CONSIDERATIONS FOR WORK WITH EXTRACELLULAR VESICLE RNA AND ITS ANALYSIS

APPLICATION NOTE

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CONTENTS

1 / INTRODUCTION

RNA regulates critical normal biological activities and is also central to pathological cellular dysregulation[1]. As important signals for cell-to-cell communication, RNAs associated with EVs (EV-RNA) are protected during transport and targeted to a recipient cell in an effective manner. A multitude of physiological roles have been attributed specifically to EV-RNA in humans, however, it is EV-RNA's diagnostic/biomarker potential harbouring the most promise to medicine and public health. Not only can signature EV-RNA profiles be linked to certain diseases, but also the presence or expression level of specific RNA sequences can be indicative of a condition's presence or level of progression. Moreover, EV-RNA roles extend beyond just human cells where for example non-eukaryotic cells, like bacteria may also release RNA-containing EVs to influence virulence and pathogen-host interactions during infection[2].

Just like other areas of EV research, EV-RNA researchers are challenged by the absence of a single standardised EV purification/characterisation method that reliably provides pure EV samples for analysis. Furthermore, there are challenges that are inherent to working with RNA, such as biases in RNA extraction/analysis tools providing a limited view of RNA composition. Together, both types of challenges slow progress in the EV-RNA field.

Here, we present considerations in experimental design when working with EV-RNA, from EV isolation, RNA extraction and quantification, requirements for different types of assays and its analysis. Hopefully this guide can help researchers in appropriate data interpretation and meaningful conclusions of EV-RNA roles and applications.

2 / EV ISOLATION FOR EV-RNA ANALYSIS

To characterise RNA content exclusively associated with EVs, EVs must be separated from other RNA-binding non-EV components, and frequently concentrated so that the quantities present are sufficient to yield RNA suitable for the sensitivity of the selected RNA assay and analysis. Each EV-containing matrix or biofluid has its own complexities when working with RNA. Some biofluids are more widely studied than others, and most information has been derived from studies of plasma/serum and culture conditioned media (CCM) samples.

Plasma samples have shown popularity in studies aimed at the discovery of diagnostic EV biomarkers. However, plasma has inherent challenges, due to the rich diversity of other RNA-binding non-EV components that may interfere with EV-RNA research. For example, plasma contains biomolecules that have been associated with miRNAs, such as High-Density Lipoproteins (HDL) and/or Low-Density Lipoproteins (LDL)[3,4] and other circulating proteins like the Argonaute complex[5]. Thus, EV isolation procedures from plasma samples must ensure that these RNA-binding contaminants are removed to acceptable standards. To achieve this, it is important to have a reliable and suitable EV purification protocol, such as SEC qEV columns. Notably, plasma HDLs[6] and circulating protein complexes[5] can be successfully separated from EVs with Size Exclusion Chromatography (SEC) **(Figure 1A).** It is also important to note that RNA-binding lipoproteins can be subject to substantial between- and within-individual variation, with regards to their abundance and composition. This is further demonstrated in a study showing RNA-associated lipoproteins were significantly increased in plasma EV preparations from individuals 4 hours after a high-fat meal[7].

Without an appropriate method for removing plasma proteins with an RNA-binding capacity, the abundance of EV-RNA is artificially overestimated, with a clear compromise of EV purity[8]. This has been demonstrated with the use of precipitation EV isolation methods, where reported RNA concentrations are approximately 2.5 ng RNA/mL of plasma, compared to 1.7 ng RNA/mL of plasma from pure EVs isolated with SEC-qEV[8].

CCM may present fewer challenges than plasma samples regarding intrinsic and variable RNA-binding contaminants, however, CCM does have its own challenges to overcome. Most cells require supplementation with Fetal Bovine Serum (FBS) for optimal growth, which contains significant amounts of FBS EVs, FBS EV-RNA and FBS non-EV-RNA. While it is common practice to deplete EVs from FBS used in culture, the depletion procedure does not eliminate all FBS RNA. In fact, only approximately 30% of all FBS RNA content can be removed, with the remaining RNA mostly associated to RNA-binding proteins[9]. Therefore, appropriate controls should be implemented at each step and throughout entire workflows; for example, for all EV isolation procedures, RNA extraction and RNA quantification and analyses should be applied to both the full media formulation used in culture **(Figure 1B and C),** and the cell CCM[9,10].

Comprehensive and comparative studies assessing RNA-binding non-EV components are needed not only for CCM, but also for other increasingly investigated biofluids such as urine, milk, and saliva. To ensure results reflect native EV-RNA composition, researchers need to be confident that impact of sample processing (EV isolation, purification and EV enrichment) on EVs and RNA degradation is minimal. Storage of EV samples is an integral part of the handling process and is highly sensitive to RNA damage, as freezing and thawing can degrade RNA[11,12]. For a detailed revision on how storage parameters impact EVs, refer to our article **Pre-analytical Storage of Extracellular Vesicles: Pre- and Post-Isolation Considerations**, where we highlight the importance of storage conditions and their effects on different samples and populations of EVs throughout the pre and post EV isolation steps.

Importantly, RNA strictly located inside EVs (luminal) is protected from RNase degradation, unlike other non-EV RNA-binding structures, or RNA loosely associated to the exterior of EVs which are degraded by RNases. Thus, to minimise RNA contamination and help in analysis of exclusive EV-RNA, RNase treatment (e.g RNase A) should be applied to purified EV preparations to eliminate all non-luminal EV-RNA. Moreover, extra treatment of purified EV preparations with Proteinase K also helps in removal of RNA-binding proteins[13].

A

	Albumin				ApoA1			
	SEC fractions							
	F7	F8	F9	F10	F7	F8	F9	F10
µg/mL ± SEM	0.16 ± 0.16	1.21 ± 0.38	10.43 ± 3.10	79.07 ± 9.69	0.10 ± 0.10	15.38 ± 6.05	83.45 ± 14.34	279.9 ± 62.22
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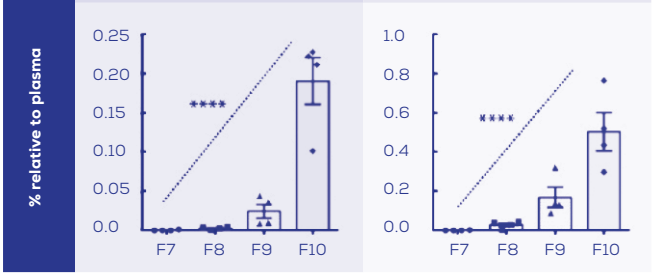
	Plasma							
µg/mL ± SEM	44509 ± 2003				64845 ± 17015			



B

RNA classes	Vesicle depleted FBS
antisense	380,533±3,688
mRNA	236,500±3,739
rRNA	37,220±3,163
miRNA	55,720±1,907
snoRNA	69,469±5,901
piRNA	51,574±1,245
Y RNA	30,538±2,772
pseudogene	12,336±341
snRNA	5,228±374
repeat	8,342±538
tRNA	2,301±86
SRP	150±3.8
vRNA	4.2±0.4
7SK	35.7±2.0
Others	110,050±2,063

C

miRNA	Vesicle depleted FBS
miR-122-5p	35,095.2
miR-1246	17,557.4
miR-148a-3p	275.6
miR-423-5p	446.7
miR-92a-3p	54.3
let-7b-5p	219.9
miR-379-5p	92.4
miR-127-3p	32.3
let-7a-5p	46.7

Figure 1. RNA-binding contaminants in EV-containing samples. **A,** Protein (Albumin) and Lipoprotein (ApoA1) contaminants (quantified by ELISA) in EV-rich fractions purified with qEV columns (Izon) from human plasma. This shows that most protein and lipoproteins are removed from EV preparations, therefore less likely to interfere with EV-RNA analysis downstream. Adapted from [14]. **B,** RNA classes from all reads mapped to human genome (determined by RNA-seq analysis) in vesicle depleted FBS. The list shows the contribution of remaining RNA-associated structures in vesicle depleted FBS normally used in EV-RNA profiling from culture conditioned media (CCM). Adapted from [9]. **C,** Most abundant miRNAs mapped to human genome (determined by RNA-seq analysis) in vesicle depleted FBS. This shows the signature miRNA profile attributed only to culture medium supplemented with vesicle depleted FBS when isolating EVs from CCM. Adapted from [9].

3 / EV-RNA EXTRACTION

Two of the most popular EV-RNA extraction methods can be categorised as organic RNA extraction[15] or solid phase extraction. Overall, organic RNA extraction is a scalable and efficient method that uses phenol and guanidine isothiocyanate to lyse EVs, separating an organic phase that contains proteins and DNA from an aqueous phase containing RNA, and followed by a final RNA precipitation step.

However, limitations of this method include the need for hazardous materials, bias towards long, low structured (lack of stable secondary structures), and low GC content RNAs[16], and an RNA preparation that may have medium-level purity.

Combining organic RNA extraction with solid phase extraction has gained popularity in EV-RNA research since it significantly improves the yield and purity of RNA preparations. Solid phase RNA extraction relies on the manipulation of RNA affinity to a solid support, usually provided by a filter in a spin column.

The material of spin columns has been developed and diversified to generate commercial kits, each with their own advantages and limitations. As the amount of RNA found in EVs is very small, it is commonly required to use an RNA carrier along with spin columns; i.e., a molecule – usually an unrelated nucleic acid – that helps in binding and recovery of the target RNA[15,17].

Common RNA carriers are yeast RNA, bacteriophage RNA, Poly (A) RNA or glycogen. RNA carriers may have no impact on PCR-based assays which detect specific sequences; however, a drawback to consider would be that assays will be using target RNA plus carrier RNA samples in functional studies, with unknown effects in cells.

Most RNA extraction methods isolate total RNA from EVs[15,18], however some methods allow modifications for preferential extraction of small RNAs (<200 nt) separated from larger (>200 nt) RNAs[18]. The qEV RNA extraction kit is an EV-RNA extraction kit that does not use hazardous materials for lysis, has silicon-carbide material in spin columns enabling the isolation of total RNA without biases for size or GC content and does not require carrier RNA for optimal yields.

4 / EV-RNA QUANTIFICATION

In general, RNA concentration can be estimated by spectrophotometry, fluorometry, and conventional or automated electrophoresis. Whilst each method has its own strengths and limitations; the selection of a quantification assay depends on how relevant the limitations are to the end point analysis. Specific/sensitive detection and accurate quantification of RNA may be desirable or required for many downstream applications, even when samples are contaminated with DNA.

Typically, fluorometric or automated electrophoresis[13,15,19] assays are used for EV-RNA quantification, due to their specificity and sensitivity, respectively. The amount of RNA obtained from EVs is a controversial topic since it is directly correlated with the preceding EV purification method; as mentioned earlier, RNA can be bound to proteins or lipoproteins that can co-isolate along with EVs under some EV isolation methods. Also, it is not uncommon to have undetectable EV-RNA concentration (below limit of detection of the chosen quantification method) from highly pure EV preparations. In these cases, consecutive RT-qPCRs assays are still likely to succeed due to the nature of PCR assay which amplifies RNA signal.

Alternatively, some of these quantification methods can be useful for qualitative analysis of EV-RNA. For example, the principle behind automated electrophoresis is that RNA fragments are quickly separated by size and visualised in a user-friendly format. In this sense, knowing the size of RNA species can indicate the abundance of rRNA, sRNA or even very small miRNA species in the EV-RNA sample **(Figure 2)**. A drawback of this analysis is that is not specific for RNA species, therefore DNA contamination is detected as RNA. Therefore, DNA digestion of RNA samples is an optional but highly recommended step, to ensure complete removal of contaminating DNA. This is normally achieved by treating the RNA sample with DNases and then an appropriate inactivation of the enzyme applied before continuing with assays[11,20,21].

Automated electrophoresis methods are also used to assess RNA quality in cellular RNA samples, an index based on 18S/28S rRNA proportions. However, RNA profiling of EVs has shown that the majority of EV-RNAs are small RNA, with little to no rRNA or without intact large 18S/28S rRNAs. Therefore, these cellular RNA quality assessments are not applicable to EV-RNA.

5 / TYPE OF EV-RNAs

Having a rigorous EV purification method can have a substantial impact on EV-RNA-based diagnostic potential, as knowing the true association of RNAs to EVs is critical to understanding the biology and roles of EV-RNA. A clear example of the importance of EV isolation procedures for EV-RNA was shown in the search for prostate cancer miRNA markers, where authors found that expression of miRNA 200c-3p and miR-21-5p from EVs could better distinguish cancer patients from healthy subjects, compared to using the same miRNAs from whole plasma[13]. EV isolation techniques which grant EVs of high purity normally compromise recovery[22], which can be a limitation for RNA down-stream analysis. Broad and basic characterisation of EV-RNA content can be also performed by the previously mentioned automated electrophoresis method, where outputs are presented as electropherograms or digital gels which provide easy interpretation of RNA sizes present in the EV-RNA sample. For example, broad RNA profiling of SEC qEV purified plasma EVs has shown predominance of sRNAs and a lack of large rRNA **(Figure 2A)**. Different available commercial kits have improved sensitivities for ultra-low EV-RNA samples (e.g. 50-250 pg/µL RNA concentration) or higher resolution in the range of specific RNA populations, such as sRNA, where miRNAs can be identified by a peak around the 20 nucleotide mark, as well as peaks for other conserved sRNAs of known sizes **(Figure 2B and 2C)**[20,23].

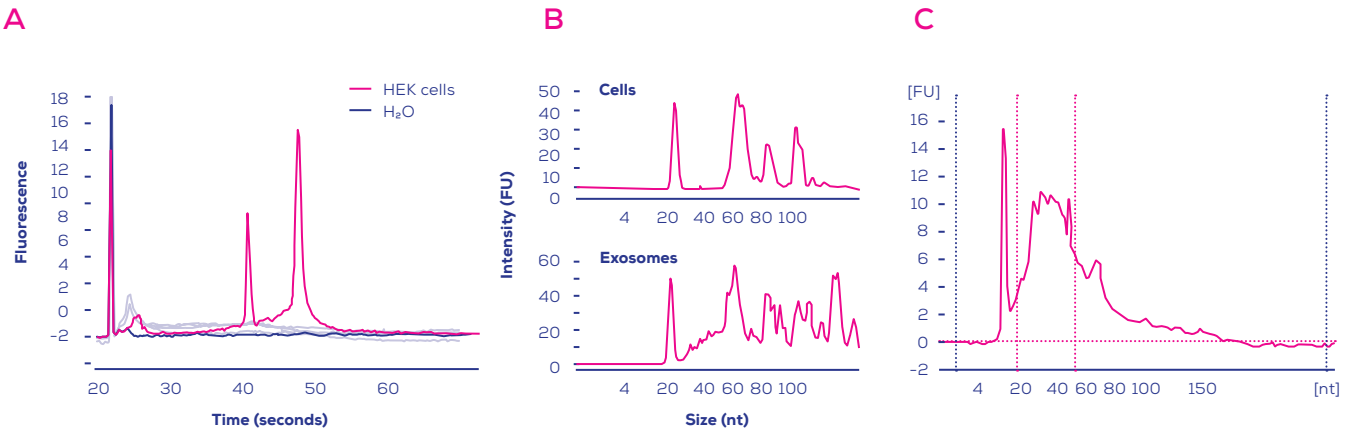


Figure 2. RNA profiles of EV-RNA analysed by automated electrophoresis. **A,** Electropherogram of EV-RNA extracted from human plasma EVs purified with qEV Isolation columns (grey line) (Izon). HEK293T (HEK) cells (pink line) and H_2O (dark blue line) were used as controls. This shows that the most abundant RNA in EV-RNA corresponds to small RNA, with a peak around 25 seconds. Adapted from[14]. **B,** Electropherogram using a small RNA chip of EV-RNA extracted from CCM small EVs (bottom) from endothelial cells (top). This shows the size distribution of sRNA in cells and EVs, where miRNAs appear in the segment between 10 and 40 nucleotides, a peak present in both samples. Adapted from[20]. **C,** Electropherogram using a small RNA chip of EV-RNA extracted from human plasma EVs. This shows the exclusive predominance of miRNA population within sRNAs in plasma EVs. Adapted from [24].

In turn, in-depth study of EV-RNA composition is usually accomplished by RNA-seq approaches and has demonstrated variable results also depending on the EV isolation method. Precipitation methods, known to enrich not only EVs but also many other contaminants, have shown a higher relative miRNA biotype content in serum samples than the SEC-qEV purification approach **(Figure 3A)**[19]. This overestimation of miRNAs in poorly purified EVs is in line with studies showing an abundance of circulating miRNAs in plasma/serum associated with lipoproteins or proteins [3,5]. Also, miRNAs are highly stable and resistant to wide range of external conditions, in biofluid and/or in EV isolation or storage, therefore their persistence might not be indicative of EVs and EV-RNA damage[25]. Moreover, small RNA-seq analysis approach in plasma EVs purified with more reliable EV isolation methods, such as SEC-qEVs, have also shown that that the majority of EV-RNA content is miRNA, but also with a small percentage being gene coding RNAs **(Figure 3B)**[14].

EV-RNA from CCM can present a great extent of variability regarding its composition, depending on the cell line. For example, stem cell EVs have shown considerably distinct miRNA signatures, providing evidence of their biological relevance and therapeutic potential[26]. Moreover, RNA profiling of EVs isolated from endothelial cells CCM has demonstrated a high abundance of miRNAs, however a substantial enrichment of Y-RNAs in EVs compared to their cellular counterparts has also been shown **(Figure 3C)**[20]. Y-RNAs are conserved mid-sized sRNAs that have been identified in EVs from many different cell types in vitro, as well as biofluids, and its presence has prompted interest in studying roles and diagnostic potential[27].

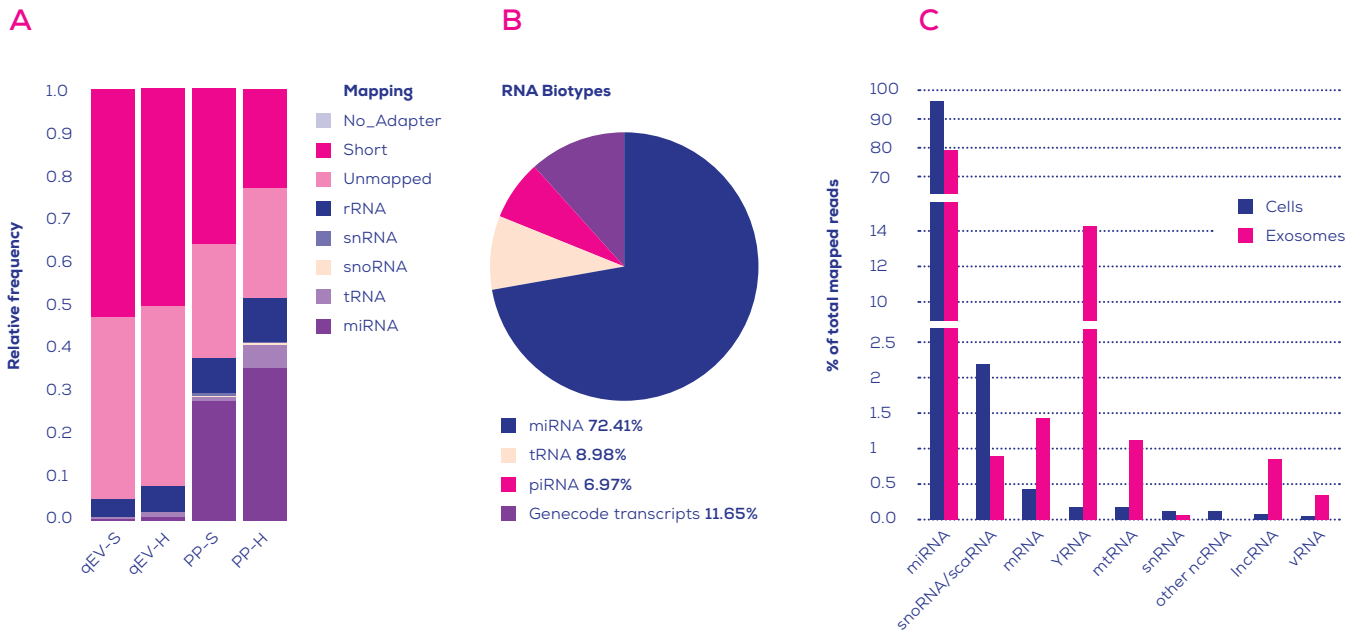


Figure 3. Relative distribution of RNA biotypes in EV-RNA. **A,** Small RNA sequencing of RNA obtained from serum EVs (sepsis or healthy volunteers) purified with qEV columns (Izon) or precipitation kit. Highest frequencies of miRNA mapping were observed in samples from precipitation kit, possibly due to miRNAs in non-EV structures. Adapted from 19. **B,** Small RNA sequencing of RNA obtained from qEV (Izon) purified plasma EVs. miRNAs represent a large percentage of the sRNA content, whereas gene coding RNAs (mRNA) comprise a small percentage. Adapted from [14]. **C,** Small RNA sequencing of RNA obtained from EVs from endothelial cells' CCM. Average percentages of mapped reads of most abundant RNA classes in cells; EVs show a clear enrichment of Y-RNA in EVs. Adapted from [20].

6 / EV-RNA ANALYSIS

EV-RNA analysis is progressing quickly as new technologies applied to fields like cellular biology, are quickly adjusted to EV work.

PCR

Quantitative PCR (qPCR) presents many advantages for EV-RNA research, as it is a quick, robust, reproducible, highly sensitive and specific method. As mentioned earlier, some highly pure EV samples might provide extremely low amounts of RNA. Therefore, depending on the origin and initial volume of EV-containing matrix, it is common end with EV-RNA yields that are below the limit of detection, even using most sensitive RNA quantification tools. In this way, EV samples from rare and small volume biofluids such as saliva, cerebrospinal fluid, embryonic, or samples taken from small animal models, may be expected to have undetectable RNA; however the amplification of signal through PCR-based assays may allow a successful detection of some targets, depending on their abundance.

To circumvent undetectable RNA amounts, known amounts of exogenous RNAs (spike-in) can be added, for quality control and normalisation purposes. For example, equal amounts of exogenous RNA added to different EV lysates before the RNA extraction allows to account for uncontrolled variability or biases to certain RNAs within the RNA extraction process. Exogenous RNA added to EV-RNA sample before qPCR allows to normalise RNA targets and to do absolute quantification when RNAs spike-in is used as standard curve. As these RNAs spike-in work as quality controls for both steps, they must represent characteristics of different types of RNAs found in EVs or at least the target RNA regarding length, structure, GC content, to account for potential biases based on these parameters in the EV-RNA extraction or PCR[28]. Thus, RNA spike-in cocktails might be needed to represent a spectrum of RNA types. Common synthetic RNA oligos used for normalisation have sequences that differ from endogenous EV-RNAs, are commercially available, and for example, include miRNAs from *Caenorhabditis elegans*[5].

A step prior to qPCR involves the conversion of EV-RNA into its complementary DNA (cDNA) in a reaction mediated by a Reverse Transcriptase (RT). Different RTs offer different attributes, such as RNase activity, fidelity, thermostability or processivity, and the ideal selection of RT will depend on the experimental requirements.

If EV-RNA concentration is known, then good practice calls for normalisation of EV-RNA input for each of the cDNA reactions[20]. In general, EV-RNA input amounts used for cDNA synthesis may range from 10 pg to 5 µg RNA, depending on RT performance. If EV-RNA concentration is unknown, EV-RNA may be normalised to volumes across samples with same processing protocols[13] or to spike-in RNA (added to the RNA before RT reaction). RTs are available for purchase as enzymes alone or as cDNA kits, which include RT, dNTPs, primers (e.g. random hexamers and/or oligos dT) and buffer.

The cDNA reaction is then followed by amplification and quantification by PCR. There are two main amplicon detection chemistries or technologies used in qPCR: SYBR Green or Taqman probes. SYBR Green is a fluorescent dye that binds non-specifically to any double-stranded DNAs or amplicons generated in a PCR reaction[20]. The Taqman approach utilises a sequence-specific probe bound to a dye that fluoresces when the amplicon is generated[23,28]. Although qPCR studies have shown that EVs contain both mRNA and sRNA (including miRNAs)[23], as shown in **Figure 4**, miRNA targets are most popular in PCR-based assays as they are more abundant in EVs, and there have been more thorough investigations into their diagnostic potential and regulatory roles.

Although less popular in all labs, another PCR technology that is significantly useful to EV-RNA research, due to its ultra-sensitivity and absolute quantification capacity, is Droplet Digital PCR (ddPCR). In general, this method generates ~20,000 droplets or small PCR reactions from one regular PCR reaction, followed by the quantification of amplification-positive droplets. This technology is particularly relevant in EV-studies aimed at improving the diagnosis of cancers that are detected far too late because of lack of markers. One example is hepatocellular carcinoma, where ddPCR has helped in detection of signature RNAs in optimised specific capture of cancer-specific EVs[29].

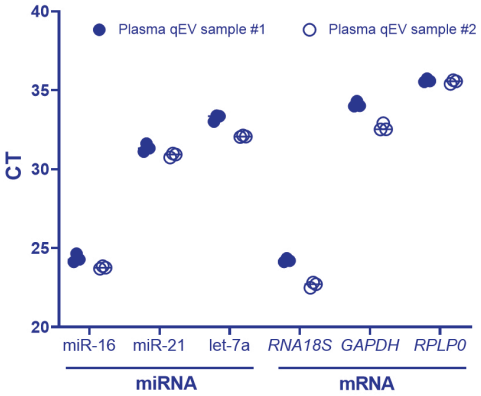


Figure 4. The detection of three miRNA and three mRNA targets in plasma EV-RNA by RT-qPCR. EV-RNA was extracted using a qEV RNA extraction kit (Izon) from human plasma EVs purified with qEV columns (Izon). The Taqman approach was used to detect miRNA targets, and the SYBR approach was used to detect mRNA targets. This shows that both types of RNA targets can be readily detected in EV-RNA samples, which had previously shown RNA yield below the limit of detection for the chosen fluorometric assay.

RNA sequencing

RNA sequencing (RNA-seq) is a powerful technique for studying EV-RNA and its discovery potential, as it is a quantitative and qualitative technique not restricted to known RNA sequences or structures (e.g. polyadenylated 3′ ends of mRNA). Yet, RNA-seq approaches can be challenging when applied to the EV field, as EV-RNA may be degraded during -sometime long - EV isolation processes and sub-optimal storage conditions or, there is ultra-low amount of RNA retrieved strictly from highly pure EVs. Therefore, there are some specific considerations to account for in RNA-seq analysis with respect to each EV-containing biofluid, that users should evaluate before RNA-seq analysis.

Due to the ultra-low amounts of EV-RNA that can sometimes be obtained, it is recommended to concentrate EV-RNA to concentration and volume inputs required specifically by the used RNA-seq library kit[19,30]. In the same way as PCRs, RNA spike-in can be useful to normalise RNA-seq data, especially relevant when dealing with uncontrolled variability during EV-RNA processing from different subject's biofluids[31]. The quality of EV-RNA plays an important role, with automated electrophoresis analysis providing great utility in quality control of EV-RNA before RNA-seq. It has been suggested that a relative abundance larger than 1% miRNA and no intact large 18S/28S rRNAs species in an EV-RNA preparation is a good indicator of sRNA enrichment and successful library preparation for small RNA-seq[24]. Specific small RNA-seq library preparation kits are commonly used[8,15,18], however total RNA-seq library preparation kits have also been used with EV-RNA[30]. RNA-seq library preparation kits usually require a range of 1-1000 ng total EV-RNA input, or a range of 10 pg -1000 ng of enriched EV-sRNA, resuspended in volumes of 3-10 µL water. Moreover, some small RNA-seq library kits allow preparation of sRNA from total RNA samples and/or allow miRNAs to be targeted specifically. It is up to the user to optimise their EV-RNA extraction or enrichment protocols according to the experimental needs and research questions. Awareness that biases also occur during library preparation is essential, including bias in EV-RNA adapter ligation step and bias in cDNA size selection[11]. Different sequencing platforms provide different amount of reads and coverage (e.g. Illumina HiSeq[15,20] or MiSeq[8]) or chemistry (e.g. Ion Torrent[24,30]), with bioinformatics analysis also having a substantial impact or bias on outputs. Altogether, RNA-seq is an incredibly powerful tool, however it must be thoroughly planned, interpreted, and data carefully compared to other studies.

Microarrays

Although RNA-seq provides the most comprehensive analysis of EV-RNA composition, it does have several challenges throughout the protocol, requiring advanced knowledge and skills. In this scenario, microarrays offer a simpler alternative for wide qualitative and relative quantitative analysis of specific RNA populations, such as is the case for the most popular RNA population investigated in EV-RNA with miRNA microarrays. There are few commercial miRNA microarray kits available in the market, ranging in the number of miRNA probes included (from 15,000 to 30,000), type of miRNAs (pre-miRNA or mature), dynamic range (covering from 3 to 4 logs difference), total RNA inputs (from 100 to 500 ng RNA) or organisms covered in a single array (human, mouse, rat). Although an EV-RNA input of 100 ng might seem hard to achieve with highly purified EV samples, it has been shown that EV-RNA inputs unable to be quantified were successfully labelled, hybridised and miRNAs identified by a kit requiring 100 ng RNA input[32]. As this is not an absolute quantification tool, normalisation strategies (usually control treatments) must be planned before microarray analysis[33]. Array-based gene expression profiling of mRNA in EVs might be less common due to their less abundant proportion in EV-RNA, however it has also demonstrated diagnostic potential, with differential gene expression found in serum and urine EVs between healthy and cancer patients[34].

7 / CONCLUDING REMARKS

Although currently there is no method able to isolate absolute pure EV populations, the importance of an efficient EV purification method cannot be stressed enough when studying EV-RNA. The common and persistent presence of RNA-binding non-EV contaminants in EV preparations can have significant impact on RNA investigation. In this RNA-driven era, it becomes more critical to understand the limitations of chosen protocols, so accurate biological conclusions can be derived, especially when the aim is to exploit EV-RNA for nanomedicine or diagnostic applications.

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