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## **Diversity and heterogeneity of extracellular RNA in human plasma**

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**Keywords:** extracellular RNAs (exRNAs); exogenous RNA; deep sequencing; extracellular vesicles; human plasma

**Abbreviations:** exRNAs - Extracellular RNAs, EVs - extracellular vesicles, SEC - size-exclusion chromatography, RNPs – ribonucleoproteins, PEG - polyethylene glycol, RNases- ribonucleases, LDL and HDL - low- and high-density lipoproteins, MVEs/MVBs - multivesicular endosomes/multivesicular bodies, ESCRT - endosomal sorting complexes required for transport, PES – polyethersulfone, PBS - phosphate-buffered saline, NTA - NanoParticle Tracking Analysis, PNK - T4 polynucleotide kinase, NEB – New England Biolabs, MN – Macherey-Nagel, SSU/LSU – small/large subunit

## ABSTRACT

Extracellular RNAs (exRNAs) are secreted by nearly all cell types and are now known to play multiple physiological roles. In humans, exRNA populations are found in nearly any physiological liquid and are attracting growing interest as a potential source for biomarker discovery. Human plasma, a readily available sample for biomedical analysis, reported to contain various subpopulations of exRNA, some of which are most likely components of plasma ribonucleoproteins (RNPs), while others are encapsulated into extracellular vesicles (EVs) of different size, origin and composition. This variation explains the extreme complexity of the human exRNA fraction in plasma. In this work, we aimed to characterize exRNA species from blood samples of healthy human donors to achieve the most comprehensive overview of the species, sizes and origins of the exRNA present in plasma fractions. Unbiased analysis of exRNA composition was performed with prefractionation of plasma exRNA followed by library preparation, sequencing and bioinformatics analysis. Our results demonstrate that, in addition to “mature”, adaptor ligation-competent RNA species (5'-P/3'-OH), human plasma contains a substantial proportion of degraded RNA fragments (5'-OH/3'-P or cycloP), which can be made competent for ligation using appropriate treatments. These degraded RNAs represent the major fraction in the overall population and mostly correspond to rRNA, in contrast to mature products, which mostly contain miRNAs and hY4 RNA fragments. Precipitation polyethylene glycol (PEG)-based kits for EV isolation yield a fraction that is highly contaminated by large RNPs and by RNA loosely bound to EVs. Purer EV preparations are obtained by using proteinase K and RNase A treatment, as well as by size-exclusion chromatography (SEC). These samples have rather distinct RNA compositions compared to PEG-precipitated EV preparations and contain a substantial proportion of exRNA of non-human origin, arising from human skin and gut microbiota, including viral microbiota. These exogenous exRNAs represent up to 75-80% of total RNA reads in highly purified extracellular vesicles, paving the way for biomedical exploitation of these non-human biomarkers.

# 1. INTRODUCTION

## 1.1 Extracellular RNA origins

In addition to intracellular RNAs, it is now well established that a multitude of RNAs also circulates outside of cells (extracellular RNAs, exRNA) [1,2]. These species are secreted by various cells via different mechanisms that are not yet fully understood [3,4]. In multicellular organisms, exRNAs are present in almost every biological fluid (urine, milk, saliva ...), the most studied of which is certainly blood [5–7]. To be stable in such an unfavorable extracellular environment rich in ribonucleases (RNases), circulating RNAs require protection from nucleolytic degradation. This protection can be ensured in two different ways.

On one hand, some RNAs, especially miRNAs, are in a “soluble state” and are included in ribonucleoprotein (RNP) complexes containing both ribonucleic acids and associated proteins [8,9], or, more rarely, as a part of particles, such as low- and high-density lipoproteins (LDL and HDL) [10] or exomeres [11].

On the other hand, RNAs are also found in particles released from the cell, termed extracellular vesicles (EVs) [12–15]. These vesicles are delimited by a lipid bilayer membrane and cannot replicate. There are evolving recommendations to characterize their subtypes, particularly in complex biological fluids. To date, there is no well-established consensus on specific markers of EV subtypes [16,17]. Nevertheless, three major types of EVs have been described according to their size and biogenesis: apoptotic bodies [18–20], microvesicles (or budding/shedding vesicles) [21–23] and exosomes [24–26]. Apoptotic bodies are 1 - 5  $\mu\text{m}$  vesicles that arise from apoptotic cells through invagination. They are composed predominantly of fragmented DNA and histones.

Microvesicles, from 100 nm to  $\sim 1 \mu\text{m}$  in size, are formed by budding of the plasma membrane, and their contents depend on where in the cell budding occurs. Their membrane composition is similar to the parental cell [23].

Microvesicles are enriched in phosphatidylserine, lipid rafts, cell lineage markers and cell surface receptors [23,27,28]. The third type of EVs, called exosomes, are particles of 50 - 150 nm in size [29] originating from the classical endocytic pathway (see reviews [25,30] for details on exosomes biogenesis and composition).

Endocytosis is commonly mediated via clathrin-coated vesicles after which endocytic vesicles progress from early to multivesicular endosomes (MVEs, also called multivesicular bodies, MVBs). MVEs often fuse with lysosomes for degradation but can also join the plasma membrane, thereby releasing exosomes to the extracellular space [29].

Their fate depends on the MVE composition. MVEs rich in cholesterol and poor in lysobisphosphatidic acid are secreted as exosomes, while when the proportion of cholesterol to lysobisphosphatidic acid is inverted, they are directed to the lysosomal pathway. Most future exosomes follow a secretory pathway involving the endosomal sorting complexes required for transport (ESCRT). Members of ESCRT, such as proteins Alix or TSG101, are considered internal exosomal markers. Moreover, transmembrane tetraspanin proteins (e.g., CD9, CD63 and CD81) are highly enriched in exosomal membranes and serve as major external exosome markers [29] (see review [31] for details on tetraspanins).

Considering these multiple secretory pathways, exRNA composition is extremely diverse and heterogeneous [18,32]. Depending on the origin of biological samples and, even more importantly, on the experimental approaches used for characterization of exRNA, the results of different studies are barely comparable. This is an important issue, since exRNAs are attractive biomarkers for pathology and disease, with growing applications in diagnostics and prognostics [33–37]. Indeed, highlighting new biomarkers from populations present in the circulating blood could improve diagnostic methodology by allowing use of liquid biopsies instead of classical invasive biopsy types.

## 1.2 Extraction of exRNA from human blood

### 1.2.1 Choosing between serum and plasma

Analysis of human blood is of great interest for clinical diagnostics, and numerous standard treatment protocols are available. Most common pretreatments consist of preparation of plasma or serum fractions from blood. Plasma is obtained by centrifugation in the presence of anticoagulants, while serum is prepared by centrifugation of coagulated samples. Technically, working with serum is easier, but it is known that platelets release many EVs containing miRNAs in response to coagulation [38], and this considerably affects the composition of exRNAs originally present in circulating blood. For this reason, plasma is the preferred source when studying exRNAs as potential biomarkers [39].

However, some popular anticoagulants, such as heparin and EDTA, are not compatible with downstream applications (e.g., polymerase chain reaction, PCR) and are generally replaced with citrate or acid citrate dextrose [40–42]. In addition, special care should be taken during these initial centrifugation steps to prevent release of platelet-derived EVs [41].

### 1.2.2 Extraction of exRNA from EVs and RNPs

Extracellular RNAs present in whole plasma, by themselves or in its subpopulations (EVs or RNPs), have to be extracted for further analysis. Classical phenol/chloroform or TRIzol extractions are not efficient enough for these types of samples [43]. In fact, the yield is rather low, and small RNAs (< 70 nt), which represent the majority of exRNAs [44], are generally lost [45–47]. Thus, column-based RNA extraction kits, such as the Nucleospin® miRNA (Macherey-Nagel) or the miRNeasy Micro kit from Qiagen, are generally used.

## 1.3 Characterization of human exRNAs in plasma

### 1.3.1 Fractionation of human plasma to obtain exRNA subpopulations

Human plasma is rather viscous and contains a high concentration of soluble proteins, mostly albumin and immunoglobulins but also lipoproteins and lipoprotein particles: HDL, LDL and chylomicrons. This complex composition should be taken into account for fractionation.

Among all subpopulations of exRNA present in plasma, the exosomal (or generally, EV) fraction is the best studied because these vesicles have a specific biogenesis, perform known biological functions and have been demonstrated to be involved in several diseases [29,48–50]. For analysis of EV-derived exRNA, cell cultures in conditioned media are generally used. From these samples, EVs are purified using relatively standardized techniques, mostly differential centrifugation steps, followed by ultracentrifugation to pellet submicrometric particles [51]. However, compared to complex and viscous plasma, conditioned media has a simple and controlled composition. Thus, these classical fractionation methods yield poor results with plasma, since protein complexes, lipoproteins and RNPs also sediment during ultracentrifugation [8,52].

Improvements can be obtained by the use of density gradient/cushion centrifugation [4], where particles are separated by their floating density allowing separation of soluble protein aggregates from EV. However, these methods are very laborious and require specific equipment and reagents [53,54].

Apart from “gold standard” ultracentrifugation protocols, many other methods for EV purification are commercially available. These methods are either based on the use of “precipitation agents” of unknown composition (Exoquick™ Exosome precipitation solution, System Biosciences, Total Exosome Isolation Reagent, Invitrogen, miRCURY™ Exosome Isolation Kit, Exiqon) or on immunoaffinity selection of EVs containing specific protein markers (usually membrane tetraspanins CD9, CD63 and CD81). However, none of these methods offers

sufficient purity of obtained EVs, which are still heavily contaminated by plasma proteins [55]. Immunoaffinity-based methods [55–57] avoid important contamination by plasma proteins and non-EV exRNA but could potentially exclude important EV populations without specific membrane markers, since specific antibodies are used for the selection step [39].

Finally, alternative techniques based on size-exclusion chromatography (SEC, qEV column, Izon) have been developed. These methods avoid protein aggregates and provide EV fractions free of protein contamination. Several comparative studies concluded that SEC is the best method for EV isolation from plasma [58–60] since only a few HDL particles were found in the size range of EVs [61]. Moreover, it is possible to obtain EV and non-EV fractions (mostly RNPs) in parallel.

Other protocols for EV purification, such as alternating current electrokinetic microarray chips, are certainly promising but require sophisticated and specific equipment [62].

In any case, for each method, it is crucial to perform either physical or biological validation after isolation/enrichment of appropriate exRNA subpopulations. Size distribution and concentration of nanoparticles are determined by Nanoparticle Tracking Analysis using a NanoSight [63], while electron microscopy establishes size and morphological characteristics of particles, such as a lipid bilayer [64–66]. One can also cite Dynamic Light Scattering that gives size distribution but does not provide information about particle concentrations [67,68]. For biological validation, immunoblotting targeting specific biomarkers, such as TSG101 (46 kDa), Alix (96 kDa) or tetraspanins CD63 (26 kDa), CD9 (26 kDa) and CD81 (26 kDa) for exosomes are a standard procedure for EVs secreted by cells in culture [69,70]. All these techniques are used routinely for EVs derived from specific cells grown in culture. However, in complex biological fluids containing a huge proportion of proteins and lipids, validation of EV preparations may present a true challenge.

### 1.3.2 Characterization of extracellular RNAs in plasma

Initial studies on the composition of exRNA from extracellular vesicles showed the presence of miRNA and more than a thousand different mRNAs [71]. These mRNAs even seem to be functional because after transfer of mouse extravesicular RNAs into human mast cells, new mouse proteins were found to be produced in the recipient cells, indicating that transferred exosomal mRNAs can be translated after entering another cell. In this study, authors used miRNA and mRNA specific microarrays for exRNA characterization. Indeed, the first methods used for RNA characterization were mostly real-time quantitative RT-qPCR [9,72,73] or hybridization-based assays [9,74]. However, these methods require the design of specific oligonucleotides to known targets. Thus, it is not possible to determine the presence of unexpected RNA transcripts. Currently, high-throughput sequencing overcomes this issue by sequencing and identifying all RNAs present in a given sample. In the last decade, more than a dozen papers have described exRNA content from plasma/serum and/or respective extracellular vesicles [5,32,34,35,75–81]. Using RNA sequencing, almost all known RNA species identified have been found in blood and extracellular vesicles: mRNA, rRNA, tRNA, miRNA, piRNA, snoRNA, vault RNA, Y RNA, lncRNA, etc. However, the reported proportion of different RNA varies dramatically from one study to another (Table 1). For instance, protein coding RNA and miRNA are described at 24.5% and 8.8% in one study, respectively [79], while another study with a similar design reported a proportion of 20% and 42%, respectively [34]. Moreover, the proportion of rRNA was reported to be low (<10%) in one study [78], while the same rRNA was found to be a major species at ~50% in another study [35]. These discrepancies most likely reflect extreme heterogeneity in sample preparation and in the techniques used to isolate and characterize extracellular vesicle fractions [82]. Distinctions may also arise from the methods used in library preparation for sequencing, as well as from the bioinformatics pipeline used for data analysis and interpretation (discussed in [83]).

Globally, it is a significant challenge to characterize exRNA content when plasma is used as source [84]. It is therefore critical to carefully design the study and to be aware of possible contamination issues, which may affect sample composition.

In this work, we approached the exhaustive characterization of exRNA from healthy human plasma samples using a combination of extraction/characterization approaches and deep sequencing, followed by extensive bioinformatics analysis. Our results demonstrate that the extreme heterogeneity of human plasma exRNA depends on the purity of isolated extracellular vesicles. Extracellular RNA contains various types of both mature and degraded RNAs, also unexpectedly including high proportions of foreign (non-human) RNAs derived from the human microbiota (bacteria, fungi and viruses). Our data show that, compared to total human plasma, highly purified human EVs contain a substantial fraction of exogenous RNAs, likely reflecting the past and current state of individual microbiota composition.

## 2. MATERIAL AND METHODS

### 2.1 Collection of human plasma samples

Plasma samples used in this study were from healthy men blood donors. They agreed to give their blood for biological research at the French Blood Transfusion Institution. Blood was collected in a pocket containing sodium citrate as anticoagulant and was immediately centrifuged for 15 min at 2,000xg at room temperature (RT). In order to remove cellular debris, the supernatant corresponding to plasma was centrifuged again for 10 min at 1,500xg at RT. Supernatant was finally centrifuged for 15 min at 16,000xg at RT. Finally, the resulting supernatant was filtered through a 0.22  $\mu\text{m}$  polyethersulfone (PES) membrane filter to obtain “pre-treated” plasma.

### 2.2 Plasma fractionation

#### 2.2.1 Ultracentrifugation

1 mL of pre-treated plasma was centrifuged for 2h at 120,000xg at 4°C in high-speed ultracentrifuge (Thermo Scientific Sorval MX120) with S45A rotor. Pellet was resuspended in 400  $\mu\text{L}$  of RNase-free water by rotation head-over-tail for 1-2 h at 4°C.

#### 2.2.2 Precipitation-based kits

Four commercially available kits based on exosome precipitation were used. Following the manufacturer’s instructions: miRCURY™ Exosome Isolation Kit – Serum and plasma (Exiqon), Total Exosome Isolation (Invitrogen), Exosome Precipitation Solution (Serum/Plasma) (Macherey-Nagel) and ExoQuick™ Exosome Precipitation Solution (System Bioscience). Briefly, according to the manufacturer’s guidelines, pre-cleared plasma (400 -- 1000  $\mu\text{L}$ ) was mixed with an appropriate volume of precipitation buffer. After a vortexing step and indicated incubation time at RT, extracellular vesicles were pelleted by centrifugation and resuspended in RNase-free water or phosphate-buffered saline (PBS) by rotation head-over-tail 1-2 h at 4°C. The kit from Exiqon provides an optional depletion of fibrinogen by thrombin treatment before EV isolation.

#### 2.2.3 Proteinase K/RNase A treatment

EV fraction enriched from 400  $\mu\text{L}$  of plasma using miRCURY™ Exosome Isolation Kit – Serum and plasma (Exiqon) was incubated with 20  $\mu\text{g}$  of proteinase K in 100 mM Tris-HCl pH 7.6, for 2 h at 30°C. Proteinase K was inactivated

by heating samples for 15 min at 70°C. Then, samples were treated with RNase A at 10 µg/mL for 15 min at RT and then stored at – 20°C.

#### 2.2.4 Size-exclusion chromatography (SEC)

EV fraction enriched from 500 µL of plasma using miRCURY™ Exosome Isolation Kit – Serum and plasma (Exiqon) was used for EV purification by SEC using the qEV column from Izon. This column allows the isolation of vesicles > 70nm in size. Briefly, after column equilibration with 1x PBS, EV from 500 µL of pre-treated plasma were loaded and then eluted with the same buffer. 500 µL fractions were collected. Fractions 7-9 contain EV with a size corresponding to exosomes.

#### 2.3 NanoSight

Particle size distribution and concentration of plasma treated with kits based on precipitation was determined by the NanoSight® LM10 instrument (Malvern). A dedicated software called NanoParticle Tracking Analysis (NTA) analyzes videos captured giving a particle size distribution and particle count based upon tracking of each particle's Brownian motion. About 1 mL of sample solution diluted from 1/20<sup>th</sup> to 1/4,000<sup>th</sup> is injected into a chamber, which is placed under an objective coupled with a camera. The movement of the particles are recorded during 60 to 90 sec in order to follow their respective shift to determine their size and their concentration in the sample.

#### 2.4 Extracellular RNA extraction

RNA were isolated by Nucleospin® miRNA (Macherey-Nagel) for whole plasma exRNA and RNA from EV isolated by precipitation kits. Concerning EV isolated by proteinase K/ RNase A treatment and SEC, miRNeasy Micro kit from Qiagen was used. Both kits were found to give the best exRNA yield (1-5 ng of exRNA/ mL of plasma) and were used following manufacturer's instructions. Both these kits are based on the same principle and include miRNA and small RNA fractions, without excluding large RNAs which may be present.

#### 2.5 Dephosphorylation/ phosphorylation treatment

Isolated RNA were 3'-end dephosphorylated using 5 U of Antarctic phosphatase (New England Biolabs) for 30 min at 37 °C. After the inactivation of the phosphatase for 5 min at 70 °C, RNA were phosphorylated at the 5'-end using 20 U of T4 polynucleotide kinase (PNK) and 1 mM ATP for 1 h at 37 °C. RNA were cleaned up from the enzymatic reactions by RNeasy MinElute Cleanup Kit (Qiagen) according to the manufacturer's recommendations, except that the volume of 96% ethanol was increased to 675 µL for small RNA binding.

#### 2.6 Library preparation

Libraries were prepared following the manufacturer's instructions using the NEBNext® Multiplex Small RNA Library Prep Set for Illumina (NEB). Libraries were cleaned up by GeneJET PCR Purification Kit (Thermo Fisher Scientific). Libraries containing a high proportion of dimers were further purified by an automated agarose gel electrophoresis (Pippin Prep™ 3 % agarose gel). The library of interest was specifically eluted in 40 µL by a size selection range between 110 to 300 bp to exclude dimer adaptors. Purified libraries were then concentrated by a concentrator plus (Eppendorf). Each library was quantified by Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific) and the quality was checked on capillary electrophoresis using a HS DNA chip (Agilent).



## 2.7 Sequencing

Sequencing of Illumina-type libraries was performed either on MiSeq (Illumina) in PE 2x75 nt mode to get paired-end information, or on HiSeq1000 (Illumina) in single-read SR50 mode.

## 2.8 Bioinformatics

Initial trimming of raw fastq files was performed by Trimmomatic, v0.32 [85], using the following parameters: first trimming with `TruSeq3-SE.fa:2:30:10 LEADING:30 TRAILING:30 SLIDINGWINDOW:4:15 MINLEN:1 AVGQUAL:30` to get distribution of all reads in population, followed by `TruSeq3-SE.fa:2:30:7 LEADING:30 TRAILING:30 SLIDINGWINDOW:4:15 MINLEN:10 AVGQUAL:30` for reads used in downstream analysis. The following reference sequences were used for analysis: human 45S pre-rRNA sequence (accession number NR\_046235), human genome build hg19 from UCSC, reference sequences for SSU and LSU rRNAs from SILVA database (<https://www.arb-silva.de/>), release 132, non-redundant SSU\_Nr99 sequences and whole LSU collection. Viral/phage reference sequences from NCBI (v68 nov 03 2014, 5794 sequences). Alignment was performed using bowtie 2 in `-local` (soft trimming) mode and following parameters: `-D 15 -R 2 -N 1 -L 10 -i S,1,1.15`.

## 3. RESULTS

### 3.1 Study design

In this study, we used deep sequencing for the exhaustive characterization of exRNA subpopulations in human blood from healthy subjects. Five plasma samples were used (Plasma #1 to Plasma #5) to evaluate the biological diversity among samples. The composition of plasma exRNA is expected to be extremely complex, since these species have diverse origins, secretory pathways and are included within lipid particles of different sizes, as well as in “soluble” RNP complexes. To achieve separation of these subpopulations, different methods for plasma fractionation were used, including precipitation-based commercial kits, size-exclusion chromatography (SEC) and ultracentrifugation methods. In addition, conversion of exRNAs into a sequencing library allows discrimination between “true” maturation products (RNAs having 5'-P/3'-OH extremities) and RNA degradation fragments arising from chemical and enzymatic pathways (5'-OH, 3'-P or cyclophosphate at their extremities). Since exRNA species are mostly small RNAs or fragments (< 70 nts), small RNA sequencing kits are the most appropriate for their conversion into a sequencing library. Here, we used the NEBNext® Multiplex Small RNA Library Prep Set for Illumina (NEB).

In brief, human plasma samples were either directly used to extract exRNA species or first fractionated by precipitation, ultracentrifugation, and/or size-exclusion chromatography and proteinase K/RNase A treatment (Figure 1) to obtain exRNAs included within vesicles. RNAs were extracted and subjected to Illumina deep-sequencing either directly or after preliminary conversion to ligation-compatible extremities with alkaline phosphatase/T4 polynucleotide kinase treatment (deP/P-treatment). Resulting libraries were sequenced in paired-end or single read modes on MiSeq and HiSeq1000 Illumina sequencers. Subsequent bioinformatics analysis aimed to identify, as much as possible, RNA species present in human plasma.

We applied the following strategy for exRNA mapping and identification:

- local (soft-trimming alignment mode): alignment to the most abundant human rRNA species to sort out reads mapping to these RNAs
- local alignment mode to hg19 human reference genome

-- non-mapped to hg19 reads were further aligned to collection of SSU/LSU rRNA sequences from the SILVA database [86] to identify the most represented RNA species from the human microbiota  
-- finally, to gain insights into the eventual presence of RNAs arising from viral/phage microbiota, non-identified reads were aligned to a virus/phage database. Complete alignment statistics for all studied samples are shown in Table S1.

Overall, this bioinformatics pipeline allowed identification of > 80% of all RNA reads present in human plasma samples, except for highly purified extracellular vesicles, where identification was slightly lower due to an important proportion of bacterial reads comprising from 60% to 80% of the population.

### 3.2 Deep sequencing analysis of exRNAs from pretreated human plasma

First, the total population of human plasma exRNAs was extracted and globally characterized. Bioanalyzer traces show an RNA peak of approximately 80-120 nt in size (Figure 2A), with the average yield of approximately 8.5 ng RNA/ml of plasma. Extracted exRNA were converted into a library by adaptor ligation at extremities with and without the preliminary deP/P-treatment step (Figure 2B) and sequenced (Table S1). Inspection of fragment sizes for exRNA extracted from two independent plasma samples (P #1 and P #2) showed a striking difference depending on the applied preliminary treatment. As anticipated, direct ligation of exRNA yielded two major peaks at 21 nt and 33 nt in length (Figure 2C), presumably corresponding to miRNA fractions and hY4 RNA fragments, respectively. These species were previously reported to be highly abundant in human plasma samples. In contrast, very different profiles were observed for total RNA fractions captured after 3'-deP/5'-P treatment. These samples showed a broad peak of small fragment sizes (12-15 nts) and a substantial proportion of longer (>50 nts) fragments. Even if the proportion of "mature" and degraded RNA fragments is impossible to evaluate using this approach, the yield of sequencing libraries clearly indicates a large domination of degradation products in the exRNA population. Size distribution showed very similar results for RNA fragments in total library preparations (Figure 2C), fragments aligned to human hg19 genome (Figure 2D) or reads unmapped for hg19 (Figure 2E).

The majority of exRNA fragments successfully mapped to the human hg19 genome, but human rRNA-derived fragments were highly abundant in the degraded fraction at the expense of miRNAs and hY4 fragment RNAs compared to mature RNA sequences (Figure 2DF).

### 3.3 Fractionation of EVs and "free" exRNAs from human plasma

In general, exRNA fractions are composed of two major subpopulations, those that are most likely included in stable RNPs and those that are encapsulated within EVs of different origins, primarily submicrometric particles. These fractions can be separated using physical methods, such as ultracentrifugation (or gradient ultracentrifugation), but many commercially available kits propose isolation of EVs without this long and laborious step. For clinical exRNA analysis, we first compared the yield and composition of exRNA fractions isolated by ultracentrifugation and precipitation-based kits.

Ultracentrifugation at 120,000 xg yielded moderate EV levels of ~100 nm in the pellet (Figure 3A) and thus, a rather low yield of associated exRNAs (Figure 3B). Indeed, the majority of plasma exRNA remained in the supernatant after ultracentrifugation (named S100), corresponding to the "RNP" fraction. Global profiles of fragment sizes were extremely similar to those previously observed for total plasma (P #2), S100 supernatant and EV fraction for both mature and degraded RNA (Figure 3C). Mapping to hg19 reads demonstrated a similar trend, indicating that there is no global selection of specific RNAs into EVs (Figure 3DE). Very similar results were obtained for another independent plasma sample (P #3, Supplementary Figure S1).

As anticipated, precipitation-based kits provided enriched fractions containing submicrometric EVs [87–89] with a slightly different size, ~80 nm, but with a higher fractionation yield compared to the ultracentrifugation approach used for P #2 (Figure 4A). We have chosen several of them, giving the best EV yield for further characterization (Exosome Isolation kit (Exiqon), ExoQuick™ Exosome precipitation solution (System Biosciences) and Exosome Precipitation solution (Serum/Plasma) from Macherey Nagel (MN)) (Figure 4B).

Protein composition analysis of the enriched fractions demonstrated a very high concentration of major plasma proteins (mostly serum albumin and immunoglobulins), which certainly coprecipitate together with EVs (Supplement Figure S2AB). Indeed, it has been demonstrated that after ultracentrifugation and precipitation-based kits, the EV fraction is contaminated by protein aggregates that give similar signals by nanoparticle tracking analysis [90]. Thus, plasma fractionation by ultracentrifugation or precipitation-based kits certainly enriches certain EV population(s) but does not provide high EV purity.

exRNA extracted from kit-precipitated EVs follow the same general trend: a minor mature fraction shows 21-22 nt and 31-33 nt peaks, while the degraded population is highly heterogeneous and spans from 9-10 nt to >50 nt (Figure 4C). As in the case of ultracentrifugation-derived EVs, mapping to different reference sequences shows a relatively low human rRNA proportion in mature products, while this proportion becomes substantial in the degraded RNA population (Figure 4D). Globally, the profile of mapped reads shows that the MN kit gives the best match to EVs prepared by ultracentrifugation. Interestingly, different kits result in variable levels of contamination with non-human rRNA, a parameter that may be used as criteria for their use in different applications (for example, analysis of human or non-human RNAs) (Supplementary Figure S3).

### 3.4 Analysis of exRNA in highly purified EVs

To gain better insights into the composition of exRNAs present in the inner compartment of plasma EV particles, we decided to apply a combined purification protocol consisting of initial enrichment of EVs using the miRCURY™ Exosome Isolation kit, followed by their purification using either SEC (qEV column, Izon) or consecutive proteinase K and RNase A treatments (Figure 1). The last technique is supposed to remove loosely bound exRNAs from RNPs and EV outer shells, while internal RNAs in EVs are protected by the lipid bilayer [91] (Supplementary Figure S2C). The yield of exRNAs extracted from these highly purified EVs was relatively low (Figure 5A). Indeed, the major fraction of exRNAs seems to be bound at the exterior shell of these particles or coprecipitated RNPs. Due to the low yield of exRNA, the analysis was performed using a preliminary deP/P-treatment. A profile of obtained libraries is shown in Figure 5B.

In contrast to kit-precipitated EVs, highly purified lipid particles have very different fragment size profiles, with rather broad and flat distribution between 10 to 40 nts without any characteristic peaks (Figure 5C). Of note, the proportion of >50 nt fragments is relatively high compared to all other samples studied.

Alignment to human genome sequences showed that only very short reads were successively mapped, while only a minor proportion of long sequences were aligned (Figure 5D). Analysis of RNA populations revealed that human RNA (<20% of total) is mostly represented by rRNA, while major proportions map to non-human rRNA sequences, and almost 40% cannot be identified (Figure 5E, see also below). These data demonstrate that highly purified EV fractions primarily contain non-human RNAs, and their composition is radically different from those obtained by ultracentrifugation or precipitation.

### 3.5 Non-human RNAs in plasma exRNA

As clearly seen in Figures 2F and 3E, different subpopulations of exRNA from human plasma contain nonnegligible proportions of non-human RNA. While this proportion is quite minor (12-20%) in total exRNA from plasma, it becomes substantial in enriched EV preparations and even predominant in highly purified EVs.

Analysis of such non-human RNA species is not straightforward, since the human microbiota genome may represent 300-fold the size of the human genome. However, we speculated that the major portion of these exRNAs comes from bacterial and eukaryotic microbiota rRNA sequences. For identification of such species, we used the SILVA database, containing millions of known rRNA sequences. Alignment was made to a nonredundant version of the SILVA database, including 695,171 sequences of Small SubUnit (SSU) and 198,843 sequences of Large SubUnit (LSU) rRNA species. Approximately 40-50% of non-human RNA reads were successfully aligned to this reduced version of the SILVA rRNA database, indicating that indeed, non-human RNA species are mostly represented by bacterial and eukaryotic rRNAs from the microbiota and even food (various fish species, rice, baker's yeasts). Composition of the bacterial rRNA population was relatively similar in different plasma samples, likely reflecting the composition of the human microbiota. The most frequent species identified were from the Proteobacteria phylum, class Gammaproteobacteria, followed by Bacteroidetes and Firmicutes. More precise identification of bacterial species was not performed, since short rRNA fragments may map to multiple closely related species.

We also attempted alignment of the remaining unidentified sequences to viral/phage RNA/DNA databases to gain insight into potential viral microbiota. Only a very minor proportion of reads was aligned to these reference sequences, but the extraction of uniquely aligned reads allowed for the partial identification of viral/phage species present in samples. Reads mapping to both human viruses and phages to commensal bacteria were detected, the most frequent of which were Pandoravirus and Herpesvirus. Interestingly, total plasma exRNA was relatively enriched in human viral sequences, while highly purified EVs (SEC column and RNase A/Proteinase K treatment) contained primarily species mapping to bacterial phages.

Inspection of selected remaining sequences nonmatching to any reference sequences demonstrated either the absence of similarity with any sequence present in actual release of nr/nt NCBI nucleotide collection or indicated bacterial 23S rRNA origin, since the reference 23S rRNA list for bacteria is still incomplete in the SILVA database.

#### 4. DISCUSSION

In this study, we demonstrate the complexity of plasma exRNA analysis. This is due to the extreme diversity of exRNA subpopulations and the presence of other molecular components in such a very complex biological fluid. In addition, there are no commonly accepted or standardized methods for sample preparation, analysis and validation, leading to global confusion in the literature. Comparison of exRNA studies is compromised due to numerous biological and technical biases. For example, several studies that published data on exRNA from EVs reported huge differences in the composition of exRNA fractions (proportions of rRNA, miRNA and so on) [34,35,78,79,92] (see Table 1). **For instance, analysis of human miRNAs, which are widely employed for biomarker discovery, may be highly biased, depending on the purification procedures used. The RNA content of the exosomes isolated by centrifugation was shown to be very similar to the RNA composition of the human plasma, demonstrating that inappropriate EV purification methods yield preparations highly contaminated with nonexosomal RNA [93]. In addition, several comparative studies highlighted strong differences in exosomal RNA content when extracellular vesicles were isolated from the same biological sample using different methods [58,88,94,95]. Not only global miRNA content was found to be different [94], but the level of individual miRNAs, such as mir let-7-d, miR-25, miR-127-3p, mir-16 and mir-451, were also distinct [94,95]. Such differences in exosomal RNA content were described for plasma-derived exosomes, and, surprisingly, also for cell-derived exosomes obtained from rather well characterized cell culture media [88]. This demonstrates that biases related to exosome purification methods may be important and may lead to lower quality results, even when the biological source is not a complex biofluid like plasma. In this study comparing ultracentrifugation, density gradient protocol and a precipitation-based kit [58], only 40% of miRNAs were common between the three**

methods. Moreover, mass spectrometry analyses indicate that precipitation methods lead to contamination with PEG and serum proteins [96]. Taken together, this shows the necessity of creating an optimized pipeline for exRNA characterization by limiting biases as much as possible. In our work, we designed each step to balance advantages and drawbacks identified for each procedure, but it is also important to consider the remaining known and unknown issues.

We demonstrate that “mature” and “degraded” populations of exRNAs are quite different in composition and relative proportion in plasma. Thus, depending on the project’s objectives, one or another fraction may (or have to) be analyzed separately. Popular methods of EV isolation by ultracentrifugation and various precipitation reagents do not obtain pure EV fractions, and lipid particles are still highly contaminated with RNA-containing RNPs. These enriched EV fractions seem to be similar to a global composition of human exRNA from whole plasma. However, EV isolation methods based on SEC and proteinase K/RNase A treatment result in quite distinct exRNA composition associated with highly purified vesicles, which mostly contain degraded fragments of non-human exRNA.

Exhaustive bioinformatics analysis of exRNA by deep sequencing is also not straightforward. First, the average size of exRNA fragments is rather small, and the proportion of very short reads < 15 nt is substantial. These fragments are difficult to identify unambiguously, since they may perfectly map to various reference sequences. In this work, we used alignment to human rRNA as a first step, expecting that such fragments may be highly abundant, which is indeed the case for degraded exRNA populations. The second alignment step attributes all additional human genome related sequences. While direct alignment of RNA-derived reads to genomic sequences seems inappropriate due to the potential absence of intronic regions, the soft-trimming alignment mode used here generally provides correct mapping, even for reads split between two exons.

Third, exRNA fractions in human plasma are highly contaminated by exRNAs of non-human origin, arising from the commensal bacterial/fungal/viral microbiota present in the human body [97,98]. It is anticipated that these non-human RNAs represent primarily highly abundant RNAs from different bacterial and eukaryotic species (rRNA, tRNAs), but no exhaustive analysis of this highly variable population has been performed. In addition, this potential microbiota’s metagenome is approximately 300-fold the size of the human genome, and the small size of exRNA fragments does not allow unambiguous sequence alignment, making annotation and analysis extremely complex. In addition, due to numerous posttranscriptional events of RNA maturation and degradation (mostly polyA and/or polyU 3’-addition), exRNA reads may not directly align to reference genomic sequences unless soft-trimming (local) alignment mode is used.

## 5. CONCLUSIONS

The results of this study show that human exRNA in human plasma exists mostly in a “free” but RNP-associated form, which protects the RNA from nuclease degradation. Ultracentrifugation and precipitation-based strategies allow partial enrichment of EV fractions; however, these preparations remain highly contaminated by associated RNPs from the “free” population. The major portion of the “free/RNP-associated” exRNAs are human RNAs, with rRNA being the most prominent constituent. Precipitation-based kits provide little enrichment of EVs, since RNA composition is rather similar for whole plasma and precipitated vesicles.

When alternative protocols based on SEC or proteinase K/RNase treatment were used, the composition of EV-associated exRNAs was revealed to be entirely different, both in size distribution and in composition. Over 80% of exRNAs in these EVs was non-human RNA, representing mostly bacterial rRNA from the human microbiota. Mature human exRNAs (with 5’-P and 3’-OH extremities such as miRNA and hY4 RNA) are enriched in the “free” population and are still present in EVs obtained using precipitation protocols due to their association and/or coprecipitation. This fraction is minor in the plasma, and the majority of exRNA has “degradation”-type

extremities (5'-OH and 3'-P or cyclophosphate). This population is more diverse, and human RNA is mostly represented by rRNA, in addition to non-human (microbiota) rRNAs.

Thus, for the analysis of human mature RNAs, such as miRNA species, the extraction of whole human plasma is recommended, while for more detailed analysis of exRNA issuing from the microbiota, preparation of highly purified EVs should be considered.

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## 7. CONTRIBUTORS

EV and YM conceived the study, AG and GD performed isolation and characterizations of EVs, , AG, LA and VM prepared libraries and performed their sequencing, AG and YM analyzed the data and wrote the manuscript.

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## FIGURE LEGENDS

Figure 1: Flow-chart for exRNA extraction and characterization.

After removal of large particles, plasma samples are used for enrichment/purification of EVs using precipitation-based kits, size-exclusion chromatography (SEC) procedures (qEV, Izon) or combined proteinase K and RNase A treatment. In SEC, nanoscale EVs are separated from smaller protein contaminants and eluted in different fractions, while combined proteinase K and RNase A treatment removes RNAs associated with RNPs and external shells of EVs. Only internal exRNAs inside of EV are still protected by the lipid bilayer. Then, enriched or purified samples were submitted for RNA extraction, dephosphorylation/phosphorylation treatment (deP/P), library preparation and RNA sequencing.

Figure 2: Characterization of exRNA from human plasma.

A – Agilent Bioanalyzer profile of exRNA extracted from human plasma on PicoRNA6000 Chip. B – Agilent Bioanalyzer analysis on High Sensitivity DNA Chip of library preparations with and without (no treatment) preliminary deP/P treatment. Bands corresponding to primer adapters and primer dimers are indicated. C – Size distribution of RNA fragments observed in total library preparations (solid line – untreated samples, dashed line – RNAs treated by deP/P) from whole plasma for two samples (P #1 and P #2). D – Size distribution of fragments aligned to the human hg19 genome. The same line style and samples are used as in panel C. E – Size distribution for reads unmapped to hg19. F – Proportions of human rRNA, other human RNAs, non-human rRNAs and unidentified fragments in exRNA from Plasma #1 and Plasma #2 samples. Proportions are shown for untreated exRNA samples (-) and after preliminary deP/P treatment (deP/P).

Figure 3: Characterization of exRNA in EVs obtained by ultracentrifugation.

A – NanoSight Nanoparticle Tracking Analysis (NTA) profile for EV fraction obtained by ultracentrifugation. B – Agilent Bioanalyzer profile of exRNAs extracted from ultracentrifugation-derived EVs on PicoRNA6000 Chip. C -- Size distribution of RNA fragments observed in total library preparations from whole plasma, plasma (P #2), plasma RNA remaining in the supernatant after ultracentrifugation (S100) and RNA extracted from extracellular vesicle (EV) fraction after ultracentrifugation (solid line – untreated samples, dashed line – RNAs treated by deP/P). D – Size distribution of fragments aligned to the human hg19 genome. The same line style and samples are used as in panel C. E – Proportions of human rRNA, other human RNAs, non-human rRNAs and unidentified fragments in exRNA samples from P #2, S100 supernatant and EV fractions. Proportions are shown for untreated exRNA samples (-) and after preliminary deP/P treatment (deP/P).

Figure 4: Characterization of exRNAs extracted from EVs prepared using precipitation-based plasma fractionation kits.

A – NanoSight Nanoparticle Tracking Analysis (NTA) profile for EV fractions obtained from Plasma #2 by miRCURY Exiqon kit, Total Exosomes isolation kit (Invitrogen) and Exosome precipitation solution (Macherey-Nagel). B – Agilent Bioanalyzer profile of exRNA extracted from precipitation-derived EVs on PicoRNA6000 Chip. C -- Size distribution of RNA fragments observed in total library preparations from EVs isolated by ExoQuick™ Exosome precipitation solution (System Biosciences) and Exiqon and Macherey-Nagel kits (solid line – untreated samples, dashed line – RNAs treated by deP/P). D – Proportions of human rRNA, other human RNAs, non-human rRNAs and unidentified fragments in EV-associated exRNA samples prepared by the same kits as in panel C. Proportions are shown for untreated exRNA samples (-) and after preliminary deP/P treatment (deP/P).

Figure 5: Characterization of exRNAs from highly purified EVs obtained by size-exclusion chromatography and successive Proteinase K/RNase A treatments.

A – Agilent Bioanalyzer profile of exRNAs extracted from highly purified EVs on PicoRNA6000 Chip. B – Agilent Bioanalyzer analysis on High Sensitivity DNA Chip of library preparations for exRNAs from 2 independent plasma samples (Plasma #2 and Plasma #5) obtained by Proteinase K/RNase A treatment and SEC. C – Size distribution of all RNA fragments observed in total library preparations from these 2 samples. D – Size distribution of RNA fragments aligned to human hg19 genome. E – Proportions of human rRNA, other human RNAs, non-human rRNAs, and unidentified fragments in highly purified EVs for the 2 samples (Plasma #2 and Plasma #5).

**Table 1 Published studies of human blood exRNA characterization**

Twelve studies on human blood/plasma exRNA characterization were compared by different parameters : type of sample and treatment, RNA extraction method, RNA sequencing and analysis method.

Sample type	Blood treatment	RNA extraction methods	RNA seq methods	Studied RNA	Majors species found	Comments/Potential issues	Reference
Exosomes from plasma	Exoquick (System Biosciences) +/- RNase A treatment	Qiagen miRNeasy micro kit	Illumina sequencing (NEBNext® Multiplex Small RNA Library Prep Set for Illumina and NEXTflex) - RNA sequencing kit (Bioo Scientific) - TruSeq Small RNA sample preparation kit (Illumina) Size selection of the libraries 140-160 bp	miRNA, rRNA, lncRNA, DNA, tRNA, miscRNA, piRNA, mRNA, snRNA, snoRNA, other	miRNA, rRNA	Kit based on precipitation include a lot of soluble extracellular RNA  RNA from RNP or lipoproteins are not sensitive to RNase A	Huang et al, 2013 [78]
Exosomes from serum	Total exosome isolation (from serum) reagent (Invitrogen)	Total exosome RNA and protein isolation kit (Invitrogen)	Ion proton system (Ion Total RNA-Seq Kit v2 [Thermofischer])	miRNA, tRNA, rRNA, mRNA, snRNA, scaRNA, snoRNA, piRNA	miRNA, mRNA, tRNA	Kit based on precipitation include a lot of soluble extracellular RNA	Li et al, 2014 [35]
Plasma	N.A.	Exiqon miRCURY RNA Isolation Kit- biofluids	Ion proton system (Ion Total RNAseq kit v2 [Thermofischer])	rRNA removal  miRNA, tRNA, snoRNA, piRNA, and other RNA RFam database	miRNA, piRNA, snoRNA		Freedman et al, 2016 [76]
Exosomes from plasma	Exoquick (System Bioscience) + Rnase A treatment	Qiagen miRNeasy micro kit	Illumina sequencing (NEBNext® Multiplex Small RNA Library Prep Set for Illumina) Size selection of the libraries 140-160 bp	miRNA, piRNA, siRNA, lncRNA, mRNA, pseudogenes, antisense RNA, rRNA, snRNA, snoRNA, misc RNA other transcribed RNA, FLU	miRNA, piRNA	Kit based on precipitation include a lot of soluble extracellular RNA  RNA from RNP or lipoproteins are not sensitive to RNase A	Yuan et al, 2016 [34]



				human cDNA and predicted RNA			
Serum	N.A.	- Qiagen Circulating Nucleic Acid Kit, - TRIzol LS Reagent, - Qiagen miRNeasy Serum/Plasma kit, - QiaSymphony RNA extraction kit - Exiqon miRCURY RNA Isolation Kit-biofluids	Illumina sequencing (TruSeq Small RNA sample preparation kit) Size selection of the libraries 5-40 bp	miRNAs, isomiRs, tDRs and osRNAs	osRNAs, tDRs, miRNA (including isomiR)	Serum: include RNA from platelets secretion: affects the composition of exRNA originally present in circulating blood	Guo et al, 2017 [75]
EV from plasma	Differential centrifugation, ultracentrifugation	Total RNA purification kit (Norgen Biotek)  RNA fragmentation by RNase III	Ion proton system (Ion Total RNA-Seq Kit v2 [Thermofisher])	rRNA removal  pseudogenes, mRNA, lncRNA, miRNA, tRNA, misc RNA, other	mRNA	Ultracentrifugation on viscous plasma procedure can co-pellet soluble extracellular RNA	Amorim et al, 2017 [79]
Whole platelet poor plasma, 16,000xg vesicles, 160,000xg vesicles and particles depleted in plasma	Differential centrifugation procedure	TRIzol reagent (Life Technologies)  dephosphorylation/ phosphorylation treatment  size selection on a gel for RNA > 19nt	SOLiD sequencing (SOLiD total RNA-Seq kit [Life Technologies])	Mitochondrial RNA, human genomic repeat consensus sequences, tRNA, rRNA, snRNA, small cytoplasmic RNA	rRNA, mRNA, mitochondrial RNA (for plasma and 16,000xg vesicles only)	Ultracentrifugation on viscous plasma procedure can co-pellet soluble extracellular RNA	Savelyeva et al, 2017 [32]
Plasma	N.A.	Thermofisher mirVana miRNA Isolation kit	Illumina sequencing (TruSeq Small RNA sample preparation kit) Gel purification: removal of dimers	rRNA, miRNA, tRNA, piRNA, RNA from ENSEMBL 75 database, non-human RNA	rRNA, miRNA		Yeri et al, 2017 [5]
Plasma	+/-Proteinase K treatment	Exiqon miRCURY RNA Isolation Kit-biofluids	Illumina sequencing (NEBNext® Multiplex Small RNA Library Prep Set for Illumina) Size selection of the libraries: RNA of 21-	rRNA, miRNA, tRNA, snoRNA, piRNA	rRNA, miRNA, tRNA		Danielson et al, 2017 [81]

			40 nt			
Plasma and serum	Detergent and Proteinase K treatment	Personalized phenol/chloroform procedure	Illumina sequencing (personalized library preparation including RNA size selection)	miRNA, rRNA, tRNA, small cytoplasmic RNA, other cytoplasmic RNA, mRNA	Plasma: miRNA, mRNA, rRNA  Serum: miRNA, scRNA, rRNA	Max et al, 2018 [77]

Abbreviations:

isomiRs- micro RNA isoforms, lncRNA – long non-coding RNA, miRNA – micro RNA, miscRNA – miscellaneous RNA, mRNA – messenger RNA, osRNAs – other miscellaneous RNA, piRNA – piwi RNA, rRNA – ribosomal RNA, siRNA – small interfering RNA, snoRNA – small nucleolar RNA, snRNA – small nuclear RNA, tDRs – transfer-derived small RNA, tRNA – transfer RNA

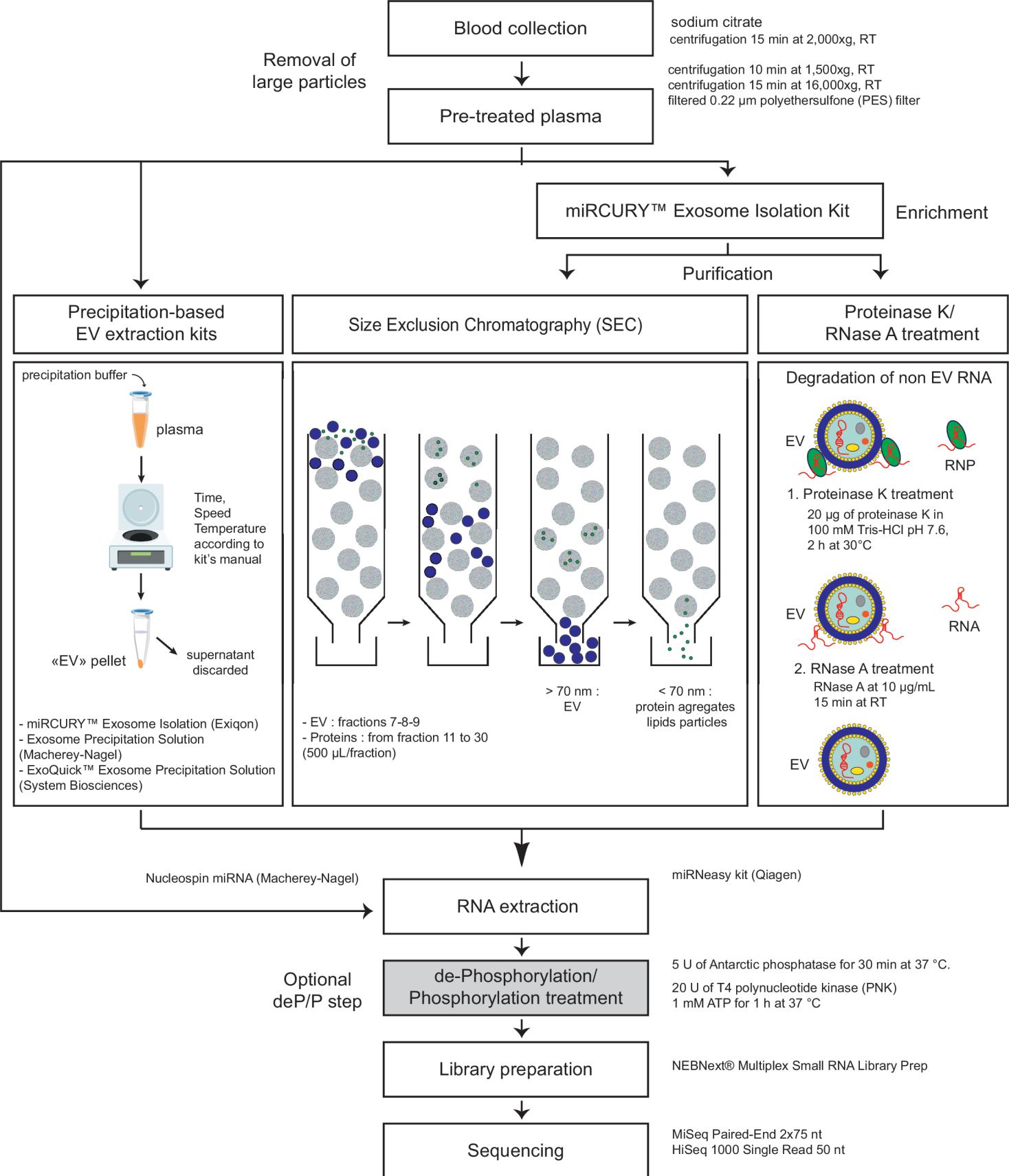


Figure 1 Galvanin et al

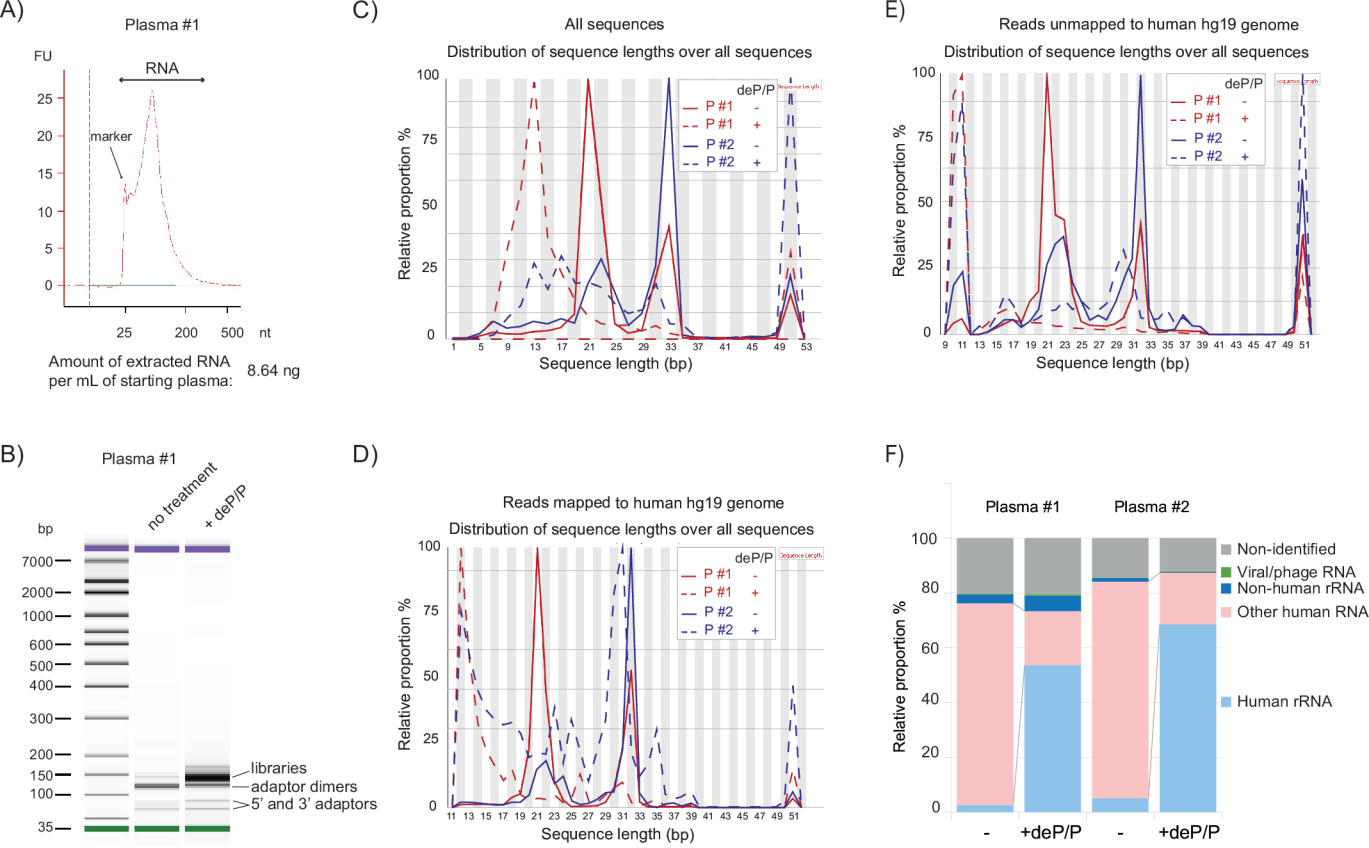


Figure 2 Galvanin et al

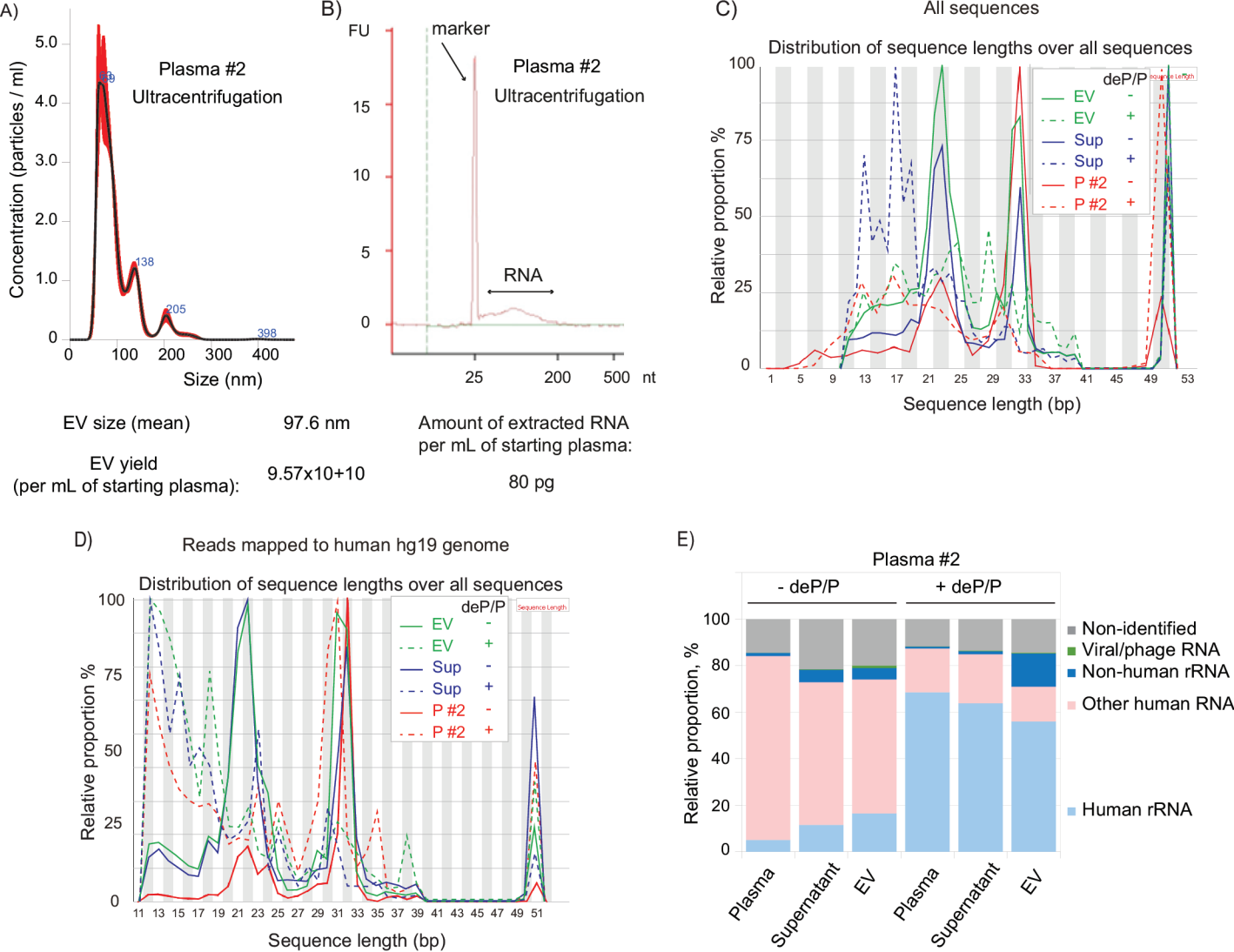


Figure 3 Galvanin et al

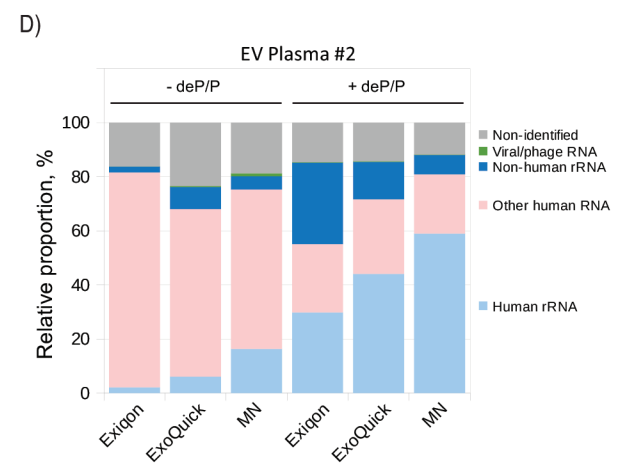
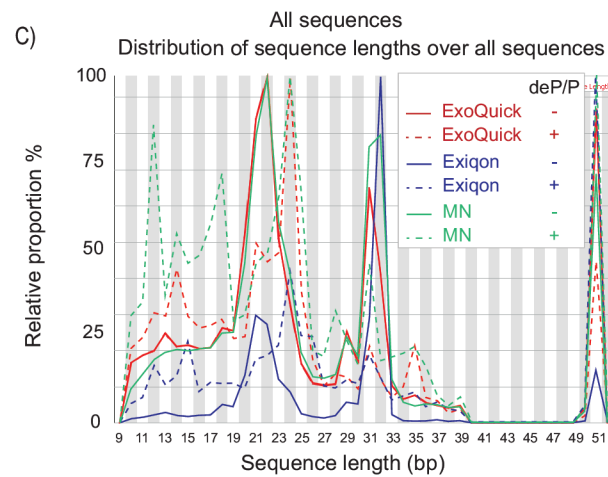
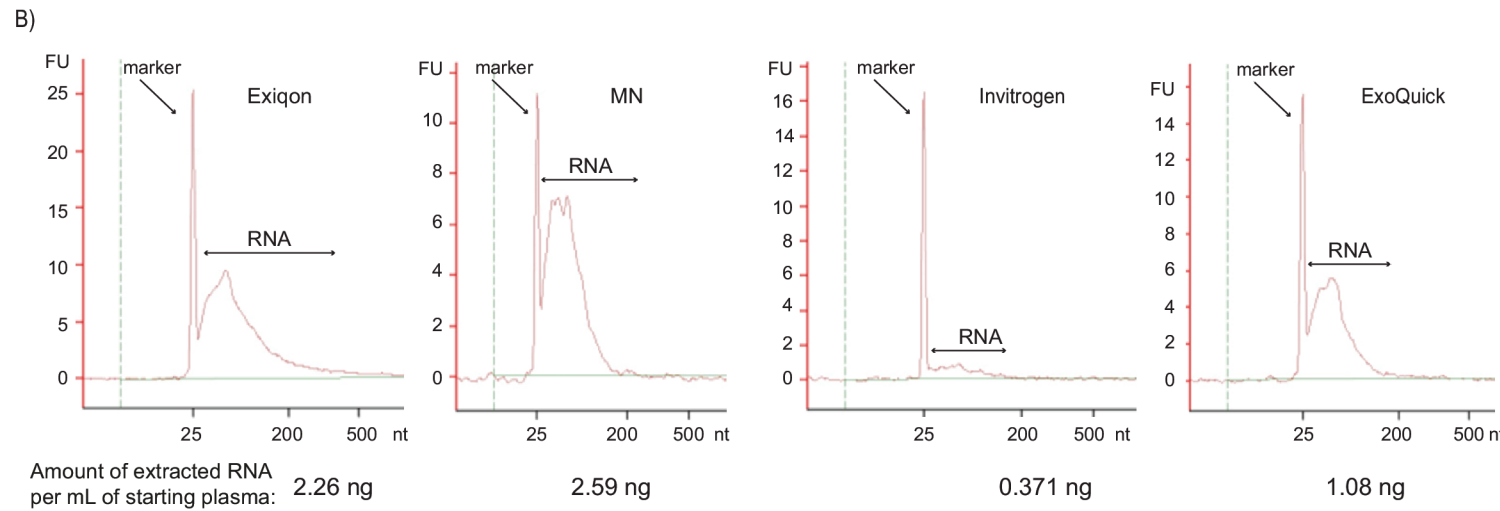
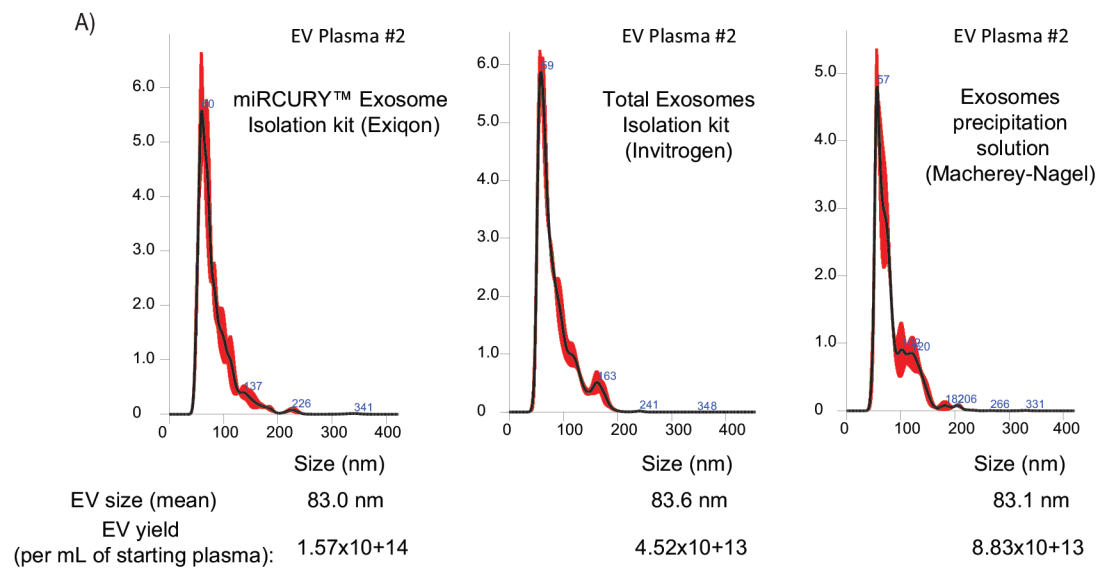


Figure 4 Galvanin et al

