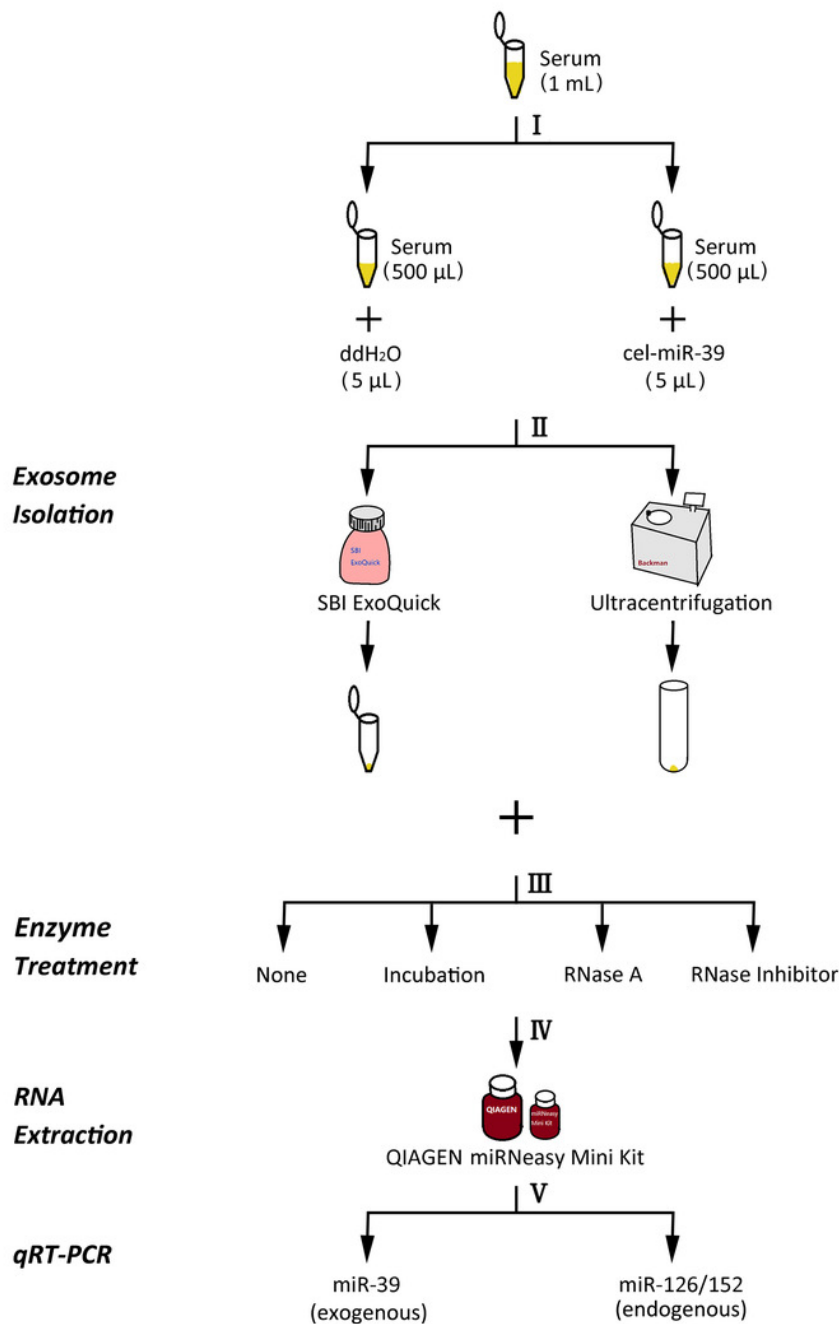
Figure 2

Figure 2. The characterization of exosomes isolated from human serum.

(a, d) The morphology of exosomes. Scale bars: 200 nm. (b, d) The particle size distribution of exosomes. (e, f) The level of exosome-associated protein, CD63, TSG101 and CD9.
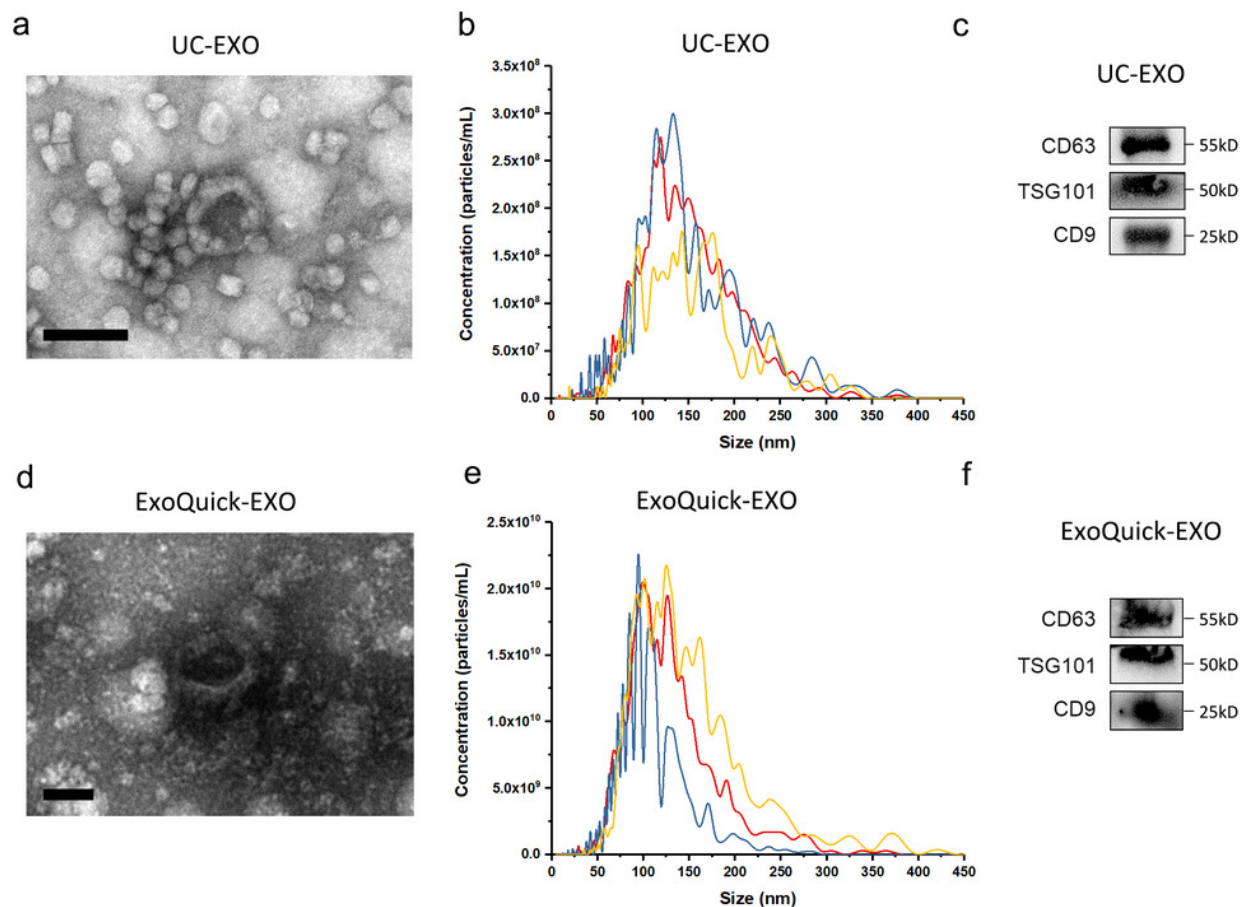
Figure 3

Figure 3. ExoQuick method co-isolates a small proportion of free miRNAs.

(a-b) High concentration (7×10^9 copies/µL) of cel-miR-39 were added in serum. Exosomes were extracted by ExoQuick method. (a) The ratio of co-precipitated cel-miR-39 in different samples to standard sample (10^9 copies/µL cel-miR-39). ***p<0.001 *vs*. Control. (b) The amount of exosomal miR-126 and miR-152 in different samples. **p<0.01, ***p<0.001 *vs*. Control. #*p*<0.05, ##*p*<0.01 *vs*. RNase Inhibitor. (c-d) Low concentration (7×10^6 copies/µL) of cel-miR-39 were added in serum. Exosomes were extracted by ExoQuick method. (c) The ratio of co-precipitated cel-miR-39 in different samples to standard sample (10^6 copies/µL cel-miR-39). (d) The amount of exosomal miR-126 and miR-152 in different samples. ***p<0.001 *vs*. Control. ###*p*<0.001 *vs*. RNase Inhibitor.

a

The ratio of cel-miR-39 copies (sample/standard)

b

c

The ratio of cel-miR-39 copies (sample/standard)

d
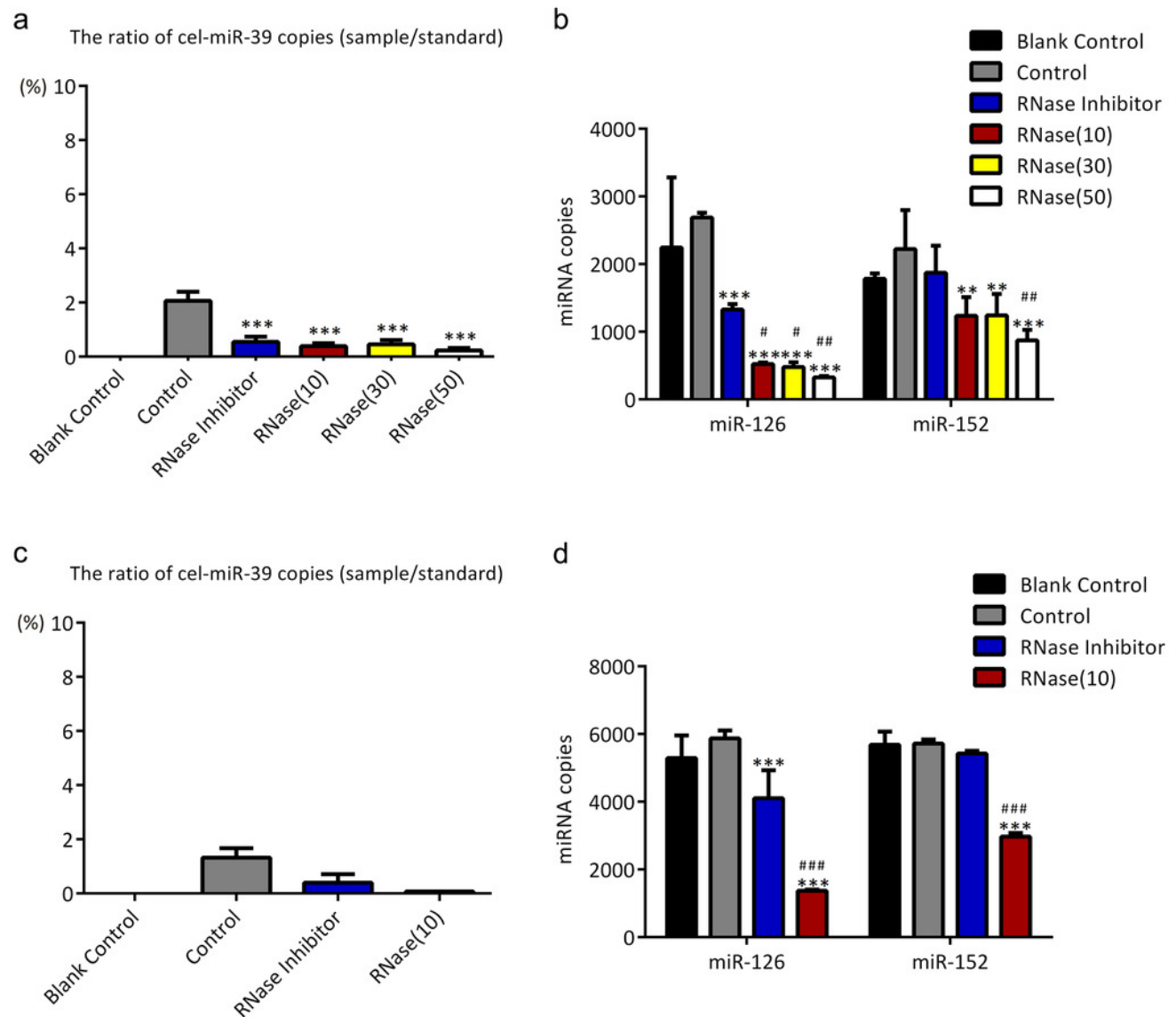
Figure 4

Figure 4. Ultracentrifugation (UC) method co-isolates almost no proportion of free miRNAs.

(a-b) High concentration (7×10^9 copies/μL) of cel-miR-39 were added in serum. Exosomes were extracted by UC method. (a) The ratio of co-precipitated cel-miR-39 in different samples to standard sample (10^9 copies/μL cel-miR-39). (b) The amount of exosomal miR-126 and miR-152 in different samples. **$p<0.01$, ***$p<0.001$ *vs*. Control. #$p<0.05$ *vs*. RNase Inhibitor.
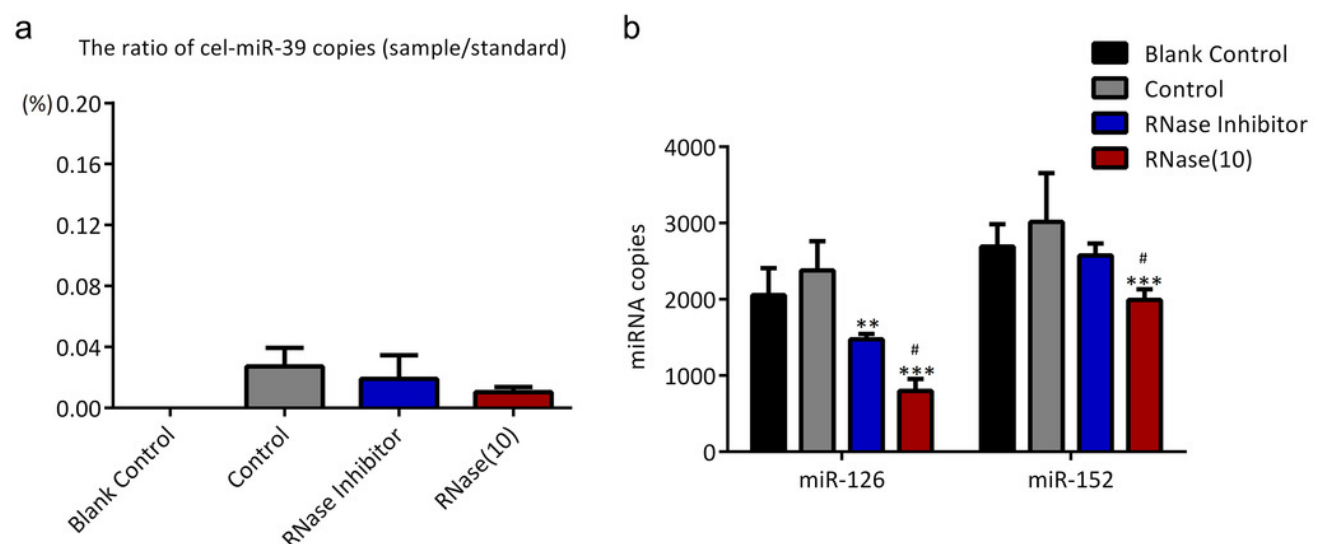
Figure 5

Figure 5. Incubation at 37℃ for 1 h reduces the amount of co-isolated free miRNAs.

(a-b) High concentration ($7×10^9$ copies/µL) of cel-miR-39 were added in serum. Exosomes were extracted by ExoQuick method. (a) The ratio of co-precipitated cel-miR-39 in different samples to standard sample (10^9 copies/µL cel-miR-39). *$p<0.001$ *vs*. Control. (b) The amount of exosomal miR-126 and miR-152 in different samples. ***$p<0.001$ *vs*. Control. ## #$p<0.05$ *vs*. Incubation.