Comparative Study on the Isolation of Exosomal RNA From Urine Using Different Methods

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Abstract

Exosomes are small vesicles of about 40 –100 nm in diameter originating from within multi-vesicular bodies, which are secreted into the extra-cellular space. The contents of these exosomes reflect the origin and the physiological status of the source cells. Exosomes are found in different bodily fluids such blood, blood derivatives, urine, amniotic fluid, and malignant ascitic fluid. Evidence has been accumulating recently that these vesicles act as cellular messengers, conveying information to distant cells and tissues within the body. Recent work has demonstrated the presence of distinct subsets of microRNAs within exosomes, which depend upon the tumour cell type from which they are secreted. For this reason, exosomal RNAs may serve as biomarkers for various diseases, including cancer. The number of exosomes varies significantly in different bodily fluids and they are usually present in low amounts. Given the small size of exosomes, there is evidence that exosomes can pass through the kidneys, into the urine. Consequently, effective detection of exosomes in urine requires both the isolation and the detection method to be sensitive and consistent. Although a significant amount of research has been done on the concentration of exosomes for RNA isolation from urine, the isolation of urine exosomal RNA remains a challenge. Exosome precipitation reagents and/ or concentrating exosomes by filtration followed by RNA isolation of urine exosomal RNA, have been widely used for that purpose. In addition to being expensive, lengthy and hazardous, these methods could result in a significant carryover contamination that may affect sensitive downstream applications such as RT-qPCR or microarrays. Here we present data of an evaluation of different sample preparation methods for urine exosomal RNA isolation. In particular, we compared a common exosome precipitation reagent and a filtration method with a silicon carbide-based non-phenol extraction method (Norgen Biotek SiC columns). Based on a sensitive R CR assay, all methods recovered all miRNA studied. However, the silicon carbide-based method showed more sensitive and consistent isolation of high quality urine exosomal miRNA.

Introduction

- Urine has been shown to be a good sample source for miRNA as it can be obtained non-invasively, in large quantities and with little training. It is also non-infectious for many pathogens, including HIV.
- Exosomes are small vesicles of about 40 –100 nm in diameter, originating from within multi-vesicular bodies, which are secreted into the extra-cellular space.
- Exosomes are found in different bodily fluids such blood, plasma, serum, urine, amniotic fluid, and malignant ascitic fluid.
- Evidence has been accumulating recently that these vesicles act as cellular messengers, conveying information to distant cells and tissues within the body.
- Exosomes may play a functional role in mediating adaptive immune responses to infectious agents and tumours, tissue repair, neural communication and transfer of pathogenic proteins.
- Recent work has demonstrated the presence of distinct subsets of microRNAs within exosomes which depend upon the tumour cell type from which they are secreted. Therefore, exosomal RNAs may serve as biomarkers for various diseases including cancer.
- Isolation of urine exosomal RNA is very challenging due to the low abundance of exosomes in urine. Efficient urine exosomal RNA isolations can be the limiting step for the success of a number of sensitive downstream applications, including reverse-transcription - real-time polymerase chain reaction (RT-qPCR).
- A fast, reliable, consistent and inhibitor-free urine exosomal RNA isolation method is very critical as exosomes can be used as biomarkers for the early detection of different cancers.

Objectives of the Study

To compare (a) RNA Quality, (b) RNA Quantity and (c) Ease of use for three different Urine Exosomal RNA isolation kits.

Materials and Methods

Urine Collection and Preparation. Urine samples were collected using Norgen's Urine Collection and Preservation Tubes (Cat# 18113). Urine samples were spun down for 10 mins at 1,000 RPM to pellet any urinary exfoliated cells. The supernatant was transferred to a fresh tube, and was then spun for an additional 10 mins at 3,000 RPM to avoid the presence of any bacterial cells.

Urine Exosomal RNA Purification Methods. Exosomal RNA was isolated from 1 mL cell-free urine using three different kits, as per the manufacturer's protocols: 1) Norgen's Urine Exosome RNA Isolation Kit 2) ExoQuick-TC Exosome Precipitation Solution 3) Amicon® Ultra-15 (MILLIPORE) followed by Norgen's Urine Total RNA Purification Maxi Kit (Slurry Format) (Cat# 29600)

RNA Isolation and Gel Electrophoresis. Subsequent to exosome precipitation using ExoQuick-TC Exosome Precipitation Solution, Exosomal RNA was isolated from the precipitated exosome pellet using Norgen's Total RNA Purification Kit (Cat# 17200). The purified Plasma exosomal RNAs were run on 1X MOPS, 1.8% formaldehyde-agarose gels for visual inspection, using 15 µL of each 100 µL elution.

RT-qPCR Analysis. The purified urine exosomal RNA was then used as a template in RT-qPCR reactions using primers specific for either the 5S rRNA, miR-16 microRNA or miR-21 microRNA. Reverse transcription was carried out to generate cDNAs from the tested miRNA using miRNA-specific stem-loop primers, whereas the 5S rRNA reverse primer was used to generate cDNA for the 5S rRNA gene. Equal portions of purified RNA (3 µL of a 100 µL elution) were used in a 20 µL cDNA reaction. **First-strand** cDNA synthesis was performed for all 3 genes using Invitrogen's Superscript II system. Three microliters from the generated cDNAs were then used as the template in qPCR reactions. Additionally, an increasing input volume (3 µL, 6 µL and 9 µL of a 100 µL elution) from RNA isolated using different kits were used as a template in the reverse transcription step followed by using 3 µL from the generated cDNAs were then used as the template in qPCR reactions.

Results and Discussion

1- Comparison of Urine Exosomal RNA Quantity and Quality. Figure 1 shows the exosomal RNA profile isolated from cell-free urine using three different methods. As expected, urine exosomal RNA is mostly small-sized RNA (red arrow). Based on Figure 1 it appears that both Norgen's Urine Exosome RNA Isolation Kit and Amicon filtration are similar, but better than ExoQuick's kit. However, based on the quantification using spectrophotometry (NanoVue, GE Healthcare), Figure 2A shows that the three tested methods yielded approximately the same quantity of exosomal RNA. Note that Amicon filtration also showed higher molecular weight bands (see green arrow to the side of the gel). This is most likely DNA contamination, as it was digestible with DNase I (data not shown). All three methods gave equivalently good and similar A260/A280 ratios of about 2.0 (Figure 2B). However, based on the RT-qPCR, Norgen's kit gave the best quality of RNA (see Figures 3 and 4).

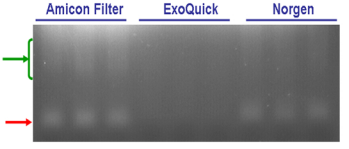


Figure 1. Resolution of urine exosomal RNA, isolated using three different methods, on a 1X MOPS, 1.8% formaldehyde-agarose gel (15µL of the 100µL elutions) for visualization.

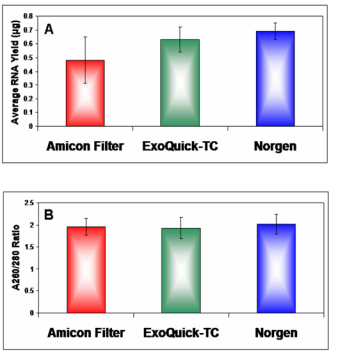


Figure 2. Quantity and quality of urine exosomal RNA isolated using Norgen's, ExoQuick's, and Amicon Filteration based on NanoVue spectrophotometry. A) Average RNA yield isolated from cell-free urine. B) A260/280 ratios for RNA isolated from cell-free urine.

2- Assessing the Quality of the Exosome-Derived Cell-Free Urine RNA using RT-qPCR Amplification. The quality of RNA isolated from cell-free urine was assessed using RT-qPCR amplification of 5S rRNA (Figure 3A), miR-21 microRNA (Figure 3B), and miR-16 microRNA (Figure 3C). Norgen's kit showed the highest RNA quality as evidenced by the lowest Ct values when compared to the Ct values generated from RNA isolated using ExoQuick's kit and Amicon filteration. This is directly correlated to the high A260/A280 ratio that was observed from RNA isolated using Norgen's kit, when compared to ExoQuick's kit and the Amicon filter (Figure 2B).

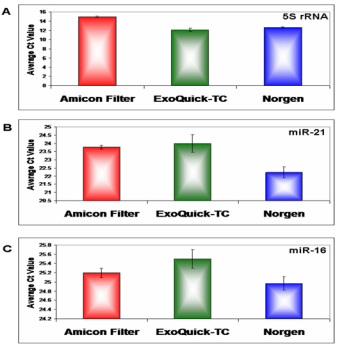


Figure 3. Assessing the quality of the exosome-derived cell-free urine RNA using RT-qPCR amplification. A) RT-qPCR using 5s rRNA primers. B) RT-qPCR using miR-21 primers. C) RT-qPCR using miR-16 primers.

3- Assessing the Quality of Exosomal RNA via RT-qPCR. To closely examine the quality of isolated RNA, we increased the input volume in the RT-qPCR reaction. It is anticipated that if the isolated RNA is of a high quality, the Ct values will decrease in proportion to the amount of input volume. When we inputted 3µl, 6µl or 9µl of isolated RNA in the RT-qPCR reaction, we found that the Ct values obtained from the Norgen's Urine Exosomal RNA Isolation kit decreased as the input volume increased. This is exactly as one would expect from good quality RNA. In contrast, in both the ExoQuick's kit and the Amicon filteration methods, the Ct values did not decrease with the increasing input volume, indicating poorer quality RNA (possibly due to PCR inhibition).

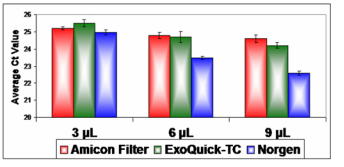
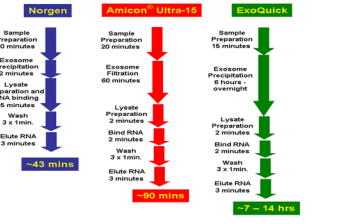


Figure 4. Assessing the quality of the exosome-derived cell-free urine RNA using increasing input volumes in the RT-qPCR reaction. Plasma exosomal RNA isolated using Norgen's Kit showed the highest quality and purity as compared to ExoQuick's and Amicon's Filter.

4- Ease of Use. It has been found that Norgen's Urine Exosomal RNA Isolation Kit was the simplest, fastest and easiest to use compared to ExoQuick's and Amicon's Filteration.



Conclusions

1. **Quantity of Urine Exosomal RNA.** All three methods yielded similar qunatity of urine exosomal RNA (Figure 2A).
2. **Quality of Urine Exosomal RNA.** Based on RT-qPCR performance (Figures 3&4), Norgen's Urine Exosomal RNA Purification kit was found to isolate RNA of higher quality than both ExoQuick's kit and Amicon filtration.
3. **Ease of Use.** Norgen's Urine Exosome RNA Kit was the simplest and easiest to use and yielded the best urine exosomal RNA quality.