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Importance of RNA isolation methods for analysis of exosomal RNA: Evaluation of different methods

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ABSTRACT

Exosomes are small RNA containing vesicles of endocytic origin, which can take part in cell-to-cell communication partly by the transfer of exosomal RNA between cells. Exosomes are released by many cells and can also be found in several biological fluids including blood plasma and breast milk. Exosomes differ compared to their donor cells not only in size but also in RNA, protein and lipid composition. The aim of the current study was to determine the optimal RNA extraction method for analysis of exosomal RNA, to support future studies determining the biological roles of the exosomal RNA.

Different methods were used to extract exosomal and cellular RNA. All methods evaluated extracted high quality and purity RNA as determined by RNA integrity number (RIN) and OD values for cellular RNA using capillary electrophoresis and spectrophotometer. Interestingly, the exosomal RNA yield differed substantially between the different RNA isolation methods. There was also a difference in the exosomal RNA patterns in the electropherograms, indicating that the tested methods extract exosomal RNA with different size distribution. A pure column based approach resulted in the highest RNA yield and the broadest RNA size distribution, whereas phenol and combined phenol and column based approaches lost primarily large RNAs. Moreover, the use of phenol and combined techniques resulted in reduced yield of exosomal RNA, with a more narrow size distribution pattern resulting in an enrichment of small RNA including microRNA.

In conclusion, the current study presents a unique comparison of seven different methods for extraction of exosomal RNA. As the different isolation methods give extensive variation in exosomal RNA yield and patterns, it is crucial to select an isolation approach depending on the research question at hand.

1. Introduction

Exosomes are small nano-vesicles of endocytic origin, which are released into the extracellular environment by many different cell types including mast cells, dendritic cells, epithelial cells and tumour cells (Raposo et al., 1997; Thery et al., 1999; Van Niel et al., 2001; Wolfers et al., 2001; Thery et al., 2009). Exosomes have also been detected in human biological fluids such as blood plasma, urine, bronchoalveolar lavage fluid, saliva and breast milk (Admyre et al., 2003; Pisitkun et al., 2004; Caby et al., 2005; Admyre et al., 2007; Palanisamy et al., 2010). Excitingly, exosomes contain RNA that can be shuttled between cells. Thus, one cell can produce exosomes that influence another cell, which has opened a new research field in cell-to-cell communication (Valadi et al., 2007; Skog et al., 2008; Palanisamy et al., 2010; Pegtel et al., 2010; Kosaka et al., 2010; Kogure et al., 2011; Montecalvo et al., 2012). Subsequently, RNA has been detected in exosomes from many different cellular sources (Valadi et al., 2007; Skog et al., 2008; Taylor and Gercel-Taylor, 2008; Kesimer et al., 2009; Michael et al., 2010; Ohshima et al., 2010; Palanisamy et al., 2010; Kosaka et al., 2010; Pegtel et al., 2010; Kogure et al., 2011; Montecalvo et al., 2012). The exosomal RNA pattern differs in the studies describing exosomes from different cellular sources. However, the technique for extracting RNA from exosomes is not uniform in these studies, which may explain some differences in the exosomal RNA pattern. Thus, there is a need to standardize the methodology to isolate RNA from exosomes.

Interestingly, the lipid composition of the cellular and exosomal membranes differ substantially which would potentially affect the RNA extraction. Exosomes have a more rigid membrane compared to the cellular membrane due to a decreased phosphatidylcholine content and enrichment in sphingomyelin and cholesterol (Laulagnier et al., 2004; Trajkovic et al., 2008; Mitchell et al., 2009). Therefore, it is possible that the methodology to extract molecules from cells and exosomes should differ to some extent. This may be especially important when characterizing the RNA content in exosomes from different cellular origins.

In different studies describing exosomal RNA, a number of alternative RNA extraction methods have been used including

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phenol based techniques (Trizol®), combined phenol and column based approaches (Trizol® followed by cleanup using the modified RNeasy®, miRNeasy and mirVana™) and pure column based techniques (RNeasy®, modified RNeasy® and miRCURY™) (Valadi et al., 2007; Skog et al., 2008; Taylor and Gercel-Taylor, 2008; Kesimer et al., 2009; Michael et al., 2010; Ohshima et al., 2010; Palanisamy et al., 2010; Pegtel et al., 2010; Kosaka et al., 2010; Kogure et al., 2011 and unpublished data).

The aim of the current study was to determine whether the difference in exosomal RNA patterns depend on the extraction method used, or if it is a true variation in exosomes originating from different cellular sources. Furthermore, we aimed to determine the most suitable RNA isolation method for exosomal RNA, which is also suitable for cellular RNA extraction. To examine this, we utilized a mast cell line (MC/9) that continuously release exosomes, which have previously been characterized (Valadi et al., 2007). Thus, we evaluated several available methods to extract the exosomal RNA with high quality, substantial yield, high purity and appropriate size distribution suitable for analysis of mRNA and small RNA including microRNA.

2. Methods

2.1. Cell culture and exosome isolation

The mouse mast cell line MC/9 (ATCC, Manassas, VA) was cultured according to the manufacturer's recommendations. To eliminate exosomes present in serum, Rat T-Stim (BD Biosciences, Erembodegem, Belgium) and fetal bovine serum (Sigma–Aldrich, St. Louis, MO, USA) were ultracentrifuged at 120,000 × *g* for 90 min using a Ti45 rotor (Beckman Coulter, Brea, CA, USA). Exosomes were prepared from the supernatant of MC/9 cells using differential centrifugations as previously described (Valadi et al., 2007). In short, cells were harvested, centrifuged at 300 × *g* for 10 min to eliminate cells and at 16,500 × *g* for 20 min, followed by filtration through 0.2 μm filters to remove cell debris and particles larger than 200 nm. Exosomes were pelleted by ultracentrifugation at 120,000 × *g* for 70 min (all steps were performed at 4 °C). The exosome pellet was dissolved in nuclease free water and subsequently split and transferred to seven different RNase free tubes for RNA isolation by seven different methods (Fig. 1). The cells were harvested and pelleted by centrifugation at 500 × *g* for 5 min ($n = 4$; 2–4 × 10^6 cells). Each exosomal sample (A–D) was isolated from a large volume of cells and then split into seven different fractions for the seven different RNA extraction methods. Thus, each exosomal sample was harvested from 85 ml cell suspension with 1–4 × 10^6 cells/ml. Cells and exosomes were then immediately lysed in respective lysing solution and continued for RNA purification.

2.2. RNA extraction methods

RNA was extracted from cells and exosomes ($n = 4$) using seven different methods; Trizol® (Invitrogen, Paisley, UK), Trizol® followed by cleanup using the modified RNeasy® Mini Kit (Qiagen, Hilden, Germany), RNeasy® Mini Kit, modified RNeasy® Mini Kit, miRNeasy Mini Kit (all three from Qiagen), miRCURY™ RNA Isolation Kit (Exiqon, Vedbaek, Denmark) and mirVana™ miRNA Isolation Kit (Ambion, Austin, TX, USA) (Table 1 and Fig. 2). Trizol®, RNeasy® Mini Kit, miRNeasy Mini Kit, miRCURY™ RNA Isolation Kit and mirVana™ miRNA Isolation Kit were all used according to the manufacturer's protocol but with the double volume lysing buffer for the RNeasy® Mini Kit. According to manufacturer's protocol, RNeasy® Mini Kit only isolates RNA molecules larger than 200 nucleotides. To also extract small RNA, a modified version of the RNeasy® Mini Kit was used. In brief, cells and exosomes were disrupted and homogenized in 700 μl RLT buffer containing 1% β-mercaptoethanol and 3.5 volumes of 100% ethanol were added to the samples prior to the use of the RNeasy® mini spin column. The samples were washed twice in 500 μl RPE buffer and eluted in RNase free water. For Trizol® followed by modified RNeasy® Mini Kit, the Trizol® protocol was followed until the phase separation and it was then continued according to the modified RNeasy® Mini Kit. The elution volume for mirVana™ miRNA Isolation Kit was 100 μl, for the other methods the RNA was eluted in a volume of 50 μl.

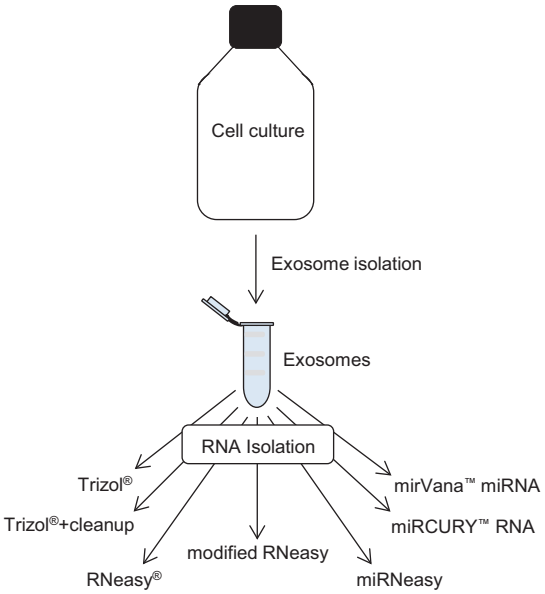


Fig. 1. Sample preparation flow chart. Cells were harvested and pelleted by centrifugation. Each exosomal sample (A–D) was isolated from a large volume of cells and then split into seven different fractions for the seven different RNA extraction methods.

2.3. RNA analyses

The RNA quality, yield, and size of exosomal and cellular total and small RNA was analyzed using capillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies, Foster City, CA, USA). For Agilent RNA, 1 μl RNA was analyzed with the Agilent RNA 6000 Nano Kit according to manufacturer's protocol. In separate experiments, total exosomal RNA was treated with or without RNase A (final concentration of 5 μg/μl, Fermentas, St. Leon-Rot, Germany) for 90 min at 37 °C and analyzed using the RNA 6000 Nano Kit. For the analyses of small RNA, 1 μl RNA (diluted to contain 10 ng total RNA or diluted 10× for all except mirVana™ that was eluted in the double volume and therefore diluted 5×) was analyzed using Agilent small RNA Kit according to manufacturer's protocol. Electropherograms were analyzed using the Agilent 2100 Expert B.02.07 software that includes data collection, presentation, and interpretation functions. The cellular and exosomal RNA purity was evaluated spectrophotometerically using a SPECTRAmax PLUS 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at the absorbance 230, 260 and 280 nm. The average A260/230 and A260/280 ratios were used to assess the presence of peptides, phenols, aromatic compounds, or carbohydrates and proteins.

The presence of microRNA was confirmed by real-time PCR. Previously isolated total RNA, using miRCURY™ and mirVana™ ($n = 3$), was DNase treated using Turbo DNase Free kit (Ambion). miRCURY™ LNA™ micro PCR System was used for first strand cDNA synthesis and real-time PCR according to manufacturer's

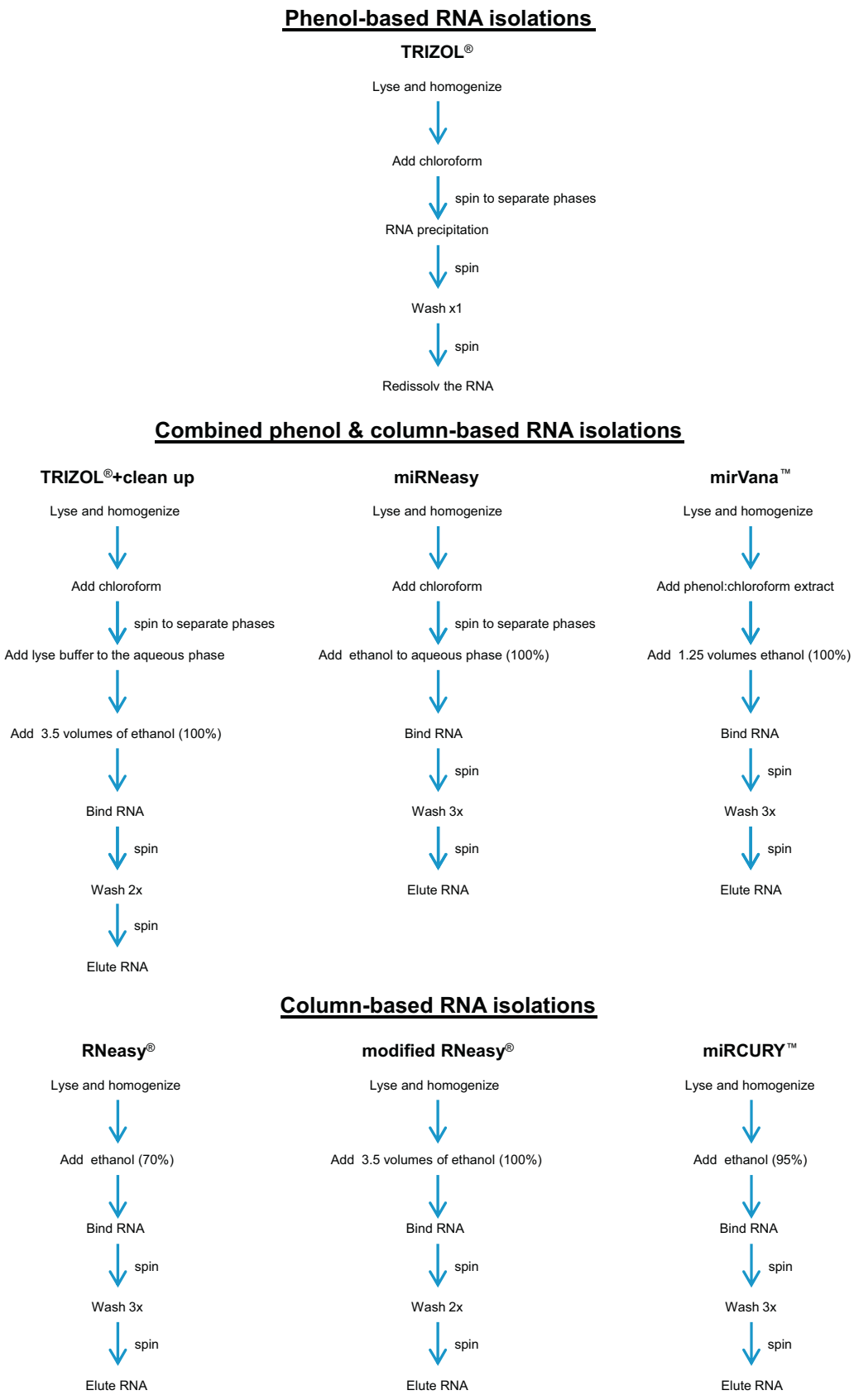


Fig. 2. Flow chart over the different RNA extraction methods. RNA from cells and exosomes was isolated using the phenol based method (Trizol®), combined phenol and column based approaches (Trizol® followed by cleanup using the modified RNeasy®, miRNeasy and mirVana™) and pure column based techniques (RNeasy®, modified RNeasy® and miRCURY™).

Table 1
RNA extraction methods evaluated.

Method	Company	Extraction technique
Trizol®	Invitrogen	Phenol/guanidine
Trizol® followed by modified RNeasy® Mini Kit (cleanup)	Invitrogen and Qiagen	Combined phenol/guanidine and column based technology
RNeasy® Mini Kit	Qiagen	Column based technology
Modified RNeasy® Mini Kit	Qiagen	Column based technology
miRNeasy Mini Kit	Qiagen	Combined phenol/guanidine and column based technology
miRCURY™ RNA Isolation Kit	Exiqon	Column based technology
mirVana™ miRNA Isolation Kit	Ambion	Combined phenol/guanidine and column based technology

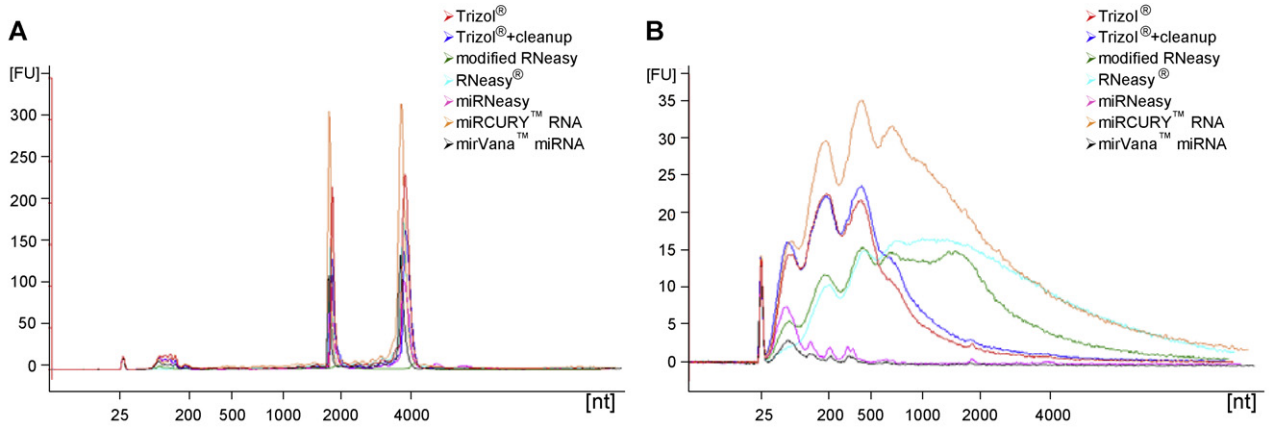


Fig. 3. Bioanalyzer analysis of cellular and exosomal total RNA. Cellular and exosomal RNA isolated with seven different methods was analyzed using RNA Nano 6000 Kit in an Agilent 2100 Bioanalyzer. The electropherograms show the size distribution in nucleotides (nt) and fluorescence intensity (FU) of total RNA in (A) cells and in (B) exosomes. The peak at 25 nt is an internal standard. (A) In cells the most dominant peaks are 18S and 28S ribosomal RNA. The ribosomal 18S and 28S RNA is a part of the algorithm, of which the RIN values are calculated and can be used as a measure of the RNA quality (RIN; 1 = totally degraded, 10 = intact). RNA isolated using all of the tested methods had intact 18S and 28S ribosomal RNA, resulting in high RIN values. (B) In exosomes, the 18S and 28S ribosomal RNA peaks are not present. The electropherogram is a representative of $n = 4$.

protocol (Exiqon). In brief, each cDNA synthesis was performed in duplicates using a fixed volume of total RNA, miR-451 specific reverse primer and first strand cDNA synthesis kit reagents and incubated for 30 min at 50 °C followed by 10 min at 85 °C. Each cDNA sample was then diluted 1:10 and used in duplicates together with miRCURY™ LNA™ SYBR® Green master mix, the Universal primer and the LNA™ PCR miR-451 specific primer. PCR was performed for 10 min at 95 °C; 10 s at 95 °C + 5 s at 60 °C for 40 cycles and finalized by a dissociation curve 5 s for each 0.5 °C. Control samples were run in parallel. The CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) was utilized for both cDNA and real-time PCR reactions. Finally, the samples were analyzed using CFX Manager™ software (Bio-Rad).

3. Results

3.1. RNA quality

Cellular and exosomal RNA was extracted using seven different RNA isolation methods (Table 1). The quality of the extracted RNA, using the different methods, was assessed by the Bioanalyzer RNA integrity numbers (RIN; 1 = totally degraded, 10 = intact). Since the algorithm is based on the ribosomal RNA and exosomes contain little or no ribosomal RNA, RIN values are only valid for cellular RNA quality assessments. The cellular RIN values were high and consistent with mean values between 8.9 and 9.8 for all the methods evaluated (Fig. 3A and Table 2).

3.2. RNA yield

To assess the RNA yield of both exosomes and cells, total RNA was analyzed with the RNA 6000 Nano Kit using an Agilent 2100 Bioanalyzer. The RNA yield differed substantially between the different RNA isolation methods both for cells and exosomes (Figs. 3A and B and 4A and B). The cellular total RNA amount ranged from 2.5 ± 0.5 μg (modified RNeasy®) to 20.2 ± 4.0 μg (miRCURY™), and when these numbers were normalized to the donor cell number, the yield ranged from 1.0 ± 0.7 to 6.3 ± 1.1 μg/million cells (Table 2). For the exosomes, the total RNA amount ranged between

Table 2
Comparison of cellular RNA isolated using seven different RNA extraction methods.

Method	RNA amount (μg)	RNA yield (μg/10^6 cells)	OD 260/280	OD 260/230	RIN value
Trizol®	14.6 (3.3)	4.3 (0.3)	2.1 (0.0)	1.8 (0.2)	9.6 (0.1)
Trizol® + cleanup	12.3 (3.1)	3.0 (0.3)	2.2 (0.1)	2.1 (0.2)	9.9 (0.1)
RNeasy®	13.4 (2.7)	4.6 (1.4)	2.3 (0.0)	2.6 (0.0)	9.8 (0.1)
Modified RNeasy®	2.5 (0.5)	1.0 (0.7)	1.9 (0.2)	1.2 (0.2)	8.9 (0.7)
miRNeasy	11.6 (2.6)	2.9 (0.3)	1.9 (0.3)	1.5 (0.3)	9.8 (0.1)
miRCURY™ RNA	20.2 (4.0)	6.3 (1.1)	2.0 (0.1)	2.0 (0.2)	9.7 (0.1)
mirVana™ miRNA	17.0 (3.2)	5.5 (1.2)	1.9 (0.0)	1.8 (0.1)	9.8 (0.0)

Cellular RNA extracted using seven different methods was analyzed using RNA Nano 6000 Kit in an Agilent 2100 Bioanalyzer. From the analysis, RNA amount, RNA yield and RNA Integrity (RIN) values were obtained. Furthermore, the RNA purity was evaluated spectrophotometerically at the absorbance 230, 260 and 280 nm. The analysis was done using RNA extracted from 2 to 4 million cells in at least three independent experiments. Mean values are shown with standard error of the mean reported in parenthesis.

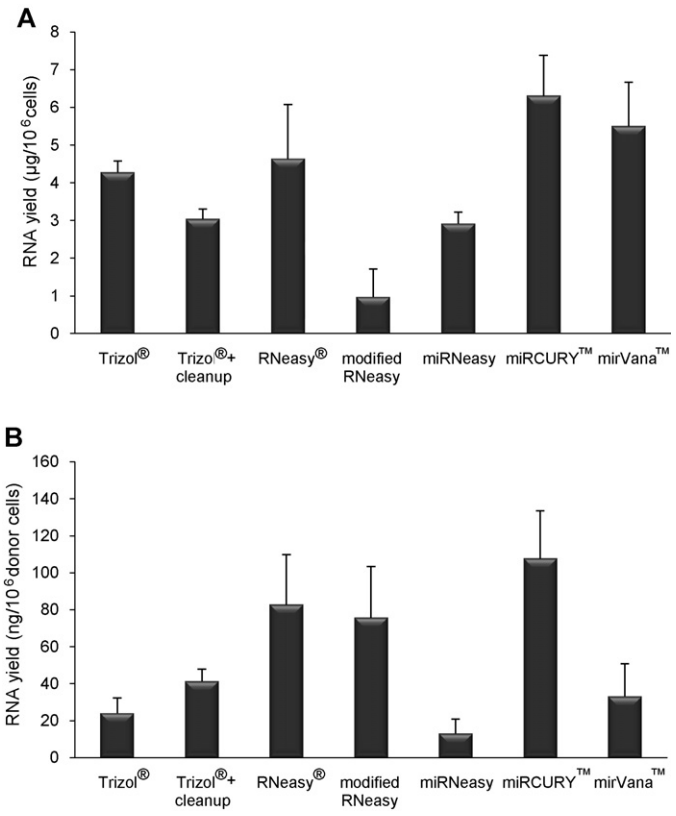


Fig. 4. Exosomal and cellular RNA yield. The yield of (A) cellular (μg/10^6 cells) and (B) exosomal (ng/10^6 donor cells) RNA for the different extraction methods, was determined using RNA Nano 6000 Kit in an Agilent 2100 Bioanalyzer. Data is presented as mean values for each method (n = 3–4) with SEM.

2.0 ± 0.7 μg (miRNeasy) and 21.8 ± 5.1 μg (miRCURY™), and when these numbers were normalized to the donor cell number, the yield ranged from 13.0 ± 7.7 to 107.7 ± 25.7 ng/million donor cells (Table 3). For both cells and exosomes the miRCURY™ resulted in the highest total RNA yield. The Trizol®, miRNeasy and mirVana™ showed the lowest total RNA yield in exosomes, while the modified RNeasy® showed the lowest yield in cells.

3.3. RNA size distribution

Bioanalyzer electropherograms of exosomal RNA patterns illustrated substantial differences in the relative presence of smaller and larger RNA molecules, depending on the method used (Fig. 3B). In short, the RNeasy®, modified RNeasy® and miRCURY™ RNA isolation methods were shown to extract RNA with a broad size distribution. The miRNeasy and mirVana™ were shown to be relatively more efficient at extracting small RNA rather than total RNA compared to the other methods evaluated. The Trizol® and Trizol® + cleanup showed no difference in RNA size pattern compared to each other, but compared to RNeasy®, modified RNeasy® and miRCURY™ these showed a more narrow size distribution pattern, favoring somewhat small RNAs.

Table 3
Comparison of exosomal RNA isolated using seven different RNA extraction methods.

Method	RNA amount (μg)	RNA yield (ng/10^6 donor cells)
Trizol®	6.4 (3.4)	24.1 (8.3)
Trizol® + cleanup	11.3 (3.4)	41.3 (6.6)
RNeasy®	15.2 (1.9)	82.8 (27.1)
Modified RNeasy	13.6 (1.8)	75.7 (27.6)
miRNeasy	2.0 (0.7)	13.0 (7.7)
miRCURY™ RNA	21.8 (5.1)	107.7 (25.7)
mirVana™ miRNA	5.0 (1.6)	33.1 (17.7)

Exosomal RNA amount and yield calculated from the results obtained from the Bioanalyzer analysis using Agilent RNA Nano 6000 Kit. The exosomes were obtained from 85 ml mast cell cultures containing 100–340 million cells. The analysis was done using RNA extracted from at least three independent experiments. Mean values are shown with standard error of the mean reported in parenthesis.

3.4. Assessment of RNA purity

Cellular RNA purity was evaluated spectrophotometerically at the absorbance 230, 260 and 280 nm (Table 2), were low A260/280 (<1.8) and A260/230 (<2.0) ratios indicate contamination. The purity as assessed by the average A260/280 ratio was high for all methods (1.9 ± 0.3–2.3 ± 0.0). However, Trizol®, modified RNeasy®, miRNeasy and mirVana™ showed lower average A260/230 ratios (1.8 ± 0.2, 1.2 ± 0.2, 1.5 ± 0.3 and 1.8 ± 0.1) compared to the other three methods as shown in Table 2. To further examine whether the difference in exosomal RNA size distribution was due to real differences in RNA content and not due to DNA contamination, the RNA samples were treated with RNase prior to analysis in the Bioanalyzer. This revealed that most or all of the nucleic acid was degraded upon RNase treatment indicating the presence of RNA and not a DNA contamination (Fig. 5A–G).

3.5. Analysis of small RNA including microRNA

Furthermore, we investigated the ability of the different RNA isolation methods to extract small RNA, including microRNA, in exosomes and cells using the small RNA kit in the Bioanalyzer instrument. The small RNA kit analyses only small RNA in the interval of 6–150 nucleotides, including the microRNA region in the size between 10 and 40 nucleotides. Since this kit has a maximum capacity of 100 ng/μl, the RNA samples were diluted 10 times prior to the analysis, which resulted in a concentration between 4 and 44 ng/μl. The analysis of small RNA in cells revealed that all methods extracted small RNA including the size of microRNA with equal ability, with the exception of RNeasy®, which only extracted the larger small RNAs (data not shown). In exosomes, all methods extracted small RNA in the size interval of 6–150 nucleotides (Fig. 6A). Unexpectedly, this was also true for the RNeasy®, which is supposed to extract primarily larger RNAs and not smaller RNAs, although it did not extract the RNA molecules with the shortest nucleotides successfully. The modified RNeasy® method extracted small RNA, including the size of microRNA, but not to the same extent as the other extraction methods. Extraction of exosomal RNA using Trizol® and miRCURY™ resulted in the highest yield of small RNA. To determine the relative amount of small RNA extracted by the different methods, the Agilent Bioanalyzer chip was loaded with the same amount of total RNA, regardless of their total yield (Fig. 6B). This experiment confirmed the variation in RNA distribution depending on the extraction method used. The relative amount of small RNA extracted using miRNeasy and mirVana™ was higher compared to the other methods, and their patterns were very similar. The Trizol® method also resulted in very similar patterns with Trizol® + cleanup, but with relatively less small RNA than miRNeasy and mirVana™.

To confirm the presence and amount of microRNA, real-time PCR was performed. For this analysis, one pure column based method (miRCURY™) and one combined phenol and column based extraction method (mirVana™), were selected on the basis of their difference in RNA pattern and yield (Figs. 6A and 7A and B). Based on previous microarray analysis, miR-451 was selected for analysis (Valadi et al., 2007). The result showed that both extraction techniques isolated miR-451, however RNA extraction with the

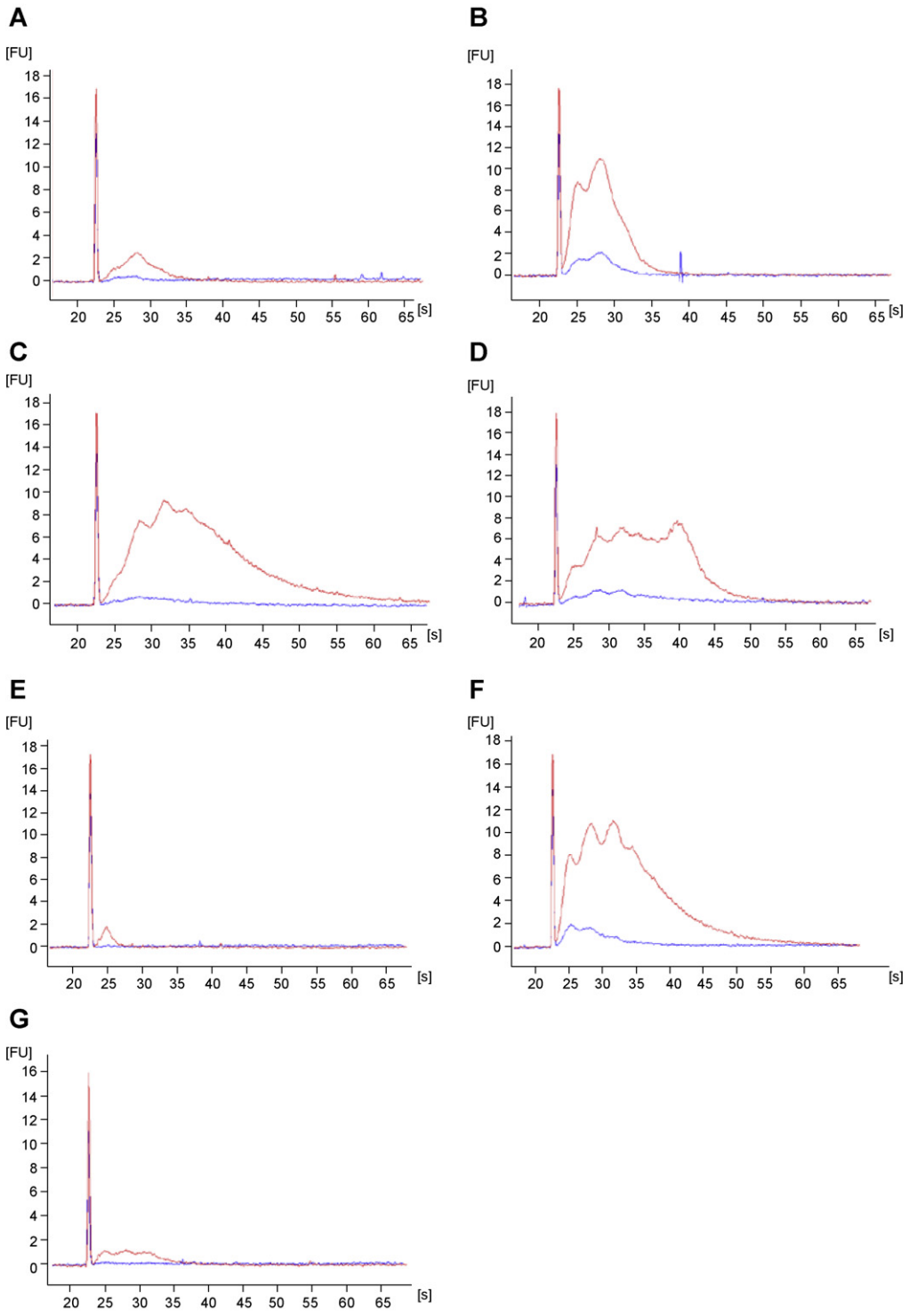


Fig. 5. Effects of RNase treatment of extracted exosomal RNA. Electropherograms of representative Agilent 2100 Bioanalyzer data of exosomal RNA extracted with the seven methods (A) Trizol®, (B) Trizol® followed by cleanup, (C) RNeasy®, (D) modified RNeasy®, (E) miRNeasy, (F) miRCURY™ and (G) mirVana™, treated with 5 μg/μl RNase A (blue peak) or vehicle (red peak) for 90 min at 37 °C. The RNase treatment resulted in elimination of the peak in the electropherogram, arguing that the Bioanalyzer peak is indeed RNA and not due to other contaminants such as DNA.

pure column based method resulted in a higher yield compared to the combined phenol and column based method (mean $C(t)$ value 33.6 ± 0.4 and 36.4 ± 1.4).

4. Discussion

In this unique study we have evaluated seven different RNA isolation methods, based on different extraction techniques from four different companies, to determine their efficiency in extracting primarily exosomal RNA. Exosomal RNA from different cellular sources has been described in a number of studies with varying RNA patterns, which may be explained by the cellular origin or extraction method. Thus, there is a need to standardize the methodology to isolate RNA from exosomes. We focused this study on the quality, yield, size distribution, purity and analysis of small RNA including microRNA in mast cell exosomes and their donor cells.

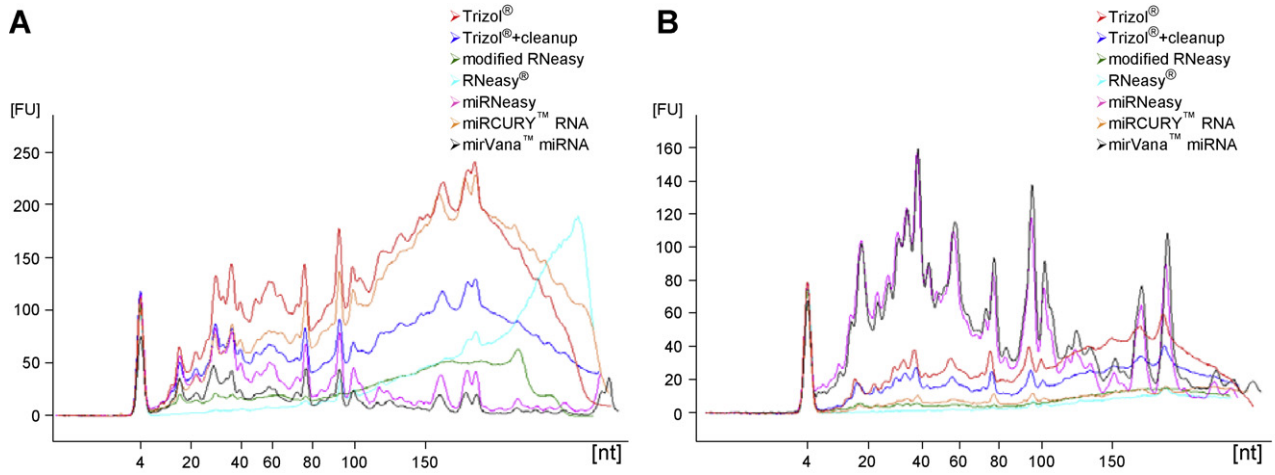
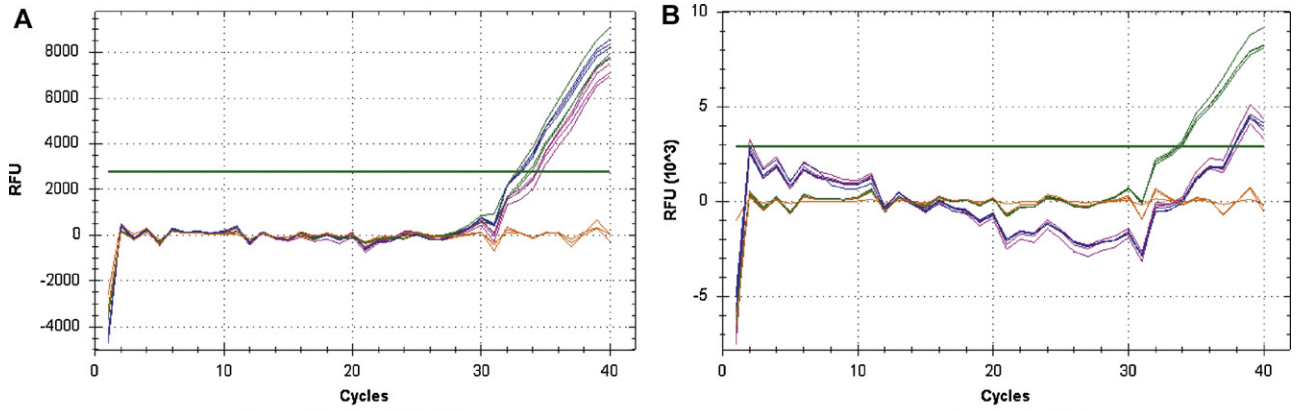


Fig. 6. Bioanalyzer analysis of exosomal small RNA. Total exosomal RNA was extracted using seven different RNA isolation methods and analyzed using small RNA Kit in an Agilent 2100 Bioanalyzer. The representative electropherograms show the size distribution of small RNA in the interval of 6–150 nucleotides, including the microRNA in the sizes between 10 and 40 nucleotides in (A) 10× diluted samples and (B) 10 ng total RNA. To determine the relative amount of small RNA extracted by the different methods, the same amount of total RNA was loaded, regardless of their total yield (Fig. 2B).



Sample	C(t) Mean
1 (green)	33.4
2 (blue)	33.0
3 (pink)	34.4

Sample	C(t) Mean
1 (green)	33.7
2 (blue)	37.8
3 (pink)	37.8

Fig. 7. Real-time PCR analysis of exosomal microRNA. The presence of miR-451 in exosomal total RNA, extracted using miRCURY™ and miRVANA™, was confirmed by real-time PCR $n = 3$. (A) miRCURY™ shows a higher miR-451 expression compared to (B) mirVana™ as seen by the $C(t)$ mean values in all three replicas.

Furthermore, this study only evaluates the RNA extraction using the different methods from MC/9 cells and exosomes, as we are interested if the differences in RNA patterns depend on the method used. The determination of the different methods ability to extract RNA from different cellular and exosomal origins are beyond the scope of this paper and are therefore not evaluated.

The quality of the extracted RNA using the different methods was assessed by the Bioanalyzer using the RIN values, where a high value indicates high quality. This algorithm can only be used when analyzing cellular RNA, since the ribosomal RNA peaks are an integral part of this algorithm and exosomes do not contain these ribosomal RNA peaks. For all the methods evaluated, the cellular RIN values were high and consistent, indicating that the extracted cellular RNA is of high quality. Based on the cellular RIN values, we also assume that the exosomal RNA is of high quality, since the experiments were performed in parallel. Therefore, the cellular RIN values are also used as a reference of the exosomal RNA quality.

Importantly, the RNA yield differed substantially between the different RNA isolation methods for both cells and exosomes. For both cells and exosomes the miRCURY™ showed the highest total RNA yield. The Trizol®, miRNeasy and mirVana™ showed the lowest total RNA yield in exosomes, while the modified RNeasy® showed the lowest yield in cells. Interestingly, the methods that showed the lowest yield in exosomes are all phenol-based (including the combined phenol and column based techniques), while the methods that showed the highest yield were all column based approaches. However, this difference between the phenol and column based techniques were not seen in the cellular RNA extractions. Importantly, the column based miRCURY™ showed the highest RNA yield, both in exosomes and in cells, suggesting good lysing ability and/or RNA binding and elution capacity of the column. The difference in RNA yield between exosomes and cells, may be explained by a more rigid membrane in exosomes due to the difference in lipid composition of the cellular and exosomal membranes, such as enrichment in sphingomyelin and cholesterol in exosomes resulting in incomplete lysing of the exosomes using the phenol based approaches (Laulagnier et al., 2004; Trajkovic et al., 2008). However, this is above the scope of this study and needs to

be further evaluated. Importantly, the aim of this study was not to compare exosomes and cells, but rather to find a method suitable for both.

Moreover, Bioanalyzer electropherograms of exosomal RNA patterns illustrates a substantial difference in the relative presence of smaller and larger RNA molecules depending on the method used. In short, the three column based RNA extraction techniques were shown to extract RNA with a broad size distribution. The pure phenol and the combined phenol and column based extraction techniques were shown to be relatively more efficient at extracting small RNA rather than total RNA, compared to the column based methods. In cells, this difference can be hard to detect as the high ribosomal RNA content masks the mRNA and small RNA expression. Exosomes may therefore be a good tool when evaluating a RNA extraction methods ability to extract total RNA, including mRNA and small RNA.

Furthermore, to examine the purity of the RNA isolated with the different methods, spectrophotometer measurement at 230, 260 and 280 nm was performed. The ratio between the absorbance at 260 and 280 nm is used to evaluate the purity of nucleic acids, a ratio lower than 1.8 may indicate the presence of proteins and peptides absorbing at 280 nm. In addition, a ratio lower than 2.0 between the 260 and 230 nm absorbance may indicate contamination by peptides, phenols, aromatic compounds or carbohydrates.

All of the methods evaluated were shown to be clear of protein and peptide contaminants, as the A260/280 ratios were high ($\geq$1.9) for all methods. Also, the A260/230 ratio was high for the combined method Trizol® + cleanup, and the column based RNeasy® and miRCURY™ indicating pure samples. However, the A260/230 ratio was low for Trizol®, modified RNeasy®, miRNeasy and mirVana™ miRNA indicating a contamination of these samples. This contamination may potentially be from phenol/guanidine due to inadequate washing steps. Interestingly, the modified RNeasy® showed much lower A260/230 compared with the original RNeasy® method. The difference between these methods is primarily an increased amount and concentration of ethanol, which is supposed to favor the isolation of small RNA but seem to result in insufficient washing of the RNA samples.

To further examine whether the difference in exosomal RNA size distribution was influenced by any contamination, we treated the RNA samples with RNase prior to analysis in the Bioanalyzer. This revealed that most or all of the RNA was degraded upon RNase treatment, demonstrating that the difference in exosomal RNA pattern between the different methods is not due to contamination, but rather due to true differences in RNA patterns.

Furthermore, we investigated the ability of the different RNA isolation methods to extract small RNA, including microRNA. For this purpose we compared equal volume total RNA from the seven different RNA isolation methods. For exosomes, all methods extracted small RNA in the size interval of 6–150 nt. Unexpectedly, this was also true for the column based technique RNeasy®, which is supposed to extract primarily larger RNAs and not smaller RNAs, although it did not extract the RNA molecules with the shortest nucleotides successfully. The modified RNeasy® method (column based technology) extracted small RNA, including microRNA, but not to the same extent as the other extraction methods, more specialized at small RNA. Extraction of exosomal RNA using Trizol® (phenol based technology) and miRCURY™ (column based technology) resulted in the highest yield of small RNA. For miRCURY™ this was not unexpected, since it also extracted the highest amount of total RNA in a wide size distribution.

To determine the relative amount of small RNA extracted by the different methods, we loaded the Bioanalyzer instrument with the same amount of total RNA, regardless of their total yield. This experiment confirmed the extensive differences in distribution depending on the extraction method used. For example, the relative amount of small RNA extracted using the combined phenol and column based techniques, miRNeasy and mirVana™, was higher compared to the other methods, and their patterns were very similar. The phenol based extraction technique Trizol® also resulted in very similar patterns with Trizol® + cleanup (combined phenol and column based technology), but with relatively less small RNA compared to miRNeasy and mirVana™. The analysis of small RNA in cells revealed that all methods extracted small RNA including microRNA with equal ability, with the exception of RNeasy®, which only extracted the larger small RNAs (data not shown).

As mentioned above, all methods isolated RNA containing small RNA as shown in the electropherograms (Fig. 6A and B). This however does not show whether the different samples contain microRNA. To confirm the presence of microRNA, real-time PCR was performed. For this analysis, the two contrasting methods, the pure column based method (miRCURY™) and the combined phenol and column based method (mirVana™), were chosen. The microRNA primer miR-451 was chosen based on our previous study, (Valadi et al., 2007) where it was shown to be one of the highest expressed microRNAs. In the electropherogram (Fig. 6A) the two methods were shown to extract small RNA in the size of microRNA. Using real-time PCR, we confirmed the presence of microRNA by looking specifically on miR-451. However, it is worth mentioning that usually a fixed concentration of RNA is used for cDNA synthesis and not a fixed volume as we used in this study. We choose to use a fixed volume as this better compares the two methods as using a fixed concentration regulates for the differences in amount. Importantly, a fixed concentration is usually used when comparing two different samples. In this case it is important to bear in mind that the normalization occurs when the exosomal sample is divided into seven equal samples. Hence, we are comparing the same sample but with two different methods. In addition this experiment was performed to validate the existence of microRNA in the two different isolation methods.

5. Summary

In conclusion, in this study we present a unique comparison of seven different methods for extraction of exosomal RNA. The methods were evaluated for their ability to extract exosomal RNA, with special focus on RNA quality, yield, size distribution, purity and analysis of small RNA. All tested methods extract RNA with high quality but with huge variation in yield and size distribution of exosomal RNA, even though the exosomes were harvested from the same cell cultures. As the different isolation methods give such an extensive variation in exosomal RNA yield and pattern, it is of great importance to choose the method carefully depending on the research question at hand, since one method can show enrichment in small RNA whereas another will show a broader RNA distribution. This is especially important in exosome research, since the quantity of exosomes harvested from different body fluids or cell cultures is often relatively limited.

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Competing interests

JL is the majority owner of a patent relating to the use of exosomes for transfer of nucleic acids (International Application No.:

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