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Title

Performance of RNA purification kits and blood collection tubes in the Extracellular RNA Quality Control (exRNAQC) study

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Conflict of interest

Carolina Fierro and Nele Nijs are employees, Thomas Piofczyk is a former employee, Pieter Mestdagh is a consultant, and Jo Vandesompele a co-founder of Biogazelle, a clinical CRO providing human

biofluid extracellular RNA sequencing. Gary P. Schroth and Scott Kuersten are employees of Illumina, providing library preparation and sequencing reagents. Promega, Qiagen and Roche sponsored blood collection tubes and/or RNA purification kits. Funders did not influence data analysis, interpretation and manuscript writing.

Abbreviations

ACD-A: BD Vacutainer Glass ACD Solution A tube; ALC: area left of the curve; Biomatrica: LBgard Blood Tube; BRISQ: Biospecimen Reporting for Improved Study Quality; bp: base pair; CCF: QIAamp ccfDNA/RNA Kit; CIRC: Plasma/Serum Circulating and Exosomal RNA Purification Kit/Slurry Format; citrate: Vacuette Tube 9 ml 9NC Coagulation sodium citrate 3.2%; DNA Streck: Cell-Free DNA BCT; EDTA: BD Vacutainer Plastic K2EDTA tube; EDTA separator: Vacuette Tube 8 ml K2E K2EDTA Separator; ERCC: Extracellular RNA Communication Consortium; exRNA: extracellular RNA; FC: fold change; gDNA: genomic DNA; LP: Library Prep Control; MAP: MagNA Pure 24 Total NA Isolation Kit in combination with the MagNA Pure instrument; MAX: Maxwell RSC miRNA Plasma and Exosome Kit in combination with the Maxwell RSC Instrument; MIR: miRNeasy Serum/Plasma Kit; MIRA: miRNeasy Serum/Plasma Advanced Kit; miRNA: microRNA; MIRV: mirVana PARIS Kit with purification protocol for total RNA; MIRVE: mirVana PARIS Kit with purification protocol for RNA enriched for small RNAs; mRNA: messenger RNA; NUC: NucleoSpin miRNA Plasma Kit; PAXgene: PAXgene Blood ccfDNA Tube; RA3: RNA 3’ adapter; RA5: RNA 5’ adapter; RC: RNA extraction Control; RNA Streck: Cell-Free RNA BCT; Roche: Cell-Free DNA Collection Tube; serum: BD Vacutainer SST II Advance Tube; SOP: standard operating procedure;

Abstract

The use of blood-based extracellular RNA (exRNA) as clinical biomarker requires the implementation of a validated procedure for sample collection, processing and profiling. So far, no study has systematically addressed the pre-analytical variables affecting transcriptome analysis of exRNAs. In the

exRNAQC study, we evaluated 10 blood collection tubes, 3 time points between blood draw and downstream processing, and 8 RNA purification methods using the supplier-specified minimum and maximum biofluid input volumes. The impact of these pre-analytics is assessed by deep transcriptome profiling of both small and messenger RNA from healthy donors' plasma or serum. Experiments are conducted in triplicate (for a total of 276 transcriptomes) using 189 synthetic spike-in RNAs as processing controls. When comparing blood tubes, so-called blood preservation tubes do not stabilize RNA very well, as is reflected by increasing RNA concentration and number of detected genes over time, and by compromised reproducibility. We also document large differences in RNA purification kit performance in terms of sensitivity, reproducibility, and observed transcriptome complexity. Our results are summarized in 11 performance metrics that enable an informed selection of the most optimal sample processing workflow for your own experiments. In conclusion, we put forward robust quality control metrics for exRNA quantification methods with validated standard operating procedures (SOPs) for processing, representing paramount groundwork for future exRNA-based precision medicine applications.

Keywords

Extracellular RNA (exRNA), pre-analytical variables, blood collection tube, RNA purification, mRNA capture sequencing, small RNA sequencing

Introduction

Biomarker studies are increasingly focusing on biofluids as an attractive resource of molecules reflecting human health or disease states. Biopsies from those human body fluids are often referred to as ‘liquid biopsies’. In contrast to tissue biopsies, they have the advantage of being minimally invasive and are compatible with serial profiling, enabling one to monitor the impact of an intervention (e.g. treatment, physical exercise) over time.

The majority of liquid biopsy biomarker studies focus on cell-free nucleic acids as candidate biomarkers. While cell-free DNA has been studied intensively and found its way to the clinic for non-invasive prenatal testing5/10/2021 11:17:00 AM, extracellular RNA (exRNA) is relatively new in the biomarker field. Nevertheless, biomarker potential has been ascribed to various RNA molecules, including microRNA (miRNA), messenger RNA (mRNA), long-non-coding RNA and circular RNA in several diseases such as cancer, autoimmune diseases, diabetes, and cardiovascular diseases[2–6]. The growing interest in exRNA as a biomarker resource requires the implementation of standardized methods for sample collection, processing and molecular profiling. Blood serum and plasma are amongst the most studied liquid biopsies and several pre-analytical variables, including blood collection tube type, needle type and blood centrifugation speed and duration, are known to influence exRNA abundance patterns (Supplemental table 1)[7–9]. Nevertheless, those pre-analytical variables are typically not reported in studies, which makes it hard to replicate findings or directly compare biomarker studies.

Over time, multiple research consortia were developed with the aim to standardize some of these pre-analytical variables, including the NIH's Extracellular RNA Communication Consortium (ERCC)[10,11], SPIDIA/SPIDIA4P[12] and CANCER-ID[13]. The ERCC aims to bundle fundamental scientific discoveries, protocols, tools and technologies that can be shared with the scientific community, and has developed standardized procedures for plasma isolation, RNA extraction, sequencing and data analysis[11]. SPIDIA4P includes different European partners that work together for standardization and improvements of pre-analytical procedures. They are currently working on an ISO standard for 'venous whole blood cell free circulating RNA'. Similarly, CANCER-ID is a European consortium that aims to establish standard protocols for clinical validation (including sample storage, sampling procedures, isolation methods) of blood-based biomarkers (e.g. microRNA and extracellular vesicles).

While it is well recognized that pre-analytical variables need to be considered when studying exRNA biomarkers, studies investigating their impact are focused on microRNAs only or are restricted to a limited number of genes (Supplemental table 1). In the Extracellular RNA Quality Control (exRNAQC)

study, we performed an extensive massively parallel sequencing-based analysis of the impact of pre-analytical variables on both small RNA and mRNA profiles. We systematically evaluated 10 blood collection tubes, 3 time points between blood draw and downstream processing, and 8 RNA purification methods using the supplier specified minimum and maximum plasma input volumes. The impact of these pre-analytical factors is firmly established using deep transcriptome profiling of all small and messenger RNAs from healthy donors' plasma or serum. Synthetic spike-in RNAs were added during and after RNA purification and a wide variety of performance metrics were evaluated (Figure 1). To the best of our knowledge, such a comprehensive analysis of pre-analytical variables in the context of exRNA profiling has not yet been performed.

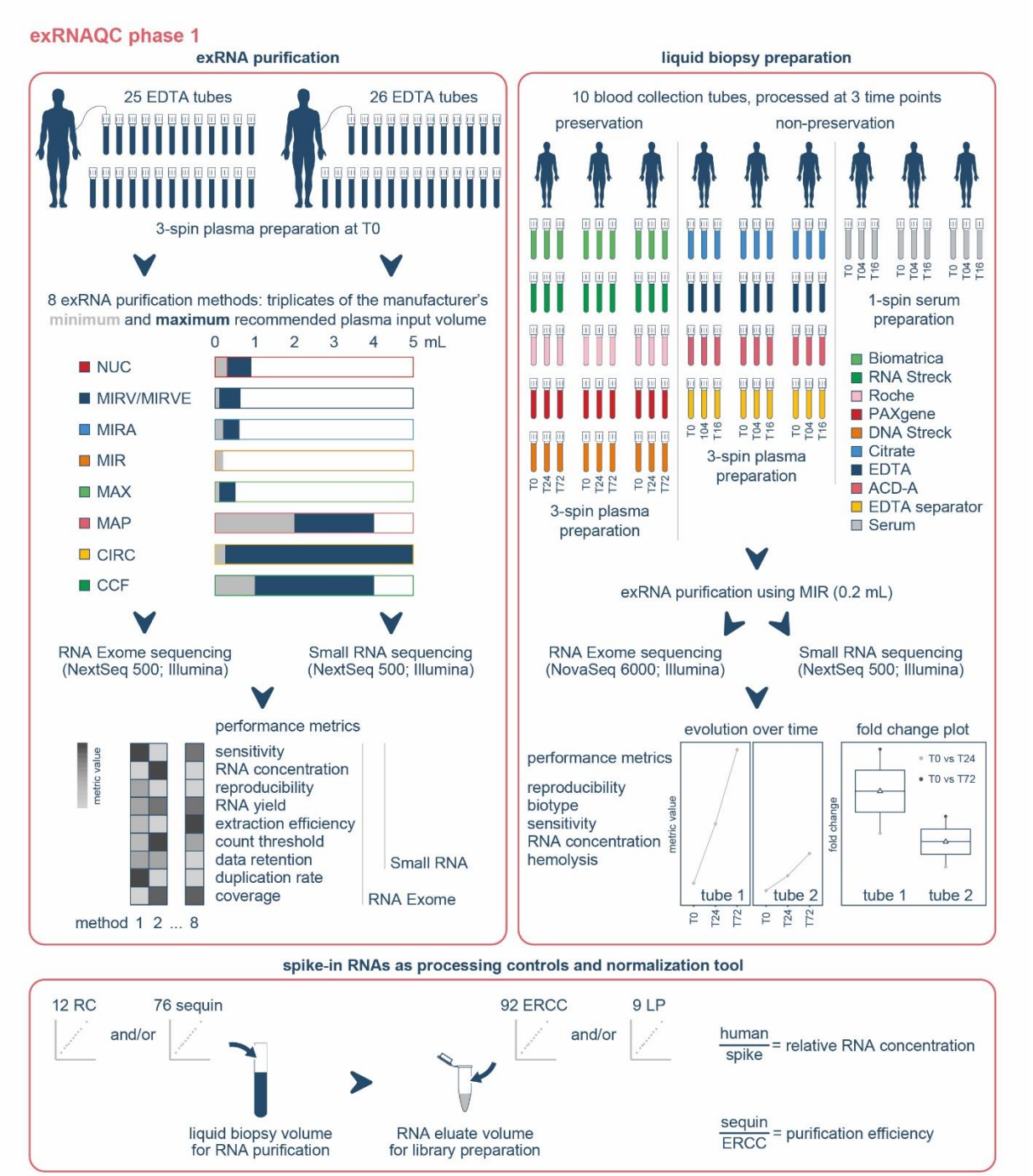

Figure 1: Overview of the exRNAQC phase 1 study design. To evaluate the impact of the 8 RNA purification methods (left panel), two blood draws from a single individual were performed to separately apply mRNA capture (study code: exRNAQC004) and small RNA (study code: exRNAQC011) sequencing. Both minimum and maximum recommended plasma input volumes were tested in triplicate. To compare RNA purification performance, 9 performance metrics were calculated. To evaluate the impact of the 10 blood collection tubes (right panel), 9 individuals were sampled, enabling

to test 3 time intervals between blood draw and downstream processing for each of the tubes. Preservation tubes were processed immediately upon blood collection (T0), after 24 hours (T24) or after 72 hours (T72). Non-preservation plasma and serum tubes were processed immediately upon blood collection (T0), after 4 hours (T4) or after 16 hours (T16). Both mRNA capture (study code: exRNAQC005) and small RNA (study code: exRNAQC013) sequencing were performed, and the data was analyzed using 5 performance metrics. To control the RNA purification and library preparation workflows, 189 synthetic spike-in RNA molecules (Sequin and Extracellular RNA Communication Consortium (ERCC) spike-ins for RNA Exome sequencing, and RNA extraction Control (RC) and Library Prep Control (LP) spike-ins for Small RNA sequencing) were used, allowing to calculate relative RNA concentrations and purification efficiency (lower panel). ACD-A: BD Vacutainer Glass ACD Solution A tube; ALC: area left of the curve; Biomatrica: LBgard Blood Tube; CCF: QIAamp ccfDNA/RNA Kit; CIRC: Plasma/Serum Circulating and Exosomal RNA Purification Kit/Slurry Format; citrate: Vacuette Tube 9 ml 9NC Coagulation sodium citrate 3.2%; DNA Streck: Cell-Free DNA BCT; EDTA: BD Vacutainer Plastic K2EDTA tube; EDTA separator: Vacuette Tube 8 ml K2E K2EDTA Separator; MAP: MagNA Pure 24 Total NA Isolation Kit in combination with the MagNA Pure instrument; MAX: the Maxwell RSC miRNA Plasma and Exosome Kit in combination with the Maxwell RSC Instrument; MIR: the miRNeasy Serum/Plasma Kit; MIRA: the miRNeasy Serum/Plasma Advanced Kit; MIRV: the mirVana PARIS Kit with purification protocol for total RNA; MIRVE: mirVana PARIS Kit with purification protocol for RNA enriched for small RNAs; NUC: the NucleoSpin miRNA Plasma Kit; PAXgene: PAXgene Blood ccfDNA Tube; RNA Streck: Cell-Free RNA BCT; Roche: Cell-Free DNA Collection Tube; serum: BD Vacutainer SST II Advance Tube.

Results

RNA purification methods strongly influence miRNA and mRNA abundance profiles

Eight different total RNA purification kits were selected for comparison: miRNeasy Serum/Plasma Kit (MIR), miRNeasy Serum/Plasma Advanced Kit (MIRA), mirVana PARIS Kit (MIRV), NucleoSpin miRNA

Plasma Kit (NUC), QIAamp ccfDNA/RNA Kit (CCF), Plasma/Serum Circulating and Exosomal RNA Purification Kit/Slurry Format (CIRC), Maxwell RSC miRNA Plasma and Exosome Kit in combination with the Maxwell RSC Instrument (MAX), and MagNA Pure 24 Total NA Isolation Kit in combination with the MagNA Pure instrument (MAP). Since most kits allow a range of blood plasma input volumes, we tested both the minimum and maximum input volume recommended by each supplier. This resulted in 15 unique combinations of kits and input volumes. To evaluate small RNA purification, we added two additional combinations of the mirVana PARIS Kit by applying an alternative protocol for specific enrichment of small RNAs (MIRVE), resulting in 17 unique kit-input volume combinations for small RNA profiling. Blood was collected from one healthy donor and three technical replicates were used for every kit-volume combination, resulting in 45 and 51 samples that were processed for RNA extraction and sequencing library preparation for mRNA capture and small RNA, respectively.

We first investigated potential DNA contamination in the RNA samples using the strandedness of the mRNA capture-seq data as a proxy. As we applied a stranded library preparation protocol, strandedness should be close to 100% in the absence of DNA contamination. Strandedness of data generated using the MAP kit, however, was considerably lower: only 70-75% and 80-85% of reads mapped to the correct strand for MAP2 and MAP4 purification, respectively, while this percentage was above 95% for all other purification methods (Supplemental figure 1c). Moreover, the small RNA-seq data from the MAP kit contained a much higher fraction of mapped reads that did not overlap annotated small RNA sequences (35 to 52% of mapped reads for MAP compared to only 1 to 6% for other purification kits) and more than 80% of these unannotated reads did not overlap with known exons. Despite DNase treatment, these findings strongly suggest residual DNA contamination in MAP kit RNA eluates and we therefore excluded this kit from further analyses.

To evaluate differences among RNA purification kits, we calculated nine different performance metrics: detection sensitivity (number of mRNAs or miRNAs detected), RNA concentration, RNA yield, extraction efficiency, reproducibility based on count threshold, data retention after filtering, reproducibility based on area left of the curve (ALC), PCR duplication rate, and transcriptome coverage

(see Methods for a detailed description of each individual metric; the last two metrics were only evaluated at the mRNA level).

In terms of **sensitivity**, the absolute **number of mRNAs and miRNAs** detected ranged from 989 to 11,322 and from 69 to 171, respectively. While a higher input volume consistently resulted in a higher number of detected mRNAs or miRNAs for a given kit (Figure 2a & b), this was not always true when comparing different kits (e.g. MIRA with 600 µl (7424 mRNAs on average) versus NUC with 900 µl plasma input (4766 mRNAs on average); Figure 2a).

The purification kit resulting in the highest relative mRNA **concentration** (CCF4) had on average a 76 times higher eluate concentration than the kit with the lowest concentration (MIRV0.1) (Figure 2c). For miRNAs, the difference was even larger, with a 238 times higher concentration in CCF4 compared to MIRVE0.1 (Figure 2d). When excluding MIRVE, which was not tested at mRNA level, the difference between the kit with the highest and lowest relative miRNA concentration was on average 29-fold. The **RNA yield** metric represents the relative amount of RNA in the total eluate volume after purification. For mRNA capture sequencing, there was on average a 30-fold difference in yield between the kit with the highest yield (CIRC5) compared to the kit with the lowest yield (NUC0.3) (Supplemental figure 2e). For small RNA sequencing, there was on average an 85-fold difference between the kit with the highest yield (MAX0.5) compared to the kit with the lowest yield (MIRVE0.1) (Supplemental figure 2f). Overall, yield differences among kits were smaller than concentration differences, as expected given that differences in eluate volumes (from 14 to 100 µl) are canceled out.

The **extraction efficiency** metric is a performance metric that, besides RNA yield, also takes into account differences in input volume for RNA purification. It is a relative measure of how well a certain kit purifies RNA from the plasma input volume. When looking at the extracellular mRNA transcriptome, the highest average purification efficiency (MAX0.1) was 10 times higher than the lowest (MIRV0.1) (Supplemental figure 2g). For small RNAs, the highest average efficiency (MAX0.1) was 25 times higher than the lowest (MIRV0.625) (Supplemental figure 2h). Note that the extraction efficiency is kit

dependent, whereby -expectedly- no differences are observed between the maximum and minimum input volume for a given kit.

For each purification kit, we determined a **count threshold** to filter noisy data based on eliminating 95% of single positive observations between technical replicates. Higher thresholds indicate higher variability. This threshold varied from 5 to 14 counts at mRNA level for CCF4 and MIRV0.1, respectively, and from 2 to 16 counts at miRNA level for MIRA0.6 and MIRVE0.1, respectively (Supplemental figure 2a & b; Supplemental table 2). A related metric, **data retention**, represents the fraction of total counts that are retained after applying the count threshold. For mRNA capture sequencing, data retention ranged from an average of 93.5% in MIRV0.1 to an average of 99.7% in CCF4 (Supplemental figure 2c). For small RNA sequencing, data retention ranged from an average of 98.8% in MIRVE0.1 to an average of 99.8% in MAX0.5 (Supplemental figure 2d).

To assess **reproducibility,** we determined the area left of the curve (ALC), a robust metric based on differences in mRNA or miRNA counts between technical replicates (see Methods and the miRQC study[14]). The higher the reproducibility, the lower the ALC value. Most kits performed equally well with respect to miRNA count reproducibility (Figure 2f) with the exception of MIRVE0.1. For mRNA, CIRC0.25 and MIRV0.1 had a lower reproducibility than the other kits while CCF4 had the best reproducibility, closely followed by CIRC5 and MIR0.2 (Figure 2e). Within a kit, the maximum input volume consistently resulted in a better reproducibility compared to the minimum input volume.

A low amount of input RNA, as is the case for plasma, typically results in mRNA capture-seq libraries with a high fraction of PCR duplicates. The average **duplication rate** ranged from 82.2% (CCF4) to 97.3% (NUC0.3) of mRNA capture sequencing reads (Supplemental figure 1a). Note that even a small difference in PCR duplication rate can have a high impact on the total number of non-duplicated reads: with CCF4, on average six times more non-duplicated reads were generated compared to NUC0.3 (Supplemental table 3).

Finally, the **transcriptome coverage** metric was used to assess the diversity of mRNA capture sequencing reads. The MIRV0.1 kit had the lowest average coverage: only 1.8% of the human Ensemble

v91 transcriptome was covered by at least one sequencing read. Purification with CCF4 resulted in the highest average coverage (17.7%, Supplemental figure 1b).

A summary plot of all performance metrics after robust z-score transformation is shown in Figure 2g & h, for mRNA and small RNA level, respectively. For each metric, a higher z-score indicates a better performance. In general, kit differences are smaller for miRNA than for mRNA (less variability in z-score and metric values). For mRNA capture sequencing, kits with a higher plasma input volume such as CIRC5 and CCF4 scored better on most performance metrics. Kits with plasma input volumes below 0.5 ml were in general less performant than other kits, with the exception of MIR0.2. Note, however, that despite the lower performance scores, MAX0.1 and MIRV0.1 were quite efficient in purifying RNA from the given 0.1 ml of plasma. Moreover, plasma input volume alone does not completely determine performance as some kits with a lower plasma input volume still perform better than kits with a higher input, for example MIRA0.6 and CCF1. For small RNAs, we mainly observed low performance in the smaller input volume kits, but there were exceptions. MAX0.5 and MIRA0.6, for example, scored surprisingly well or even better compared to kits with a much larger plasma input volume such as CIRC5 and CCF4. In contrast to mRNA capture sequencing, more plasma input for a given kit did not always result in better small RNA sequencing performance (see CIRC5 vs CIRC0.25).

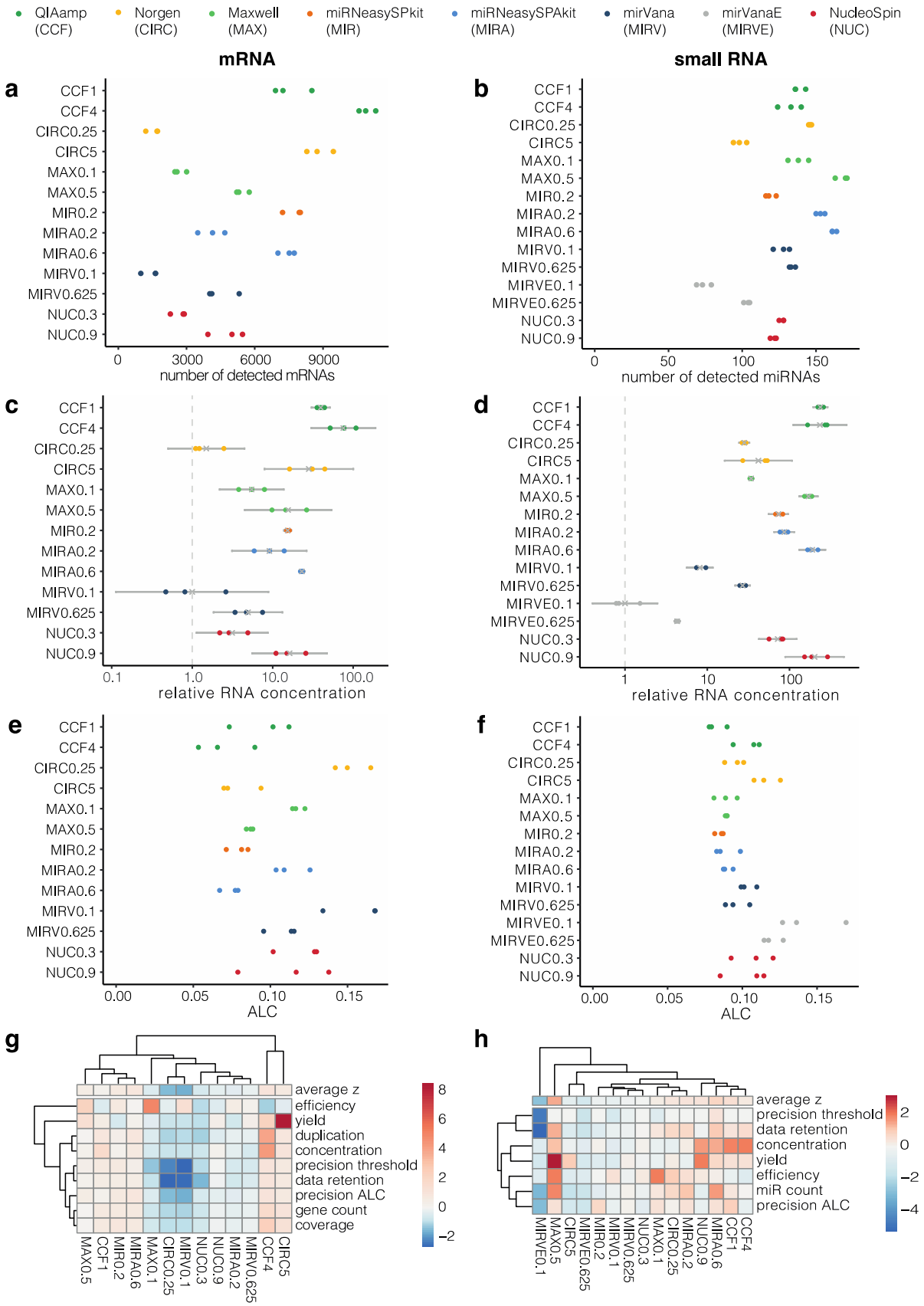

Figure 2: Performance of RNA purification kits for mRNA capture sequencing (mRNA) and small RNA sequencing (miRNA). a&b: absolute number of mRNAs and miRNAs, resp., that reach count threshold;

c: relative endogenous RNA concentration at mRNA level (ratio of endogenous RNA to ERCC spikes); d: relative endogenous miRNA concentration (ratio of endogenous miRNA to LP spikes); c&d: values are log rescaled to the lowest mean of all kits and transformed back to linear space, 95% confidence interval is shown; e&f: reproducibility between technical replicates based on ALC (smaller ALC indicates better reproducibility) at mRNA and miRNA level, resp.; g&h: overview of all performance metrics at mRNA capture and small RNA sequencing level, resp., after transforming the values to robust z-scores where a higher z-score indicates a better performance, rows and columns of heatmap are clustered according to complete hierarchical clustering based on Euclidean distance, average z refers to the mean of robust z-scores for a specific purification kit. Number that follows the abbreviation of the purification kit is the plasma input volume (in ml).

Blood preservation tubes are not suitable for exRNA analysis

Eleven different blood collection tubes were selected, belonging to two categories: tubes that are not designed to stabilize nucleic acids (which we termed “non-preservation tubes”; n = 6), and so-called “preservation tubes” (n = 5) that are specifically designed to allow more time between the blood draw and plasma preparation. The selected non-preservation tubes were the BD Vacutainer Plastic K2EDTA tube (EDTA), Vacuette Tube 8 ml K2E K2EDTA Separator (EDTA separator), BD Vacutainer Glass ACD Solution A tube (ACD-A), Vacuette Tube 9 ml 9NC Coagulation sodium citrate 3.2% (citrate), and BD Vacutainer SST II Advance Tube (serum). The preservation tubes were the Cell-Free RNA BCT (RNA Streck), Cell-Free DNA BCT (DNA Streck), PAXgene Blood ccfDNA Tube (PAXgene), Cell-Free DNA Collection Tube (Roche) and LBgard Blood Tube (Biomatrica). For each of the blood collection tubes, we recruited three healthy volunteers and three time intervals between blood draw and plasma preparation were chosen: immediately (T0), time point 1 (4 hours for non-preservation tubes, 16 hours for preservation tubes), and time point 2 (16 hours for non-preservation tubes and 72 hours for preservation tubes). This resulted in 90 samples that were subsequently processed for RNA extraction, mRNA capture-seq and small RNA-seq. To evaluate tube stability over time, we calculated 5 different

294 performance metrics: (1) hemolysis, (2) relative RNA concentration, (3) number of mRNA or miRNA
295 genes detected, (4) fraction of counts mapping on mRNAs or miRNAs, and (5) reproducibility (ALC) (see
296 Methods for a detailed description of each metric). Stability of each metric over time was evaluated as
297 a fold change between time point 0 and time point 1 and between time point 0 and time point 2 as
298 exemplified in Supplemental figure 3. If processing time has no impact on any of the above-described
299 methods, respective fold changes should be close to one. For each blood tube, the average fold change
300 of each performance over time is shown in Figure 3**.**
301 **Hemolysis** was quantified based on absolute absorbance at 414 nm and evaluated by visual inspection
302 during liquid biopsy preparation. For the non-preservation tubes, hemolysis measurements were
303 below the generally accepted absorbance threshold of 0.2[15,16] across all donors and time points
304 (Supplemental figure 4a, Supplemental figure 5a and **Supplemental figure** 6). Oppositely, for all
305 preservation tubes except the Biomatrica tube, plasma showed to be hemolytic for at least one donor
306 at T0. At T72, the Biomatrica hemolysis measurements also exceeded the 0.2 threshold. Despite the
307 low absorbance values, we did observe up to two-fold differences in function of time: mean fold
308 changes in non-preservation tubes ranged from 1.05 to 2.04, in preservation tubes from 1.19 to 2.08
309 (Supplemental figure 7a & Supplemental figure 8a).
310 **Relative RNA concentration** in non-preservation tubes remained quite stable over time, with a 1.23 to
311 1.48 fold increase in mRNA mass and 1.57 to 2.97 fold increase in miRNA mass (Supplemental figure
312 7b & Supplemental figure 8b). Unexpectedly, RNA concentration was less stable in preservation tubes,
313 with fold changes of 1.84 to 4.03 and 1.75 to 10.50 for mRNA and small RNA, respectively. While RNA
314 concentration did not change substantially between time points for the RNA Streck tubes, the relative
315 RNA concentration at the individual time points for these tubes was substantially lower compared to
316 the other tubes (on average 4.97-fold lower for mRNA and 10.36-fold lower for small RNA
317 (Supplemental figure 4b & Supplemental figure 5b).
318 The **absolute number of mRNAs and miRNAs** in non-preservation tubes remained relatively constant
319 over time: mean fold changes ranged from 1.29 to 1.59 and from 1.10 to 1.36 at mRNA and small RNA

sequencing level, respectively. In preservation tubes, the mean fold change ranged from 1.86 to 4.01 and from 1.08 to 1.67, for mRNA and miRNA, respectively (Supplemental figure 7c & Supplemental figure 8c). Furthermore, and similar to the RNA concentration, the absolute number of mRNAs and miRNAs was substantially lower in DNA Streck and RNA Streck tubes compared to the others (mean number of mRNAs: 385 and 840 for RNA Streck and DNA Streck, respectively; mean number of miRNAs: 60 for RNA Streck) (Supplemental figure 4c & Supplemental figure 5c).

The **fraction of total counts mapping to mRNAs and miRNAs** (Supplemental figure 4d & Supplemental figure 5d) in non-preservation tubes remained fairly constant over time: mean fold changes ranged from 1.08 to 1.14 and from 1.13 to 1.47, for mRNA and miRNA, respectively. For the preservation tubes, the mean fold changes were higher: from 1.69 to 2.28 and from 1.38 to 4.52, for mRNA and miRNA, respectively (Supplemental figure 7e & Supplemental figure 8e).

Reproducibility remained stable over time for both preservation and non-preservation tubes: mean fold changes ranged from 1.06 to 1.18 (Supplemental figure 7d & Supplemental figure 8d).

As noted above, the relative RNA concentration and number of detected mRNAs or miRNAs were considerably lower when using the preservation tubes DNA Streck and RNA Streck compared to the others. We also observed that the fraction of reads mapping to the correct strand was lower for these tubes compared to other tubes (see strandedness in https://github.com/OncoRNALab/exRNAQC). Moreover, library construction for RNA isolated from these two tubes resulted in libraries with an insufficient library yield for equimolar pooling. Therefore, these tube types seem unfit for blood plasma-based exRNA analysis at the evaluated time points. In general, the stability of the performance metrics over time was substantially higher for the non-preservation tubes compared to the preservation tubes (Figure 3).

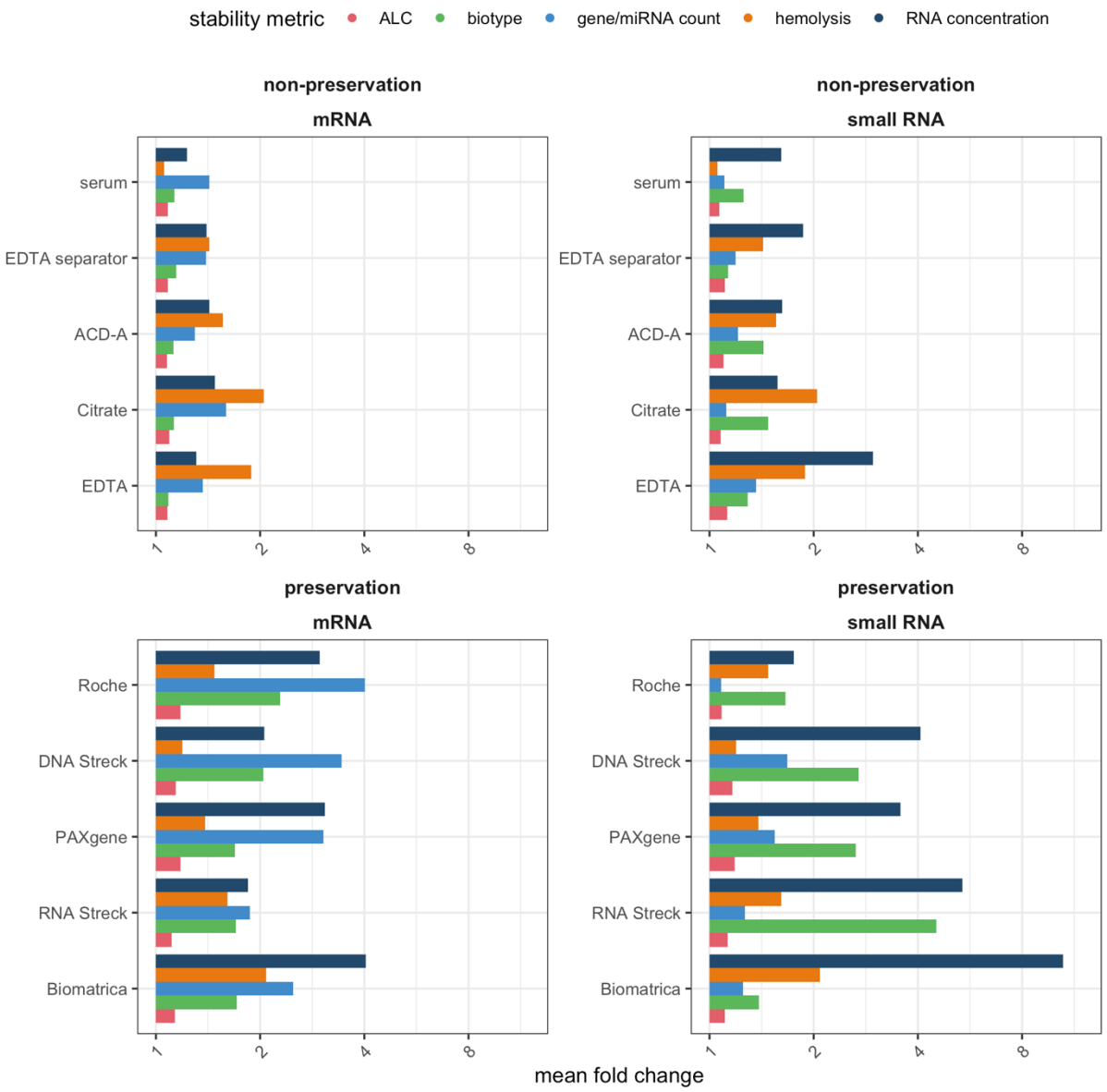

Figure 3. Summary of mean fold changes (FC) between time point 1 (centrifugation step 4 hours after blood collection for non-preservation tubes; 24 hours for preservation tubes) and time point 0 vs. time point 2 (centrifugation step 16 hours after blood collection for non-preservation tubes; 72 hours for preservation tubes) and time point 0, per tube and per metric, for mRNA capture sequencing (**left**) and small RNA sequencing (**right**). Ideally, the mean FC of the stability metrics approaches 1, indicating that there is little change from baseline and the blood collection tube performs well across time. **Legend:** "gene/miRNA count" represents stability of the absolute number of protein coding genes (mRNA) or absolute number of miRNAs (small RNA), "RNA concentration" corresponds to the stability of the relative RNA concentration as determined by number of endogenous reads vs Sequin spike-in

RNA (mRNA) or the stability of the relative RNA concentration as determined by number of endogenous reads vs RC spike-in RNA (small RNA), "hemolysis" corresponds to stability of the absorbance of light at 414 nm (mRNA and small RNA), "biotype" corresponds to the stability of the fraction of all counts mapping to mRNAs (i.e. the protein coding fraction) or the stability of the fraction of all counts mapping to micro RNAs (small RNA), "ALC" corresponds to the area left of the curve, a reproducibility metric (mRNA and small RNA). Non-preservation tubes are the BD Vacutainer Plastic K2EDTA tube (EDTA), Vacuette Tube 8 ml K2E K2EDTA Separator (EDTA separator), BD Vacutainer Glass ACD Solution A tube (ACD-A), Vacuette Tube 9 ml 9NC Coagulation sodium citrate 3.2% (citrate), and BD Vacutainer SST II Advance Tube (serum). The preservation tubes are the Cell-Free RNA BCT (RNA Streck), Cell-Free DNA BCT (DNA Streck), PAXgene Blood ccfDNA Tube (PAXgene), Cell-Free DNA Collection Tube (Roche) and LBgard Blood Tube (Biomatrica). Note that different donors were sampled and that tubes were processed at different time points for preservation and non-preservation tubes.

Discussion

In the extracellular RNA Quality Control (exRNAQC) study, we examined eight RNA purification methods and ten blood collection tubes as pre-analytical variables affecting exRNA quantification, using both mRNA as well as small RNA sequencing. Eight kits marketed for RNA purification from serum or plasma, and 10 blood collection tubes commonly used in the clinic available at study initiation were selected for investigation. More than 1.4 liter of blood was collected from 11 different healthy donors in order to conduct all experiments in triplicate, resulting in 276 extracellular transcriptomes. To control the RNA purification and library preparation workflows, 189 synthetic spike-in RNA molecules (Sequin and ERCC spike-ins for mRNA sequencing, and RC and LP spike-ins for small RNA sequencing) were used. We previously demonstrated the importance of using these spike-in RNAs for deep sequencing-based quantification of exRNA[17,18], and further confirmed their critical importance in the current exRNAQC study. Here, spike-in RNAs were used to assess the relative RNA yield and concentration, and to determine the extraction efficiency of the different RNA purification methods.

379 Importantly, we do not only provide full access to the data and analysis pipelines (European Genome-
380 phenome Archive (EGA): EGAS00001005263 and ArrayExpress,
381 https://github.com/OncoRNALab/exRNAQC), but also supply the research community with consistent
382 and standardized pre-analytics information to better interpret, compare and reproduce our results. To
383 this purpose, the transcriptomes are annotated with multiple pre-analytical variables, including the
384 Biospecimen Reporting for Improved Study Quality (BRISQ) elements[19,20] (Supplemental table 4).
385 Overall, these aspects make the exRNAQC study not only the largest, but also the most comprehensive
386 sequencing-based evaluation of pre-analytical factors affecting exRNA analysis so far. Although all
387 eight tested RNA purification kits are marketed for extraction of exRNA from serum or plasma,
388 unexpectedly large performance differences were observed for both small RNA and, to a greater
389 extend, mRNA. With most exRNA kits specifically developed for microRNA quantification, it is not very
390 surprising that the kit performance at miRNA level is more homogenous than at mRNA level. We clearly
391 noted that the mRNA purification performance was linked to the biofluid input and eluate volume.
392 More specifically, a higher biofluid input volume resulted in higher relative mRNA concentrations. This
393 association did not hold true for microRNA, as exemplified by CCF1 and CCF4. Also, RNA purification
394 kits with a large eluate volume typically showed a high yield but low relative RNA concentration. For
395 these kits, condensing the eluate volume prior to library preparation could potentially increase their
396 overall performance. Kits with a high extraction efficiency did not always result in better RNA
397 quantification results because of limited input volumes. If these kits would accommodate a larger
398 plasma input volume (while maintaining their extraction efficiency), their overall performance could
399 also improve. Note, however, that the efficiency of some kits decreased when using the maximum
400 input volume compared to the minimum (e.g. CCF). Finally, we want to emphasize the importance of
401 removing co-purified genomic DNA (gDNA) from the extracted RNA samples before proceeding to
402 exRNA quantification[21]. We observed high-level gDNA contamination in RNA-eluates produced with
403 the MAP kit despite applying a commonly used gDNA removal strategy that worked well for the other
404 RNA kits. This gDNA contamination is most likely due to an incompatibility between the RNA elution

buffer and the gDNA removal reagents. Alternative gDNA removal strategies should be used before applying the MAP RNA extraction kit for exRNA analysis.

To evaluate the impact of the blood collection tube on downstream exRNA sequencing, biofluids (serum and plasma) were prepared at three different time points upon blood collection to assess potential changes in exRNA content due to blood storage at room temperature. To set a reference, each tube type was also processed immediately after blood collection. For non-preservation tubes, we set the processing time points at 4 and 16 hours to mimic same-day processing and next-day processing, real-life situations often happening in clinics. For preservation tubes that are specifically marketed to stabilize extracellular nucleic acids for 7 up to 14 days, more extreme time points for plasma preparation were selected, i.e. 24 and 72 hours upon blood collection. Surprisingly, in terms of stability over time, preservation tubes performed far worse than non-preservation tubes (including serum), as reflected in increasing RNA concentrations and number of detected genes over time and by compromised reproducibility. While preservation tubes were stored at room temperature for longer duration compared to non-preservation tubes, storage time was still substantially shorter than advertised for these tubes. In addition, exRNA concentrations were much lower and hemolysis levels remarkably higher in some of these tubes compared to non-preservation tubes, even at baseline (i.e. immediate processing upon blood draw). Although hemolysis may induce changes in exRNA content, the observed instability of the performance metrics over time for these tubes cannot solely be explained by differences in hemolysis over time. In this context, it is worth mentioning that, between individuals and across time points, we observed substantial differences in the amount of plasma that could be prepared from the preservation tubes, an issue that was reported before[22]. This also points towards performance instability (over time). Based on these findings, we conclude that the studied preservation tubes are not suitable for exRNA analysis at the examined time intervals. We invite blood collection tube manufacturers to increase their efforts to develop a plasma or serum tube that preserves the transcriptome for at least 3 days.

We are currently extending the exRNAQC study with a second phase, results of which are not shown in this paper, in which we aim to assess possible interactions between pre-analytic variables. To this purpose, three non-preservation blood collection tubes (serum, EDTA and citrate) and two RNA purification kits were selected for further evaluation. The tube selection was based on the superior performance of these tubes as well as their widespread availability in the clinic. The kit selection was based on both sensitivity (number of detected protein coding genes or miRNAs) and reproducibility (pairwise comparison of gene counts in technical replicates). Plasma input volume was used as an additional criterium, as we included at least one kit which requires less than one milliliter plasma. Because of the differences in kit performance on mRNA and miRNA level, the kits that were selected for each biotype separately are MAX0.5 and MIRA0.6 for small RNA sequencing, and MIR0.2 and CCF2 for mRNA capture sequencing (Supplemental figure 9).

In the exRNAQC study phase 1, we demonstrate that the selection of RNA purification method and blood collection tube substantially impacts mRNA and miRNA quantification by evaluation of 11 performance metrics. Here, 8 commercially available RNA purification methods and 10 blood collection tubes were studied, but the proposed framework and metrics can also be used to evaluate the performance of more recently developed RNA purification methods and blood collection tubes. Note that the metrics solely assess technical performance, and that the impact of the pre-analytics on biomarker detection was not addressed in this study. In addition, for small RNA sequencing, we only focused on the analysis of microRNAs. While important, analysis of other types of small RNAs was beyond the scope of the exRNAQC study. Based on the findings presented here, we highly recommend a) standardizing sample collection and processing, b) carefully annotating and reporting pre-analytics, and c) making use of synthetic spike-in RNA molecules for deep sequencing-based analyses of exRNA. This is crucially important for interpretation and comparison of all exRNA study results, and will enhance the reproducibility of exRNA research, as a starting point for biofluid based biomarker studies.

Materials and methods

Donor material and liquid biopsy preparation

Sample collection was approved by the ethics committee of Ghent University Hospital (Belgian Registration number B670201733701) and written informed consent was obtained from 11 healthy donors. Venous blood was collected from an elbow vein after disinfection with 2% chlorhexidine in 70% alcohol. In total, 10 different blood collection tubes were used: the BD Vacutainer SST II Advance Tube (referred to as serum in this study; Becton Dickinson and Company, 366444), BD Vacutainer Plastic K2EDTA tube (EDTA; Becton Dickinson and Company, 367525), Vacuette Tube 8 ml K2E K2EDTA Separator (EDTA separator; Greiner Bio-One, 455040), BD Vacutainer Glass ACD Solution A tube (ACD-A; Becton Dickinson and Company, 366645), Vacuette Tube 9 ml 9NC Coagulation sodium citrate 3.2% (citrate; Greiner Bio-One, 455322), Cell-Free RNA BCT (RNA Streck; Streck, 230248), Cell-Free DNA BCT (DNA Streck; Streck, 218996), PAXgene Blood ccfDNA Tube (PAXgene; Qiagen, 768115), Cell-Free DNA Collection Tube (Roche; Roche, 07785666001), and LBgard Blood Tube (Biomatrica; Biomatrica, M68021-001). Immediately after blood draw, blood collection tubes were inverted five times and all tubes were transported to the laboratory for plasma or serum preparation. Tubes were immediately processed or at 4h, 16h, 24h or 72h upon blood collection. Details on the different blood draws and plasma/serum preparations are available in the Supplemental Materials and Methods.

RNA isolation and gDNA removal

In total, 8 different exRNA extraction methods, including 6 spin column-based kits and 2 automated extraction procedures, were used according to the manufacturer's manual: the miRNeasy Serum/Plasma Kit (abbreviated to MIR in this study; Qiagen, 217184), miRNeasy Serum/Plasma Advanced Kit (MIRA; Qiagen, 217204), mirVana PARIS Kit (MIRV; Life Technologies, AM1556), NucleoSpin miRNA Plasma Kit (NUC; Macherey-Nagel, 740981.50), QIAamp ccfDNA/RNA Kit (CCF; Qiagen, 55184), Plasma/Serum Circulating and Exosomal RNA Purification Kit/Slurry Format (CIRC; Norgen Biotek Corp., 42800), Maxwell RSC miRNA Plasma and Serum Kit (Promega, AX5740 and

AS1680) in combination with the Maxwell RSC Instrument (MAX; Promega, AS4500), and MagNA Pure 24 Total NA Isolation Kit (Roche, 07658036001) in combination with the MagNA Pure 24 instrument (MAP; Roche, 07290519001). Per 100 µl liquid biopsy input volume, 1 µl sequin spike-in controls (Garvan Institute of Medical Research[23]) and/or 1 µl RNA extraction Control (RC) spike-ins[24] (IDT) were added to the lysate for TruSeq RNA Exome Library Prep sequencing and/or TruSeq Small RNA Library Prep sequencing, respectively (see Supplemental Materials and Methods). To maximally concentrate the RNA eluate, minimum eluate volumes were used, unless otherwise recommended by the manufacturer. For evaluation of the different extraction methods, both the minimum and maximum recommended plasma input volumes were tested in triplicate. Details on the exRNA extraction methods, and sequin and RC spike-in controls are available in the Supplemental Materials and Methods.

gDNA removal of RNA samples for TruSeq RNA Exome Library Prep sequencing was performed using HL-dsDNase (ArcticZymes, 70800-202) and Heat & Run 10X Reaction Buffer (ArcticZymes, 66001). Briefly, 2 µl External RNA Control Consortium (ERCC) spike-in controls (ThermoFisher Scientific, 4456740), 1 µl HL-dsDNase and 1.4 µl reaction buffer were added to 12 µl RNA eluate, and incubated for 10 min at 37 °C, followed by 5 min at 55 °C. To RNA samples used for both TruSeq RNA Exome Library Prep sequencing and TruSeq Small RNA Library Prep sequencing, also 2 µl Library Prep Control (LP) spike-ins[25] (IDT) were added to the RNA eluate before starting gDNA removal and 1.6 µl reaction buffer was used. RNA samples solely used for TruSeq Small RNA Library Prep sequencing were not DNase treated. Here, 2 µl LP spike-ins were added to 12 µl RNA eluate before starting library preparation. ERCC and LP spike-in control details are available in the Supplemental Materials and Methods.

TruSeq RNA Exome sequencing

mRNA libraries were prepared starting from 8.5 µl RNA eluate using the TruSeq RNA Exome Kit (Illumina, 20020189, 20020490, 20020492, 20020493, 20020183), according to the manufacturer's

protocol with following adaptations: fragmentation of RNA for 2 min at 94 °C, second strand cDNA synthesis for 30 minutes at 16 °C (with the thermal cycler lid pre-heated at 40 °C), and second PCR amplification using 14 PCR cycles. Upon the first and second PCR amplification, libraries were validated on a Fragment Analyzer (Advanced Analytical Technologies), using 1 µl of library. Library concentrations were determined using Fragment Analyzer software for smear analysis in the 160 to 700 base pair (bp) range. Library quantification was qPCR-based, using the KAPA Library Quantification Kit (Kapa Biosystems), and/or based on NanoDrop 1000 measurements. Details on library preparation protocol, library quantification, pooling and sequencing are available in the Supplemental Materials and Methods and in Hulstaert *et al.*[18]

TruSeq Small RNA Library Prep sequencing

Small RNA libraries were prepared starting from 5 µL RNA eluate using the TruSeq Small RNA Library Prep Kit (Illumina, RS-200-0012, RS-200-0024, RS-200-0036, RS-200-0048), according to the manufacturer's protocol with following adaptations: the RNA 3' adapter (RA3) and the RNA 5' adapter (RA5) were 4-fold diluted with RNase-free water, and the number of PCR cycles was increased to 16[17,26]. Samples were divided across library prep batches according to index availability. For each batch, 3 µl of small RNA library from each sample was pooled prior to automated size selection using the Pippin prep (Sage Sciences, CDH3050). Size selected libraries were quantified using qPCR, and sequenced on a MO flow cell (Illumina, NextSeq 500) using loading concentrations ranging from 1.2 to 2.4 pM. Differences in read distribution across samples were subsequently used to re-pool individual libraries in order to obtain an equimolar pool. After size selection on a Pippin prep and qPCR quantification, these pools were sequenced on a HO flow cell (Illumina, NextSeq 500, NextSeq 500/550 High Output Kit v2.5, 20024907) using loading concentrations ranging from 1.2 to 3 pM.

Data analysis

The study resulted in four sequencing data sets and the raw, processed and metadata were submitted to the European Genome-phenome Archive (EGAS00001005263) and ArrayExpress. RNA Exome and Small RNA sequencing of the RNA purification kit study were identified with study codes exRNAQC004 and exRNAQC011, respectively. RNA Exome and Small RNA sequencing of the blood collection tube study were identified with study codes exRNAQC005 and exRNAQC013, respectively. A high-level summary of the sequencing statistics can be found in Supplemental table 3-Supplemental table 7. Detailed pre-analytics information (for the BRISQ elements[19,20]) can be found in Supplemental table 4.

Quality control and quantification of TruSeq RNA Exome Library Prep sequencing data

In case of adapter contamination indicated by FASTQC[27] (v0.11.8), adapters were trimmed with Cutadapt[28] (v1.18; 3' adapter R1: 'AGATCGGAAGAGCACACGTCTGAACTCCAGTCA'; 3' adapter R2 'AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT'). Read pairs with a low a base calling accuracy (< 99% in at least 80% of the bases in both mates) were discarded. Subsequently, FASTQ files were subsampled with Seqtk[29] (v1.3) to the lowest number of reads pairs obtained in the experiment (floored to a million). Since the low amount of input RNA resulted in a high number of duplicates (Supplemental table 3 and Supplemental table 6), we removed these duplicates using Clumpify[30] dedupe (v38.26) with the following specifications: paired-end mode, 2 substitutions allowed, kmersize of 31, and 20 passes). For duplicate removal, only the first 60 bases of both reads were considered to account for the sequencing quality drop at the end of the reads. Strand-specific transcript-level quantification of the deduplicated FASTQ files was performed with Kallisto[31] (v0.44.0). For coverage and strandedness analysis, mapped reads were obtained by STAR[32] (v2.6.0c) using the default parameters (except for --twopassMode Basic, --outFilterMatchNmin 20 and --outSAMprimaryFlag AllBestScore). For all exons coverage information was retrieved by the genomeCoverageBed and intersectBed functions of BEDTools[33] (v2.27.1). Strandedness information was obtained with RSeQC[34] (v2.6.4). The reference files for all analyses were based on genome build hg38[35] and transcriptome build Ensembl v91[36,37]. Spike annotations were added to both genome and transcriptome files.

Quality control and quantification of TruSeq Small RNA Library Prep sequencing data

First, adaptor trimming (3' adapter: TGGAATTCTCGGGTGCCAAGG) was performed using Cutadapt[28] (v1.16) with a maximum error rate of 0.15 and discarding reads shorter than 15 bp and those in which no adaptor was found. Subsequently, low quality reads were filtered out (Q20 in less than 80% of the bases) by FASTX-Toolkit[38] (v0.0.14). Filtered FASTQ files were subsampled to the minimum number of reads in the experiment (Supplemental table 5 and Supplemental table 7) using Seqtk[29] (v1.3). Reads were collapsed with FASTX-Toolkit and LP and RC spike reads (including possible fragments) were annotated. The non-spike reads were mapped with Bowtie[39] (v1.2.2, with additional parameters -k 10 -n 1 -l 25) considering only perfect matches. Mapped reads were annotated by matching the genomic coordinates of each read with genomic locations of miRNAs (obtained from miRBase[40–45], v22) and other small RNAs (tRNAs obtained from UCSC GRCh38/hg38; snoRNA, snRNA, MT_tRNA, MT_rRNA, rRNA, and miscRNA from Ensembl, v91).

Defining performance metrics

The statistical programming language R[46] (v4.0.3) was used throughout this section and all scripts can be found at GitHub (https://github.com/OncoRNALab/exRNAQC). Depending on the study (kit or tube selection), different metrics were used which are briefly explained below. For each part of the study, more in-depth descriptions of the metrics and results are also available through GitHub.

- *Count threshold (kit & tube study)*

In order to distinguish signal from noise we made use of pairwise count comparisons across three technical replicates for the kit study. We defined a count threshold for each RNA purification method and biotype in a similar manner as defined in the miRQC study[14]. Specifically, a threshold that reduces the fraction of single positives in technical replicates by at least 95 % (single positives are cases where a given gene has a zero value in one replicate and a non-zero value in the other one). This threshold can be used as a reproducibility metric between technical replicates. For each kit-volume combination, the median threshold of the three pairwise replicate comparisons was used (Supplemental table 2). As the tube study did not have technical replicates and RNA purification always happened with the

583 miRNeasy Serum/Plasma Kit, the median thresholds of MIR0.2 (3 counts for small RNAs; 6 counts for
584 mRNAs) were applied here as well.

585 • *Data retention (kit study)*

586 Data retention is defined as the percentage of gene counts remaining after applying the count
587 threshold as filter, therefore giving information about the fraction of counts lost by applying the cut-
588 off.

589 • *Sensitivity or gene count (kit & tube study)*

590 We defined sensitivity as the number of different protein coding genes or miRNAs picked up above the
591 count threshold.

592 • *Relative RNA concentration (kit & tube study)*

593 The same plasma was used throughout the entire purification kit experiment. By adding equal amounts
594 of ERCC and LP spikes (for mRNA and small RNA, respectively) after RNA extraction, we were able to
595 calculate relative endogenous RNA concentrations in the eluate. For instance, in cases of low
596 endogenous RNA content after RNA purification, relatively more ERCC and LP spikes will be sequenced.
597 By dividing the total sum of endogenous counts by the sum of ERCC or LP spikes, we could therefore
598 compare the relative RNA concentrations in the eluate of the different extraction methods.
599 For the tube experiment, we were interested impact of the different tubes on the RNA concentration
600 in plasma. By adding equal amounts of Sequin and RC spikes (for mRNA and small RNA, respectively)
601 before RNA extraction, we were able to calculate relative endogenous RNA concentrations in the
602 plasma. For instance, in cases of low endogenous RNA content before extraction, relatively more
603 Sequin and RC spikes will be sequenced. By dividing the total sum of endogenous counts by the sum
604 of Sequin or RC spikes, we could therefore compare the relative RNA concentrations in plasma of the
605 different tubes.

606 • *Relative RNA yield extraction (kit study)*

607 Multiplying the relative RNA concentration by the eluate volume gives the relative RNA yield in the
608 total eluate.

- *Relative extraction efficiency (kit study)*

Correcting the relative RNA yield for the plasma input volume (dividing yield by input volume) gives an idea of the theoretical RNA extraction efficiency of the method.

- *Reproducibility based on area left of the curve (kit & tube study)*

As described in the miRQC study[14], the area left of the cumulative distribution curve (ALC) was calculated by comparing the actual cumulative distribution curve of log2 fold changes in gene or miRNA abundance between pairs of replicates to the theoretical cumulative distribution (optimal curve). Less reproducibility between samples results in more deviations from this optimal curve and therefore larger ALC-values.

- *Duplication rate (kit study)*

Duplication rate was obtained by dividing the number of reads after Clumpify duplicate removal (see methods) by the number of reads after subsampling, therefore giving information about the unique reads generated after sequencing.

- *Coverage (kit study)*

Coverage is the percentage of bases from the total transcriptome covered by at least one sequencing read.

- *Hemolysis (tube study)*

Hemolysis was measured with Nanodrop (absorbance of light at 414 nm) in plasma across all tubes.

- *Fraction mRNAs or miRNAs (tube study)*

Fraction of total counts that go to mRNA (RNA Exome data) or miRNAs (small RNA sequencing data).

Transform performance metrics into robust z-scores (kit study)

Individual scores for performance metrics were transformed to z-scores in the kit study. As the standard z-score is sensitive to outliers, we used a robust z-score transformation, based on the median ($\mu_{1/2}$) and median absolute deviation ($MAD = median_i(|X_i - median\ X_{1\ldots n}|)$), instead. The general formula for robust z-score calculation is shown below:

$$robust\ zscore = \frac{x - \mu_{1/2}}{s}$$

Where s is a scaling factor that depends on the MAD. In case MAD is not zero: $s = MAD * 1.4826$. If MAD equals zero, s approximately equals the standard deviation: $s = meanAD * 1.2533$, with $meanAD = mean_i(|X_i - mean\ X_{1\ldots n}|)$.[47,48]

Accounting for size selection bias (kit study)

For the small RNA library prep, the three technical replicates of each extraction method were divided over three different pools. Next, pippin prep size selection for miRNAs occurred on each pool individually. To account for size selection bias (which resulted in consistently lower sequencing counts in the second pool), we each time down-sampled the miRNA counts of the other two replicates to the sum of miRNA counts of the replicate in the second pool. Down-sampling was based on reservoir sampling - random sampling without replacement (subsample_miRs.py script on https://github.com/OncoRNALab/exRNAQC).

Fold change analyses for stability over time assessment (tube study)

In order to evaluate tube stability across time points, we determined several performance metrics per blood collection tube at different time points. We then calculated, for every tube and donor, the fold chance across different time points (each time relative to the base point at T0, so excluding T24-72 and T04-16). Given that there are 3 donors and 3 time points per tube, this resulted in six fold change values per tube. An example is shown in Supplemental figure 3.

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759

760 **Figure legends**

761

762 Within main text.

763

764 **Supplemental table legends**

765

766 **Supplemental table 1. Available literature on the influence of pre-analytics on RNA sequencing data,**
767 **including studies on plasma and/or serum.** The pre-analytics from these studies are categorized into

768 different groups: number of blood tubes; hemolysis measured (yes/no); the fluid (serum/plasma or
769 both); number of centrifugation protocols; number of RNA isolation kits; the RNA type; the gene
770 expression analysis method; other pre-analytics.

771 **Supplemental table 2. Filter threshold of different RNA purification methods.** Kit: RNA purification
772 kit abbreviation; mRNA threshold: median threshold that removes 95% of single positive genes
773 between technical replicates; miRNA threshold: median threshold that removes 95% of single positive
774 miRNAs between technical replicates. More explanation on these thresholds in methods section
775 "Count threshold". NA: Not applicable.

776 **Supplemental table 3. RNA Exome sequencing data statistics of RNA purification kit experiment**
777 **(exRNAQC004).** UniqueID: RNA identifier; SampleID: combination of kit abbreviation and technical
778 replicate number; raw_reads: number of sequenced reads pairs; qcfiltered_reads: number of read
779 pairs after quality filtering; post_subsampling: number of read pairs after subsampling;
780 post_deduplication: number of read pairs after Clumpify duplicate removal; duplicate_prct: % of
781 duplicates in subsampled reads; kallisto_prct_alignment: % of duplicate removed reads that were
782 pseudoaligned; strandedness_prct: % of reads on correct strand (stranded protocol).

783 **Supplemental table 4. Pre-analytical variable annotation for all samples included in the exRNAQC**
784 **study.** In the first tab, the different pre-analytical variables are listed, and for each of them a
785 description is provided. Note that the pre-analytics are categorized into three groups, i.e. variables
786 linked to the blood draw (with prefix B_), biofluid preparation (with prefix L_) or RNA purification (with
787 prefix R_). This tab also includes a description of the BRISQ elements[19,20]. In the following tabs,
788 annotated samples are listed per experiment (the RNA Exome sequencing of the RNA purification kit
789 study (exRNAQC004), the RNA Exome sequencing of the blood collection tube study (exRNAQC005),
790 the Small RNA sequencing of the RNA purification kit study (exRNAQC011), or the Small RNA
791 sequencing of the blood collection tube study (exRNAQC013)).

792 **Supplemental table 5. Small RNA sequencing data statistics of RNA purification kit experiment**
793 **(exRNAQC011).** UniqueID: RNA identifier; SampleID: combination of kit abbreviation and technical

794 replicate number; raw_reads: number of sequenced (single-end) reads; qcfiltered_reads: number of
795 reads after quality filtering; post_subsampling: number of reads after subsampling; aligned_reads:
796 number of subsampled reads aligned to reference genome; spike_reads: number of reads aligned to
797 spikes; prct_aligned: % of subsampled reads aligned to reference genome; prct_aligned_plus_spikes:
798 % of subsampled reads aligned to reference genome or to spikes.

799 **Supplemental table 6. RNA Exome sequencing data statistics of blood collection tube experiment**
800 **(exRNAQC005).** UniqueID: RNA identifier; SampleID: combination of tube abbreviation, donor number
801 (biological replicate), and time point; raw_reads: number of sequenced reads pairs; qcfiltered_reads:
802 number of read pairs after quality filtering; post_subsampling: number of read pairs after subsampling;
803 post_deduplication: number of read pairs after Clumpify duplicate removal; duplicate_prct: % of
804 duplicates in subsampled reads; kallisto_prct_alignment: % of duplicate removed reads that were
805 pseudoaligned; strandedness_prct: % of reads on correct strand (stranded protocol).

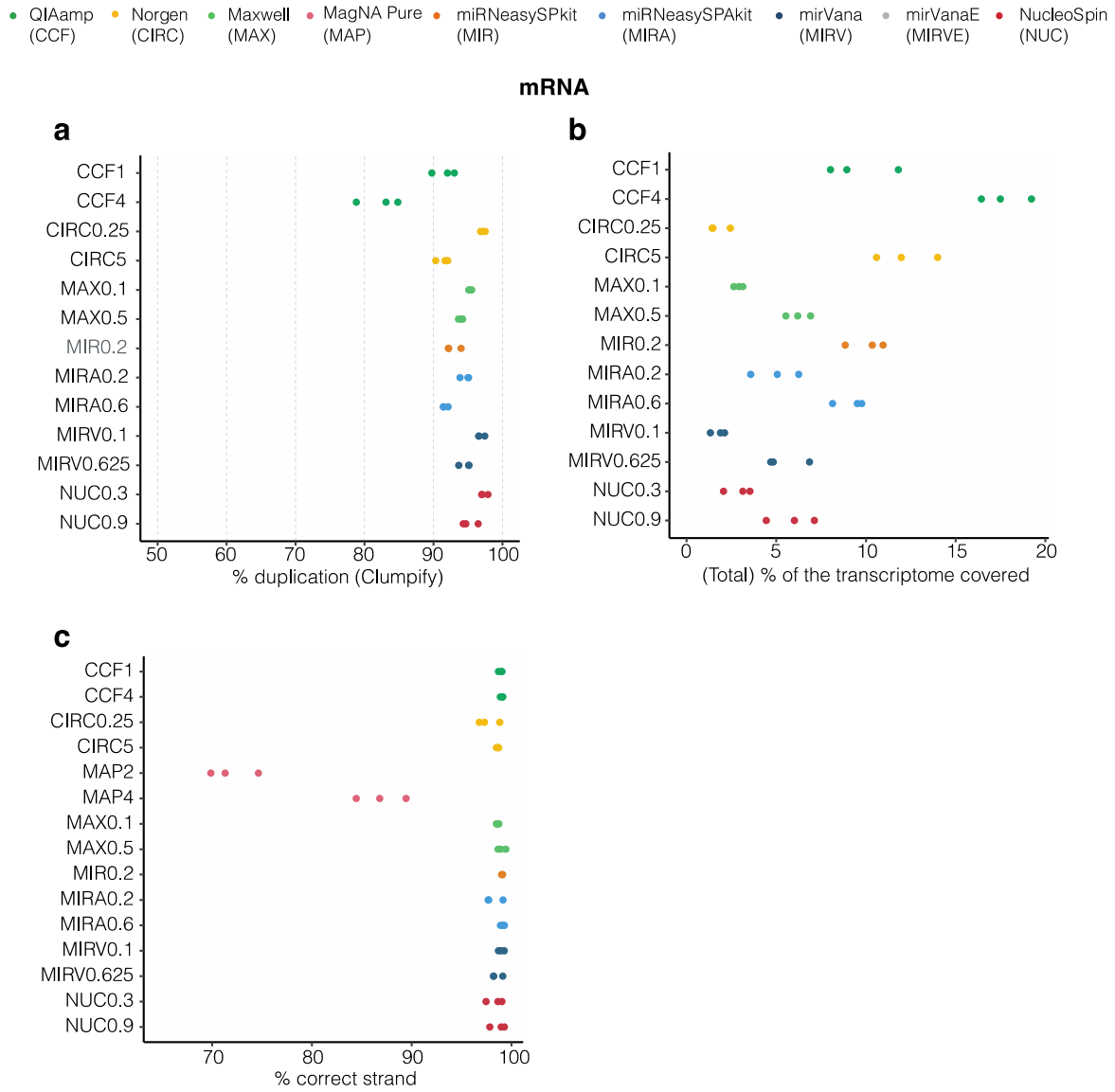
806 **Supplemental table 7. Small RNA sequencing data statistics of blood collection tube experiment**
807 **(exRNAQC013).** UniqueID: RNA identifier; SampleID: combination of tube abbreviation, donor number
808 (biological replicate), and time point; raw_reads: number of sequenced (single-end) reads;
809 qcfiltered_reads: number of reads after quality filtering; post_subsampling: number of reads after
810 subsampling; aligned_reads: number of subsampled reads aligned to reference genome; spike_reads:
811 number of reads aligned to spikes; prct_aligned: % of subsampled reads aligned to reference genome;
812 prct_aligned_plus_spikes: % of subsampled reads aligned to reference genome or to spikes.

813 **Supplemental table 8. Capture probes for Sequin and External RNA Control Consortium (ERCC) spike-**
814 **in controls.** Oligos to capture the Sequin and ERCC spike-in controls are listed. For each oligo, the
815 probe_ID, sequence, GC content (%), melting temperature (Tm in °C), ΔG and binding position in the
816 Sequin or ERCC spike-in sequence.

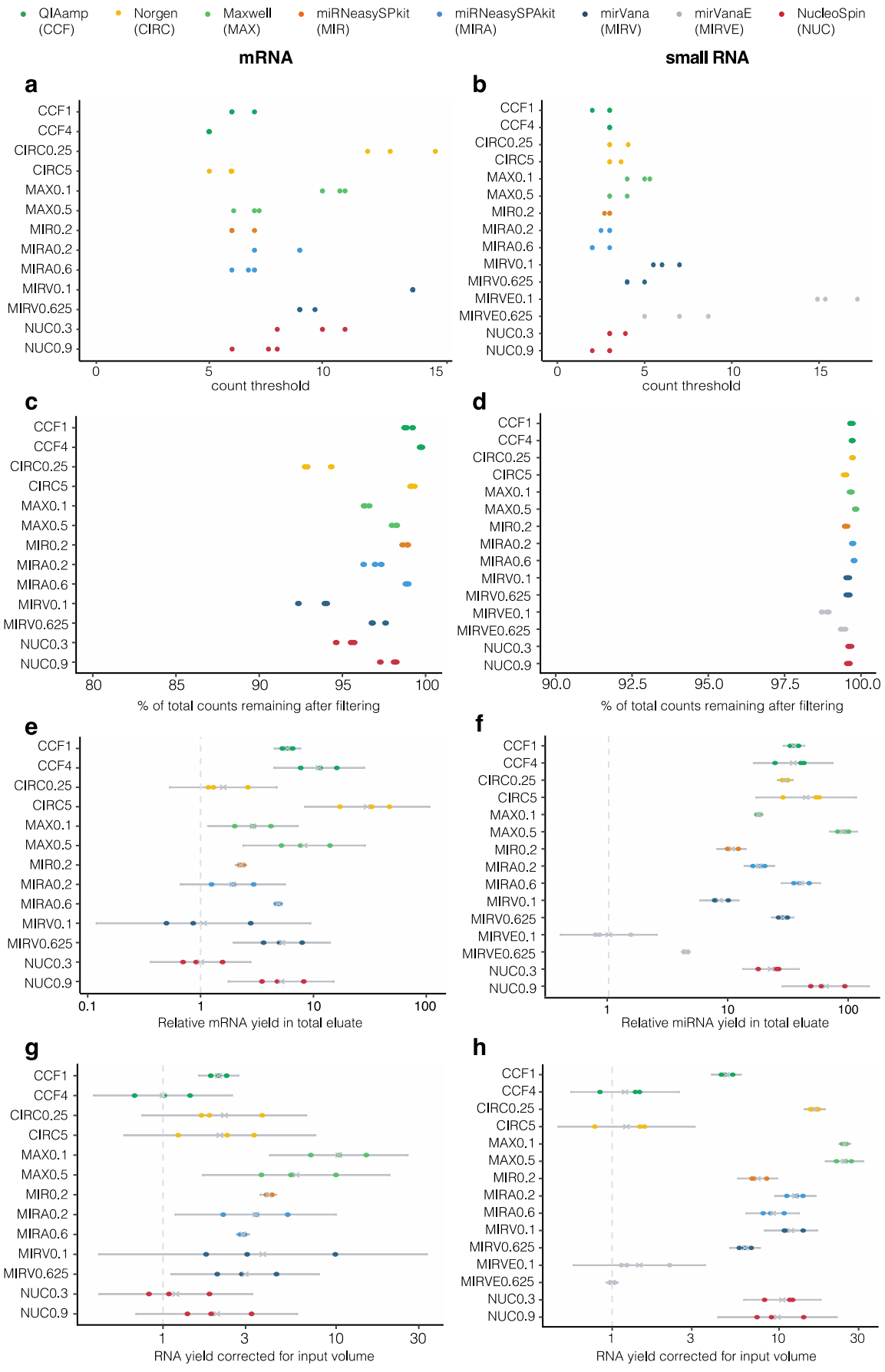
817

818 **Supplemental figures and legends**

819

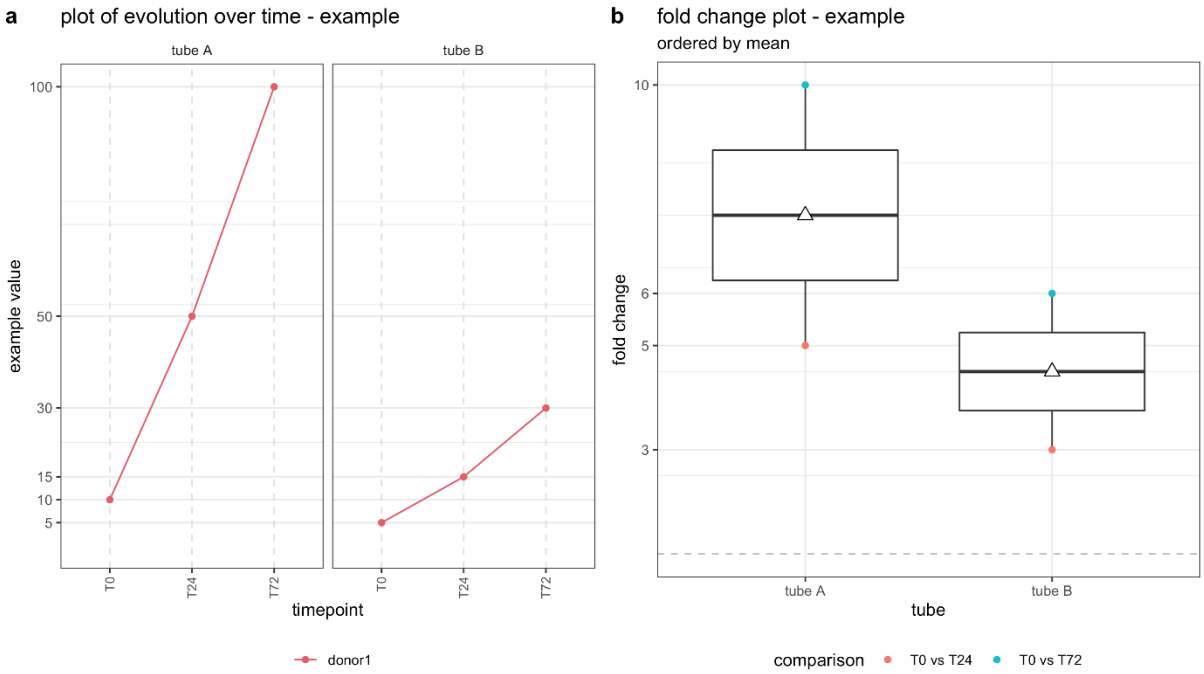


Supplemental figure 1: Performance of RNA purification kits on duplication rate, transcriptome coverage and strandedness at mRNA capture sequencing (mRNA) and small RNA sequencing (small RNA) level. a: percentage of read duplicates found by Clumpify after subsampling; b: percentage of bases in the total transcriptome that are covered at least once; c: percentage of reads on correct strand according to strand-specific protocol; Number that follows the abbreviation of the purification kit is the plasma input volume (in ml).



828

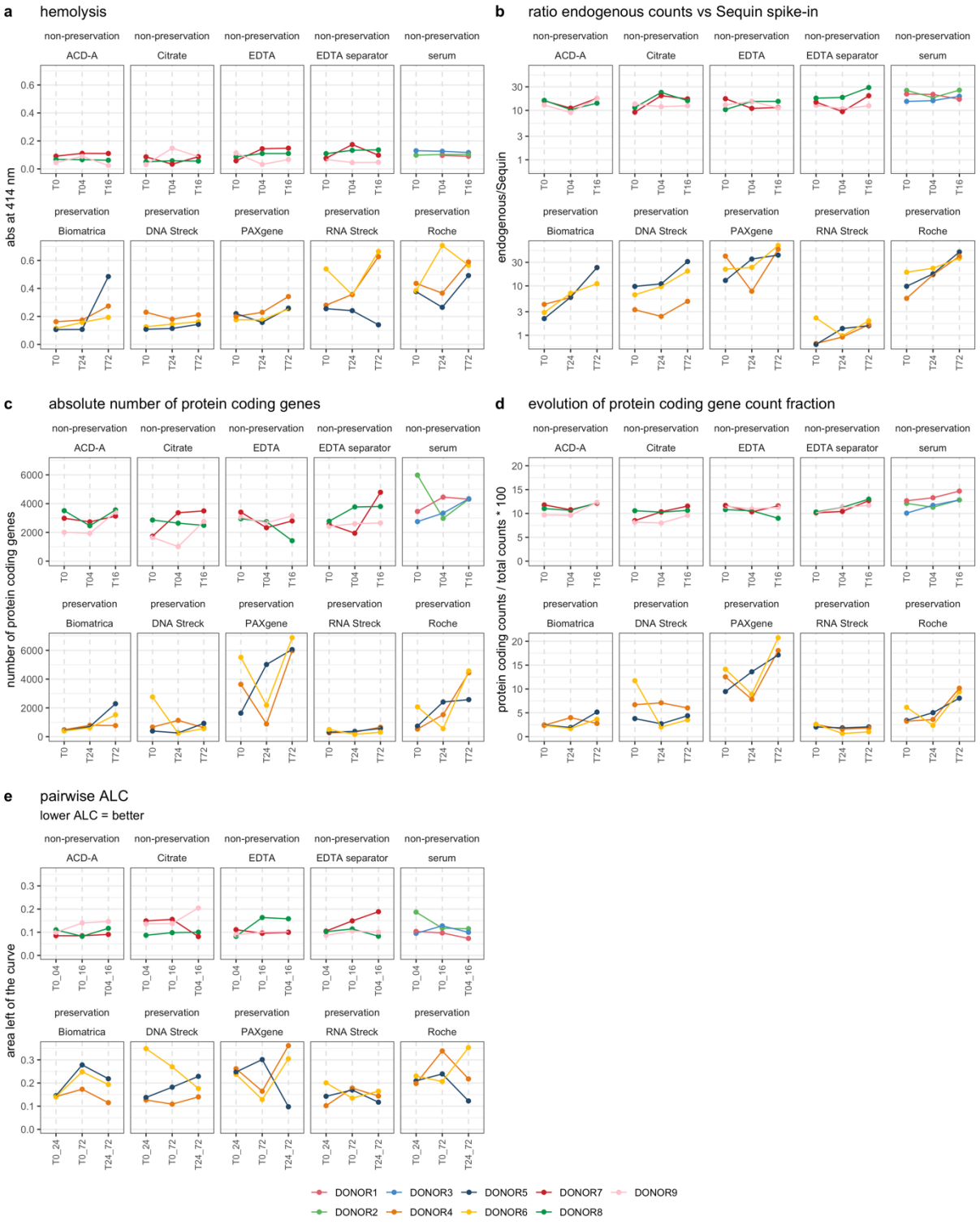
Supplemental figure 2: Performance of RNA purification kits on filter threshold, data retention, yield, and efficiency at mRNA capture sequencing (mRNA) and small RNA sequencing (small RNA) level. a&b: count threshold required to eliminate at least 95% of single positive genes or miRNAs, resp., between technical replicates; c&d: data retention – % of total counts that are kept after applying count threshold; e&f: relative mRNA and miRNA yield, resp., obtained by correcting the RNA concentration for eluate volume, values are log rescaled to the lowest mean of all kits and transformed back to linear space, 95% confidence interval is shown; g&h: relative mRNA and miRNA extraction efficiency, resp., obtained by correcting the RNA yield for input volume, values are log rescaled to the lowest mean of all kits and transformed back to linear space, 95% confidence interval is shown. Number that follows the abbreviation of the purification kit is the plasma input volume (in ml).



840

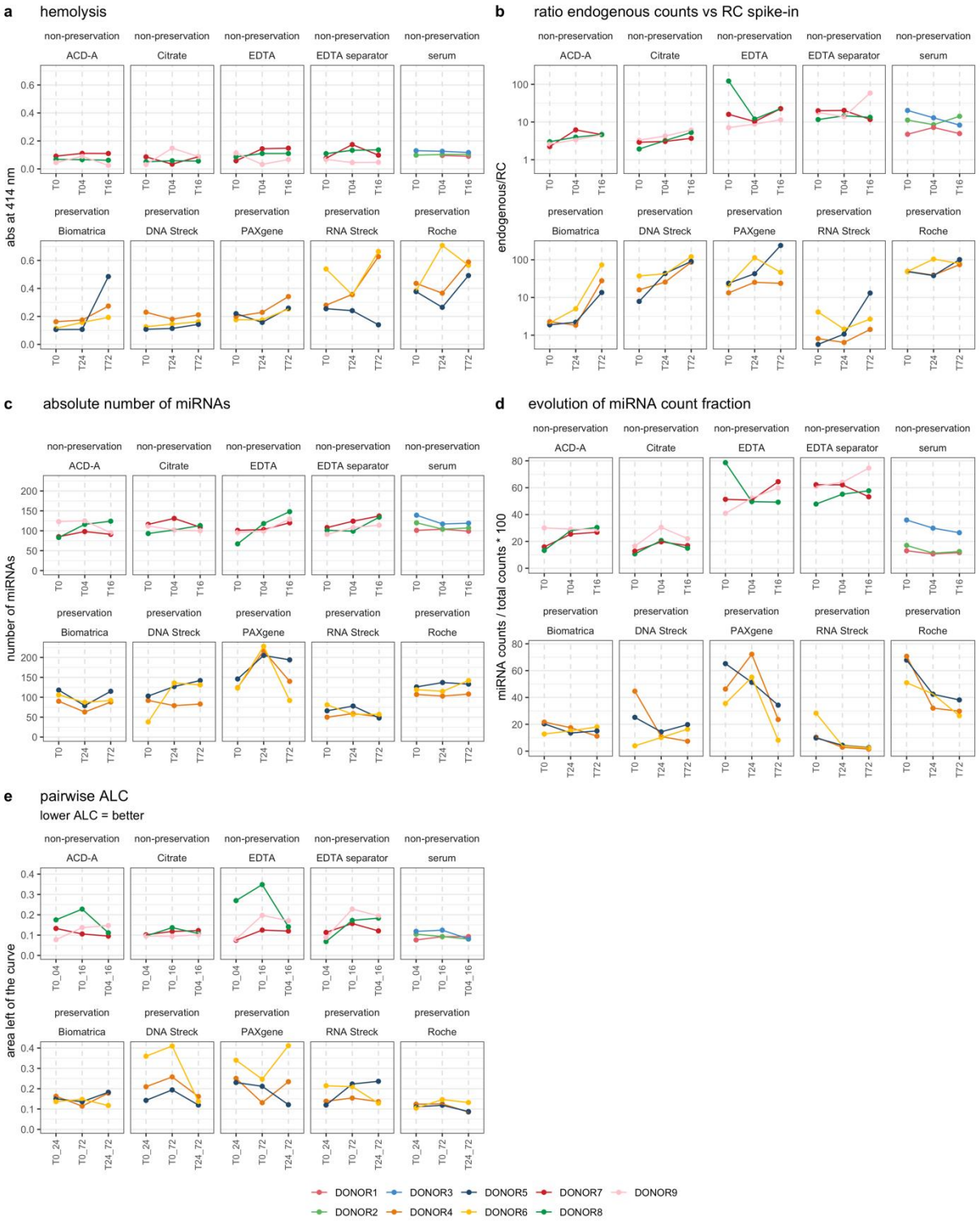
841 **Supplemental figure 3. Illustrative example of quality control metric evolution over time** for one
842 donor, two tubes and three time points (a) and corresponding boxplot of the fold changes per tube
843 (b). T0: plasma prepared immediately after blood draw, T24, T72: plasma prepared 24 hours and 72
844 hours after blood draw, respectively. The white triangle on the boxplot corresponds to the mean.
845 Reproduced from Van Paemel *et al.*[49].

846



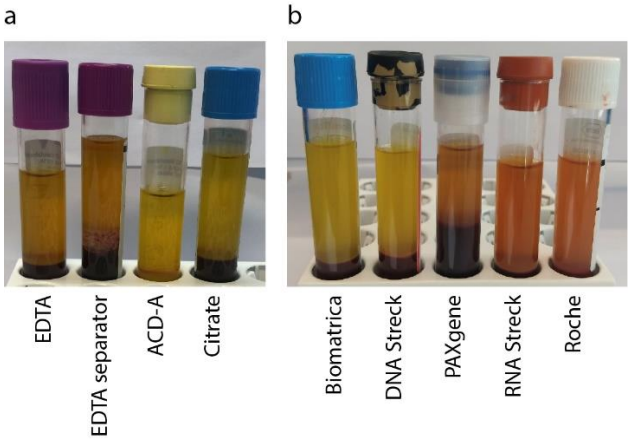
Supplemental figure 4. Performance metrics of blood tubes over time at mRNA level. a: evolution of hemolysis in plasma, measured by absorbance at 414 nm with Nanodrop; b: Evolution of relative RNA concentration calculated based on number of endogenous counts vs Sequin spike-in RNA; c: evolution the number of the absolute number of protein coding genes; d:evolution of the fraction of counts

852 mapping to mRNAs vs. all counts; e: evolution of the pairwise area left of the curve, a reproducibility
853 metric. T0: plasma prepared immediately after blood draw. T04, T16, T24, T72: plasma prepared 4
854 hours, 16 hours, 24 hours and 72 hours after blood draw, respectively. Note that different donors were
855 sampled and that tubes were processed at different time points for preservation and non-preservation
856 tubes.
857



Supplemental figure 5. Performance metrics of blood tubes over time at small RNA level. a: evolution of hemolysis in plasma, measured by absorbance at 414 nm with Nanodrop; b: evolution of relative RNA concentration calculated based on number of endogenous counts vs RC spike-in RNA; c: evolution the number of the absolute number of micro RNAs; d: evolution of the fraction of counts mapping to

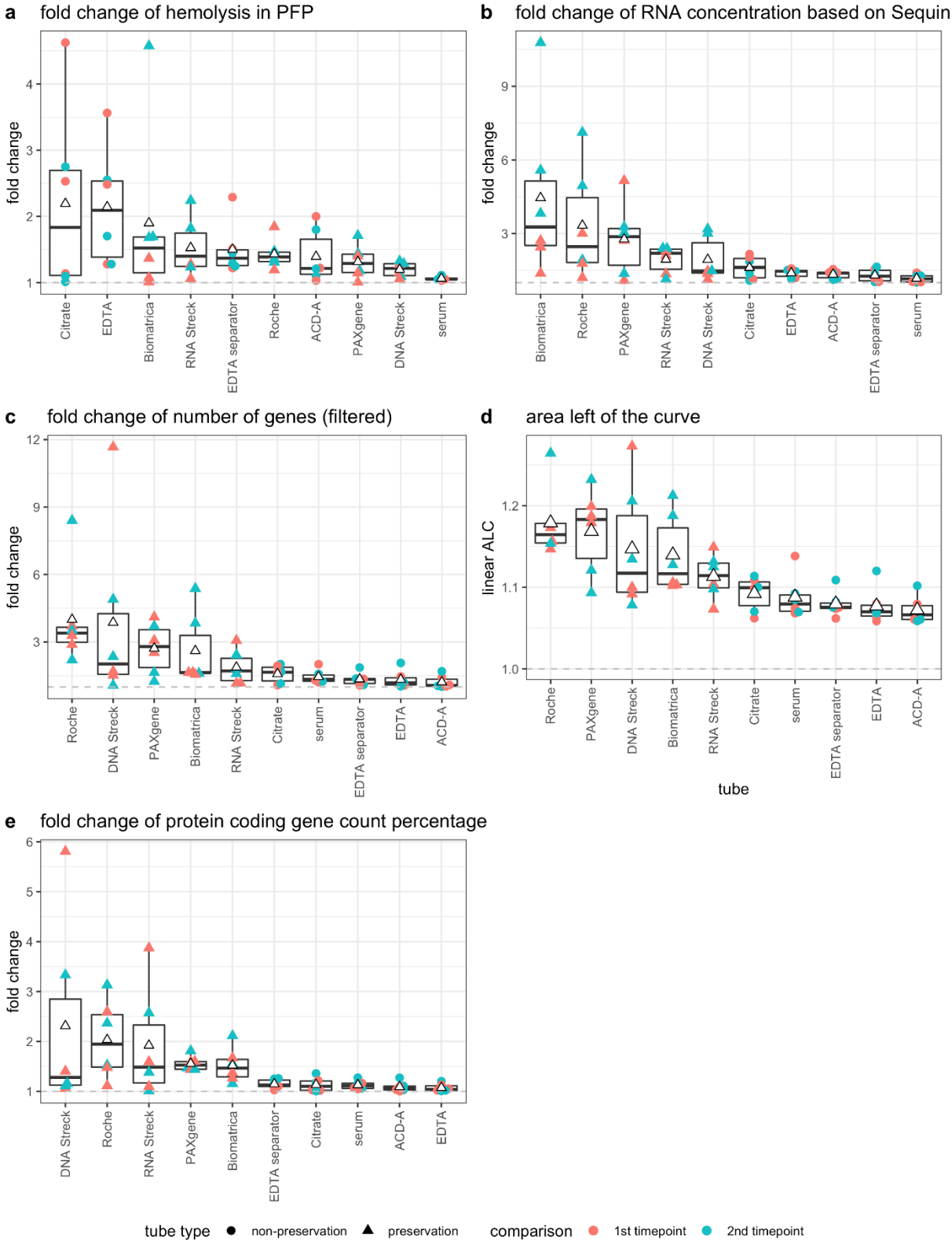
863 micro RNAs vs. all counts; e: evolution of the pairwise area left of the curve, a reproducibility metric.
864 T0: plasma prepared immediately after blood draw. T04, T16, T24, T72: plasma prepared 4 hours, 16
865 hours, 24 hours and 72 hours after blood draw, respectively. Note that different donors were sampled
866 and that tubes were processed at different time points for preservation and non-preservation tubes.
867



868

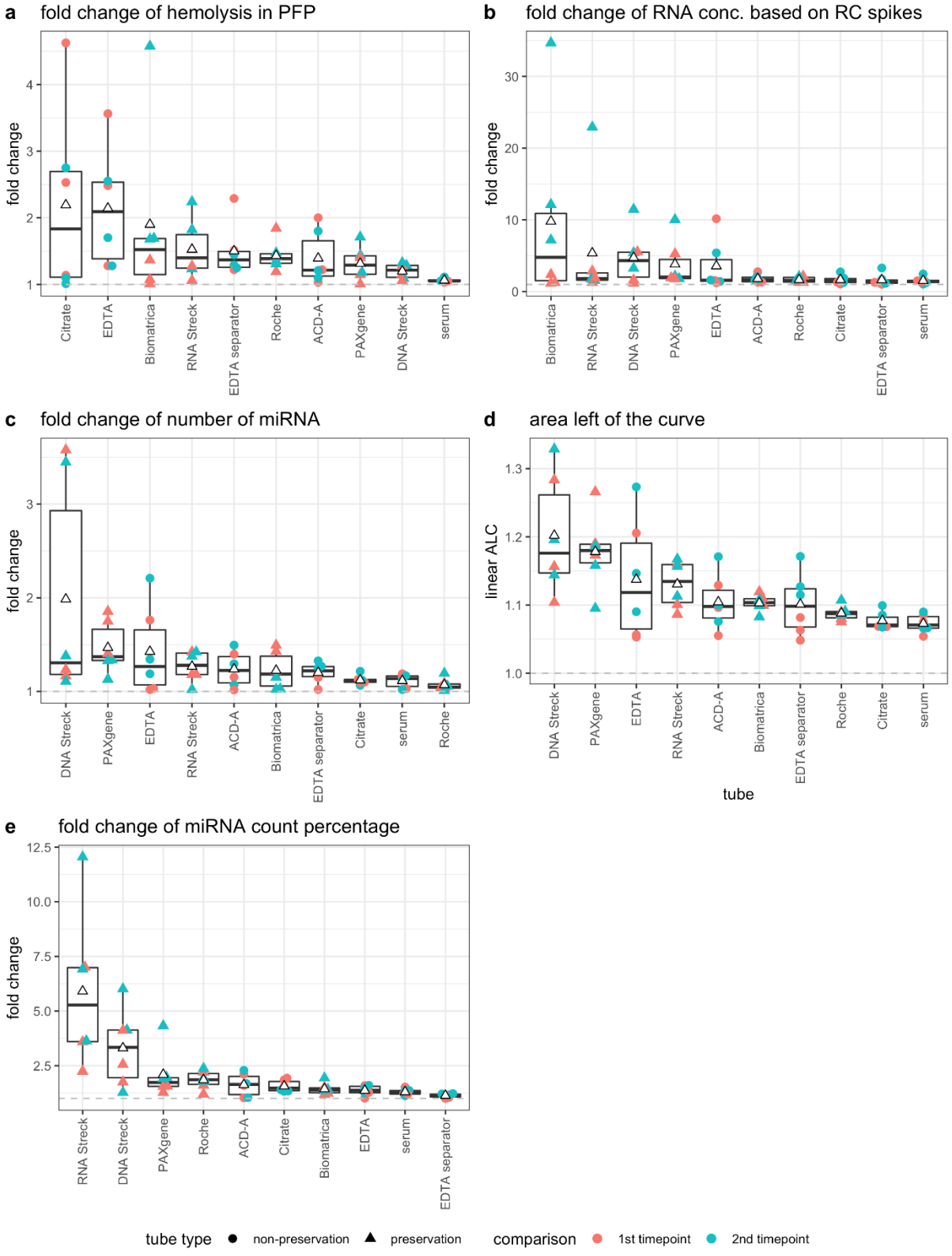
869 **Supplemental figure 6. Example of hemolysis in preservation tubes.** (a) Visual inspection of non-
870 preservation plasma tubes of DONOR7 (Supplemental figure 4a and 5a) and (b) of preservation plasma
871 tubes of DONOR5 (Supplemental figure 4a and 5a) at time point T0. For DONOR5, plasma from the
872 PAXgene, RNA Streck and Roche tube showed to be hemolytic, which is in line with the NanoDrop
873 measurements (Supplemental figure 4a and 5a).

874



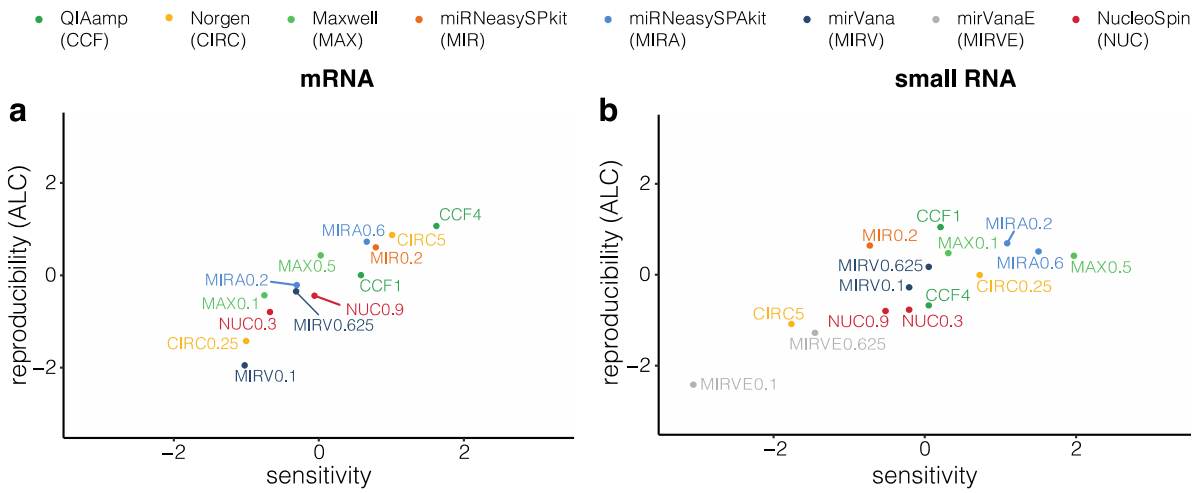
Supplemental figure 7. Fold changes over time at mRNA level for each blood collection tube metric. a: boxplot of the fold change within each donor across time points, per tube, for hemolysis in platelet-free plasma, as measures by absorbance at 414 nm with Nanodrop; b: boxplot of fold change of relative

879 RNA concentration, based on the ratio of endogenous reads vs Sequin spike-in RNA reads; c: boxplot
880 of the fold change of the number of genes, after filtering genes with counts fewer than 6 reads; d: area
881 left of the curve, transformed from log2 to linear scale; e: boxplot of the fold change of the fraction of
882 the counts mapping to protein coding genes vs. all counts. The white triangle on the boxplot
883 corresponds to the mean of the fold change. 1st time point corresponds to the comparison of T04 vs.
884 T0 (non-preservation tubes: BD Vacutainer Plastic K2EDTA tube (EDTA), Vacuette Tube 8 ml K2E
885 K2EDTA Separator (EDTA separator) , BD Vacutainer Glass ACD Solution A tube (ACD-A), Vacuette Tube
886 9 ml 9NC Coagulation sodium citrate 3.2% (citrate), and BD Vacutainer SST II Advance Tube (serum))
887 or T24 vs. T0 (preservation tubes: Cell-Free RNA BCT (RNA Streck), Cell-Free DNA BCT (DNA Streck),
888 PAXgene Blood ccfDNA Tube (PAXgene), Cell-Free DNA Collection Tube (Roche) and LBgard Blood Tube
889 (Biomatrica)). 2nd time point corresponds to the comparison of T16 vs. T0 (non-preservation tubes) or
890 T72 vs T0 (preservation tubes). T0: plasma prepared immediately after blood draw. T04, T16, T24, T72:
891 plasma prepared 4 hours, 16 hours, 24 hours and 72 hours after blood draw, respectively. Note that
892 different donors were sampled and that tubes were processed at different time points for preservation
893 and non-preservation tubes.
894



Supplemental figure 8. Fold changes over time at small RNA level for each blood collection tube metric. a: boxplot of the fold change within each donor across time points, per tube, for hemolysis in platelet-free plasma, as measures by absorbance at 414 nm with Nanodrop; b: boxplot of fold change

of relative RNA concentration, based on the ratio of endogenous reads vs RC spike-in RNA reads; c: boxplot of the fold change of the number of micro RNAs, after filtering miRNAs with counts fewer than 3 reads; d: area left of the curve, transformed from log2 to linear scale; e: boxplot of the fold change of the fraction of the counts mapping to micro RNAs vs. all counts. The white triangle on the boxplot corresponds to the mean of the fold change. 1st time point corresponds to the comparison of T04 vs. T0 (non-preservation tubes: BD Vacutainer Plastic K2EDTA tube (EDTA), Vacuette Tube 8 ml K2E K2EDTA Separator (EDTA separator) , BD Vacutainer Glass ACD Solution A tube (ACD-A), Vacuette Tube 9 ml 9NC Coagulation sodium citrate 3.2% (citrate), and BD Vacutainer SST II Advance Tube (serum)) or T24 vs. T0 (preservation tubes: Cell-Free RNA BCT (RNA Streck), Cell-Free DNA BCT (DNA Streck), PAXgene Blood ccfDNA Tube (PAXgene), Cell-Free DNA Collection Tube (Roche) and LBgard Blood Tube (Biomatrica)). 2nd time point corresponds to the comparison of T16 vs. T0 (non-preservation tubes) or T72 vs T0 (preservation tubes). T0: plasma prepared immediately after blood draw. T04, T16, T24, T72: plasma prepared 4 hours, 16 hours, 24 hours and 72 hours after blood draw, respectively. Note that different donors were sampled and that tubes were processed at different time points for preservation and non-preservation tubes.



915

916 **Supplemental figure 9: Kit selection for exRNAQC phase 2 for mRNA (a) and small RNA (b)**

917 **sequencing.** Selection based on robust z-scores for sensitivity and reproducibility metrics; Number that

918 follows the abbreviation of the purification kit is the plasma input volume (in ml).

919

920 **Supplemental Materials and Methods**

921 Supplemental Materials and Methods are described in a separate document.

922