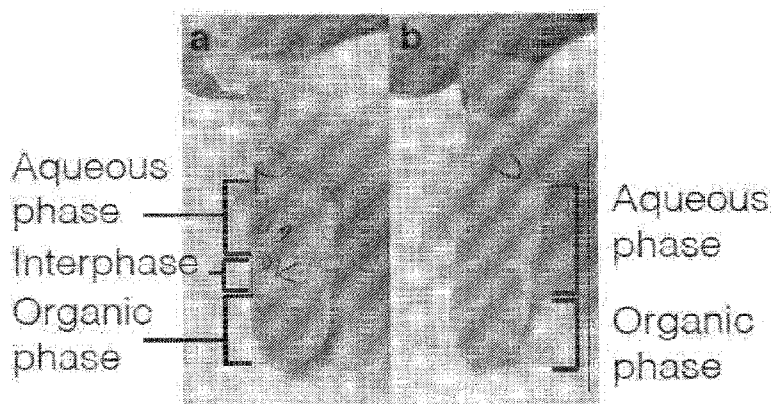




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(54) Title: METHOD OF RNA ISOLATION FROM CLINICAL SAMPLES

FIGURE 2 A
Manual



(57) Abstract: The disclosure provides methods for isolating nucleic acids from a biological fluid. In one aspect, the disclosure provides a method for isolating RNA. In another aspect, the disclosure provides a method for isolating DNA. In yet another aspect, the disclosure provides a kit for isolating RNA and/or DNA.

METHOD OF RNA ISOLATION FROM CLINICAL SAMPLES

CROSS-REFERENCE TO RELATED APPLICATIONS

5

This application claims the benefit of prior US Provisional Application No. 62/099,112, filed December 31, 2014, which is hereby incorporated by reference in its entirety.

10

BACKGROUND

RNA impacts nearly every aspect of gene expression and many human diseases are caused by or result in mistakes in RNA metabolism, e.g. mutations in pre-mRNAs lead to splicing defects or degradation of mRNAs by trigger nonsense-mediated mRNA decay. It has
15 been shown that in addition to RNA's fundamental roles in information transfer from DNA to protein, RNA molecules play crucial roles in gene regulation as their stability or rate of protein synthesis is regulated by mRNA binding proteins or ribonucleoprotein complexes (RNPs), e.g. microRNA-containing RNPs. Accordingly, RNAs present a possible new class of biomarkers.

In a medical context, biomarkers are indicators for the presence or severity of disease and
20 are usually detected in tissue samples or body fluids by various assays. Proteins, nucleic acids, lipids and other metabolites are commonly utilized in order to evaluate or diagnose a patient's status. In order for all biomarker substances to be analyzed ex vivo, they need to be extracted from the host body fluid, tissue material or cell sample, processed and analyzed accordingly. Ideally, all experimental steps, consequently leading to clinically valuable results, are performed
25 with great caution to minimize any bias introduced by extraction or specific evaluation procedures.

Ribonucleic acids show potential to serve as biomarkers in today's clinical routine for the diagnosis of various types of cancer but also cardiovascular, kidney or autoimmune diseases. Ribonucleotide biomarkers presently studied are often based on small RNA types like
30 microRNAs, isolated from body fluids or tissue samples, or longer ribonucleic acids, based on the study of long noncoding RNAs (lncRNA) generally isolated from tissue or cell samples. In order to analyze ribonucleic acids by using various techniques they need to be isolated, purified and protected from ubiquitously expressed ribonucleases. Common methods identifying specific,

possibly disease-related ribonucleic acids in biological samples include but are not limited to: Northern blotting, RT-PCR, microarray and sequencing analysis. After clinically relevant and significant differences in RNA composition between samples of diseased patients and healthy controls were found, the change in abundance of one or more specific RNAs can then be used as
5 a biomarker in order to detect this disease pattern in future applications.

The process of isolating RNA raw material is one of the most crucial steps concerning the detection of (micro)RNA-based biomarkers. Where stable RNA isolation procedures are vital to obtain meaningful results, multiple reports pointed out that differences in RNA isolation and library preparation procedures introduced substantial biases during sample analysis. Numerous
10 studies compared these differences and pointed out inconsistencies during RNA isolation utilizing frequently used commercial kits or components like TRIzol or column based approaches. Considering these substantial biases introduced during the isolation procedure, deviations in yield after RNA extraction were shown to cause significant variability in qPCR and microarray-based results when several already available RNA isolation techniques were used and
15 compared. In summary, significant discrepancies in RNA isolation, preparation procedures, and downstream analysis were highlighted in recent literature, possibly caused by the utilization of potentially unstable and uncontrolled RNA purification approaches.

Isolating ribonucleic acids (RNA) from biological samples is a frequently utilized method in biochemical, medical and biological research. Since circulating microRNAs (miRNA) in
20 various body fluids were initially proposed to potentially serve as biomarkers in clinical routine, the immanent need for successful small RNA purification procedures concerning these samples arose.

The isolation of small RNAs from biological sample material, however, is not trivial as prevalence is generally low and intrinsic ribonucleases (RNases) naturally present in body fluids
25 rapidly degrade and destroy unprotected RNA. Furthermore, the isolation and purification procedures currently described in the literature or offered through commercial kits provide varying results terms of ribonuclease protection, automation capabilities, and homogeneity of RNA extracted from multiple samples.

Therefore, there remains a need for improved methods to isolate RNA from nuclease-rich
30 and RNA-poor human clinical samples, which maximizes yield and preserves RNA integrity.

SUMMARY OF THE INVENTION

In one aspect, the disclosure provides a method for isolating RNA from a biological fluid. The method includes contacting the biological fluid with a denaturing solution comprising at least one of reducing agent and detergent to form a biological fluid mixture; contacting the
5 biological fluid mixture with a protease to form a protease treated biological fluid mixture; contacting the protease treated biological fluid mixture with an organic extraction solution, forming a solution having an aqueous phase containing the RNA and an organic phase; binding the RNA to a silica based solid phase by contacting the aqueous phase with said silica based solid phase; contacting the silica based solid phase with a first wash solution comprising alcohol,
10 chaotropic agent, and reducing agent; and eluting the RNA from the silica based solid phase comprising contacting the silica based solid phase with an aqueous solution to provide isolated RNA.

In another aspect, the disclosure provides a kit for isolating RNA from a biological fluid. The kit includes a denaturing solution; an organic extraction solution; a nucleic acid binding
15 solution; and a first wash solution comprising alcohol, chaotropic agent, and reducing agent.

In another aspect, the disclosure provides a method for isolating DNA from a biological fluid. The method includes contacting the biological fluid with a denaturing solution comprising at least one of reducing agent and detergent to form a biological fluid mixture; contacting the biological fluid mixture with a protease to form a protease treated biological fluid
20 mixture; contacting the protease treated biological fluid mixture with an organic extraction solution, forming a solution having an first aqueous phase containing RNA and an organic phase containing DNA; contacting the organic phase with a DNA extraction solution containing a buffer and a chaotropic agent to form a second solution having a second aqueous phase containing DNA; binding the DNA to a silica based solid phase by contacting the second
25 aqueous phase with said silica based solid phase; contacting the silica based solid phase with a first wash solution comprising alcohol, chaotropic agent, and reducing agent; and. eluting the DNA from the silica based solid phase comprising contacting the silica based solid phase with an aqueous solution to isolate DNA.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING

Figures 1A-1C depict the use of spike-in calibrators to find optimized conditions for RNA isolation from biofluids. A mix of synthetic 5' ³²P-labeled spike-in ssRNA (19, 24 nt, 21 nt 2'-O-methyl RNA and 45 nt) and ssDNA (31 nt) oligonucleotides was used to monitor damages resulting from ribonuclease activity during and after purification. Different oligonucleotide combinations (see each individual input lane for each experiment) of less than 0.1 pmol per oligo were added to the initial denaturation condition (a, b: lane 3 – 6, c: lane 1 – 6) or to extraction agents (A, B: lane 1,2). The concentration labels in this figure refer to the resulting concentrations of the aqueous conditions after mixing the sample with the denaturing solutions.

Figure 1A depicts a hot initial denaturation significantly improves RNA intactness in biofluid sample extracts using protocols that combine organic extraction with nucleic acid precipitation.

Reaction conditions: RNA isolation using organic extraction combined with ethanol precipitation. Samples used in this setup could either be serum samples or water controls. 150 µl samples were either directly extracted with 3 volumes of Trizol LS reagent (Thermofisher Scientific) (lane 1,2), or mixed 1:1 with 50°C-pre-heated denaturation solutions containing either 4% SDS, 4 mM EDTA, 20 mM TRIS, pH 7.5 (lanes 3, 4) or 8M GITC, 80 mM Na citrate, 0.13 g sarcosyl, 50 mM 2-mercaptoethanol (lanes 5, 6), incubated at at 50°C for five minutes, cooled to room temperature for two minutes, followed by organic extraction with 3 volumes of Trizol LS (lanes 3, 4) or acidic phenol (lanes 5, 6). One additional sample (v = 125 µl was mixed with an 375 µl of 8M GITC, 80 mM Na citrate, 0.13 g sarcosyl, 33 mM 2-mercaptoethanol without thermal denaturation (lanes 7, 8), followed by extraction with 3 volumes of acidic phenol. Phase separation in all samples was induced by addition of 0.2 vol. of chloroform. After adding 1/10 volume of 3 M Na acetate pH 4.6, the aqueous phases were reextracted with 1 vol. of phenol / chloroform / isoamylalcohol (25:24:1) and precipitated with 3 vol. of ethanol. Samples were incubated on ice for 30 min., spun at 12000 xg for 30 min. Then supernatants were carefully removed and pellets were air-dried until glossy and resuspended in 20 µl water. 5 µl aliquots were loaded.

Figure 1B depicts hot initial denaturation significantly improve RNA intactness in biofluid sample extracts using protocols that combine organic extraction with column purification.

Reaction conditions: A 150 µl serum sample was extracted with 3 vol. Trizol (lane 1), or
5 mixed 1:1 with 50°C-pre-heated denaturation solutions containing either 4% SDS, 4 mM EDTA, 20 mM TRIS, pH 7.5 (lane 3) or 8M GITC, 80 mM Na citrate, 0.13g sarcosyl, 50 mM 2-mercaptoethanol (lane 5), incubated at 50°C for five minutes, cooled to room temperature for two minutes, followed by organic extraction with 3 volumes of Trizol LS (lanes 3) or acidic phenol (lanes 5). Phase separation in all samples was induced by addition of 0.2 vol. of
10 chloroform. After addition 1/10 vol. of 3 M Na acetate pH 4.6 and 1.5 vol. of ethanol, the aqueous supernatants were subjected to column purification using the RNeasy minElute kit for serum / plasma using buffer RWP and RPE. Samples were eluted from the columns in 20 µl water. Column flow through fractions of aqueous phases were subjected to nucleic acid precipitation by adding additional 1.5 vol. of ethanol, followed by cooling on ice for 30 min.,
15 centrifugation at 12000xg for 30 min., aspiration of the supernatant, air-drying of the pellets until they look glossy, followed by dissolving the pellets in 20 µl water. 5-µl aliquots of eluate and flow through fractions were loaded.

Figure 1C depicts optimized RNA extraction conditions combining detergent-based initial denaturation, proteinase K digestion and organic extraction followed by column
20 purification provide superior protection of RNAs.

Reaction conditions: A 250 µl serum sample mixed 1:1 with 50°C-pre-heated denaturation solutions containing either 4% SDS, 4 mM EDTA, 20 mM TRIS, pH 7.5 (lane 3) or 8M GITC, 80 mM Na citrate, 0.13 g sarcosyl, 50 mM 2-mercaptoethanol (lane 5), incubated at 50°C for two minutes, followed by addition of 50µg proteinase K and proteolytic digestion for
25 15 minutes at 50°C. After digestion, solutions were cooled to room temperature for two minutes, followed by organic extraction with 1.5 volumes of 4M GITC extraction solution or acidic phenol (lanes 5). Phase separation in all samples was induced by addition of 0.2 vol. of chloroform. After addition 1/10 vol of 3 M Na acetate pH 4.6 and 1.5 ethanol, the aqueous supernatants were subjected to column purification using the RNeasy minElute kit for serum /

plasma using buffer RWP and RPE. Samples were eluted from the columns in 20 µl water. 5µl fractions from frozen eluates and of 4°C overnight incubations of eluates were loaded.

Figures 2A-2B depict the interphase during extraction. Avoiding interphase formation after organic extraction of biofluid samples by proteolytic digestion with proteinase K. By performing protein digestion with proteinase K in a denaturation buffer, interphase formation typically observed in serum and plasma samples after subsequent organic extractions (a, c) can be prevented or significantly reduced (b, d). This has important implications:

1. It allows phase separation to be achieved at lower centrifugal speeds; 3700 xg or less is sufficient for complete separation of phases with prior proteinase K treatment. In contrast, complete separation of phases was not achieved at 12000 xg, the speed recommended for comparable phase separations, without proteinase K treatment.

2. It allows the time of phase separation by centrifugation to be significantly reduced to 5 min or less. In contrast, protocols for organic extraction without prior proteinase K treatment frequently recommend centrifugation times of 15 min and more.

3. Implications 1 and 2 in samples treated with proteinase K allow for the maximization and timely optimization of aqueous phase recovery, thereby maximizing RNA recovery using standard microtitre deep-well plates (e.g. eppendorf 96 standard DeepWell Plate), in inexpensive general purpose centrifuges (e.g. Sorvall legend RT) equipped with a swing-out rotor (Sorvall Heraeus 6434 / 75006434 Swingout Rotor) and microplate adapters.

Although it is also possible to recover the aqueous phase from biofluid samples processed without proteinase K treatment (C), due to the reduced compactness of interphases at centrifugal speeds of 3700 xg or less, the volume of the aqueous phase is reduced to approximately 50% of the total aqueous phase volume under these conditions. This cannot be overcome by longer centrifugation times. High-speed centrifugation e.g. 12000 xg would compact interphases (A) further, but requires different centrifugation equipment and consumables.

4. Implications 1. and 2. in proteinase K-treated samples simplify automatic aspiration of aqueous phases containing RNA using automated liquid handling equipment (e.g. epMotion 5075) since no special sensory mechanism is required to prevent aspiration of interphases.

Aspiration of interphases could potentially clog the apparatus, lead to carryover of impurities such as ribonucleases, or clog columns used in subsequent column-based RNA purification steps.

5. Implications 1. and 2. in PNK-treated samples enable DNA recovery from organic phases obtained by initial organic extraction by simplifying automated re-extraction of existing organic phases with suitable reextraction solution, followed by subsequent second phase separation in the absence of interphases and allowing automated aspiration of resulting aqueous phases which then contain the DNA.

Figures 3A-3B: Depict (A.) GITC concentration controls partitioning of ^{32}P -labeled ssDNA during organic extraction. Using extraction solutions containing 1.5 M GITC (5.91g GITC ($M_r = 118.2$ g/mol), 290 μl 4 M citric acid, 150 μl 4 M NaOH, 0.13 g sacrosyl ($M_r = 293.4$ g/mol), 3.06 ml water, 25 ml phenol (citrate-buffered pH 4.3) in a total vol. of 34 ml) this product remained in the aqueous phase, with a resulting GITC concentration of approximately 1.7 M after phase separation (Lane 1 and 3). Using an extraction solution with 3.0 M GITC (see previous formulation, but use of 11.81 g GITC and do not add water) the ssDNA was observed to partition into the organic phase, apparent by its absence in the aqueous phase and a detectable amount of radioactivity in the resulting organic phase using a β -counter. *Reaction conditions:* The aqueous phase consisted of 450 μl heparin plasma, 125 μl buffer P, 3 μl proteinase K stock solution and 25 μl 4 M NaCl (see protocol, formulations in section 2) and was processed according to the current version of the protocol, with the modified volumes given here. Approximately 603 μl of aqueous phase were mixed with 750 μl organic extraction agent, followed by 1 min incubation at 65 °C and 1 min incubation at 4 °C. During the incubation at 65 °C formation of a single phase was observed which is expected to contain approximately 0.8 M GITC and 1.6 M GITC for samples extracted with 1.5 M and 3.0 M GITC-containing extraction solution, respectively. Phase separation was induced by adding 150 μl chloroform (0.2 vol. of the original organic phase, followed by mixing for 1 min at 4 °C in a *eppendorf* thermomixer at 1200 rpm and 5-minute centrifugation at 12000xg in tabletop centrifuge at 4°C. (B.) Separation of RNA and DNA in biofluid samples using two sequential organic extractions. The RNA isolation method described here utilizes the above finding of GITC dependence on DNA partitioning (see A): In a first organic extraction an extraction solution containing 1.6 M GITC (solution ED2, section 2.6.3.1) is used to allow DNA molecules to partition into the organic phase. RNA remains in the aqueous phase under these conditions, which is aspirated and subjected to column

purification and subsequent RNA elution (lane 1,2; 5,6; 9,10, 13,14). In a second extraction step, the organic phase is extracted with an aqueous solution containing 4M GITC (solution EA3, section, 2.6.5.1) which causes the DNA to redistribute from the organic phase into the resulting aqueous phase, followed by subsequent column purification and DNA elution (lane 3,4; 7,8; 11,12; 15,16). The current extraction protocols use slightly modified volumes of aqueous and organic solutions than those originally used (see figure A and section 3.3 for comparison). However, the resulting concentrations (summarized in section 4) are virtually identical. Trace amounts of ³²P-labeled RNA spikes in the DNA-containing fractions in the fully automated procedure (lanes 7,8; 11,12; 15,16), are a consequence of incomplete aspiration of aqueous phases phase after the first organic extraction due to shortcomings of our automated liquid handling system and are not expected to interfere with DNA analysis. If a higher level of purity is desired, an additional wash step with an aqueous solution containing 1.5 M GITC may help to avoid RNA carryover.

All lanes in this figure show aliquots of aqueous phases from organic extractions that were subjected to a column purification as described in the protocol, section 3.3.4), prior to gel loading.

Figure 4 depicts sequence length distributions of miRNAs, tRNAs, scRNAs, mRNAs and unmapped sequences in serum, plasma and urine. a) Average sample compositions considering only selected sRNA categories. For plasma miRNA represents 72.8%, scRNA represents 2.7%, mRNA represents 3.6%, rRNA represents 2.2%, and tRNA represents 0.3%. For serum miRNA represents 43.4%, scRNA represents 6.9%, mRNA represents 4.9%, rRNA represents 9.1%, and tRNA represents 0.3%. For urine miRNA represents 18.6%, scRNA represents 2.1%, mRNA represents 6.4%, rRNA represents 6.8%, and tRNA represents 20.3%. b) Total sequence length distributions in selected sRNA categories based on biofluid sample averages. c) relative sequence length distributions based on biofluid sample averages. d) Relative sequence length distributions for unmapped sequences using individual samples. For b), c), and d), miRNA correspond to 22, tRNA corresponds to 30, mRNA correspond to 33, and scRNA corresponds to 32.

Figures 5A-5C depict miRNA composition in biofluid samples (plasma, serum, and urine). 282 individual samples of kidney transplant patients, (Figure 5A) 96 plasma and (Figure

5B) 72 serum samples of eight individuals participating in a referencing study were processed using input volumes of 450 μ l. Isolation of (Figure 5C) urine samples sRNA was semi-automated, serum and plasma samples were processed using the automated protocol. Biofluid samples of each cohort were organized in batches of 24 samples (numbered bars). The final 17 samples of the urine cohort did not fill a batch and were hence added to batches of two other urine sample cohorts.

Log₂-transformed calibrator heatmaps of ten synthetic 5'P-, 3'OH spike-in RNAs absent in the human genome were added at to the denaturation buffer at the beginning of RNA isolation (calibrator set 1) and at the beginning of sRNA-based cDNA library generation (calibrator set 2) allow to detect low RNA recovery yields and/or damage by RNases by absence of the typical abundance pattern observed in other samples (e.g. batch 12).

Log₂-transformed heatmaps showing the combined most abundant 85% miRNAs across all samples. miRNA abundance data of each cohort was arranged by unsupervised clustering with pHeatmap of the bioconductor package using the Manhattan clustering method and complete clustering of miRNA annotations only, the sequence of samples across the Y-axis corresponds to the position of the sample during RNA isolation.

Figures 6A-6B depict unsupervised clustering of calibrators and miRNAs from 96 human plasma sample isolations helps to identify biological replicates of certain individuals. Sequencing data plasma samples from 8 individuals taken at 12 different timepoints over the course of two months (described in Fig. 5) was subjected to unsupervised clustering with pHeatmap of the bioconductor package using the Manhattan clustering method and complete clustering for both miRNA annotations and sample order. Sample annotation categories such as timepoints, individuals, gender, adapters used for barcoding and batches were included. (Figure 6A) clustering of the combined sets 1 and 2 of calibrators. (Figure 6B) most abundant 90% of miRNAs combined across all samples.

Figures 7A-7C depict identification of samples with high BK-virus activity. a) Differential expression analysis, comparing average abundance values of miRNAs and their fold-change comparing two populations of 282 samples, of which one population tested virus-positive by blood-based antibody test or rt-pcr-based assay of cells from urine, while the other population tested virus negative. Statistically significant fold changes are shown as red dots ($p_{adj} < 0.01$). b)

boxplots of bkv miRNA abundance in populations which tested bkv-positive by antibody test, bkv-positive by rt-pcr based assay, or bkv-negative. c) unsupervised clustering bkv miRNA in 282 urine samples using before-mentioned populations.

Figure 8 depicts loss of PK activity following 0-5 min pre-incubation of PK stock solution at room temperature. For this assay, FBS was incubated for 3, 10 or 30 min with a PK stock solution (tube a, b, c). To evaluate appropriate storage conditions for PK stock solution, the proteolytic activity of PK was evaluated after pre-incubating PK stock solution at RT for 0 (tube a), 2 (tube b) and 5 (tube c) min.

Figure 9 depicts UV absorbance spectra for Qubit HS RNA dye diluted 1:20 and 1:200, (-) RNA.

Figure 10 depicts UV absorbance spectra for Qubit HS RNA dye (+/-) HEK293 total RNA dye diluted 1:20 and 1:200.

Figure 11 depicts intensity of the fluorescent signal from Qubit HS RNA dye in the presence of rRNA, DNA and a mixture of RNA and DNA in solution.

Figure 12 depicts a plot showing the fluorescence intensity of Qubit HS RNA dye when associated with rRNA, tRNA and mRNA, respectively, in increasing concentrations of the RNA.

Figure 13 depicts Qubit fluorophore is sensitive to RNA sequence and structure.

Figure 14 depicts effect of total RNA length on Qubit HS RNA fluorescence assay.

Figure 15 depicts reported linear range for Qubit RNA HS assay measurements.

Figure 16 depicts linear range of Qubit RNA HS Assay dye with HEK293 total RNA. The values on the x-axis correspond to mass of input RNA.

Figure 17 depicts 2'-OMe-let-7a shows a similar fluorescence enhancement compared to all-ribo let-7a, and 10x lower fluorescence enhancement than total RNA.

Figure 18 depicts a dilution series of total RNA and 2'-OMe-let-7a standard show expected linearity. The ratio of the slopes can be used to calculate total RNA concentrations.

Figure 19 depicts a dilution series of total RNA and the 2'-OMe-let-7a standard show expected linearity. The total RNA series yields standard curves with an average slope of 7, and the 2'-OMe-let-7a series yields an average slope of 0.55. The slopes differ by an average factor of 13. This ratio is used to determine total RNA concentrations. RNA sample concentrations can
 5 be calculated from the Qubit RFU units using the following equation:

$$c_{\text{sample}}(\text{ng}/\mu\text{l}) = \frac{\text{RFU}(\text{sample}) - \text{RFU}(\text{reagent blank})}{V(\mu\text{l}) \cdot 7/\text{ng}}$$

Figure 20 depicts a representative standard curve used to calculate RNA concentration using the Quant-iT™ RiboGreen® RNA Reagent.

DETAILED DESCRIPTION

In one aspect, the disclosure provides a method for isolating RNA from a biological
 10 sample.

As used herein, "RNA" is defined as at least two ribonucleotides covalently linked together. The RNA may be any type of RNA. Examples include mRNA, tRNA, rRNA, shRNA, circRNA, scaRNA, scRNA, snRNA, siRNA or Piwi-interacting RNA, or a pri-miRNA, pre-miRNA, miRNA, snoRNA, long ncRNAs, anti-miRNA, precursors and any variants thereof.
 15 Further examples of RNA include RNA of a virus, or RNA sequences derived from a virus genome. Even further examples include RNA of a bacteria or In one embodiment, the RNA is a short RNA molecule derived from a degraded source, such as, for example, degraded mRNA, degraded rRNA, and degraded tRNA.

RNA may be single stranded or double stranded, or may contain portions of both double
 20 stranded and single stranded sequence. RNA may be synthesized as a single stranded molecule or expressed in a cell (in vitro or in vivo) using a synthetic gene. RNA may be obtained by chemical synthesis methods or by recombinant methods.

RNA also encompasses the complementary strand of a depicted single strand. Many variants of RNA may be used for the same purpose as a given RNA. Thus, RNA also
 25 encompasses substantially identical RNA and complements thereof. A single strand provides a probe that may hybridize to a target sequence under stringent hybridization conditions. Thus, RNA also encompasses a probe that hybridizes under stringent hybridization conditions.

In one embodiment, RNA is a short RNA. A “short” RNA refers to RNA that has a maximum number of bases in length of about 500, 400, 300, 200, 100, 90, 80, 70, 60, 50, 40, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, or 21 bases. The short RNA has a minimum number of bases in length of about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 bases.
5 Any minimum amount can be combined with any maximum amount to define a range for a short RNA.

In another embodiment, the RNA is between 5-500 nucleotides, 5-250 nucleotides, 5-100 nucleotides, or 5-50 nucleotides.

Exemplary short RNAs include mRNA, tRNA, rRNA, shRNA, circRNA, scaRNA,
10 scRNA, snRNA, siRNA or Piwi-interacting RNA, or a pri-miRNA, pre-miRNA, miRNA, snoRNA, long ncRNAs, anti-miRNA, precursors and any variants thereof. Further examples of RNA include RNA of a virus, or RNA sequences derived from a virus genome. Even further examples include RNA of a bacteria or In one embodiment, the RNA is a short RNA molecule derived from a degraded source, such as, for example, degraded mRNA, degraded rRNA, and
15 degraded tRNA.

In a preferred embodiment, the RNA acid is microRNA (miRNA). MicroRNA molecules are known in the art (see, for example, Bartel, *Cell*, 2004, 116, 281-297 for a review on microRNA molecules). The definitions and characterizations of microRNA molecules in the article by Bartel are hereby incorporated by reference. Such molecules are derived from genomic
20 loci and are produced from specific microRNA genes.

miRNAs are typically small RNA molecules of generally about 13-33, 18-24, or 21-23 nucleotides in length. The miRNA may also have a total of at about 5-40 nucleotides in length. These microRNAs are non-coding RNAs which are cleaved from hairpin precursors. miRNAs are naturally 5' phosphorylated and carry 2', 3' dihydroxyl termini. The sequence of the miRNA
25 may comprise the sequence of a miRNA disclosed in U.S. patent application Ser. Nos. 11/384,049, 11/418,870 or 11/429,720, the contents of which are incorporated herein, or variants thereof.

A source of the short target RNA is a biological sample. A “biological sample” as used herein refers to a sample of “biological tissue or solid” or “biological fluid” that includes nucleic

acids. Such samples include, but are not limited to, tissue or fluid isolated from animals or plants, including plant or animal derived cell suspensions, and cell free extracts of animal or plant origin. Biological samples also include viruses or unicellular organisms.

It has been contemplated that the biological sample may contain bacteria, virus, amoeba,
5 or fungus.

Furthermore, the biological sample may include bacterial, fungal, or human cell cultures.

Examples of biological tissue include sections of tissues such as biopsy and autopsy samples, frozen sections taken for histologic purposes, abcess material, hair, and skin. Examples of tissue include tissue from the brain, muscle, heart, fat, pancreas, lung, spleen, and bone.

10 In a preferred embodiment, the biological sample is a biological fluid. Examples of biological fluids include blood, a blood fraction, plasma, serum, urine, pleural effusion, pericardial effusion, sinovial fluid, mucus, ascitic fluid, amniotic fluid, stool, tears, saliva, cerebrospinal fluid, cervical secretions, vaginal secretions, abcess discharge, puss, endometrial secretions, gastrointestinal secretions, bronchial secretions, sputum, secretions from ovarian cyst,
15 sperm, seminal fluid, secretions from the breast, cell line. In a preferred embodiment, the biological samples include urine, serum, and plasma.

In another embodiment, the plasma sample includes citrate-, EDTA-, and heparin-plasma samples.

20 “Biological fluids” as defined herein also include a biological tissue or solid that has been physically or chemically disrupted, e.g. by maceration or homogenation, as is known in the art, to form a liquid sample.

When the biological sample is a tissue or in solid form, or viscous, the biological sample can also be homogenized or disrupted to maximize contact between the extraction solvent and the biological sample. Methods to homogenize tissue are commonly known in the art. Such
25 methods include chemical or mechanical means. Mechanical means include grinding, shearing, freeze-thaw, beating, shocking, sonicating, or a combination thereof. For example, grinding techniques can be accomplished through manual means such as a mortar and pestle. Automated methods for disrupting tissue can be used as well to lyse cells, and are commonly known in the

art. Examples of automated homogenizers include HT Homogenizer from OPS Diagnostics, the Homogenizer from Invitrogen, or Stomacher paddle mixer from Seward Laboratory Systems.

Any remaining insoluble materials can be cleared by any known means. For example, the homogenized material can be centrifuged and the supernatant is used.

5 A biological sample may be provided by removing a sample of cells from an animal, or plant, but can also be accomplished by using previously isolated cells (e.g., isolated by another person, at another time, and/or for another purpose), or by performing the methods described herein in vivo. "Animal" as used herein refers to any animal, including fish, amphibians, reptiles, birds, and mammals, such as mice, rats, rabbits, goats, cats, dogs, cows, apes, and humans.

10 The method includes contacting the biological sample with a denaturing solution to form a biological fluid mixture.

The denaturing solution includes at least one of a detergent and reducing agent.

A "detergent" as defined herein is any substance that reduces the surface tension of water, and is used synonymously with the term "surfactant". Examples of detergents suitable for the
15 claimed invention include anionic, nonionic, and zwitterionic detergents. In our specific application, detergents are used which disrupt the tertiary structure of most proteins including ribonucleases and thereby inactivate their function.

Preferred anionic detergents include sulfates of C₆-C₂₀ alkylalcohols and N-C₆-C₂₀-acylaminoacids. Examples of anionic detergents include sodium dodecyl sulfate (SDS), sodium
20 lauryl sarcosin(sarcosyl), and sodium deoxycholate. The preferred detergent from this group includes SDS and sarcosyl.

Preferred non-ionic detergents include polymers of poly-(C₂-C₆)-alkoxy-(C₂-C₆)-alkylenes, esters and ethers of poly-(C₂-C₆)-alkyleneglycols and glycosides of C₆-C₂₀-alkylalcohols. Specifically preferred non-ionic detergents include Synperonic (block copolymer
25 made from poloxyethylene and polyethoxypropylene, Pharmacia), Tween 20 (polyethylene glycol (20) sorbitan monolaurate), ThesitTM (dodecylpolyethylene glycol ether), NP-40 (ethylenephenolpolyethyleneglycolether), TritonX-100 (polyethylene glycol (9-10) p-t-oktylphenol) and glycosidic detergents, e.g. octyl:β-D-glucopyranoside.

Preferred zwitterionic detergents are N-C₁-C₁₆-alkyl-ammonio-C₂-C₆-alkyl-sulfonates. Examples of zwitterionic detergents include Zwittergent 3-08 (N-octyl-N,N-dimethyl-3-amino-1-propane sulfonate) Zwittergent 3-12 (N-docecanyl-N,N-dimethyl-3-ammonio-1-propane sulfonate), CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate) and
5 CHAPSO (3-cholamidopropyl)-dimethylamino]-2-hydroxy-1-propane sulfonate).

In one embodiment, the denaturing solution includes detergent at a concentration of about 10-70% (w/v), more preferably from 20-50 % (w/v). The concentration of detergent in the biological fluid mixture is 1-10% (w/v), more preferably 5-8% (w/v). The detergent may have a concentration of at least about 1%, at least about 4%, at least about 10%, or at least about 15%
10 (w/v). In another preferred embodiment, the detergent in the denaturing solution is SDS and is present at a concentration of at least about 5% (w/v). In a preferred embodiment, the detergent in the mixture is SDS and is present at a concentration of at least about 4% (w/v). In another preferred embodiment, the detergent in the denaturing solution is SDS and is present at a concentration of at least about 3% (w/v). In another preferred embodiment, the detergent in the
15 denaturing solution is SDS and is present at a concentration of at least about 2% (w/v).

A "reducing agent" as defined herein is an element or compound that loses (or "donates") an electron to another chemical species in a redox chemical reaction. Examples of reducing agents suitable for the claimed invention include dithiothreitol (DTT), 2-Mercaptoethanol (BME), and *tris*(2-carboxyethyl)phosphine (TCEP). In some embodiments, the preferred
20 reducing agent is TCEP.

The denaturing solution may also include a buffer. As used herein, buffers suitable for the claimed invention include biological buffers. Examples of biological buffers include TRIS, MPOS, MES, HEPES, citrate, and phosphate buffer.

The denaturing solution may contain other additives, to include
25 ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), and metal salts. As defined herein, "metal salts" include NaCl, KCl, CaCl₂ and MgCl₂.

In one embodiment, the denaturing solution contains SDS, TRIS, and EDTA. In a preferred embodiment, the denaturing solution contains from about 20-40 % SDS, 10-100 mM TRIS buffer, 10-50 mM EDTA, and 100 – 500 mM BME.

In a preferred embodiment, the concentration of BME in the mixture of biological fluid mixture is about 1-100 mM. In a preferred embodiment, the concentration of BME in the mixture is 25-75 mM.

5 The denaturing solution may contain a visualization agent to monitor the mixing/agitation and phase extraction steps. The visualization agent allows visual inspection of the homogeneity of the aqueous phase. The visualization agent allows visual inspection of phase separation because the visualization agent partitions into the organic phase leaving an uncolored aqueous phase. Examples of a visualization agent include methylene blue.

10 Once the biological sample has been contacted with the denaturing solution, a protease is added to the resulting mixture. Contact with the protease creates a protease treated biological fluid mixture.

A “protease” as defined herein is an enzyme that hydrolyses peptide bonds. Conventional proteases may be used. It has been unexpectedly discovered that a protease will still be functional with such components being present in the mixture of the biological sample, protease, 15 and denaturing solution in the conditions as set forth herein. Such conditions are not tolerated by, for example, RNases. Proteinase K is a preferred example.

It is preferred that the specific activity of the protease be high to degrade proteins in what can be a protein-rich biological sample and thereby protect the RNA from ribonucleases. The specific activity as determined by the Chromozym assay of the protease in the mixture of 20 biological sample and denaturing solution is for example at least about 0.1 U/ml, at least about 1U/ml, at least about 2.5U/ml, at least about 5 U/ml, or at least about 10 U/ml. In another embodiment, the specific activity of the protease in the mixture is between 0.1 and 1000 U/ml.

In one embodiment, the denaturing solution is heated to at least about 45 °C, at least about 50 °C, at least about 55 °C, or at least about 60 °C prior to contact with the biological 25 sample. In some embodiments, the denaturing solution is heated to at least about 65 °C prior to contact with the biological sample. Upon addition of the biological sample to the denaturing solution, the mixture is further incubated at a temperature at least about 45 °C, at least about 50 °C, at least about 55 °C, or at least about 60 °C. In another embodiment, the mixture is incubated

for at least about 2 minutes, at least about 5 minutes, at least about 10 minutes, or at least about 15 minutes.

The incubation step may be performed while agitating the mixture. Agitation is defined as any condition that causes turbulence or shearing within the mixture. Examples of agitation
5 include pipetting, shaking, rocking, vibrating, or stirring.

The method further includes contacting the biological sample with an organic extraction solution to form an aqueous phase and an organic phase. The organic extraction solution includes at least one of phenol, chaotropic agent, phase separation inducing agent, and detergent.

A “chaotropic agent” as defined herein is a substance which disrupts the three
10 dimensional structure in macromolecules such as proteins, DNA, or RNA and denatures them. Examples of suitable chaotropic agents for use in the claimed invention include: sodium perchlorate, sodium trichloroacetate, sodium trifluoroacetate, sodium iodide, urea, and guanidine. In one embodiment the chaotropic agent is guanidine, in the form of guanidinium thiocyanate (GITC) or guanidinium hydrochloride (GuHCl).

15 As used herein, phase separation inducing agents are agents that promote or induce the formation of an organic phase. Such phase separation inducing agents are known in the art. Examples of such agents include chloroform, or dichloromethane. In a preferred embodiment, the phase separation inducing agent is chloroform.

In the alternative, if phase inversion is desired (top: organic phase, bottom aqueous
20 phase), heptanol or other heavier alcohols (e.g. alcohols having 6 or more carbons). Examples of other suitable alcohols include hexanol, octanol, nonanol, and decanol.

In one embodiment, the organic extraction solution contains a chaotropic agent at a concentration of less than about 10 M, less than about 5M, or less than about 3M. In another embodiment, the chaotropic agent has a concentration between 1M and 10M, more preferably
25 between 1M and 5M.

In one embodiment, the mixture obtained after contacting the biological sample with the organic extraction solution contains a chaotropic agent at a concentration of less than about 3M,

less than about 2M, or less than about 1M. In a preferred embodiment, the chaotropic agent has a concentration between 0.5-3M.

It has been unexpectedly discovered that by lowering the overall chaotropic agent concentration to less than 2 M, in some embodiments less than 3 M, it was possible to deplete
5 virtually all DNA from the aqueous phase while simultaneously retaining desired RNA. In particular, it is possible to deplete virtually all DNA in at least the size range of 30 bases to 2000 bases from the aqueous phase while simultaneously retaining the desired RNA

It has been further unexpectedly discovered that, due to the efficient denaturing step as described above, the ratio of aqueous to organic phase in the organic extraction solution can be
10 reduced. For example, the chaotropic agent concentration as described above, in combination with a mixing ratio of organic phase: aqueous phase can be less than 2 to 1, less than 1 to 1, or less than 0.75 to 1 (the phase separation inducing agent is between 0.1 to 0.5 volumes, more preferably between 0.3 and 0.5 volumes with regard to the organic phase). In another embodiment, the mixing ratio is 0.45-2.5 to 1, more preferably from 1-2 to 1. Commercially
15 available products utilize ratios of 3:1 and 4:1, or even higher.

As defined herein, DNA includes genomic DNA, cDNA, or a hybrid wherein the DNA may contain combinations of deoxyribo- and ribo-nucleotides, and combinations of bases including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine hypoxanthine, isocytosine and isoguanine. Further examples of DNA include DNA of a virus, or DNA
20 sequences derived from a virus genome. In one embodiment, the DNA is a short DNA molecule derived from a degraded source, such as, for example degraded DNA. DNA may be single stranded or double stranded, or may contain portions of both double stranded and single stranded sequence. DNA may be synthesized as a single stranded molecule or expressed in a cell (in vitro or in vivo) using a synthetic gene. DNA may be obtained by chemical synthesis or synthesis
25 methods or by recombinant methods.

In one embodiment, the biological sample is further contacted with phase separation inducing agent after contact with the organic extraction solution.

In another embodiment, the organic extraction solution contains isoamyl alcohol.

Phase separation may also include methods of physical separation, such as centrifugation. Such methods are well known in the art. Traditional methods of RNA isolation result in interphase formation between the aqueous and organic phases. The interphase is a separate layer between the aqueous phase and organic phase and often appears white and often includes
5 proteinaceous material. The interphase can interfere with isolation of the aqueous phase. The inventors have unexpectedly discovered that the addition of protease to a mixture of a biological sample and the denaturing solution as described above prior to the organic extraction step as described herein permits the reduction of the ratio of organic to aqueous phase during the organic
10 extraction step as described herein as well as removes or significantly reduces interphase formation. Thus, less organic solvent may be used.

In addition, the denaturing and protease treatment steps allow less force to be required to separate the aqueous and organic phases, for example centrifugation under lower relative centrifugal forces (RCF). Without wishing to be bound by theory, the biological sample may be protein-rich and have hydrophobic and hydrophilic groups (amino acids). Upon denaturation,
15 proteins unfold and interact with both hydrophobic (organic) and hydrophilic (aqueous) phases, thereby acting as emulsifiers. The interphase is considered to be an emulsion containing both hydrophobic as well as hydrophilic properties. One way of overcoming the formation of an emulsion is to degrade proteins into peptides. Depending on their hydrophobicity these peptides will either partition into the organic phase or aqueous phase and will no longer act as emulsifiers
20 and interphase formation is reduced. As a consequence, the centrifugation force needed to achieve phase separation is reduced, and the amount of aqueous phase, which contains the desired RNA, is maximized.

The aqueous phase is contacted with a nucleic acid binding solution. The nucleic acid binding solution contains at least one of an alcohol and reducing agent. In some embodiments,
25 the aqueous phase is contacted with a nucleic acid binding solution containing at least one of an alcohol, at least one salt, a chaotropic agent, and a reducing agent.

In some embodiments, the concentration of the reducing agent in the nucleic acid binding solution is at least 0.05 mM, at least 0.5 mM, or at least 1mM. In another embodiment, the concentration of the reducing agent in the solution is less than 100 mM, less than 50 mM, less

than 25 mM, or less than 10 mM. In some embodiments, the reducing agent has a concentration between 1-10 mM, or 3-8 mM.

The alcohol has a concentration of at least about 70 %, at least about 80 %, at least about 90 %, at least about 95 %, or at least about 99 % (v/v). As used herein, alcohols include, for
5 example, methanol, ethanol, propanol, butanol, and mixtures thereof.

The nucleic acid binding solution may contain other additives, to include ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), and metal salts. Examples of suitable metal salts include NaCl, KCl, CaCl₂, and MgCl₂.

In one embodiment, the nucleic acid binding solution contains from 80-99% (v/v)
10 alcohol, 5-20 mM of a first salt, 0.5-10 mM of a second salt, 0.1 -10 M chaotropic agent, and 0.1-10 mM reducing agent. In another embodiment, the solution contains from 90-99% (v/v) alcohol, 5-10 mM of a first salt, 0.5-5 mM of a second salt, 0.1 -5 M chaotropic agent, and 2-7 mM reducing agent. In one embodiment, the alcohol is isopropanol, the first salt is MgCl₂, the second salt is CaCl₂, the chaotropic agent is GITC, and the reducing agent is TCEP.

15 In one embodiment, the nucleic acid binding solution includes 50-80% isopropanol and 0.5-5 M GITC. In another embodiment, the nucleic acid binding solution includes 60-70% isopropanol and 0.5-2.5 M GITC.

The aqueous phase is contacted with a silica-based solid phase. The term “solid phase” is not intended to imply any limitation regarding its form or structure. The solid phase may be
20 porous, non-porous, permeable, or impermeable. The silica-based solid phase can be in the form of a matrix, beads, particles, fibers, gels, filters, sheets, or a membrane. The silica-based solid phase may be in the form of a column. In one embodiment, the silica is in the form of a magnetic particle.

Examples of suitable columns include Zymo-SpinTM I columns and Qiagen RNeasy Mini
25 Spin Columns.

A vacuum manifold such as a QIA vac 24 Vacuum Manifold is preferably used for convenient vacuum processing of spin columns in parallel. Samples and wash solutions can be drawn through the column membranes by vacuum instead of centrifugation, providing greater

speed, and reduced hands-on time in the extraction and purification of RNA from cells and tissues.

The column bound silica-based solid phase may be in the form of a 96 well plate. An example of a suitable plate includes Zymo-Spin™ I-96 Plate.

5 According to another embodiment, after the aqueous phase is contacted with the silica-based solid phase, the silica-based solid phase is washed at least one of a first wash solution and second wash solution.

The silica-based solid phase with bound RNA acid is contacted with a first wash solution. The first wash solution includes alcohol, chaotropic agent, and a reducing agent.

10 In one embodiment, the concentration of the reducing agent in the first wash solution is at least 0.05 mM, at least 0.5 mM, or at least 1mM. In another embodiment, the concentration of the reducing agent in the solution is less than 100 mM, less than 50 mM, less than 25 mM, or less than 10 mM. In some embodiments, the reducing agent has a concentration between 1-20 mM, 2-10 mM, or 3-8 mM.

15 In one embodiment, the alcohol is present in a concentration between 50-90% or 60-80% (v/v). In a preferred embodiment, the reducing agent is present in a concentration between 1 and 20 mM. In another preferred embodiment, the reducing agent is present in a concentration between 2mM and 10mM.

20 In one embodiment, the first wash solution includes at least one salt, a detergent, chaotropic agent, alcohol, and a reducing agent. In a preferred embodiment, the first wash solution includes NaCl, MgCl₂, CaCl₂, Triton X-100, GITC, isopropanol, and TCEP. In one embodiment, the first wash solution contains from 0.1-10 mM triton X-100, from 10-20 mM NaCl, from about 0.5-10 mM MgCl₂, from 0.1-10 mM CaCl₂ from 0.1-5 mM chaotropic agent; and from 40-80% (v/v) alcohol.

25 In one embodiment, the first wash solution includes at least one salt, a detergent, chaotropic agent, alcohol, and a reducing agent. In one embodiment, the first wash solution contains from 0.5 mM Triton-X-100, 18 mM NaCl, from 1.3 – 5.4 mM MgCl₂, from 0.4 – 1.8 mM CaCl₂; from 0.36 M GITC agent; and from 40-80% (v/v) alcohol. In a preferred

embodiment, the first wash solution includes NaCl, MgCl₂, CaCl₂, Triton X-100, GITC, isopropanol, and TCEP.

In one embodiment, the first wash solution includes Qiagen™ buffer RWT supplemented with TCEP. For example, buffer RWT may be supplemented with between 2mM and 10 mM
5 TCEP.

The silica-based solid phase with bound RNA is optionally contacted with a second wash solution. The second wash solution includes alcohol, and a reducing agent. In a preferred embodiment, the second wash solution further contains a chaotropic agent in an amount equal to or less than that in the first wash solution.

10 In one embodiment, the alcohol is present in a concentration between 50-95% (v/v), 60-90% (v/v).

In one embodiment, the concentration of the reducing agent in the second wash solution is at least 0.05 mM, at least 0.5 mM, or at least 1mM. In another embodiment, the concentration of the reducing agent in the solution is less than 100 mM, less than 50 mM, less than 25 mM, or
15 less than 10 mM. In some embodiments, the reducing agent has a concentration between 1-20 mM, 2-10 mM, or 3-8 mM.

In one embodiment, the second wash solution includes Qiagen™ buffer RPE supplemented with TCEP. For example, buffer RPE may be supplemented with between 1mM and 10 mM TCEP.

20 The first and second wash solution may contain other additives. For example, the first and second wash solution may include ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), detergents, or metal salts. Examples of suitable metal salts include NaCl, KCl, CaCl₂, and MgCl₂.

In one embodiment, silica-based solid phase is contacted with at least one alcohol after
25 contact with the first wash solution or the second wash solution. In one embodiment, the silica-based solid phase may be contacted with an alcohol having a concentration of at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 99%, or 100% (v/v).

In a preferred embodiment, after contact with the first wash solution or the second wash solution, the silica-based solid phase is contacted with a solution of 100% alcohol. In another preferred embodiment, after contact with the first or second wash solution, the silica-based solid phase is contacted with a solution of 100% alcohol and a solution having at least about 70% alcohol.

In a preferred embodiment, after contact with the first wash solution or the second wash solution, the silica-based solid phase is contacted with a solution of 100% alcohol. In another preferred embodiment, after contact with the second wash solution, the silica-based solid phase is contacted with a solution of 100% alcohol and a solution having at least about 80% alcohol.

In some embodiments, the silica based wash solution is contacted with any combination and any number of a first wash solution and second wash solution.

The RNA bound to the silica-based solid phase may be eluted from the solid phase by contacting the solid phase with an aqueous elution solution, such solutions are known in the art. The aqueous elution solution may contain at least one of water, a buffer, and a RNA preservative. RNA preservatives are commonly known in the art. An example of a suitable RNA preservative includes RNase inhibitor.

Elution may include methods of physical elution, such as subjecting the silica-based solid phase to vacuum or centrifugation. Such methods are known in the art.

In another aspect, the DNA partitioned into the organic phase described above may be further processed to isolate DNA contained therein.

In one embodiment, the organic phase described above is contacted with a DNA extraction solution containing a chaotropic agent and a buffer to form a mixture to form a second aqueous phase and a second organic phase. In one embodiment, the buffer is sodium citrate and the chaotropic agent is GITC. In one embodiment, solution contains at least 3M GITC, preferably at least 4 M GITC.

The mixture is agitated. The second aqueous phase is separated from the second organic phase. Separation of the second aqueous phase and second organic phase may be accomplished by physical means, including a centrifuge.

The separated second aqueous phase containing the DNA is contacted with a solution containing alcohol, at least one salt, a chaotropic agent, and a reducing agent to form a mixture. In one embodiment, the solution contains from 80-99% (v/v) alcohol, 5-20 mM of a first salt, 0.5-10 mM of a second salt, 0.1 -10 M chaotropic agent, and 0.1-10 mM reducing agent. In another embodiment, the solution contains from 90-99% (v/v) alcohol, 5-10 mM of a first salt, 0.5-5 mM of a second salt, 0.1 -5 M chaotropic agent, and 2-7 mM reducing agent. In one embodiment, the alcohol is isopropanol, the first salt is MgCl_2 , the second salt is CaCl_2 , the chaotropic agent is GITC, and the reducing agent is TCEP.

This mixture is agitated.

The mixture containing the DNA is then contacted with a silica-based solid phase.

The silica-based solid phase with bound RNA acid is contacted with a first wash solution. The first wash solution includes alcohol, chaotropic agent, and a reducing agent.

In one embodiment, the concentration of the reducing agent in the first wash solution is at least 0.05 mM, at least 0.5 mM, or at least 1mM. In another embodiment, the concentration of the reducing agent in the solution is less than 100 mM, less than 50 mM, less than 25 mM, or less than 10 mM. In some embodiments, the reducing agent has a concentration between 1-20 mM, 2-10 mM, or 3-8 mM.

In one embodiment, the alcohol is present in a concentration between 50-90% or 60-80% (v/v). In a preferred embodiment, the reducing agent is present in a concentration between 1 and 20 mM. In another preferred embodiment, the reducing agent is present in a concentration between 2mM and 10mM.

In one embodiment, the first wash solution includes at least one salt, a detergent, chaotropic agent, alcohol, and a reducing agent. In a preferred embodiment, the first wash solution includes NaCl , MgCl_2 , CaCl_2 , Triton X-100, GITC, isopropanol, and TCEP. In one embodiment, the first wash solution contains from 0.1-10 mM triton X-100, from 10-20 mM NaCl , from about 0.5-10 mM MgCl_2 , from 0.1-10 mM CaCl_2 from 0.1-5 mM chaotropic agent; and from 40-80% (v/v) alcohol.

In one embodiment, the first wash solution includes at least one salt, a detergent, chaotropic agent, alcohol, and a reducing agent. In one embodiment, the first wash solution contains from 0.5 mM Triton-X-100, 18 mM NaCl, from 1.3 – 5.4 mM MgCl₂, from 0.4 – 1.8 mM CaCl₂; from 0.36 M GITC agent; and from 40-80% (v/v) alcohol. In a preferred
5 embodiment, the first wash solution includes NaCl, MgCl₂, CaCl₂, Triton X-100, GITC, isopropanol, and TCEP.

In one embodiment, the first wash solution includes Qiagen™ buffer RWT supplemented with TCEP. For example, buffer RWT may be supplemented with between 2mM and 10 mM TCEP.

10 In one embodiment, the first wash solution includes at least one salt, a detergent, chaotropic agent, alcohol, and a reducing agent. In a preferred embodiment, the first wash solution includes NaCl, MgCl₂, CaCl₂, Triton X-100, GITC, isopropanol, and TCEP.

In one embodiment, silica-based solid phase is contacted with at least one alcohol after contact with the first wash solution. In one embodiment, the silica-based solid phase may be
15 contacted with an alcohol having a concentration of at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 99%, or 100% (v/v).

In a preferred embodiment, after contact with the first wash solution, the silica-based solid phase is contacted with a solution of 100% alcohol. In another preferred embodiment, after contact with the second wash solution, the silica-based solid phase is contacted with a solution of
20 100% alcohol and a solution having at least about 70% alcohol.

In some embodiments, the silica-based solid phase may be washed with a second wash solution as described above.

In a preferred embodiment, after contact with the first wash solution or the second wash solution, the silica-based solid phase is contacted with a solution of 100% alcohol. In another
25 preferred embodiment, after contact with the second wash solution, the silica-based solid phase is contacted with a solution of 100% alcohol and a solution having at least about 80% alcohol.

The DNA bound to the silica-based solid phase may be eluted from the solid phase by contacting the solid phase with an aqueous elution solution, such solutions are known in the art.

The aqueous elution solution may contain at least one of water, a buffer, and a DNA preservative. DNA preservatives are commonly known in the art.

Elution may include methods of physical elution, such as subjecting the silica-based solid phase to vacuum or centrifugation. Such methods are known in the art.

5 In the specification, numerous specific details are set forth in order to provide a thorough understanding of the present embodiments. It will be apparent, however, to one having ordinary skill in the art that the specific detail need not be employed to practice the present embodiments. In other instances, well-known materials or methods have not been described in detail in order to avoid obscuring the present embodiments.

10 Throughout this specification, quantities are defined by ranges, and by lower and upper boundaries of ranges. Each lower boundary can be combined with each upper boundary to define a range. The lower and upper boundaries should each be taken as a separate element.

 Reference throughout this specification to “one embodiment,” “an embodiment,” “one example,” or “an example” means that a particular feature, structure or characteristic described
15 in connection with the embodiment or example is included in at least one embodiment of the present embodiments. Thus, appearances of the phrases “in one embodiment,” “in an embodiment,” “one example,” or “an example” in various places throughout this specification are not necessarily all referring to the same embodiment or example. Furthermore, the particular features, structures or characteristics may be combined in any suitable combinations and/or sub-
20 combinations in one or more embodiments or examples. In addition, it is appreciated that the figures provided herewith are for explanation purposes to persons ordinarily skilled in the art and that the drawings are not necessarily drawn to scale.

 As used herein, the terms “comprises,” “comprising,” “includes,” “including,” “has,”
“having,” or any other variation thereof, are intended to cover a non-exclusive inclusion. For
25 example, a process, article, or apparatus that comprises a list of elements is not necessarily limited to only those elements but may include other elements not expressly listed or inherent to such process, article, or apparatus.

 Further, unless expressly stated to the contrary, “or” refers to an inclusive “or” and not to an exclusive “or”. For example, a condition A or B is satisfied by any one of the following: A is

true (or present) and B is false (or not present), A is false (or not present) and B is true (or present), and both A and B are true (or present).

Additionally, any examples or illustrations given herein are not to be regarded in any way as restrictions on, limits to, or express definitions of any term or terms with which they are
5 utilized. Instead, these examples or illustrations are to be regarded as being described with respect to one particular embodiment and as being illustrative only. Those of ordinary skill in the art will appreciate that any term or terms with which these examples or illustrations are utilized will encompass other embodiments which may or may not be given therewith or elsewhere in the specification and all such embodiments are intended to be included within the scope of that term
10 or terms. Language designating such non-limiting examples and illustrations includes, but is not limited to: “for example,” “for instance,” “e.g.,” and “in one embodiment.”

In this specification, groups of various parameters containing multiple members are described. Within a group of parameters, each member may be combined with any one or more of the other members to make additional sub-groups. For example, if the members of a group are
15 a, b, c, d, and e, additional sub-groups specifically contemplated include any one, two, three, or four of the members, e.g., a and c; a, d, and e; b, c, d, and e; etc.

EXAMPLES

The present embodiments of the disclosure are illustrated in further details by the following non-limiting examples

2 Materials and buffers

2.1 Procedural considerations regarding number of samples and manual, semi-automated or automated processes

The volumes and amounts of all solutions and consumables were chosen for parallel processing of 24 biofluid samples unless stated otherwise. Twenty four (24) samples are typically processed
25 in a single multiplexed library preparation and sequencing reaction using 24 different barcoded 3' DNA adapters. The RNA purification procedure consists of (1) an initial denaturation step with detergent at an elevated temperature, followed by (2) an enzymatic digestion of protein, (3) an organic extraction step to remove hydrophobic peptides and other hydrophobic substances, and (4) a column purification step.

There are three protocols using different degrees of automation:

A. Manual purification using either a vacuum manifold or centrifugation in order to pass solutions through columns.

B. Semi-automated purification where the lysis step and the organic extraction step are carried out manually while the column purification is performed using an automated liquid handling system.

C. Fully automated purification where all steps are performed using the fully equipped liquid epMotion 5075 liquid handling system (see section 1.4 for equipment).

Formulations of buffers and solutions are provided for 8, 24, and 96 extractions. For manual RNA isolations any number of samples up to a total of 24 samples are done at once. For semi-automated sample processing denaturation, digestion, and organic extraction in batches of up to 24 samples, one batch at a time. After organic extraction, samples can be stored in binding buffer for an intermitted period of up to three hours until all batches (of up to 4) are denatured and extracted. Multiple batches are then subjected to automated column purification. For semi-automated or fully automated setups, samples are processed in multiples of 8 samples at a time, since microtiter plates and filter plates of manifolds are typically organized in a 12 x 8 well format. Using a total of 96 samples (4 x 24 samples) takes full advantage of this form factor.

2.2 Chemicals

- 2-Propanol (also named: isopropanol, *Fisher Sci*, Cat# 314, M_r 60.1 g/mol)
- Acetic acid, glacial (*Fisher Sci*, Cat# A38-212, M_r 60.05 g/mol)
- 2-Mercapthoethanol (also named: β -mercaptoethanol, *Sigma*, Cat# M3148, M_r 78.13 g/mol)
- Brilliant Blue R, powder (*Sigma*, Cat# B0149, M_r 825.99 g/mol)
- Bromphenol blue, technical grade (*Sigma*, Cat# B6131, M_r 691.94 g/mol)
- Calcium dichloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, *Appllichem*, Cat# A1873, M_r 147.02 g/mol)
- Citric acid monohydrate, crystalline (*Fisher Sci*, Cat# A1112-12, M_r 210.14 g/mol)

- Disodium ethylenediaminetetraacetic acid dihydrate ($\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$, *Sigma*, Cat# E6635, M_r 372.24 g/mol)
- Glycerol (*Fisher Sci*, Cat# G31-1, M_r 92.09 g/mol)
- Guanidium thiocyanate (GITC, *Sigma*, Cat# 50981, M_r 118.16 g/mol)
- 5 - Magnesium dichloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, *Applichem*, Cat# A1036, M_r 203.3 g/mol)
- Mini-PROTEAN TGX 4-20% SDS PAGE gel, 15-well, 15 μl (*Bio-Rad*, Cat# 456-1096)
- Phenol solution, saturated with 0.1 M citrate buffer, pH 4.3 ± 0.2 (*Sigma*, Cat# P4682, M_r 94.11 g/mol) or phenol, water saturated, stabilized, pH 4.0 (*Applichem*, Cat# A1624, M_r 94.11 g/mol)
- 10 - Phenylmethylsulfonyl fluoride (PMSF, *Sigma*, Cat# P7626, M_r 174.19 g/mol)
- Sarcosyl (*Fisher Sci*, Cat# BP234, M_r 293.38 g/mol)
- Sodium chloride (NaCl , *Fisher Sci*, Cat# S271500, M_r 58.44 g/mol)
- Sodium dodecyl sulfate (SDS, *Fisher Sci*, Cat# BP166-500, M_r 288.38 g/mol)
- 15 - Sodium hydroxide, pellets (NaOH , *Fisher Sci*, Cat# S318-1, M_r 40.00 g/mol)
- Tri(2-carboxyethyl)phosphine hydro-chloride, 10 g ($\text{TCEP} \cdot \text{HCl}$, *Hampton Research*, Cat# HR2-801, M_r 286.65 g/mol)
- Tris base (*Fisher Sci*, Cat# BP152, Stock# 336,000, M_r 121.14 g/mol)
- Tris-HCl (*Sigma*, Cat# T3253, M_r 157.60 g/mol)
- 20 - Tris/glycine buffer (10x) (*Bio-rad*, Cat# 161-0734)

2.3 Enzymes

- Proteinase K (recombinant, *Storeroom*, 100 mg, from Roche Ref # 03 115 879 001, Cat# 301104)

2.4 Oligonucleotides

Calibrator pool 1 consists of 10 equimolar concentrated 5' phosphorylated 3' hydroxyl 22-nt RNA oligonucleotides, which were ordered from Dharmacon at a 0.05 μmol scale. They have no match to the human or mouse genome.

Calibrator (synthesis no.)	Sequence	Extinction coefficient ($\text{L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$)
01 (cali_01_rc)	pUCCACGACGUCUCAUGUAUUUC	191700
02 (cali_04_rc)	pGGGUACCAUACCGGUUGUCUUA	201900
03 (cali_17_rc)	pUCAUGAGUCCGUACCUUGAUUG	201900
04 (cali_18_rc)	pAUCAUUUACGAUUCGGAGCUGU	203100
05 (cali_20_rc)	pGAUAGUUCGGGAUCGCUGUAAC	208400
06 (cali_24_rc)	pUGCUACUCCGAUCUUUAGCCUC	182900
07 (cali_25_rc)	pAGGGCCCUUUAGGCACUAAUAG	209500
08 (cali_27_rc)	pGUAGCUGUCAGUACGUUCGUGC	198000
09 (cali_43_rc)	pUCUAGUUGCGUGAUGGAGAGAA	218000
10 (cali_44_rc)	pAGCCGCAUUUCGUAGUGAUUU	204300

5 p: 5' phosphate

- Measure the concentrations of each calibrator using a spectrophotometer.
- The preparation of diluted solutions of the calibrator pools requires the use of a carrier oligonucleotide to prevent surface adsorption during preparation of the dilution series in the nanomolar concentration range. For example, an 11-nt oligodeoxynucleotide, 5'-TCGAAGTATTC, at a final concentration of 500 nM may be used.
- Prepare 50 μl of a concentrated stock containing 1 μM of each calibrator RNA, which does not require addition of carrier DNA.

10

- Dilute the 1 μM calibrator each stock 1:10 into 500 nM TCGAAGTATTC resulting in a concentration of 0.1 μM each calibrator (10 μl calibrator stock plus 90 μl 500 nM 11-nt carrier DNA).
- Further dilute the 0.1 μM calibrator pool 1:10 in 500 nM TCGAAGTATTC resulting in a calibrator concentration of 10 nM each calibrator (50 μl calibrator dilution plus 450 μl 500 nM 11-nt carrier DNA).
- Further dilute the 10 nM calibrator pool 1:10 in 500 nM TCGAAGTATTC resulting in a calibrator concentration of 1 nM each (50 μl calibrator dilution plus 450 μl 500 nM 11-nt carrier DNA).
- Further dilute the 1 nM calibrator pool 1:10 in 500 nM TCGAAGTATTC resulting in a calibrator concentration of 100 pM each (50 μl calibrator dilution plus 450 μl 500 nM 11-nt carrier DNA).
- Further dilute the 100 pM calibrator pool 1:10 in 500 nM TCGAAGTATTC resulting in a calibrator concentration of 10 pM each (50 μl calibrator dilution plus 450 μl 500 nM 11-nt carrier DNA). **This dilution will be used for plasma and serum samples.**
- Dilute the 10 pM calibrator pool one last time 1:10 to reach a calibrator concentration of 1 pM each (50 μl calibrator solution plus 450 μl 500 nM 11-nt carrier DNA). **This dilution will be used for urine samples.**

Example for using calibrator pool 1:

- The suggested final amount of calibrator per sample is 0.2 μM each oligoribonucleotide for urine and 2 attomol each oligoribonucleotide for serum or plasma per 450 μl of input biofluid.

For urine add 2.8 μl 1 pM each oligoribonucleotide of “Calibrator pool 1” to 1.467 ml of buffer P with 2-mercaptoethanol and methylene blue added to obtain 14 aliquots of 105 μl of denaturing buffer with 0.2 attomol of each RNA per aliquot:

- 2.8 μl x 1 pM of each “calibrator pool 1” RNA / 14 aliquots
- $$= 2.8 \times 10^{-6} \text{ l} \times 10^{-12} \text{ M of each “calibrator pool 1” RNA / 14 aliquots}$$
- $$= 2.8 \times 10^{-18} \text{ mol of each “calibrator pool 1” RNA / 14 aliquots}$$

= 2.8 attomol of each “calibrator pool 1” RNA / 14 aliquots

= 0.2 attomol of each “calibrator pool 1” RNA / aliquot.

2.4 Equipment

- Qubit 2.0 Fluorometer (Invitrogen)
- 5 - A set of pipettes (20 µl, 200 µl, 1000 µl), (e.g. Gilson)
- Repeater pipette (e.g. *eppendorf* M4)
- Spectrofluorometer or fluorescence microplate reader

A. For manual processing/semi-automated processing

- Vacuum manifold 24x, (e.g. *QIAGEN*, QIAval 24 Plus Vacuum Manifold, Cat# 19413)
- 10 - Table top centrifuge (e.g. *SORVALL* Legend Micro 21R centrifuge), set to 4°C
- Table top centrifuge (e.g. *SORVALL* Biofuge pico) or *eppendorf* centrifuge, at room temperature
- *eppendorf* thermomixer set to 10°C
- *eppendorf* thermomixer set to 60°C

15 B. For semi-automated and automated processing

- epMotion 5075 or similar liquid handling system with vacuum manifold option, for fully automated processing also equipped with one thermoplate heater/cooler and the thermomixer option
- Single-channel dispensing tool TS 50 (Cat# 960001010)
- 20 - Single-channel dispensing tool TS 300 (Cat# 960001028)
- Single-channel dispensing tool TS 1000 (Cat# 960001036)
- Eight-channel-dispensing tool TM 50-8 (Cat# 960001044)
- Eight-channel-dispensing tool TM 300-8 (Cat# 960001052)

- Eight-channel-dispensing tool TM 1000-8 (Cat# 960001061)
- Gripper (Cat# 960002270)
- Gripper holder (Cat# 960002211)
- Thermoblock for PCR 96 wells (Cat# 960002083)
- 5 - Thermorack for 24 Safe Lock tubes (Cat# 960002067)
- Deep well plate 96/2000 µl (Cat# 951033561)
- Eppendorf heat sealer (5390 000.024, from Sigma Aldrich)
- Eppendorf heat sealing foil (Cat# 0030127.845, from Sigma Aldrich)
- Centrifuge with holder for 96-well plates (e.g. *SORVALL* Legend RT), set to 4°C

10 2.5 Consumables

2.5.1 RNA extractions from biofluids

Items listed for processing of 24 samples. For manual, semi-automated or automated processing of 48, 72 or 96 samples, multiply by two, three or four, respectively. When performing semi-automated or automated purification only one Zymo-spin™ I 96 filter plate is needed for up to

15 96 samples.

A. For manual processing

- Filter pipet tips (10 µl, 200 µl, 1000 µl)
- 48 x 2 ml *eppendorf* Lo-Bind tubes (VWR, DNA LoBind Tube 2.0 ml, PCR clean, CS/250 (5x PK/50); 22431048, Cat# 470202-584)
- 20 - 24 x 1.5 ml siliconized tubes (VWR, G-Tube® Snap Cap Siliconized Microcentrifuge Tubes, Cat# 22179-004)
- 24 x Zymo-Research Zymo-Spin™ I columns (*Zymo-Research*, Zymo-Spin™ I columns, 50, Cat# C1003-50),

B. For semi-automated and automated processing

- 1x Zymo-Research Zymo-SpinTM I-96 filter plate, Cat# C2004
- 26 x 2 ml microcentrifuge tube
- 380 µl RNase free water
- 1 ml deep well plate 96 well, green border (Eppendorf, Cat# 0030 502.230, Cat#
5 951032760
- 2 ml deep well plate 96 well, green border (Eppendorf, Cat# 0030 502.337, Cat#
951033561)
- 250 µl twin.tec 96 well, semi-skirted, colorless pcr plate (Eppendorf, Cat# 0030 128.575,
Cat# 951020303)
- 10 - epMotion® PCR clean reservoir 100 ml (Eppendorf, Cat# 960051017)

C. Optional. For automated initial sample addition using width adjustable multichannel pipette

- Rainin EA8 1200XLS multichannel and adjustable spacer electronic pipette (Cat#
15 17012331)
- -Rainin LTS 1 ml Filter RT-L1000FLR Low Retention Tips (Cat# 17007954)

2.5.2 Qubit assay

- Qubit RNA HS assay kit (Invitrogen, Cat# Q32852), including Qubit RNA HS assay
dilution buffer and Qubit RNA HS assay dye
- 20 - 2'-OMe-let-7a oligoribonucleotide standard (A₂₆₀ = 0.5)
- 1.5 ml siliconized tubes
- 0.5 ml Qubit assay tubes (500 tubes, *Invitrogen*, Cat# Q32856) or Axygen PCR-05-C
tubes (VWR, part number 10011-830).

2.5.3 Quant-iTTM RiboGreen® RNA assay

- Quant-iT™ RiboGreen® RNA Assay Kit (Life Technologies, Cat# R11490)
 - Quant-iT™ RiboGreen® RNA Reagent
 - 20X TE Buffer, RNase-free
 - Ribosomal RNA standard, 16S and 23S rRNA from E. coli (100 µg/ml)
- 5 - Costar™96-well clear-bottom plates (Fisher Scientific, Cat# 07-200-565)

2.6 Buffers, reagents and enzyme solutions

All buffers should be prepared in advance and stored under the recommended conditions to minimize degradation and microbial growth. All buffers are made using MilliQ deionized and sterile filtered water, from now on referred to as 'water'.

10 2.6.1 Stock solutions

The following stock solutions will be required to prepare working solutions used during the isolation procedure.

2.6.1.1 1 M Tris-HCl, 1000 ml

15 In a 1000 ml glass bottle equipped with a magnetic stir bar, weigh out 157.6 g of Tris-HCl (M_r 157.60 g/mol). Add approx. 800 ml water and dissolve powder.

Transfer solution to 1000 ml graduated cylinder, fill up to 1000 ml with water and return solution to 1000 ml bottle.

Store at room temperature.

2.6.1.2 1 M Tris base, 1000 ml

20 In a 1000 ml glass bottle equipped with a magnetic stir bar, weigh out 121.1 g of Tris base (M_r 121.14 g/mol). Add approx. 800 ml water and dissolve powder.

Transfer solution to 1000 ml graduated cylinder, fill up to 1000 ml with water and return solution to 1000 ml bottle.

Store at room temperature.

2.6.1.3 1 M Buffer Tris-HCl (pH 6.8), 50 ml

Combine 47.4 ml of 1 M Tris-HCl and 2.6 ml of 1 M Tris base in a 50 ml *FALCON* tube.

Volume (Tris base) = $(10^{(\text{pH}-\text{pKa})}/(1+10^{(\text{pH}-\text{pKa})})) \times 1000$ ml; pKa = 8.06 at 25°C.

Dispense into aliquots.

5 Store at room temperature.

2.6.1.4 1 M Buffer Tris-HCl (pH 7.5), 50 ml

Combine 39.2 ml of 1 M Tris-HCl and 10.8 ml of 1 M Tris base in a 1000 ml glass bottle.

Volume (Tris base) = $(10^{(\text{pH}-\text{pKa})}/(1+10^{(\text{pH}-\text{pKa})})) \times 1000$ ml; pKa = 8.06 at 25°C.

Dispense into aliquots.

10 Store at room temperature.

2.6.1.5 1 M Buffer Tris-HCl (pH 8.0), 50 ml

Combine 26.7 ml of 1 M Tris-HCl and 23.3 ml of 1 M Tris base in a 1000 ml glass bottle.

Volume (Tris base) = $(10^{(\text{pH}-\text{pKa})}/(1+10^{(\text{pH}-\text{pKa})})) \times 1000$ ml; pKa = 8.06 at 25°C.

Dispense into aliquots.

15 Store at room temperature.

2.6.1.6 0.5 M EDTA (pH 8.0), 1000 ml

In a 1 l graduated cylinder, add 186.1 g of Na₂EDTA·2H₂O (M_r 372.24 g/mol) to 800 ml water.

Stir vigorously on a magnetic stirrer. Adjust pH to 8.0 with NaOH pellets (~20 g). Add pellets slowly and wait for pH to stabilize. Expect EDTA to dissolve fully before pH can reach 8.0.

20 Adjust volume to 1 liter.

Store at room temperature.

Note: The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to pH 8.0 by adding NaOH.

2.6.1.7 4 M NaOH, 50 ml

Place an appropriately sized tube holder on a scale and tare with a 50 ml *FALCON* tube. Add 8.00 g NaOH pellets (M_r 40.00 g/mol) to this tube. Add water to reach a final weight of 56.3 g. Close lid and vortex until a clear solution has formed.

Store at room temperature.

5 2.6.1.8 4 M Citric acid, 50 ml

Place an appropriately sized tube holder on a scale and tare with a 50 ml *FALCON* tube. Add 42.03 g citric acid monohydrate (M_r 210.14 g/mol) to this tube. Add water to reach a final weight of 64.1 g. Close lid and vortex until a clear solution has formed.

Store at room temperature.

10 2.6.1.9 1 M $MgCl_2$, 50 ml

Place an appropriately sized tube holder on a scale and tare with a 50 ml *FALCON* tube. Add 10.17 g of $MgCl_2 \cdot 6H_2O$ (M_r 203.30 g/mol) to this tube. Add water to reach a final weight of 53.1 g. Close lid and vortex until a clear solution has formed.

Store at room temperature.

15 2.6.1.10 1 M $CaCl_2$, 50 ml

Place an appropriately sized tube holder on a scale and tare with a 50 ml *FALCON* tube. Add 10.95 g of $CaCl_2 \cdot 6H_2O$ (M_r 219.08 g/mol) to this tube. Make sure to return the $CaCl_2 \cdot 6H_2O$ powder to its recommended storage temperature of 4°C immediately after use. Add water to reach a final weight of 53.7 g. Close lid and vortex until a clear solution has formed.

20 Store at room temperature.

2.6.1.11 5 M NaCl, 50 ml

Dissolve 14.61 g NaCl (M_r 58.44 g/mol) in 45 ml water in a 50 ml *FALCON* tube. Adjust the volume to 50 ml with water and vortex.

Store at room temperature.

25 2.6.1.12 0.5 M TCEP, 5 ml

Dissolve 0.72 g TCEP·HCl in 4 ml water in a 15 ml *FALCON* tube. Adjust the volume to 5 ml with water and vortex. Wrap the labeled tube with aluminum foil in order to protect the solution from light. TCEP is light sensitive.

Store at 4°C for up to one week.

5 2.6.1.13 0.63 M SDS solution (20%), 10 ml

Dissolve 2.00 g SDS (M_r 288.37 g/mol) in 8 ml water in a 15 ml *FALCON* tube. To dissolve the SDS, remove the cap of the 15 ml *FALCON* and place upright (using a *FALCON* tube stand) inside the microwave. Heat with 4 x 5 s pulses at 1250 W. The temperature of the solution must not rise above lukewarm; close tube, check and invert after every pulses. Since the volume will
10 decrease as the powdered SDS dissolves, readjust volume to exactly 10 ml with water. Heat the solution again with 1-3 x 5 s pulses at 1250 W inside the microwave using the above procedure and precautions. Do not vortex as the solution will easily foam.

Store at room temperature.

2.6.1.14 Coomassie brilliant blue protein gel staining solution, 1000 ml

15 In a 1 l flask, weigh 500 mg of brilliant blue powder. Then, add 400 ml ethanol, 100 ml acetic acid and fill up to 1 l with water. Close lid and mix by inverting the bottle.

Store at room temperature.

2.6.1.15 Coomassie gel de-staining solution, 1000 ml

In a 1 l flask, add 200 ml methanol to 100 ml acetic acid and fill up to 1 l with water. Close lid
20 and mix by inverting the bottle.

Store at room temperature.

2.6.2 Buffers for sample protein degradation

2.6.2.1 Buffer P (Proteolysis), 50 ml

Never vortex this buffer as it will foam! Only gently invert tubes. For 380 samples. Buffer can be
25 stored at room temperature.

In a 50 ml *FALCON* tube combine:

Reagent or solution	Amount	Buffer concentration
Sodium dodecyl sulfate (SDS)	15 g	30% (w/v)
1 M Buffer Tris-HCl (pH 7.5)	3.3 ml	66 mM
0.5 M EDTA (pH 8.0)	1.98 ml	19.8 mM

Add Buffer Tris-HCl, EDTA solution and 15 ml water in a 50 ml *FALCON* tube. Weigh out SDS powder and add to the previously added solutions. Adjust volume to roughly 50 ml with water.

- 5 Mix by gently inverting the tube multiple times. Adjust volume again to 50 ml with water. Close the tube tightly and invert for 6 hours at room temperature until a clear solution has formed. Adjust volume again to exactly 50 ml with water and invert multiple times to prepare a homogenous solution.

Be prepared to add 2-mercapthoethanol to a Buffer P aliquot just before use.

- 10 Store at room temperature. Do not autoclave.

Refrigeration will cause SDS to precipitate. DO NOT refrigerate. If precipitate forms at room temperature, use 1-3 x 10 s pulses at 1250 W in the microwave and invert repeatedly until all precipitates are dissolved.

2.6.3 Buffers for protein and detergent removal by organic extraction

- 15 2.6.3.1 Buffer ED2, 24.5 ml

This buffer is less acidic and approaches pH 4.5. For 8, 24 and 48 extractions prepare given amount in a 50-ml *FALCON* tube. For 96 samples prepare 2x the amount for 48 extractions in two separate 50-ml *FALCON* tubes. Use filter pipet tips to avoid contamination. Be prepared to add 2-mercapthoethanol to Buffer ED2 just before usage (see section 3.1.2, step 9).

- 20 In a 50 ml *FALCON* tube combine:

Reagent or solution	8 extr.	24 extr.	2x 48	Buffer conc.
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			extr. = 96 extr.	
4 M Citric acid	74 μ l	222 μ l	2x 444 μ l	37.4 mM
4 M Sodium hydroxide solution (NaOH)	92 μ l	275 μ l	2x 550 μ l	
Sarcosyl	33 mg	100 mg	2x 200 mg	0.4% (w/v)
Guanidium thiocyanate (GITC)	1.5 g	4.51 g	2x 9.02 g	1.6 M
Phenol solution, saturated with 0.1 M citrate buffer (pH 4.3)	6.4 ml	19.1 ml	2x 38.2 ml	80% (v/v)

Add citric acid and NaOH solutions to a 50 ml *FALCON* tube, then add GITC and sarcosyl powder. Dissolve all components by adding 19.1 ml buffer equilibrated phenol, close tube and mix by inverting until a clear, colorless liquid is formed. Do not adjust pH or volume, the expected final volume is about 47.5 ml. Short-term storage at 4°C, long-term storage at -20°C.

- 5 Buffer ED may contain 0.15 M NaCl. In the alternative, NaCl is added either before the lysis step or during the addition of the protease since protease activity is higher in the presence of 0.2-0.35 M NaCl.

2.6.4 Buffers for RNA purification using Zymo-Research columns and the vacuum manifold or the Zymo-SpinTM 1-96 plate

- 10 This buffer minimizes precipitation of peptides and clogging of columns, which was observed when aqueous supernatants from organic extractions were applied using other buffers. VB2G appears to work well for all sample types thereby eliminating the need to specifically adjust divalent cation concentrations.

2.6.4.1 Buffer VB2G (Vacuum-manifold Binding) – for serum, plasma (EDTA and citrate) and urine

For 8 and 24, and 32 extractions prepare given amount in a 50-ml *FALCON* tube. For 96 samples prepare 3x the amount for 32 extractions in three separate 50-ml *FALCON* tubes (8 extr. = 15 ml, otherwise 50 ml).

Reagent or solution	8 extr.	24 extr.	3x 32 extr. = 96 extr.	Buffer conc.
Isopropanol (100%)	12.2 ml	36.8 ml	3x 49.1 ml	98.2% (v/v)
1 M MgCl ₂	84.5 µl	271.5 µl	3x 362 µl	7.2 mM
1 M CaCl ₂	28.2 µl	84.5 µl	3x 121 µl	2.4 mM
GITC	1.5 g	4.4 g	5.9 g	1 M
Add before use: 0.5 M TCEP	123 µl	367 µl	500 µl	5.0 mM

Do not adjust volume. Combine isopropanol, MgCl₂ and CaCl₂ solutions and GITC. Mix well and store solution at room temperature. **Before use, add 1% 0.5 M TCEP.** Upon storage, precipitate may form. Shake well before use. It has not been tested how long TCEP will remain stable; according to the literature it should be stable for at least a week.

2.6.4.2 Buffer EWL wash buffer for silica columns during RNA or DNA purification

EWL aqueous concentrate, 50 ml:

Reagent or solution	
5 M NaCl	600 µl
1 M MgCl ₂	450 µl
1 M CaCl ₂	150 µl
Triton X-100	750 µl
GITC	7.08 g

Dissolve GITC in 20 ml of Water, add NaCl, MgCl₂, CaCl₂ solution, add TRITON X-100. Fill up to a final volume 50 ml with water.

EWL Stock solution:

Reagent or solution	8 extr.	24 extr.	96 extr.	Buffer conc.

			= 2x 50 ex	
EWL aqueous concentrate	5 ml	15 ml	4x 15 ml	1x
Isopropanol	11.7 ml	35 ml	4x 35 ml	
Add before use: 0.5 M TCEP	150 µl	0.5 ml	4x 0.5 ml	5 mM

Do not adjust volume. Combine EWL aqueous concentrate and Isopropanol. Mix well and store solution at room temperature. Before use, add TCEP. Shake well before use. It has not been tested how long TCEP will remain stable; according to the literature it should be stable for at least a week.

2.6.5 Buffers for DNA re-extraction from organic phase

2.6.5.1 Buffers EA3, 24.5 ml

For 8, 24 and 48 extractions prepare given amount in a 50-ml *FALCON* tube. For 96 samples prepare 2x the amount for 48 extractions in two separate 50-ml *FALCON* tubes. Use filter pipet tips to avoid contamination.

In a 50 ml *FALCON* tube, combine:

Reagent or solution	8 extr.	24 extr.	96 extr.	Buffer concentration
4 M Citric acid	74 µl	222 µl	2x444 µl	35.5 mM sodium citrate
4 M Sodium hydroxide (NaOH)	110 µl	330 µl	2x660 µl	
Guanidium thiocyanate (GITC)	3.55 g	10.64 g	2x21.27g	4.00 M
H ₂ O	7.5 ml	22.5 ml	45 ml	

Add citric acid and NaOH solutions first, then add GITC. Dissolve all components by adding H₂O, close tube and mix by inverting until a clear, colorless liquid is formed. Do not adjust pH or volume. The final volume is approximately 50 ml. For short-term storage keep a convenient volume of a working stock at 4°C.

2.6.6 *Proteinase K* solutions *and quality control assay*

2.6.6.1 PK (Proteinase K) stock solution (20 mg/ml), 1 ml

Use filter pipet tips to avoid contamination.

First, prepare proteinase K storage buffer, which is then used to dissolve the crystalline enzyme.

- 5 In a 50 ml *FALCON* tube, combine:

Reagent or solution	Amount (ml)	Buffer concentration
Glycerol (100%)	25	50% (v/v)
1 M CaCl ₂	1.5	30 mM
1 M Buffer Tris-HCl (pH 8.0)	2.5	50 mM

- Start by adding 25 ml 100% glycerol (reading from the graded 50 ml tube), then add water to 40 ml followed by all other stock solutions. Mix gently and thoroughly by inverting the tube many times. Adjust volume to 50 ml. Mix again and readjust volume if necessary. Prepare 1 ml
- 5 aliquots in 1.5 ml *ependorf* tubes, and store at -20°C.

- To prepare PK stock solution, pre-chill the above proteinase K storage buffer on ice. Weigh 20 mg of proteinase K (*Roche Diagnostics, recombinant*) in one 1.5 ml *ependorf* tube, then add 1 ml pre-chilled proteinase K storage buffer and immediately transfer to a thermal pack pre-cooled to -20°C. Prepare 80 µl aliquots in pre-chilled 1.5 ml tubes (enough for 24 extractions)
- 10 and store at -20°C

PK stock solution loses activity within minutes should it ever reach room temperature. Always prepare and keep PK stock solution in a thermal pack or other suitable container pre-chilled to -20°C.

2.6.6.2 Buffer PKQC (Proteinase K Quality Control), 10 ml

- 15 In a 15 ml *FALCON* tube, combine:

Reagent or solution	Amount (ml)	Buffer concentration
1 M Buffer Tris-HCl (pH 7.5)	1	100 mM
0.5 M EDTA (pH 8.0)	0.25	12.5 mM
5 M NaCl	0.3	150 mM
SDS solution (20%)	0.5	1% (v/v)

Add all solutions and adjust volume to 10 ml with water. Mix by inverting the tube.

Store at room temperature.

2.6.6.3 1 M Phenylmethylsulfonyl fluoride (PMSF) solution, 1 ml

Dissolve 0.17 g of PMSF powder in 1 ml of 100% DMSO by vortexing.

Store at -20°C.

5 2.6.6.4 Stop mix solution, 10 x 10 µl

SDS gel loading buffer with PMSF.

In a 1.5 ml eppendorf tube, add 8 µl of 1 M PMSF solution to 100 µl of 4x SDS loading buffer. Mix well by vortexing. Store at room temperature.

2.6.7 *Gel loading buffer*

10 2.6.7.1 SDS gel loading buffer (4x), 20 ml

In a 50 ml *FALCON* tube, combine:

Reagent or solution	Amount	Buffer concentration
1 M Buffer Tris-HCl (pH 6.8)	4 ml	200 mM
SDS powder	1.6 g	8%
Glycerol (100%)	8 ml	40%
Bromophenol blue	8 mg	0.4 mg/ml
2-mercaptoethanol	4 ml	2.6 M

Start by adding 8 ml 100% glycerol, then add water to 11 ml followed by all other stock solutions. Mix gently and thoroughly by inverting the tube many times. Adjust volume to 20 ml.

15 Mix again and readjust volume if necessary. Store at room temperature.

3 Procedures

3.1 Introduction

The following RNA extraction protocol was developed to isolate RNA from human urine, serum or citrate-, heparin- and EDTA-plasma samples. The presence of endogenous ribonucleases in these body fluids requires protective steps to recover intact RNA from otherwise protein-bound and thereby protected extracellular RNA. Be aware that biofluids are rich in RNases and DNases and that any spills or contamination of pipetmans, centrifuges or tube holder carry the risk of contaminating and degrading recovered RNA or DNA otherwise free of nucleases.

Using a vacuum manifold, the column-based RNA isolation procedure is at least 2-times faster compared to the usual centrifuge spin protocols.

For sample processing, the specimen is initially mixed with Buffer P (Proteolysis) and heated in order to denature ribonucleoprotein complexes, vesicle-enclosed RNA and RNases. Subsequent proteinase K digestion at 60°C efficiently degrades protein including RNases, eliminating any possibility for RNA degradation due to renaturation of RNases.

A subsequent organic extraction, using custom-made buffers containing guanidinium isothiocyanate (GITC) and phenol/chloroform, allows the transfer of the majority of DNA and hydrophobic peptide fragments into the organic phase while retaining RNA within the aqueous phase.

The solubility of the RNA is subsequently lowered by the addition of isopropanol and divalent cations to the aqueous phase. This solution is applied to a *Zymo-Spin*TM I column and handled on the vacuum manifold. Several wash steps ensure the removal of residual DNA, peptides and salts while RNA of 19-nt or longer is retained on the column matrix.

The bound RNA is eluted from the columns in volumes as small as 15 µl, thereby yielding a maximum final RNA concentration for subsequent procedures.

3.2 Organization of samples and workflow

The following recommendations (especially points 1-3) are intended for the organization of large sample collections into batches of 24 samples, which are subsequently used for small RNA-

based multiplexed cDNA library preparation and high-throughput sequencing. They may however also be relevant for other types of RNA characterization.

1. Avoid combining different biofluid type samples (serum/plasma with urine) into one batch of 24 samples.

5 2. Include “standardized” samples, which are present at least once in each batch in order to assess the level of reproducibility between batches.

3. If necessary, randomize your samples to avoid introducing biases in ligation, amplification or sequencing caused by adapters or sample categories (healthy or diseased), e.g. avoid assigning samples from one category to the same slot of 24 across
10 different batches, therefore barcoding them with the same 3’ adapter.

4. Avoid having sample categories coincide with individual batches, e.g. avoid having one sample category (healthy) that is only present in one batch and another (diseased) that is present only in the other batch.

5. Make sure that all samples contain enough input material (preferably, sample is least a
15 minimum of 475 µl, even more preferably the sample size is 500 µl).

6. Organize the samples you want to use for extraction, make sample lists and print stickers to label the sample tubes.

7. Assess the amount of reagents needed for your purification scale. Make sure you have all the solutions and additives that are required. An overview is given in Table 1S.

20

Reagent (ml)	Number of Samples (Multiples of 8)					
	RNA isolation only			RNA and DNA isolation combined		
	8	24	96	8	24	96
Buffer P working solution	1.0	3.0	11.3	1.0	3.0	11.3
Proteinase K working solution	0.3	0.9	3.1	0.3	0.9	3.1
Buffer ED2 working solution	5.2	15.6	52.2	5.2	15.6	52.2
EA3	-	-	-	5.5	16.5	10.5
VB2G + TCEP	12.1	36.0	133.2	24.2	72.0	266.4
EW + TCEP	19.8	59.4	198.0	39.6	118.8	396.0
100% ethanol	9.8	24.4	98.0	19.6	48.8	196.0
80% ethanol	5.0	15.0	50.0	10.0	30.0	100.0

Table 1S: Overview of reagent solutions needed for RNA isolation of 8, 24 or 96 samples.

3.3 Manual RNA extraction of up to 24 samples

DO NOT cool, heat or centrifuge samples unless instructed by the protocol. If not specified,
 5 carry out steps at room temperature. Use thermomixers for both heating and mixing. The term
 ‘tube holder’ refers to a piece of equipment of the *eppendorf thermomixer* and *thermomixer R*
 series that allows the transfer of 24 tubes at a time between two mixers. Use filter pipet tips at all
 times to minimize contamination of pipette shafts with ribonucleases from the sample. Clean
 your workplace and all technical equipment with 70% ethanol prior to starting to reduce possible
 10 contamination by particles carrying RNases. Change gloves frequently or wash gloved hands and
 dry them using a paper towel, especially after handling nuclease active sample material. Keep
 centrifuges and pipets clean. Mix working solutions by vortexing unless otherwise instructed
 when adding additives such as methylene blue, 2-mercaptoethanol, and TCEP.

Before choosing the sample denaturation option in this protocol (see 0 step 5), please also
 15 consider looking into the alternative sample denaturation protocol (section 3.3.2), which
 combines sample aspiration, which combines sample aspiration, addition of Proteinase K
 working solution, and mixing with buffer P into a single work step using a programmable Rainin
 multichannel pipette. This approach may be used in automated sample processing.

When using the repeater pipette be careful not to aspirate air bubbles. Also test the dispense function at least once before applying aspirated solutions to samples since every first dispense tends to not contain the entire adjusted volume.

Once started, the procedure should be carried out continuously. Try to avoid pauses during the preparation procedure. Do not process more than 24 samples at a time.

Duration: Initial preparation: 30 min. Extraction: 30 min per batch of 24 samples. Column purification: 60 min per batch of 24. Optional DNA purification and column purification: 90 min per batch of 24.

3.3.1 Preparation of reagents and consumables (manual processing)

1. Organize your samples (see section 3.2) but keep them frozen at this time.
2. Preheat one heatblock to 60°C and a second heatblock to 10°C. Make sure these temperatures are reached.
3. Label a first set of 8x or 24x 2 ml *ependorf Lo-Bind* tubes, e.g. 1.1-1.24 (set 1).
4. Label a second set of 8x or 24x 2 ml *ependorf Lo-Bind* tubes, e.g. 2.1-2.24 (set 2).
- Optional:** If DNA isolation is intended label a third set of 8x or 24x 2 ml *ependorf Lo-Bind* tubes, e.g. 3.1-3.24 (set 3).

5. Prepare Buffer P working solution:

For 8 samples, aliquot 1 ml of Buffer P with 5 µl methylene blue stock into one 2 ml tube and add 19 µl β-mercaptoethanol and 1.9 µl “calibrator pool 1”, either c = 1 pM each oligonucleotide (urine) or c = 10 pM each oligonucleotide (serum or plasma).

For 24 samples, aliquot **two aliquots** of 1.47 ml of Buffer P with 7.4 methylene blue stock into two separate 2 ml tubes and to each aliquot add 28.0 µl β-mercaptoethanol and 2.8 µl “calibrator pool 1”, c = 1 pM each oligonucleotide (urine) or c = 10 pM each oligonucleotide (serum or plasma).

Make sure that buffer P working solution is properly mixed by tumbling or vortexing and is free of bubbles. Buffer P solution may get discolored if kept too long and the level of reducing agent may have dropped due to oxidation. Centrifuge buffer P at room temperature.

6. Aliquot 8x or 24x 105 µl of Buffer P working solution (prepared in step 5) into set 1 tubes.
7. Aliquot 8x, or 24x, or 96x 1200 µl (twice 600 µl) of Buffer VB2G with TCEP added (0) into each tube set. **Optional:** if DNA re-extraction from the organic phase is intended, prepare & fill third set of tubes (set 3) accordingly.
- 5 8. Label 8x or 24x 1.5 ml siliconized tubes with sample identification number (e.g. 1.1 to 1.24, 2.1 to 2.24, etc.). Keep lids closed. The eluted RNA will be collected in these tubes at the end of the purification process. **Optional:** For DNA re-extraction from organic phase: prepare another set of tubes.
9. **Prepare Buffer ED working solution:**
10 **For 8 samples**, aliquot 6.75 ml Buffer ED2 (9x master mix) in a 15 ml *FALCON* tube. Before continuing with RNA extraction, add 23.6 µl 2-mercaptoethanol to Buffer ED.
For 24 samples, aliquot 18.75 ml Buffer ED2 (25x master mix) in a 50 ml *FALCON* tube. Before continuing with RNA extraction, add 65.5 µl 2-mercaptoethanol to Buffer ED.
10. **Prepare EWL working solution**
15 **For 8 samples**, aliquot 17 ml EWL stock solution and add 170 µl of 0.5M TCEP (section 2.6.4.2) into 50 ml *FALCON* tube.
For 24 samples, aliquot 50 ml EWL stock solution and add 500 µl of 0.5M TCEP added (section 2.6.4.2) into 50 ml *FALCON* tube.
Optional: if DNA re-extraction from the organic phase is intended, prepare twice the
20 amounts listed before.
11. **Prepare 100% and 80% ethanol**
For 8 samples, prepare 10 ml 80% ethanol and 10 ml 100% ethanol in 15 ml *FALCON* tubes. **For 24 samples**, prepare 30 ml 80% ethanol and 30 ml 100% ethanol in 50 ml *FALCON* tubes.
25 **Optional:** if DNA re-extraction from the organic phase is intended, prepare twice the amounts listed before.
12. **Prepare Proteinase K working solution:**
For 8 samples, aliquot 250 µl 5 M NaCl and 30 µl proteinase K stock (20 mg/ml) into a 1.5 ml reaction tube, mix gently by pipetting.

For 24 samples, aliquot 750 µl 5 M NaCl and 90 µl proteinase K stock (20 mg/ml) into a 1.5 ml reaction tube, mix gently by pipetting. If a multichannel pipette is to be used for sample aspiration/mixing/proteinase K addition, aliquot PK/NaCl solution as 105 µl fractions into 8 tubes.

5 *Dispose Proteinase K working solution, and prepare a fresh working solution for the next isolation. The 20 mg/ml proteinase K stock should always be kept at -20°C using suitable equipment (cooling containers, etc.). At this concentration the enzyme quickly loses activity at all other temperatures. Aliquots of proteinase K solution that reached room temperature should be disposed.*

10 **13. Optional: For DNA re-extraction from organic phase:**

For 8 samples, prepare 6.75 ml buffer EA3 in a 15 ml *FALCON* tube.

For 24 samples, prepare 18.75 ml buffer EA3 in a 50 ml *FALCON* tube.

Also: prepare a third set of tubes (step 4), double the amount of VB2G (step 7), EWL working solution (step 10), 80% ethanol & 100% ethanol (step 11).

15 **3.3.2 Denaturation and proteolytic digestion of samples to inactivate ribonucleases**

1. Organize your workspace: Have all three tube sets ready in individual tube holders. Tube set 1 containing buffer P working solution, tube set 2 and (optional for DNA isolation) tube set 3 filled with TCEP-added VB2G. Have all required pipettes and tips in place.

20 If you want to use a repeater pipette for applying Proteinase K working solution (step 6), wrap a 1-ml repeater pipette tip in aluminum foil inside a zip lock bag and pre-chill in -20°C freezer for each batch of samples that you want to process.

2. Preheat one *eppendorf* thermomixer to 60°C and a second *eppendorf* thermomixer to 10°C. Make sure these temperatures are reached.

25 3. Thaw biofluid samples. For samples in 1.5 ml microcentrifuge tubes thawed with warm air, e.g. by placing sample tubes in tube holders that allow airflow to pass through inside a incubator/shaker pre-heated to 37°C shaking at 130 rpm for 5-10 min. Watch samples during thawing procedure, remove from incubator when thawed and store at room temperature.

Homogenize samples by inverting tube racks with sample tubes several times to allow

precipitates to dissolve and liquid fractions to mix. Thawed samples are typically maintained at room temperature. DO NOT put thawed urine samples on ice, since this will cause precipitation of salts and other compounds in some samples.

If the provided sample volume is very low, spin tubes at 50xg for 10 s to minimize losses due to adhesion of sample to the lids.

Organize sample tubes in a separate tube rack and check sample order.

4. Preheat all 8 or 24 2-ml tubes (tube set 1) containing 105 µl of Buffer P working solution (prepared in section 3.3.1, step 4 & 5) to 60°C in *eppendorf* thermomixer for 2 min. Keep tubes at 60°C in *eppendorf* thermomixer until instructed to remove them. At the end of the incubation period open all lids of tube set 1 and of your sample tubes.

5. **Addition of biofluid samples:**

Alternative 1: Switch *eppendorf* thermomixer to interval mixing mode at 60°C featuring a 3 s mixing phase at 1200 rpm followed by a 3 s pause. During the mixing phase add 450 µl of sample (plasma, serum or urine) to a “set 1” tube containing Buffer P working solution. Aspirate the next sample and position pipette tip with next sample into the next “set 1” tube during mixing pause. You may need to adjust the pause time according to your work speed.

Alternative 2: Switch *eppendorf* thermomixer to continuous mixing mode at 60°C but do not turn on mixing. Add 450 µl biofluid sample to “set 1” tube and immediately after addition of each sample, press quick mixing button for 3 s. Repeat by adding the next biofluid sample into the next “set 1” tube. Upon each sample addition, press the quick mixing button to allow rapid mixing.

Partially mixed solutions may expose RNA released from proteins to more stable, partially denatured ribonucleases followed by its degradation.

Alternative 3: Do not switch on mixing function of *eppendorf* thermomixer. Add 450 µl biofluid sample to “set 1” tube and mix by pipetting up and down at least five times. This can be done reproducibly using automated single channel or multi channel pipettes.

If using mixing by eppendorf thermomixer shaking DO NOT EXCEED the recommended mixing speed or time to prevent spillage of the sample. It may be necessary to determine the optimal speed for mixing when using different equipment. Make sure the sample is properly mixed and does not spill out during mixing with open lids!

6. Proteolytic digestion:

Alternative 1: Adjust *eppendorf* thermomixer to 1200 rpm but do not turn on. Add 28 µl Proteinase K working solution (prepared 0, step 12) to each sample. Without closing the tube, mix by activating the thermomixer for a 2 s pulse at 1200 rpm after each addition.

- 5 **Alternative 2:** For faster addition of proteinase K allowing for higher throughput use a repeater pipette and a pre-chilled 1.0-ml repeater tip (from step 1) to dispense Proteinase K working solution (prepared 0, step 12). Avoid formation of air-bubbles when loading solution. Set dispense volume to 30 µl. Switch *eppendorf* thermomixer to interval mixing mode at 60°C featuring a 2 s mixing phase at 1200 rpm followed by a 2 s pause and position repeater pipette
- 10 above the tube during pause time while ejecting before or during mixing phase. Strictly aim for the liquid to be ejected to the center of the tube, not the tube wall. The viscous PK stock solution sticking to the tube wall at high temperatures will be rendered inactive otherwise.
- DO NOT EXCEED suggested mixing speed or time to prevent spillage of the sample. It may be necessary to determine the optimal speed for mixing when using different equipment. Make sure*
- 15 *the sample is properly mixed and does not spill out during mixing with open lids!*

7. Close all lids, perform protease digestion for 10 min at 60°C, during this period shake gently at 550 rpm. Then stop *eppendorf thermomixer* mixing but do not turn off heating.

8. Organic extraction

- 20 **Alternative 1:** Open tubes and add 513 µl of Buffer ED2 working solution containing 2-mercaptoethanol (from 0 step 9). Close tubes after addition, mix vigorously at 1400 rpm for 30 s.

- Alternative 2:** Use repeater pipette to dispense Buffer ED2 working solution containing 2-mercaptoethanol (from 0 step 9). Set dispensing volume to 550 µl and aim for the liquid to be ejected against the lower half of the tube wall, not the liquid interface.
- 25 Aiming directly at the center of the tube will result in spilling of tube contents. Close tubes after addition, mix vigorously at 1400 rpm for 30 s.

9. Transfer entire tube holder (with 24 samples) to second *eppendorf* thermomixer previously set to 10°C. Cool down sample by mixing vigorously at 1400 rpm for 1 minute.

10. Centrifuge samples in tube holder for 30 s at 50xg to prevent contamination when opening tubes for next step.
11. Add 103 µl chloroform to each tube. Close tubes, mix vigorously at 1400 rpm for 30 s at 10°C. *[or: Close tubes, vortex tubes by hand for 30 s at full speed before proceeding. If previously 550 µl of Buffer ED2 working solution had been added using a repeater pipette use 110 µl chloroform instead, the chloroform: ED2 ratio should be 1:5.*
12. Centrifuge all tubes at full speed in a microcentrifuge at 4°C for 5 min.
13. Remove all tubes from the centrifuge, keep at room temperature from now on.
14. Transfer 650 µl from the upper aqueous phase to numbered tubes containing Buffer VB2G (containing TCEP as prepared in section 3.3.1 step 7). Try to avoid carrying over any white interphase material if present. **Optional:** Keep the organic phase if DNA re-extraction is intended.
15. Vortex. A precipitate may form. DO NOT centrifuge at high speed as an RNA pellet may form that remains behind in the tube.

3.3.3 *Optional: Manual DNA re-extraction from organic phases*

1. Add 500 µl of Buffer EA3 to each organic phase (from section 3.3.2, step 14). Close tubes after addition, mix vigorously at 1400 rpm for 30 s.
When using the repeater pipette to dispense Buffer EA3, aim for the liquid to be ejected against the lower half of the tube wall, not the liquid interface. Aiming directly at the center of the tube will result in spilling of tube contents.
2. Centrifuge all tubes at full speed in a microcentrifuge at 4°C for 5 min.
3. Remove all tubes from the centrifuge, keep at room temperature from now on.
4. Transfer 600 µl from the upper aqueous phase to numbered tubes containing buffer VB2G, prepared in section 3.3.1, step 7. DO NOT forget to add TCEP in advance. Try to avoid carrying over any white interphase material if present.

5. Vortex. A precipitate may form. DO NOT centrifuge at high speed as an RNA pellet may form that remains behind in the tube.

3.3.4 Column purification of nucleic acids (for both DNA and RNA isolation)

1. Load the vacuum manifold with 8 or 24 Zymo-Spin™ I columns for parallel processing of all samples.
2. Connect the vacuum manifold to a vacuum source (in-house lab vacuum valve is sufficient). Turn on vacuum, leave on at all times until prompted to turn off.
3. Carefully pour the isopropanol-containing solution/suspension of each tube (from section 3.3.2, step 15) onto the respective column mounted on the manifold.
- 10 4. Wash spin columns with 900 µl EWL working solution (from section 3.3.1 step 10), dispense quickly to remove remaining liquid drops on column walls.
5. Wash spin columns for a second time with 900 µl EWL working solution with (from section 3.3.1 step 10), using previous conditions.
6. Wash spin columns with 900 µl 100% ethanol (from section 3.3.2, step 11) using previous conditions.
- 15

Omitting this step will decrease sample yield at molecular ranges smaller than 45 nt.

7. Wash spin columns with 500 µl 80% ethanol using previous conditions (from section 3.3.2, step 11).
8. Wash spin columns with 500 µl 80% ethanol using previous conditions (from section 3.3.2, step 11).
- 20
9. Turn off vacuum. Carefully remove the first column to release the vacuum.
10. Transfer all Zymo-Spin™ I columns into fresh 2-ml collection tubes (use any 2-ml microcentrifuge tube) and place into a microcentrifuge at room temperature.
11. Spin at full speed ($\geq 13,000$ rpm) for 5 min to dry silica matrix.

12. Carefully transfer columns into earlier pre-numbered, siliconized 1.5 ml collection tubes (prepared in section 3.3.2, step 8). Discard the 2 ml collection tubes with any liquid inside.

13. To elute, apply 20 µl 10 mM TRIS-HCl, pH 7.4 directly onto the filter matrix.

5 14. Place all *Zymo-Spin*TM I column-collection tube assemblies into a microcentrifuge.

15. Incubate for 1 min.

16. To collect eluates, spin at full speed ($\geq 13,000$ rpm) for 1 min.

17. Approximately 18 µl of liquid are collected in each siliconized tube (step 12), which is stored at -20°C. Discard *Zymo-Spin*TM I columns after elution.

10 18. Store eluates at -20°C.

3.4 Semi-automated RNA extraction of up to 96 samples

DO NOT cool, heat or centrifuge samples unless instructed by the protocol. If not specified, carry out steps at room temperature. Use thermomixers for both heating and mixing. Tube holder refers to a piece of equipment of the *epENDORF thermomixer* and *thermomixer R* series that
15 allows the transfer of 24 tubes at a time between two mixers. Use filter pipet tips at all times. Clean your workplace and all technical equipment with 70% ethanol prior to starting to reduce possible contamination by particles carrying RNases. Change gloves frequently or wash gloved hands and dry them using a paper towel, especially after handling nuclease active sample material. Keep centrifuges and pipets clean. Mix working solutions by vortexing unless
20 otherwise instructed when adding additives such as methylene blue, 2-mercaptoethanol, and TCEP.

Before choosing the sample denaturation option in this protocol (see 3.3.2 step 5), please also consider looking into the alternative sample denaturation protocol (section 3.6), which combines sample aspiration, addition of Proteinase K working solution, and mixing with buffer P into a
25 single work step using a programmable Rainin multichannel pipette. This approach may be used in automated sample processing.

When choosing to use the repeater pipette be careful not to aspirate air bubbles when aspirating solutions. Also test the dispense function at least once before applying aspirated solutions to samples since every first dispense tends to not contain the entire adjusted volume.

Once started, the procedure should be carried out continuously. Try to avoid pauses during the preparation procedure. Organize your samples in batches of 24 samples (if applicable). For sample denaturation, RNA extraction and optional DNA extraction do not process more than 24 samples at a time (manual part). For column purification do not process more than 96 samples at a time (automated part).

Duration: Initial preparation: 30 min for 96 samples. Extraction: 30 min per batch of 24 samples. Automated column purification: 90 min. Optional DNA purification and automated column purification: 120 min.

3.4.1 Preparation of reagents and consumables (semi-automated processing)

1. Preheat one heatblock to 60°C and a second heatblock to 10°C. Make sure these temperatures are reached.

2. Label one set of 8x or 24x or 96x 2 ml *eppendorf Lo-Bind* tubes, e.g. 1-24, set 1.

3. When running in low or intermediate throughput (8 to 24 samples), label another set of 8x or 24x 2ml *eppendorf Lo-Bind* tubes, e.g. 1-24, set 2, and place into epMotion 5075 tube holder. For higher throughput use a 2 ml 96x deep well plate, labeled as set 2.

Optional : If DNA isolation is intended, label a third set or prepare another 2 ml 96x deep well plate accordingly (set 3). When processing up to 48 samples, you may use the second half of the 2 ml 96x deep well plate.

4. **Prepare Buffer P working solution:**

For 8 samples, aliquot 1 ml of Buffer P with 5 µl methylene blue into one 2 ml tube and add 19 µl 2-mercapthoethanol and 1.9 µl “calibrator pool 1”, either c = 1 pM each oligonucleotide (urine) or c = 10 pM each oligonucleotide (serum or plasma). **For 24 samples**, prepare **two aliquots** of 1.47 ml of Buffer P with 7.4 methylene blue stock in two separate 2 ml tubes and to each aliquot add 28.0 µl β-mercaptoethanol and 2.8 µl “calibrator pool 1”, c = 1 pM each oligonucleotide (urine) or c = 10 pM each

oligonucleotide (serum or plasma).

For 96 samples, aliquot 11 ml buffer P with 55 μ l methylene blue into a 15 ml tube and add 209 μ l 2-mercaptoethanol and 20.9 μ l “calibrator pool 1”, $c = 1$ pM each oligonucleotide (urine) or $c = 10$ pM each oligonucleotide (serum or plasma).

5 *It is important buffer P is properly mixed by tumbling or vortexing and is free of bubbles. Buffer P solution may get discolored if kept too long and the level of reducing agent may have dropped due to oxidation. Buffer P is centrifuged at room temperature.*

5. Use epMotion 5075 script “Xx_BufferP_aliq “ to dispense 8x or 24x or 96x 105 μ l of Buffer P with additives from step 4 into tube set 1 where X=8, 24, or 96.

10 6. Use epMotion 5075 to aliquot 1200 μ l (twice 600 μ l) of TCEP-added buffer VB2G (0) into a 2-ml 96x deep well plate (set 2) using the script “S1VBadd only 96s1” for 96 samples. For 48 or 24 samples use the S1 file ending in 48samp or 24samp, respectively).
Optional: if DNA re-extraction from the organic phase is intended, prepare and fill a third set of tubes (set 3) accordingly.

15 *After dilution in the experimental setup by addition of 650 μ l aqueous RNA sample, as obtained after organic extraction, Buffer concentrations correspond to: VB2G: 15 mM $MgCl_2$ and 5 mM $CaCl_2$, approx. 1 M GITC.*

20 7. Label 96 well PCR plate “elution plate” with sample identification information. Seal plate with adhesive plastic film. The eluted RNA will be collected in this plate at the end of the purification process. **Optional:** For DNA re-extraction from organic phase: prepare another PCR plate OR use the second half of the PCR plate (for up to 48 samples).

8. **Prepare Buffer ED2 working solution:**

For 8 samples aliquot 6.75 ml Buffer ED2 (9 x master mix), add 23.6 μ l 2-mercaptoethanol and fill into 100 ml tub reservoir.

25 **For 24 samples** aliquot 18.75 ml Buffer ED2 (25 x master mix), add 65.5 μ l 2-mercaptoethanol and fill into 100 ml tub reservoir.

For 96 samples aliquot 75 ml Buffer ED2 (100 x master mix), add 262 μ l 2-mercaptoethanol and fill into 100 ml tub reservoir.

9. Prepare EWL working solution

For 8 samples, aliquot 17 ml EWL stock solution, add 170 μ l TCEP and fill into a 100 ml tub reservoir.

For 24 samples, aliquot 50 ml EWL stock solution, add 500 μ l TCEP and fill into a 100 ml tub reservoir.

For 96 samples, aliquot 2x 100 ml EWL stock solution, add 2x 1000 μ l TCEP, and fill each aliquot into 100 ml tub reservoir.

Optional: if DNA re-extraction from the organic phase is intended, prepare twice the amounts listed before.

10. For 8 samples, prepare 10 ml 80% ethanol and 100% ethanol, fill solutions into 100 ml tub reservoirs.

For 24 samples, prepare 30 ml 80% ethanol and 100% ethanol, fill solutions into 100 ml tub reservoirs.

For 96 samples, prepare 100 ml 80% ethanol and 100% ethanol, fill solutions into 100 ml tub reservoirs.

Optional: if DNA re-extraction from the organic phase is intended, prepare twice the amounts listed before.

11. Prepare Proteinase K working solution:

For 8 samples, aliquot 250 μ l 5 M NaCl and 30 μ l proteinase K stock (20 mg/ml) into a 1.5 ml reaction tube, mix gently by pipetting.

For 24 samples, aliquot 750 μ l 5 M NaCl and 90 μ l proteinase K stock (20 mg/ml) into a 1.5 ml reaction tube, mix gently by pipetting. If a multichannel pipette is to be used for proteinase K addition, aliquot PK/NaCl solution as 105 μ l fractions into 8 separate 1.5 ml tubes.

For 96 samples, aliquot 2750 μ l 5 M NaCl and 330 μ l proteinase K stock (20 mg/ml) into a 5 ml reaction tube, mix gently by pipetting. If a multichannel pipette is to be used for sample proteinase K addition, aliquot PK/NaCl solution as 385 μ l fractions into 8 separate 1.5 ml tubes.

Dispose Proteinase K working solution, and prepare a fresh working solution for the next isolation. The 20 mg/ml proteinase K stock should always be kept at -20°C using suitable equipment (cooling containers, etc.). At this concentration the enzyme quickly loses

activity at all other temperatures. Aliquots that reach room temperature should be disposed of right away.

12. Optional: For DNA re-extraction from organic phase:

For 8 samples, prepare 6.75 ml buffer EA3 in a 100 ml tub reservoir.

5 **For 24 samples,** prepare 18.75 ml buffer EA3 in a 100 ml tub reservoir.

For 96 samples, prepare 75 ml EA3 in a 100 ml tub reservoir.

Also: prepare, double the amount of VB2G (step 6), EWL (step 9), 80% & 100% ethanol (step 10) and prepare a third set of tubes or another 96-well plate filled with VB2G (step 6)

10 **3.4.2 Initial denaturation and proteolytic digestion of sample to inactivate ribonucleases - manual part**

1. Organize your workspace: Have all three tube sets ready in individual tube holders. Tube set 1 containing buffer P working solution, tube set 2 and (optional for DNA isolation) tube set 3 filled with buffer VB2G with TCEP added. Have all required pipettes and tips in place.

If you want to use a repeater pipette for applying Proteinase K working solution (see 0 step 6), wrap a 1-ml repeater pipette tip in aluminum foil inside a zip lock bag and pre-chill in -20°C freezer for each batch of samples that you want to process.

2. Preheat one heatblock to 60°C and a second heatblock to 10°C. Make sure these temperatures are reached.

3. Thaw biofluid samples. For samples in 1.5 ml microcentrifuge tubes are thawed with warm air, e.g. by placing sample tubes in tube holders that allow airflow to pass through inside a incubator/shaker pre-heated to 37°C shaking at 130 rpm for 5-10 min. Watch samples during thawing procedure, remove from incubator when thawed and store at room temperature.

Homogenize samples by inverting tube racks with sample tubes several times to allow precipitates to dissolve and liquid fractions to mix. Thawed samples are maintained at room temperature. Urine samples are never thawed on ice, since this will cause precipitation of salts and other compounds in some samples.

If the provided sample volume is very low, spin tubes at 50xg for 10 s to minimize losses due to adhesion of sample to the lids.

Organize sample tubes in a separate tube rack and check sample order.

4. Preheat all 8 or 24 2-ml tubes (tube set 1) from one batch containing 105 µl of Buffer P working solution (prepared in section 3.4.1, step 4 & 5) to 60°C in *eppendorf* thermomixer for 2 min. Keep tubes at 60°C in *eppendorf* thermomixer at all times until instructed to remove them. At the end of the incubation period open all lids of “set 1” tubes and the biofluid samples.

5. **Addition of biofluid samples:**

Alternative 1: Switch *eppendorf* thermomixer to interval mixing mode at 60°C featuring a 3 s mixing phase at 1200 rpm followed by a 3 s pause. During the mixing phase add 450 µl of sample (plasma, serum or urine) to a “set 1” tube containing Buffer P working solution. Aspirate the next sample and position the pipette tip with next sample into the next “set 1” tube during mixing pause. You may need to adjust the pause time according to your work speed.

Alternative 2: Switch *eppendorf* thermomixer to continuous mixing mode at 60°C but do not turn on mixing. Add 450 µl biofluid sample to “set 1” tube and immediately after addition of each sample, press quick mixing button for 3 s. Repeat by adding the next biofluid sample into the next “set 1” tube. Upon each sample addition, press the quick mixing button to allow rapid mixing.

Partially mixed solutions may expose RNA released from proteins to more stable, partially denatured ribonucleases followed by its degradation.

Alternative 3: Do not switch on mixing function of *eppendorf* thermomixer. Add 450 µl biofluid sample to “set 1” tube and mix by pipetting up and down at least five times. This can be done reproducibly using automated single channel or multi channel pipettes.

If mixing by eppendorf thermomixer DO NOT EXCEED the recommended mixing speed or time to prevent spillage of the sample. It may be necessary to determine the optimal speed for mixing when using different equipment. Make sure the sample is properly mixed and does not spill out during mixing with open lids!

6. Proteolytic digestion:

Alternative 1: Adjust *eppendorf* thermomixer to 1200 rpm but do not turn on. Add 28 μ l Proteinase K working (prepared 0, step 12) to each sample. Without closing the tube, mix by activating the thermomixer for a 2 s pulse at 1200 rpm after each addition.

5 **Alternative 2:** For faster addition of proteinase K allowing for higher throughput use a repeater pipette and a pre-chilled 1-ml repeater tip (from step 1) to dispense Proteinase K working solution (prepared 0, step 12). Avoid formation of air-bubbles when loading solution. Set dispense volume to 30 μ l. Switch *eppendorf* thermomixer to interval mixing mode at 60°C featuring a 2 s mixing phase at 1200 rpm followed by a 2 s pause and position
10 repeater pipette above the tube during pause time while ejecting before or during mixing phase. Strictly aim for the liquid to be ejected to the center of the tube, not the tube wall. The viscous PK stock solution sticking to the tube wall at high temperatures will be rendered inactive otherwise.

*DO NOT EXCEED suggested mixing speed or time to prevent spillage of the sample. It may
15 be necessary to determine the optimal speed for mixing when using different equipment. Make sure the sample is properly mixed and does not spill out during mixing with open lids!*

7. Close all lids, perform protease digestion for 10 min at 60°C, during this period shake gently at 550 rpm. Then stop *eppendorf thermomixer* mixing but do not turn off heating.

8. Organic extraction

20 **Alternative 1:** Open tubes and add 513 μ l of Buffer ED2 working solution containing 2-mercaptoethanol (from 0 step 9). Close tubes after addition, mix vigorously at 1400 rpm for 30 s.

Alternative 2: Use repeater pipette to dispense Buffer ED2 containing 2-mercaptoethanol (from 0 step 9). Set dispensing volume to 550 μ l and aim for the liquid
25 to be ejected against the lower half of the tube wall, not the liquid interface. Aiming directly at the center of the tube will result in spilling of tube contents. Close tubes after addition, mix vigorously at 1400 rpm for 30 s.

9. Transfer entire tube holder (with 24 samples) to second *eppendorf* thermomixer previously set to 10°C. Cool down sample by mixing vigorously at 1400 rpm for 1 min.

10. Centrifuge samples in tube holder for 30 s at 50xg to prevent contamination when opening tubes for next step.
11. Add 103 µl chloroform to each tube. Close tubes, mix vigorously at 1400 rpm for 30 s at 10°C. *[or: Close tubes, vortex tubes by hand for 30 s at full speed before proceeding. If previously 550 µl of Buffer ED2 working solution had been added using a repeater pipette use 110 µl chloroform instead, the chloroform: ED2 ratio should be 1:5.*
12. Centrifuge all tubes at full speed in a microcentrifuge at 4°C for 5 min.
13. Remove all tubes from the centrifuge, keep at room temperature from now on.
14. Transfer 650 µl from the upper aqueous phase to numbered tubes or 2-ml deep well plate containing buffer VB2G with TCEP added 0, step 6. Try to avoid carrying over any white interphase material if present. **Optional:** Keep the organic phase if DNA re-extraction is intend
15. Vortex the tubes, or heat-seal the plate and mix by inverting 10 times. A precipitate may form. DO NOT centrifuge at high speed as an RNA pellet may form that remains behind in the tube.

3.4.3 *Optional: Manual DNA re-extraction from organic phase, manual part*

1. Add 500 µl of Buffer EA3 to each tube (from section 3.4.2, step 14). Close tubes after addition, mix vigorously at 1400 rpm for 30 s.
When using the repeater pipette to dispense Buffer EA3, aim for the liquid to be ejected against the lower half of the tube wall, not the liquid interface. Aiming directly at the center of the tube will result in spilling of tube contents.
2. Centrifuge all tubes at full speed in a microcentrifuge at 4°C for 5 min.
3. Remove all tubes from the centrifuge, keep at room temperature from now on.
4. Transfer 600 µl from the upper aqueous phase to deep well plate containing buffer VB2G with TCEP added (see 0 step 6). Try to avoid carrying over any white interphase material if present.

5. Vortex. A precipitate may form. DO NOT centrifuge at high speed as an RNA pellet may form that remains behind in the tube.

3.4.4 Column purification of nucleic acids, automated part (both RNA and DNA isolation)

1. Load the vacuum manifold with an unused *Zymo-I* filter plate for parallel processing of all samples. Make sure that “set 2” or “set 3” plate is placed into epMotion 5075. “Set 2” and “set 3” plate refer to VB2G-plate mixed with aqueous phase from organic extraction (section 3.4.2, step 15) or organic re-extraction (section 3.4.3, step 5), respectively.
2. Start epMotion 5075 automated exRNA isolation script “S6-ldfill1+2 96sl” in the folder labeled “RNA only 96sl”. From here on out, the script names given will be for 96 samples in this folder. For 24 or 48 samples, find the corresponding script in the folders “DNA + RNA 24 samples” or “RNA 48 samples”, respectively.
3. epMotion 5075 loads first portion (1 ml) of isopropanolic aqueous phase (from section 3.4.2, step 15) onto *Zymo-I* filter plate followed by application of 600 mPa vacuum for 6 min. Confirm that all liquid was applied to the filter plate or apply vacuum for a longer time.
4. epMotion 5075 loads first portion (1 ml) of isopropanolic aqueous phase (from section 3.4.2, step 15) onto *Zymo-I* filter plate followed by application of 600 mPa vacuum for 7 min and 900 mPa vacuum for 1 min. Confirm that all liquid was applied to the filter plate or apply vacuum for a longer time.
5. Start epMotion 5075 automated exRNA isolation script “S10-Wash pt1 96sl” to apply 970 µl Buffer EWL working solution (from section 3.4.1, step 9) to sample-loaded columns (step 3), followed by application of 800 mPa vacuum for 7 min and high-vacuum for 1 min.
6. epMotion 5075 loads columns with 970 µl Buffer EWL working solution (from 3.4.1, step 9), and applies 600 mPa vacuum for 7 min, followed 900 mPa vacuum for 1 min. Confirm that all liquid was applied to the filter plate or apply vacuum for a longer time.
7. Start epMotion 5075 automated exRNA isolation script “S11-Wash pt2 elute 96sl” to load columns in filter plate with 970 µl 100% ethanol (section 3.4.1, step 10), followed

by vacuum for 7 min and high-vacuum for 1 min.

Omitting this step will result in sample loss, especially at molecular ranges smaller than 45 nucleotides.

- 5 8. epMotion 5075 loads columns with 500 µl 80% ethanol (section 3.4.1, step 10), followed by vacuum for 5 min and high-vacuum for 1 min.
9. epMotion 5075 loads columns with 500 µl 80% ethanol (section 3.4.1, step 10), followed by vacuum for 5 min and high-vacuum for 1 min.
10. Remove *Zymo-I* filter plate from vacuum manifold, seal it with adhesive foil (no heat seal) and place it above 96 well PCR plate. Place this assembly into centrifuge basket for 10 96 well plates and place into a centrifuge. Insert an appropriate counterweight to balance weight inside centrifuge rotor.
11. Spin at full speed (e.g. 3700xg) for 5 min to dry silica matrix.
12. Remove seal and place *Zymo-I* filter plate back into epMotion 5075 vacuum manifold. Discard the liquid in 96 well PCR plate used for collection. If sealed and stored clean, 15 this 96 PCR plate can be reused for step 8 & 9 in another purification.
13. To elute RNA, have epMotion 5075 apply 21 µl 10 mM TRIS, pH 7.4 directly onto the filter matrix.
14. Remove *Zymo-I* filter plate from vacuum manifold, seal it with an adhesive seal (no heat seal) and place it above 96 PCR plate. Place this assembly into centrifuge basket for 96 20 well plates and place into a centrifuge. Insert an appropriate counterweight to balance weight inside centrifuge rotor.
15. Incubate for 1 min.
16. To collect eluates, spin *Zymo-I* at full speed (3700xg) for 5 min.
17. Approximately 18 µl of liquid are collected in each siliconized tube, which is stored at - 25 20°C. Discard *Zymo-I* filter plate or label the used sections of the plate for later purifications.

18. Seal PCR plate and store eluates at -20°C.

3.5 Fully automated RNA extraction of up to 96 samples

DO NOT cool, heat or centrifuge samples unless instructed by the protocol. If not specified, carry out steps at room temperature. Clean your workplace, the epMotion 5075 and all technical
5 equipment with 70% ethanol prior to starting to reduce possible contamination by particles carrying RNases. Change gloves frequently or wash gloved hands and dry them using a paper towel, especially after handling nuclease active sample material. Keep centrifuges and pipets clean.

Before choosing the sample denaturation option in this protocol (see 3.5.2 step 5), please also
10 consider looking into the alternative sample denaturation protocol (section 3.6), which combines sample aspiration/ addition of Proteinase K working solution and mixing with buffer P into a single work step using a programmable Rainin multichannel pipette. This approach may be used in automated sample processing.

When choosing to use the repeater pipette be careful not to aspirate air bubbles when aspirating
15 solutions. Also test the dispense function at least once before applying aspirated solutions to samples since every first dispense tends to not contain the entire adjusted volume.

Once started, the procedure should be carried out continuously. Try to avoid pauses during the preparation procedure. Organize your samples in batches of 24 samples (if applicable). Do not process more than 96 at a time.

20 Duration: Initial preparation: 30 min. for 96 samples. Extraction: 60 min for 96 samples.
Automated column purification: 90 min. Optional DNA purification: 120 min for 96 samples.

3.5.1 Preparation of reagents and consumables (fully automated processing)

1. Organize your samples (see section 3.2) but keep them frozen at this time.
2. Preheat one heatblock to 60°C and a second heatblock to 10°C. Make sure these
25 temperatures are reached.

3. Preparation of Buffer P working solution:

For 8 samples, aliquot 1 ml of Buffer P with 5 µl methylene blue stock into one 2 ml

tube and add 19 μ l β -mercaptoethanol and 1.9 μ l “calibrator pool 1”, either $c = 1$ pM each oligonucleotide (urine) or $c = 10$ pM each oligonucleotide (serum or plasma).

For 24 samples, prepare **two aliquots** of 1.47 ml of Buffer P with 7.4 methylene blue stock in two separate 2 ml tubes and to each aliquot add 28.0 μ l β -mercaptoethanol and 2.8 μ l “calibrator pool 1”, $c = 1$ pM each oligonucleotide (urine) or $c = 10$ pM each oligonucleotide (serum or plasma).

For 96 samples, aliquot 11 ml buffer P with 55 μ l methylene blue stock into a 15 ml tube and add 209 μ l 2-mercaptoethanol and 20.9 μ l “calibrator pool 1”, $c = 1$ pM each oligonucleotide (urine) or $c = 10$ pM each oligonucleotide (serum or plasma).

Make sure that buffer P working solution is properly mixed by tumbling or vortexing and is free of bubbles. Buffer P working solution may get discolored if kept too long and the level of reducing agent may have dropped due to oxidation.

Label a 1-ml deep well plate “set 1”. Use epMotion 5075 script “S1-P+VBadd96s1” to aliquot 105 μ l buffer P with additives from step 1 into a 1-ml deep well plate.

4. Label 2-ml 96x deep well plate “set 2”. Use epMotion 5075 script “S1-P+VBadd96s1” to aliquot 1200 μ l (twice 600 μ l) of TCEP-added buffer VB2G into a 2-ml 96x deep well plate (set 2). **Optional:** if DNA re-extraction from the organic phase is intended, prepare and fill a third plate (“set 3”) accordingly using epMotion script “S1-VBadd only 96s1” in the folder “DNA 2ndext 96s1”.

5. Label 96 well PCR plate “elution plate” with sample identification information. Seal plate with adhesive plastic film. The eluted RNA will be collected in this plate at the end of the purification process. **Optional:** For DNA re-extraction from organic phase: prepare another PCR plate OR use the second half of the PCR plate (for up to 48 samples).

6. Prepare Buffer ED working solution:

For 8 samples aliquot 6.75 ml Buffer ED2 (9 x master mix), add 23.6 μ l 2-mercaptoethanol and fill into 100 ml tub reservoir.

For 24 samples aliquot 18.75 ml Buffer ED2 (25 x master mix), add 65.5 μ l 2-mercaptoethanol and fill into 100 ml tub reservoir.

For 96 samples aliquot 75 ml Buffer ED2 (100 x master mix), add 262 µl 2-mercaptoethanol and fill into 100 ml tub reservoir.

7. **Prepare EWL working solution**

For 8 samples, aliquot 17 ml EWL stock solution, add 170 µl TCEP and fill into a 100 ml tub reservoir.

For 24 samples, aliquot 50 ml EWL stock solution, add 500 µl TCEP and fill into a 100 ml tub reservoir.

For 96 samples, aliquot 2x 100 ml EWL stock solution, add 1000 µl TCEP, and fill each aliquot into 100 ml tub reservoir.

Optional: if DNA re-extraction from the organic phase is intended, prepare twice the amounts listed before.

8. **For 8 samples**, prepare 10 ml 80% ethanol and 100% ethanol, fill solutions into 100 ml tub reservoirs.

For 24 samples, prepare 30 ml 80% ethanol and 100% ethanol, fill solutions into 100 ml tub reservoirs.

For 96 samples, prepare 100 ml 80% ethanol and 100% ethanol, fill solutions into 100 ml tub reservoirs.

Optional: if DNA re-extraction from the organic phase is intended, prepare twice the amounts listed before.

9. **Prepare Proteinase K working solution:**

For 8 samples, aliquot 250 µl 5 M NaCl and 30 µl proteinase K stock (20 mg/ml) into a 1.5 ml reaction tube, mix gently by pipetting.

For 24 samples, aliquot 750 µl 5 M NaCl and 90 µl proteinase K stock (20 mg/ml) into a 1.5 ml reaction tube, mix gently by pipetting. If a multichannel pipette is to be used for sample proteinase K addition, aliquot PK/NaCl solution as 105 µl fractions into 8 tubes.

For 96 samples, aliquot 2750 µl 5 M NaCl and 330 µl proteinase K stock (20 mg/ml) into a 5 ml reaction tube, mix gently by pipetting. If a multichannel pipette is to be used for sample proteinase K addition, aliquot PK/NaCl solution as 385 µl fractions into 8 tubes.

Dispose Proteinase K working solution, and prepare a fresh working solution for the next

isolation. The 20 mg/ml proteinase K stock should always be kept at -20°C using suitable equipment (cooling containers, etc). At this concentration the enzyme quickly loses activity at all other temperatures. Aliquots of proteinase K that reach room temperature should be disposed.

5 **10. Optional: For DNA extraction from organic phase:**

For 8 samples, prepare 6.75 ml buffer EA3 in a 100 ml tub reservoir.

For 24 samples, prepare 18.75 ml buffer EA3 in a 100 ml tub reservoir.

For 96 samples, prepare 75 ml EA3 in a 100 ml tub reservoir.

Also: double the amount of EWL working solution (step 9), 80% & 100% ethanol (step
10 10), VB2G and prepare another 96-well plate filled with VB2G (step 4).

3.5.2 Denaturation and proteolytic digestion of samples to inactivate ribonucleases

1. Organize your workspace: Have all deep well plate sets ready. Plate “set 1” is filled with Buffer P working solution, “set 2” and (optional for DNA isolation) “set 3” is filled with buffer TCEP-added VB2G. Have all required filled reservoir tubs: 2-mercaptoethanol-added ED2 working solution, chloroform, TCEP-added EWL working solution, 80%
15 ethanol, 100% ethanol (and EA3 if doing DNA extractions) and place them into epMotion 5075. Make sure pipette tools and tips are in place. Check that epMotion’s liquid and consumable waste containers are empty.
2. Start epMotion 5075 automated exRNA isolation script “S2-ED2+chloEx 96sl” in the
20 folder labeled “RNA only 96sl”. From here on out, the script names given will be for 96 samples in this folder. For 24 or 48 samples, find the corresponding script in the folders “DNA + RNA 24 samples” or “RNA 48 samples”, respectively. It will take about 10 min for the heat blocks to reach their assigned temperatures 65°C and 10°C. During this time you can proceed with the next step.
- 25 3. Thaw your samples. For biofluid samples in 1.5 ml microcentrifuge tubes thawed with warm air, e.g. by placing sample tubes in tube holders that allow airflow to pass through inside a incubator/shaker pre-heated to 37°C shaking at 130 rpm for 5 to 10 min. Watch thawing process, remove samples from incubator once thawed.
Homogenize samples by inverting tube racks with sample tubes several times to allow

precipitates to dissolve and liquid fractions to mix. Thawed samples are maintained at room temperature. DO NOT put thawed urine samples on ice, since this will cause precipitation of salts and other compounds in some samples. Manually fill samples into a 0.5 ml deep well plate (input plate) with at least 475 µl of sample per well.

5 If the provided sample volume is very low, spin tubes at 50xg for 10 s to minimize losses due to adhesion of sample to the lids.

4. Before proceeding, make sure that the epMotion heatblocks have reached their temperatures 65°C and 10°C. Use epMotion 5075 to preheat 1-ml lysis plate containing 105 µl of Buffer P working solution (prepared in section 3.5.1, step 3, 4) to 65°C in
10 *eppendorf* thermomixer for 2 min.

5. Place 0.5 ml input plate filled with biofluid samples into epMotion 5075. Press “Continue” to continue script “S1.5-Sampleadd_BufferP” 450 µl of biofluid sample will be transferred into set 1 deep well plate (sample denaturation), followed by quick mixing at 1200 rpm for 10 s.

15 Note: DO NOT EXCEED this speed to prevent spillage of the sample. It may be necessary to determine the optimal speed for mixing when using different equipment. Make sure the sample is properly mixed!

6. epMotion 5075 adds 28 µl Proteinase K working solution to each lysis reaction, followed by mixing at 1200 rpm for 1 min.

20 7. epmotion 5075 mixes samples for 8 min at 65°C at 450 rpm.

8. epMotion 5075 adds 517 µl of Buffer ED2 working solution with 2-mercaptoethanol added (from section 3.5.1, step 5) to each sample position, followed by mixing at 800 rpm for two min.

9. epMotion 5075 transfers set 1 plate (denaturation plate) to thermoblock at 10°C and cools plate and sample for 8 min.
25

10. epMotion 5075 adds 103 µl chloroform to each “set 1” sample position, followed by 5 mixing tasks by up and down pipetting.

11. Remove denaturation plate ("set 1") from epMotion 5075 and check lysis plate for spillage/splashing. Remove any splashed liquid by carefully patting the top of the plate with a Kim wipe. Seal plate with adhesive seal made of aluminum foil.
12. Centrifuge "set 1" plate at 3700xg for 5 min at 4°C. Check that two phases well separated
5 phases formed: The lower (organic) phase should be blue, there should be no interphase, and the top (aqueous) phase should be colorless.
13. Carefully remove sealing foil and place the plate back into epMotion 5075. Start epMotion 5075 automated exRNA isolation script "S3-aquaasp1 96s1".
14. epMotion 5075 transfers 645 µl from aqueous phase (top) of the lysis plate (including
10 some air) to "set 2" deep well plate (RNA binding plate) containing buffer VB2G, (prepared in section 3.5.1, step 4). Step 3.5.4 for RNA purification is done prior to re-extraction DNA (3.5.3).

3.5.3 Optional: automated DNA re-extraction from organic phase

The following steps should be carried out continuously, try to avoid pauses during the
15 preparation procedure.

1. Run epMotion 5075 automated exRNA isolation script "S4-2ndorgex 96s1".
2. epMotion 5075 adds 500 µl of Buffer EA3 to each tube (from section 3.5.2, step 14).
3. Heat seal the multiwell plate by pressing down on the foil with the sealer for at least 2 s. Mix by inverting 10 times.
- 20 4. Centrifuge multiwall plate at full speed in a microcentrifuge at 4°C for 5 min.
5. Remove multiwell plate from the centrifuge, keep at room temperature from now on.
6. Use epMotion 5075 script "S5-aquaasp2 96s1" to transfer 600 µl from the upper (aqueous) phase to "set 3" deep well plate containing Buffer VB2G with TCEP added, prepared in section 3.5.1 step 4, followed by five-fold mixing by pipetting up and down.

3.5.4 Column purification of nucleic acids – automated part

1. Load the vacuum manifold with an unused *Zymo-I* filter plate for parallel processing of all samples. Make sure that “set 2” or “set 3” plate is placed into epMotion 5075. “Set 2” and “set 3” plate refer to VB2G-plate mixed with aqueous phase from organic extraction (section 3.5.2 step 14) or organic re-extraction (section 3.5.3, step 6), respectively.
2. Start epMotion 5075 automated exRNA isolation script “S6-ldfill1+2 96s1”.
3. epMotion 5075 loads first portion (1 ml) of isopropanolic aqueous phase (from section 3.5.2, step 14, or 3.5.3, step 6) onto *Zymo-I* filter plate, followed by application of 600 mPa vacuum for 6 min. Confirm that all liquid was applied to the filter plate or apply vacuum for a longer time.
4. epMotion 5075 loads second portion (1 ml) of isopropanolic aqueous phase, followed by application of 600 mPa vacuum for 7 min and 900 mPa vacuum for 1 min. Confirm that all liquid was applied to the filter plate or apply vacuum for a longer time.
5. Start epMotion 5075 automated exRNA isolation script “S10-Wash pt1 96s1” to load columns with 970 µl Buffer EWL with TCEP added (from section 3.5.1, step 7), and applies vacuum for 7 min, followed by high-vacuum for 1 min. Confirm that all liquid was applied to the filter plate or apply vacuum for a longer time.
6. epMotion 5075 loads columns with 970 µl Buffer EWL with TCEP added (from section 3.5.1, step 7), and applies 600 mPa vacuum for 7 min, followed 900 mPa vacuum for 1 min. Confirm that all liquid was applied to the filter plate or apply vacuum for a longer time.
7. Start epMotion 5075 automated exRNA isolation script “S11-Wash pt2 elute 96s1” to load columns with 970 µl 100% ethanol (section 3.5.1, step 8), followed by vacuum for 7 min and high-vacuum for 1 min.
Omitting this step will result in sample loss, especially at molecular ranges smaller than 45 nucleotides.
8. epMotion 5075 loads columns columns with 500 µl 80% ethanol (section 3.5.1, step 8), followed by vacuum for 7 min and high-vacuum for 1 min.

9. epMotion 5075 loads columns with 500 µl 80% ethanol (section 3.5.1, step 8), followed by vacuum for 7 min and high-vacuum for 1 min.
10. Remove *Zymo-I* filter plate from vacuum manifold, seal it with adhesive foil (no heat seal) and place it above 96 well PCR plate. Place this assembly into centrifuge basket for 96 well plates and place into a centrifuge. Insert an appropriate counterweight to balance weight inside centrifuge rotor.
11. Spin at full speed (e.g. 3700xg) for 5 min to dry silica matrix.
12. Remove seal and place *Zymo-I* filter plate back into epMotion 5075 vacuum manifold. Discard the liquid in 96 well PCR plate used for collection. If sealed and stored clean, this 96 PCR plate can be reused for step 8 & 9 in another purification.
13. To elute RNA, have epMotion 5075 apply 21 µl 10 mM TRIS, pH 7.4 directly onto the filter matrix.
14. Remove *Zymo-I* filter plate from vacuum manifold, seal it with an adhesive seal (no heat seal) and place it above 96 PCR plate. Place this assembly into centrifuge basket for 96 well plates and place into a centrifuge. Insert an appropriate counterweight to balance weight inside centrifuge rotor.
15. Incubate for 1 min.
16. To collect eluates, spin at full speed e.g. 3700xg) for 5 min.
17. Approximately 18 µl of liquid are collected in each siliconized tube, which is stored at -20°C. Discard *Zymo-I* filter plate. Or label which part has been used and save unused columns for additional purifications.
18. Seal PCR plate and store eluates at -20°C.

3.6 Alternative denaturation procedure

A 96-deep well lysis plate filled with 105 µl aliquots of buffer P (section 3.5.1, step 3) is preincubated at 65°C for 2 min.

Using a Rainin multichannel electronic pipette with adjustable spacing, aspirate 28 µl of Proteinase K working solution (section 3.5.1, step 9) from 8 1.5 ml microcentrifuge tubes, followed by a brief rinse of the pipette tips with water. Next, 100 µl of air are aspirated followed by 450 µl of biofluid sample from 8 1.5 or 2 ml microcentrifuge tubes. During this step the

5 PK/NaCl and biofluid are aspirated into the same tip, but the two liquids do not mix.

The spacing of the pipette is then adjusted such that the tips fit into the 2-ml deep well plate filled with buffer P working solution. The contents of the tips are added to the first column and mixed with the buffer P working solution by pipetting up and down 5 times. This is repeated twelve times until all 96 samples have been added.

10 The deep well plate containing the denaturation samples/proteolytic digestions is then inserted into epMotion 5075, and the RNA isolation is continued at step 7 (section 3.5.2).

4 Effective concentrations during purification and parameters influencing purification efficiency

Denaturation step:

15 To each sample of 450 µl biofluid, 105 µl Buffer P to 25 µl 5 M NaCl, 3 µl proteinase K were added to obtain a final volume of 583 µl and a final working concentration of approx. 12 mM Buffer Tris-HCl (pH 7.5), 215 mM NaCl, 2 mM EDTA, 50 mM 2-mercapthoethanol and 5.4% SDS during extraction. Lower Proteinase K activity was observed at a lower NaCl concentration of 50 mM.

20 - NaCl is not added to Buffer P before the biofluid sample is added because the resulting high NaCl and SDS concentrations would lead to precipitation of Na SDS.

Proteolysis step:

Proteinase K stock loses activity within minutes if not at cold temperatures. Proteinase K stock is maintained at -20°C at all times. However, proteinase K diluted to approximately 2.1 mg/ml in

25 5 M NaCl can tolerate higher temperatures, and this stock is maintained on ice at the time of preparation or at room temperature when it is added to the samples.

- To accelerate proteolysis and obtain a more complete denaturation of ribonucleases, the digest is performed at 60°C. An incubation temperature of 65°C was chosen for the automated setup to

compensate for the fact that the heat transfer is less optimal when using the epMotion thermomixer 5075.

Organic extraction step:

After denaturation and proteolysis, addition of 513 µl buffer ED2 working solution containing
5 37.4 mM Na Citrate, 0.4% sarcosyl, 1.6 M GITC and 80.4% Phenol, brought the effective concentrations to approximately 3.0% SDS, 6.6 mM TRIS, 1.0 mM EDTA, 120 mM NaCl, 18.5 mM Na Citrate, 0.2% sarcosyl, 0.8 M GITC, 30 mM 2-ME and 40% phenol at total volume of approximately 1040 µl. Phase separation is induced by adding 103 µl of chloroform, followed centrifugation.

10 After phase separation, the effective concentrations in the aqueous phase are approximately 12.5 mM TRIS, 1.9 mM EDTA, 225 mM NaCl, 35 mM Na Citrate and 1.5 M GITC.

- Make sure that chloroform is added at a ratio of 1:5 with regards to ED2 working solution to partition DNA into the organic phase while keeping RNA in the aqueous phase.

- Noticeable interphase after organic extraction likely indicates failure incomplete proteolytic
15 digestion. Prepare a fresh Proteinase K stock and working solution.

If a more stringent organic extraction is required, e.g. to address remaining RNase activity observed during purification, 750 µl of ED2 working solution can be added and phase separation can be induced by addition of 150 µl chloroform. If this variant is used, make sure to maintain the same ratio of aqueous phase to VB2G working solutions, e.g. mix approx. 650 µl of aqueous
20 phase obtained after aqueous phase with 1600 µl of VB2G.

Binding to silicate columns

After mixing approximately 525 µl of aqueous phases from organic extractions with buffer VB1G working solution resulting effective concentrations are approximately 10.5 mM sodium citrate, 0.3 mM EDTA, 68.5 mM NaCl, 5.0 mM MgCl₂, 2.1 mM CaCl₂, 0.9 M GITC, 68.3%
25 isopropanol.

Please make sure the isopropanol concentration is at least 66% to avoid losses of RNAs shorter than 25 nt due to incomplete column binding.

Make sure that the GITC concentration is at least 0.9 M to avoid precipitation of peptides in the aqueous phase upon addition of isopropanol.

Organic re-extraction

The concentrations of the aqueous phase after re-extraction of the organic phase with solution
5 EA3 are expected to resemble those of solution EA3 which contains 4 M GITC.

Please make sure that you use an aqueous solution containing at least 3 M GITC to partition DNA from the organic phase into the aqueous phase.

5 Proteinase K quality control assay

5.1.1 Introduction

10 This assay evaluates the proteolytic activity of a batch of proteinase K (PK) stock solution (prepared in section 2.6.6.1) using fetal bovine serum (FBS) as protein substrate for degradation.

It compares proteolytic activity of fresh PK stock solution (prepared in section 2.6.6.1) stored at -20°C and PK stock solutions incubated at room temperature for 2 and 5 min, respectively. To compare proteolytic potency, all three PK stock solutions will be incubated with substrate (FBS)
15 for 3, 10 and 30 min, respectively.

To stop the reaction at a precise time point, a stop mix solution containing PMSF protease inhibitor and SDS is used.

PK activity is assessed for every new commercial PK powder batch to verify that it has the desired activity for follow-up applications. PK solutions may lose activity even when incubated
20 shortly at room temperature or above. PK stock solutions are prepared and stored at -20°C at all times and using pre-chilled thermal packs while PK stock solutions are handled.

5.1.2 Setup

Always keep PK stock solution at -20°C in a thermal pack if not deliberately instructed to do otherwise.

25 1. Preheat *eppendorf* thermomixer to 50°C.

2. In a 1.5 ml *eppendorf* tube, prepare a 4x master mix as proposed in the table below and mix by vortexing. Aliquot 148.5 μ l of this master mix into three 1.5 ml *eppendorf* tubes labeled with 'a', 'b' and 'c'. Keep tubes at room temperature.

Reagent or solution	<i>Per sample, 1x (μl)</i>	<i>Master mix, 4x (μl)</i>
FBS	120	420
Buffer PKQC	28.5	114

5

3. To prepare a negative control sample, transfer 30 μ l of the master mix prepared in step 2 into one 10 μ l aliquot of stop mix solution (prepared in section 2.6.6.4).
4. Label two 1.5 ml *eppendorf* tubes with 2 and 5 min, respectively. Place them into an appropriate tube rack at room temperature.

10 5.1.3 Quality control assay

5. Prepare a timer.
6. Start the timer and swiftly transfer 5 μ l PK stock solution in one 1.5 ml *eppendorf* tube labeled in step 4 and kept at room temperature. Incubate 5 min at room temperature.
7. 3 min after completing step 6, swiftly transfer 5 μ l PK stock solution in one 1.5 ml *eppendorf* tube labeled in step 4 and kept at room temperature. Incubate 2 min at room temperature.
8. Start the assay reaction by adding 1.5 μ l PK stock solution (chilled at -20°C) to tube 'a' (prepared in step 2), 1.5 μ l 2 min pre-incubated PK stock solution (from step 7) to tube 'b' (prepared in step 2), and 1.5 μ l 5 min pre-incubated PK stock solution (from step 6) to tube 'c' (prepared in step 2). Briefly mix by vortexing and immediately transfer all three tubes to the 50°C *eppendorf* thermomixer.
9. After 3 min, 10 min and 30 min, transfer 30- μ l aliquots of each tube (a, b, c) into the according 10 μ l stop mix tube (prepared and labeled in section 0).

15

20

	PK stock solution, time at RT (min)		incubation at 50°C (min)		stop mix tube label
tube a (prepared in step 2)	0	→	3	→	a3
			10		a10
			30		a30
tube b (prepared in step 2)	2	→	3	→	b3
			10		b10
			30		b30
tube c (prepared in step 2)	5	→	3	→	c3
			10		c10
			30		c30

10. Denature all sample tubes (from step 9) at 90°C for 3 min.

11. Prepare a mini-PROTEAN TGX 4-20% gradient gel for SDS PAGE in the appropriate running chamber using a 1x dilution of the 10x Tris/glycine buffer (*Bio-rad*).

5 12. Load 2 µl of each denatured sample on this gel.

13. Run the gel at 150 V for 50 min until the blue dye ran to the bottom edge of the gel.

10 14. To stain the gel, disassemble the plastic scaffold and place gel in a plastic tray. Add 50 ml Coomassie brilliant blue protein gel staining solution and keep on shaker at room temperature and shake gently for one hour. Make sure the entire gel is covered in staining solution. To de-stain, remove the staining solution and add 50 ml of Coomassie gel de-staining solution. Keep on shaker at room temperature, shake gently and make sure that the gel is covered completely in solution at all times. De-stain for 1-3 h, change de-staining solution once in-between.

5.1.4 Exemplary results

On a 4-20% PAGE gel, running FBS only shows two major bands at 68 and 60 kDa, respectively Figure 8. Using a correctly stored PK stock solution, a 30 min incubation time is sufficient to completely degrade the most prominent 68 kDa FBS band (Figure 8; tube a, 30 min). Decreasing this incubation time generally results in less FBS degradation, hence the increase protein band strengths (Figure 8; tube a, b & c, 3-10 min). Pre-incubation of PK stock solutions at room temperature for 2 or 5 min impairs the expected degradation process of the 68 kDa band (Figure 8; tube b & c).

6 RNA concentration determination

6.1.1 Nanodrop (ThermoScientific)

The Nanodrop photometer allows for the determination of RNA concentrations in sample volumes of 0.5 – 2 μ l. Typically 1 μ l is used to avoid variability in the reading. The reported upper and lower concentration limits for accurate detection are 4 ng/ μ l and 14 μ g/ μ l, respectively, according to the manufacturer's specifications. Samples in an expected concentration range greater than 10 ng/ μ l should be photometrically evaluated whereas samples with suspected lower concentrations (0.1-4 ng/ μ l), or samples containing possible UV-absorbent contaminants (e.g. protein, phenol) should be processed using the Qubit RNA HS assay.

6.1.2 Qubit RNA HS assay dye (Invitrogen)

The Qubit 2.0 fluorometer is a small instrument used for low concentration DNA, RNA, and protein quantification (US Patent No. 7,776,529). The Qubit RNA HS assay utilizes a methionine-substituted cyanine dye (**Error! Reference source not found.**). The Qubit HS RNA assay has some advantages over UV-absorbance-based quantification methods (e.g. Nanodrop; ThermoScientific). Since it is a fluorescence-based method, it is very sensitive and UV-absorbing compounds unresponsive to the fluorophore do not interfere.

In absence of nucleic acid, the dye alone shows little fluorescence emission in the 600-700 nm range (Figure 9). Addition of RNA to diluted Qubit HS RNA dye increased fluorescence intensity within this range (Figure 10).

The fluorescent adduct formed with RNA is approximately 10 times than for DNA. The substituted group on the methine bridge is believed to confer specificity to RNA over DNA (Figure 11). The fluorescence intensity increases with increasing concentration of RNA or DNA.

The dye demonstrates an equal affinity for the three different species of rRNA, tRNA, and mRNA as long as these are composed of pools of variable sequence (Figure 12).

Fluorescence emission of the dye is dependent on the RNA sequence and presumably also secondary structure of the RNA. Short oligoribonucleotides (22 nt) display rather variable
5 fluorescence, but when combined, the average mirrors the signal observed for HEK293 total RNA at the same nucleic acid concentration (Figure 13).

Hydrolyzed total RNA shows comparable fluorescent enhancement compared to non-hydrolyzed total RNA, i.e. the length of the RNA is not critical (Figure 14).

Invitrogen recommends the assay for the undiluted input RNA concentrations between 5-100 ng
10 per ml. Sample volumes of 5 - 10 10 µl are used and the detection range for 1-80 ng input total RNA in that volume (Figure 15) is verified.

This means that in order to accurately report the concentration of a RNA solution of 0.1 ng/µl, 10 µl of the solution needs to be combined with 190 µl of Qubit working solution to yield sufficient fluorescence increase over background to be measured accurately.

15 6.1.2.1 Calibration standards for the Qubit RNA HS assay

Fully 2'-OMe-modified oligoribonucleotide resistant to nuclease degradation as calibration standard for the Qubit RNA HS Assay (Invitrogen) is used herein. 2'-OMe-let-7a-1 RNA is purchased from IDT DNA (\$170 for 100 nmol). Enter below sequence into custom DNA oligonucleotide synthesis form (IDT considers 2'-OMe a DNA and not an RNA modification).
20 'm' before base designates 2'-OMe modification. This particular RNA sequence shows about 13x lower fluorescence than total RNA at the same concentration, otherwise the fluorescence signal is linear with increasing concentrations (Figure 16).

6.1.2.2 Preparation of RNA standards for concentration measurements using the Qubit fluorometer

- 25 1. Determine the concentration of the 2'-OMe let-7a calibration standard by measuring the absorbance on the Nanodrop. Alternatively, a 1:100 dilution of the standard can also be measured using the traditional UV spectrophotometer at 260 nm.

2. Dilute the calibration standard in water to produce 1000 μl of standard of $\text{OD}_{260} = 4$ (Table. 1). Measure the mass of volumes to accurately determine the final expected A260 absorbance.
3. Dilute 125 μl of this $\text{OD}_{260} = 4$ standard with 875 μl water to arrive at 1000 μl of an $\text{OD}_{260} = 0.5$ standard (Table. 1). This concentration produces fluorescence values 1.5x above background with the Qubit HS assay and corresponds to approximately 2.5 ng/ μl total RNA.
4. Make 50 μl aliquots of both 2'-OMe-let-7a $\text{OD}_{260} = 4$ and $\text{OD}_{260} = 0.5$ calibration standards. Store aliquots both short and long-term at -20°C .

Table 2S Example preparation of 2'-OMe-let-7a-1 standard for Qubit

$C_1 V_1 = C_2 V_2$	C_1	C_2	V_2	V_1	$V_2 - V_1$		
$V_2 = \frac{C_1}{C_2} \times V_1$	concentrated standard OD_{260} (AU)	diluted standard OD_{260} (AU)	final volume (μl)	concentrated standard (μl)	water (μl)	mass dH ₂ O (g)	final mass (g)
$V_2 = \frac{4}{70} \times 1000 \mu\text{l}$	70	4	1000	57.2	942.8	0.94	1.00
$V_2 = \frac{0.50}{4} \times 1000 \mu\text{l}$	4	0.50	1000	125	875	0.88	1.00

A260 to ng/μl conversion							
$A = \epsilon \times l \times c$	A	ϵ	l	c			
$c = \frac{A}{\epsilon \times l}$	OD ₂₆₀ (AU)	extinction coefficient $\left(\frac{L}{\text{mol} \times \text{cm}}\right)$	path length (cm)	concentration (M)	concentration (μM)	molecular mass (g/mol)	concentration (ng/μl)
$c = \frac{0.5}{228000 \times 1}$	0.5	228000	1	2.19×10^{-6}	2.19	7089.6	15.5

6.1.2.3 Measuring samples with the Qubit 2.0 fluorometric assay

General information

The assay requires a 2 min incubation of the RNA with the diluted assay dye before measurement on the Qubit device. The fluorescence signal of the Qubit RNA HS assay dye is stable for 3 h at RT after the addition of RNA. To work within this timeframe, plan an appropriate number of samples to measure together in parallel. (Sets of 24 samples or less are recommended)

All tubes must be inserted into the Qubit device in the same orientation.

This assay is very sensitive to temperature fluctuations. Do not warm the tubes in your hand. When measuring a sample multiple times remove tube from device for 5 s before re-measuring.

Disregard all concentration readouts and low concentration errors given on the Qubit screen. Concentrations will be calculated from the data that is found in the exported measurement report.

Setup

1. Set up and label the necessary number of siliconized tubes needed for samples. Additionally, a single 0.5 ml PCR tube will be used repeatedly for all measurements taken with the Qubit.
2. Prepare 199 μl Qubit working solution for each sample, 1 standard, and an additional 10% extra to accommodate for variation in aliquoting the solution. Prepare the Qubit working solution by diluting the Qubit RNA HS assay dye 1:200 in Qubit RNA dilution buffer using a clean plastic tube. For example, ~200 μl for 8 samples and 1 standard, with

an additional 10% extra yields 2 ml of working solution (10 µl of Qubit reagent plus 1,990 µl of Qubit buffer).

3. Transfer 199 µl Qubit working solution to each individual labeled siliconized tube.
4. Load 199 µl Qubit working solution into the 0.5 ml Qubit assay tube. This blank sample
5 will be used to determine the background and measure the calibration standard. The minimum volume in a tube that can be measured accurately is approximately 130 µl.)
5. On the Home Screen of the Qubit, press **RNA**, and then press **RNA** as the assay type. On the Standards Screen, the Qubit will prompt you to choose between reading new standards and using the previous calibration. Press **No** to use the last calibration. 2'-OMe-
10 let-7a is used as our calibration standard, so one initial calibration with the Invitrogen-supplied standards is only needed to bypass the Standards Screen.

Calibration method (chose one)

Short calibration – 10 min

15 A single 2'-OMe-let-7a calibration standard measurement is generally sufficient to ensure that the Qubit assay and device are working properly for measurements of low RNA concentrations (less than 10 ng/µl). Proceed to step 11.

Full calibration curve – 20 min

20 To measure samples of higher concentrations (greater than 10 ng/µl), that you do not want to measure on the Nanodrop, measure a full calibration standard curve. Additionally, it may be necessary to reevaluate expected RFU values with new batches of dye. Proceed to step 16.

Short calibration: measure background and 2'-OMe-let-7a Qubit calibration standard

1. Read the blank sample, 199 µl of the Qubit working solution without RNA, 3 times to determine the background. Remove tube from device for at least 5 s between measurements.
- 25 2. Add 1 µl of the 2'-OMe-let-7a standard to the Qubit assay tube used to determine the background, containing 199 µl of Qubit working solution, and vortex tube 2-3 s. Follow with a short spin. Allow the Qubit assay tube to incubate at room temperature for 2 min.

3. Read the 2'-OMe-let-7a calibration standard on the Qubit.
4. Transfer readings to computer using a flash drive.
5. Average the background readings from the blank sample. The RFU values for each reading should be stable (± 3 RFU units). The average background reading should be 30-35 RFU and the 2'-OMe-let-7a calibration standard value should be approximately 15-20 RFU above the background. This value corresponds to approximately 2.5 ng/ μ l total RNA.

If the 2'-OMe-let-7a calibration standard measurement does not appear above the background measurement, or if the value deviates significantly from a 15-20 RFU increase above background, it may be necessary to measure a full calibration standard curve. To do so, continue to step 15. Otherwise, continue to step 22.

Full calibration: measure a 3-point 2'-OMe-let-7a Qubit calibration standard curve

1. Prepare a second standard value by adding 1 μ l more of the 2'-OMe-let-7a standard to the same Qubit assay tube amounting to 2 μ l standard/199 μ l Qubit working solution. Mix by vortexing 2-3 s, follow with a short spin, and allow tube to incubate for 2 more min.
2. Read this second 2'-OMe-let-7a calibration standard three times on the Qubit.
3. Prepare a third standard value by repeating steps 15 and 16. (3 μ l standard/199 μ l Qubit working solution).
4. Prepare a fourth standard value by adding 1 μ l of the higher concentration calibration standard (OD = 4) to the same Qubit assay tube amounting to 4 μ l standard/199 μ l Qubit working solution, final OD = 5.5. This value corresponds to approximately 35 ng/ μ l total RNA.
5. Discard the diluted calibration standard.
6. Transfer readings to computer using flash drive.
7. Plot a standard curve with the averaged values. The curve should be linear and have a high correlation coefficient. Calibration standard values should increase in consistent steps.

After calibration standards measurements are complete, measure samples

1. Add 1 μl of samples to siliconized tubes containing 199 μl of Qubit working solution and mix by vortexing 2-3 s. Follow with a short spin.
2. Allow tube to incubate for 2 min at room temperature.
- 5 3. Transfer the first diluted sample from the siliconized tube to the Qubit assay tube.
4. Read your diluted sample.
5. Transfer diluted sample back to a labeled siliconized tube.
6. Repeat steps 3-5 for additional samples.
7. When finished, transfer readings to computer using flash drive.
- 10 8. Copy the three RFU values for each sample to the provided template table. Write the volume of sample added to the “RNA added (μl)” column. The average RFU will be calculated for each sample. If this value is not above background, add an additional 1 μl and return to step 20 (2 μl sample + 199 μl Qubit working solution). NOTE: If there is not a change in RFU after the second addition, assume the RNA concentration to be less
- 15 than 1 ng/ μl .
9. Calculate sample RNA concentrations using the equation provided below.

If 1 μl of sample was measured $V=1$, if an additional 1 μl of sample was added $V=2$.

RNA sample concentration calculation:
$c_{\text{sample}}(\text{ng}/\mu\text{l}) = \frac{\text{RFU}(\text{sample}) - \text{RFU}(\text{reagent blank})}{V (\mu\text{l}) \cdot 7/\text{ng}}$

6.1.2.4 Example Qubit measurements with 1-point 2'-OMe-let-7a calibration

RNA	Total vol. (μ l)	RNA added (μ l)	RNA total added (μ l)	RNA total added (ng)	RFU 1	RFU 2	RFU 3	Average RFU (AU)	STD	Expected 2'-OMe-let-7a standard values (AU)	C_{sample} (ng/ μ l)
Reagent blank	199	0	0	0	30.93	31.07	31.02	31.01	0.07		
2'-OMe-let-7a	200	1	1	15.5	47.72	48.18	48.22	48.04	0.28	blank+(P)*	2.4
HEK293 total RNA	200	1	1		40.11						1.3
HEK293 total RNA	201	2	2		50.67						$2.7/2 = 1.35$

*P is in the range of 10 to 20 and may need to reevaluate this value with new batches of Qubit dye

6.1.2.5 Example Qubit measurements with 4-point 2'-OMe-let-7a calibration curve

*P is in the range of 10 to 20 and may need to reevaluate this value with new batches of Qubit dye.

RNA	Total vol. (μl)	RNA added (μl)	RNA total added (μl)	RNA total added (ng)	RFU 1	RFU 2	RFU 3	Average RFU (AU)	ST D	Expected 2'-OMe-let-7a standard values (AU)	C _{sample} (ng/μl)
Reagent blank	199	0	0	0	30.93	31.07	31.02	31.01	0.07		
2'-OMe-let-7a	200	1	1	15.5	47.72	48.18	48.22	48.04	0.28	blank+(P)*	2.4
2'-OMe-let-7a	201	1	2	31	60.33	60.88	61.24	60.82	0.46		4.3
2'-OMe-let-7a	202	1	3	46.5	75.34	75.52	77.37	76.08	1.12		6.4
2'-OMe-let-7a	203	1	4	170.5							~35
HEK293 total RNA	200	1	1		40.11						1.3
HEK293 total RNA	201	1	2		50.67						2.7/2 = 1.35

5

6.1.3 Ribogreen assay (Invitrogen)

The Quant-iT Ribogreen assay from Invitrogen offers a similar sensitivity of 1 ng/ml at the lower limit as the Qubit HS RNA assay from Invitrogen. The Ribogreen has a higher range (upper limit according to the manufacturer 1 μg/ml) and, more importantly for our purposes, can be scaled up more easily for a large number of samples.

10

Materials

- Quant-iT™ RiboGreen® RNA Assay Kit (Life Technologies, Cat# R11490)
- Quant-iT™ RiboGreen® RNA Reagent
- 20x TE Buffer, RNase-free
- Ribosomal RNA standard, 16S and 23S rRNA from E. coli (100 µg/ml)

Costar™ 96-Well Clear-Bottom Plates (Fisher Scientific, Cat#07-200-565)

*Procedure***Preparing the RNA Standards**

1. Prepare 125 µl of 2 µg/ml ribosomal RNA standard by adding 2.5 µl of the included RNA Standard (100 µg/ml) to 122.5 µl 1X TE Buffer.
2. Prepare the dilution series shown in Table 1S1, starting with the 2 µg/ml RNA standard, in an 8-well PCR strip.

Table 1S1. Dilution series for quantifying RNA isolated from biofluids.

Dilution Number	Volume of 1X TE (µl)	Volume transferred from previous dilution (µl)	Dilution concentration (µg/ml)	Final concentration in plate(ng/ml)
1	-	122 (2 µg/ml stock)	2.00	50.00
2	48	72 (from dilution #1)	1.20	30.00
3	35	70 (from dilution #2)	0.80	20.00
4	33	55 (from dilution #3)	0.50	12.50
5	48	32 (from dilution #4)	0.20	5.00
6	45	30 (from dilution #5)	0.08	2.00
7	25	25 (from dilution #6)	0.04	1.00
8 (blank)	50	0	0.00	0.00

Sample RNA Quantification

The assay plate setup is performed using the epMotion 5075 liquid handling system.

3. Prepare a 1:2000 dilution of the Quant-iT™ RiboGreen® RNA Reagent in 1X TE Buffer. Make sure that the RiboGreen has fully thawed before making the dilution. The dye is light-sensitive, so wrap the tube in foil and use within a few hours of preparation.
5
4. The first column of the 96-well assay plate will contain the 8 RNA dilutions prepared in step 2. The standards are diluted 20-fold from their starting concentrations to their final concentrations in the plate, as shown in Table 1S1. The final volume in each well will be 200 µl, so use the EpMotion 5075 to transfer 10 µl of each standard to the first column.
10 Add 90 µl of 1X TE Buffer for a total volume of 100 µl.
5. The isolated RNA of unknown concentration will be added to the subsequent columns. First use the EpMotion 5075 to transfer 97 µl of 1X TE Buffer to the appropriate number of wells in the assay plate. Next, transfer 3 µl of the isolate to each well for a total volume of 100 µl.
- 15 6. Add 100 µl of the 1:2000 RiboGreen® RNA Reagent prepared in step 3 to all wells, for a final volume of 200 µl.
7. Mix thoroughly by shaking the plate for 1 minute at 1200 rpm.
8. Cover the plate from light by wrapping it in foil and incubate for 2 min at room temperature.
- 20 9. Measure the fluorescence of the samples using a fluorescence microplate reader (excitation: 480 nm, emission: 530 nm). Generate a standard curve using the concentrations of the standards in the plate (Table 1S1) and their measured absorbances. The curve should be linear in this concentration range (Figure 1).
10. Calculate the RNA concentration of each sample from the standard curve. The amount of
25 RNA from each sample used for cDNA library generation should be equal as to not limit sequencing depth of individual samples. If there is significant variance in the RNA

content of each sample to be used in library preparation, adjust the concentration accordingly.

7. Steps of method development

I. Isolating DNA and RNA from biofluids can be challenging due to their low of exRNA and high levels of RNase activity in these samples. Fluctuations in high-quality RNA recovery from biofluids (plasma and serum) prompted to examine the RNA isolation process. Using a set of radiolabeled (^{32}P -labeled) oligonucleotides (ssRNA, ssDNA), spikeins, transcripts (RNA), PCR products (dsDNA) and restriction fragments (dsDNA), as well as a set of 22-nt spike-in calibrators, steps that limited RNA recovery and intactness were detected and optimized. The spike in were applied to the denaturant or extraction solution prior to addition of a biofluid samples. Typical organic extraction protocols used to denature and remove ribonucleases were insufficient in preventing RNA spike-in degradation in certain biofluid samples, as exemplary shown for a serum sample processed using a standard Trizol LS based protocol (Fig 1a, lane 2).

II. In contrast, a 5-minute initial denaturation at 50°C with a buffered detergent, 2% (w/v) sodium dodecylsulfate (SDS) solution, followed by TRIZOL extraction and ethanol precipitation could significantly reduce RNA spike-in damage (lane 4). Likewise, a 5-minute hot initial denaturation with a buffered chaotroph and a reducing agent, 4M guanidinium iso thiocyanate (GITC) solution with 25 mM 2-mercaptoethanol, followed by organic extraction and ethanol precipitation could prevent degradation of RNA spike-ins to a similar extent (lane 6), while treatment with the same reducing agent and a harsher, 6M, GITC concentration at room temperature failed to achieve this (lane 8). In contrast, RNA spike-ins in water controls processed in parallel using the same conditions did not show any degradation (Fig. 1A, lanes 1, 3, 5, 7), suggesting that residual intrinsic RNase activities in the serum are likely responsible for the degradation of the RNA spike ins and that thermal denaturation at 50°C alone or in combination with chaotrophs, detergents and/or reducing agents used effectively reduce these activities to a level where no obvious damage is observed.

III. In order to simplify the purification effort, 50°C preincubations were combined with column-based RNA purification. While damage of ^{32}P -RNA spike-ins appeared to be lower in column-purified RNA samples even in the Trizol sample (Fig 1b, lane 1,2), the initial thermal denaturation step with denaturants and reducing agents, followed by either Trizol or

phenol/chloroform extraction appeared to protect the spike-ins further against ribonuclease activity, as suggested by the absence of degradation product in the eluates and flow through fraction (lanes 3-6). However, the column-based RNA purification method showed substantial losses of 19 to 24 nt RNA spike ins, when Trizol was used as an extraction agent. (Fig. 1b, lane 2, 4) or wash fractions.

As a next step the thermal denaturation with 2% buffered SDS buffer or GITC and reducing agent with proteolysis followed by column purification were combined. Proteinase K digestion removes or markedly reduces interphase formation after organic extraction and phase separation (Fig 2b). Interphases at this step tended to be very prominent in blood-based biofluids (Fig. 2a), probably due to high protein concentrations in plasma and serum.

Via Greater interphase reduction and higher protection against lower levels of RNA spike in degradation in SDS-treated & proteinase K digested samples (Fig 1C, lane 4 vs. lane 2) suggested higher lead to the preference of SDS-based solutions in initial denaturation over GITC-based formulations over the and lead to the development of SDS-based denaturing buffer P which has been used as initial denaturing agent from here on (see Total RNA isolation from urine, serum, citrate-, EDTA- and heparin-plasma samples, section 2.6.2.1).

VIb. Column binding of small 19-nt and 24-nt ³²P-labelled spikes ins could be improved by increasing the GITC concentration during organic extraction (Fig. 1c, lanes 1,3). High GITC concentrations in the extraction buffer, however, appeared to abolish DNA partitioning into the organic phase and hence prevented separation of DNA and RNA, while at low concentrations, separation of RNA and DNA could be achieved (Fig 3a). High GITC concentration in commercial extraction solutions such as Trizol LS may be the reason, why these agents under all conditions tested also failed to separate these two nucleic acids (Fig 1a, 1-4, Fig. 1b, 1,3).

V. The GITC dependence on DNA partitioning lead to the development of organic extraction solution ED2 and aqueous re-extraction solution AE3, (see Total RNA isolation from urine, serum, citrate-, EDTA- and heparin-plasma samples, section 2.6.3.1 and 2.6.5.1) which can be used to sequentially purify DNA and RNA: First an organic extraction is performed using a low concentration of GITC, which allows ³²P-labelled DNA spike-ins to partition into the organic phase, while ³²P-labelled spike-ins remain in the aqueous phase, which and are subjected to column purification. Then, the organic phase is re-extracted with an aqueous buffer containing

4M GITC, which causes the ³²P-labelled DNA spike-ins to relocate into the aqueous phase, which is consequently aspirated and subjected to a similar column purification (Fig. 3b).

VI. The resulting lower GITC in the aqueous phase containing RNA required adjustments in the column binding conditions of nucleic acids, which could be overcome by replacing ethanol by isopropanol, increasing the isopropanol concentration to approximately 66% and adding divalent cations.

VII. The concomitant increase in precipitation of presumably aqueous peptides due to lower GITC / higher alcohol concentration increased the tendency of silica columns used in nucleic acid purification to clog. This could be overcome by adding GITC to isopropanol and led to the development of binding buffer VB2G.

VIII. Finally, unusually high and / or persistent RNase activities in biofluids present in certain biofluid samples such as certain urine samples which reproducibly degrade ³²P-RNA RNA spike ins during RNA isolation could be overcome by increasing the SDS concentration and temperature in the initial denaturation step and adding by adding 5 mM TCEP reducing agent to the binding and wash buffers. Our routine method now uses an initial SDS concentration of 5.5 mM SDS at 60°C for 10 min. incubation time. Under these conditions proteinase K is still sufficiently active to prevent interphase formation after organic extraction / phase separation (see Total RNA isolation from urine, serum, citrate-, EDTA- and heparin-plasma samples, section 3).

8. Library preparation and sRNA sequencing of biofluids isolated with the current RNA isolation protocol.

RNA isolation protocol presented here were used to isolate RNA from three biofluid types, including 96 human EDTA plasma samples, 72 human serum samples, and 182 human urine samples. Prior to RNA isolation, samples were organized in batches of 24 with up to four batches processed in parallel (see chapter 3.2). RNA quality and recovery efficiencies were monitored using two different synthetic RNA calibrator sets, which were spiked to the 1) denaturant solution prior to contacting the biofluid (calibrator set 1) and 2) to the barcoded 3' adapter ligation reaction during cDNA library preparation (set 2). The sample batches were ligated using a set of 24 individual 3' DNA adapters which introduce a specific 3' barcode to each RNA molecule in a sample. Barcoding is done prior to cDNA library generation and allowed to to

combine up to 24 samples into one sequencing reaction, the resulting reads read contains all sequences from the batch of 24 samples. During cDNA library preparation step, selection was applied to a size range of 19 - 30 nt for urine-based RNAs and 19 – 45 nt for serum- and plasma-based RNAs. It was chosen to specifically enrich for small RNAs such as miRNAs, with a
 5 typical length of 22 nt without enriching too much information from molecules that are too short for to be unambiguously identified in the reference transcriptome based on the human reference genome assembly GRCh37,hg 19. Barcoding, multiplexing and small RNA cDNA library preparation was followed by single end Illumina HiSeq sequencing yielding up to 150 million reads per batch and typically 1 – 2 million reads per sample, although the range can greatly vary
 10 from a about 14.000 to almost 59 million reads per individual sample in the case of urine (summarized in table 1a, detailed lists available in tables 1c, d and e, for plasma, serum and urine, respectively). Sequence reads were demultiplexed and mapped against our curated human reference transcriptome to obtain abundance data of miRNAs, tRNAs, scRNAs, rRNAs, mRNAs and other RNA classes.

15 9. RNA composition of biofluids isolated with the current RNA isolation protocol.

The protocol sRNA cloning protocol described herein was used was designed for enriching and cloning of 22-nt miRNAs by selecting 5'phosphorylated 3'OH RNA molecules in a size range of typically less than 50 nucleotides. Although most other intact ncRNAs are larger and RNase-degraded fragments do not usually have a matching phosphorylation signature at their 5' and 3'
 20 ends, sequences of other RNA classes in each biofluid samples were observed as well. Due to the selection criteria however, the proportions observed and reported here do not reflect the actual RNA composition in the biofluid samples; due to favoring of miRNAs the abundance of other RNA types is in fact expected to be much higher. After correcting for technical reads, such as adapter-ligation products without sample insert, calibrators, and markers which can differ in
 25 proportion depending on the amount of RNA isolated from a biofluid sample, the RNA composition shows a high degrees of variability (summarized in Table 2a, detailed lists available in Tables 1b, c and d, for plasma, serum and urine, respectively). The biofluid with the most defined RNA composition appears to be human plasma, with a miRNA content of approximately 75%, typically less than 3% rRNA, scRNA, and mRNA, less than 0.5% tRNA and typically
 30 15% of hg19-unmapped sequences. Serum has a lower proportion of miRNAs (med. approximately 45%) and higher proportions than plasma but typically less then 10% rRNA,

scRNA, tRNA, and mRNA. Urine is the least defined biofluid with an even lesser proportion of miRNAs (med. approx. 16%) and typically less than 10% of rRNA, tRNA and mRNA and a median proportion of hg19-unmapped sequences of approximately 45%. However, relative abundance of miRNAs, tRNAs and unmapped sequences can reach almost every proportion from less than 2% to more than 85%. In two of 282 urine samples, 16-4, and 16-19, the unmapped sequences contributed to more than 95% of the total sequences, with more than 50 million reads in total. Using averaged RNA composition data for the categories miRNA, tRNA, rRNA, scRNA, mRNA and unmatched samples and their distribution of insert lengths throughout all samples for each biofluid type (summarized 4a – d) found distinctive fragments of each RNA class were observed. In agreement with the biological definition for miRNAs, miRNA-derived sequences show a peak of 22nt length in all biofluids, while tRNA based fragments in serum and urine show a peak at 30 nt (Fig. 4b,c). scRNAs show a peak at 32 nucleotides. The lengths distribution of mRNA-based fragments begins with a wide peak a 17 nt and ends at 25 nt. Most fragments are smaller than 22 nt. rRNA fragments show peak at 17, followed by a declining tail which covers the range up to about 40 nt. The average size distributions of the unmapped RNA fragments is are flat curves which shows an increase towards 22nt, a local maximum, followed by long tails to approximately 40 nt.

While average size distributions of ncRNA types represent their distributions in all originating samples well, unmapped sequences show a much higher degree of variability with respect to their average length distributions (Fig. 4d) which appears to be highest in urine: Here, several samples show deviating size distributions with additional maxima at 30 to 33 nucleotides, similar to distributions in tRNA, and scRNA. The large contribution of unmapped sequences prompted us to analyze their source of origin on a per-sample basis for selected samples with more than 2 million total reads and more than 80% unmapped sequences (14-16, 16-4, 16-16, and 16-19).

Two randomly selected samples with less than 25% unmapped sequences (13-6, and 14-17) were also included. Unmapped reads longer than 25 nt were used in a contig-building process followed by a BLASTN search using the assembled contigs as queries against the NCBI non-redundant database. Several target hits include rRNAs and tRNAs genes of non-human origin. For unambiguously identified targets, taxonomic information was assigned to queries; assignments of similar or identical orders, genera and species were then combined and endowed with total read counts of sequences substantiating their queries. An overview of the most prevalent sources of unmapped RNA is given in Table 3. Interestingly, the composition of

organisms found in the selected samples vary considerably:

16-4, a sample of 56 million unmapped reads, the highest number observed here, has major contributions from a few taxonomic orders, mainly enterobacteria (48.9% of all unmapped reads), providing evidence for a handful of genera. One of its main contributors appears to be from the *Klebsiella* genus (support by 165 individual contig assignments), with *Klebsiella pneumoniae* as the most likely candidate (53 individual supporting assignments). Although this organism usually causes systemic infections originating in the lungs, this bacterium has been reported to cause urinary tract infections in immunocompromized hosts such as diabetic patients and can cause emphysematous pyelonephritis and urosepsis.

In sample 16-19 with almost 50 million unmapped reads, evidence for members of more than ten taxonomic orders of bacteria was found, including proteobacteria, high GC gram positive bacteria, fusobacteria and firmicutes, with more than 20 contributing genera and species and without evidence for a predominant contributor.

In sample 14-16 the main contributions of unmapped sequences (38.8% of all unmapped reads) appear to originate from the taxonomic order of Firmicutes, with *Lactobacillus* (35.9% of all unmapped reads) as the most likely genus. *Lactobacillus acidophilus* is a major part of the vaginal flora.

In sample 13-6 evidence, a sample with 23.3% unmapped reads, evidence was found for the presence of amoeba (5% of all unmapped reads), with *vermamoeba vermiformis* (4.9% of all unmapped reads), as the most likely candidate. This amoeba belongs to the group of free living amoeba which can commonly be found in water supplies, industrial waters and cooling towers and isn't known as a human pathogen.

It was unexpectedly discovered that evidence from RNA from Eudicots an order of flowering plants, which contains many fruit and vegetable families including soy, broccoli, mango and cashew were observed. In at least two samples, 16-16 and 16-19, evidence for trace amounts of RNA derived from the 5.8S ribosomal RNA of *Anacardium occidentale* (cashew) were observed, with E values of 10^{-79} and 10^{-81} respectively, suggesting that assignable fragments of food-derived RNA at least under certain conditions prevail the human metabolism and can be found in the urine of human subjects. Using our method of contig-assisted RNA identification, between 6.5% and 53.6% of unmapped reads could be identified. This high level of assigned data

supports the assumption that that the non-hg19 mapable RNA present in urine mainly originate from organisms in the urine and are not an artifactual entities of side reactions (template switch) or by products of spike-in RNAs.

10. miRNA composition of biofluids

- 5 miRNA and spike-in calibrator abundance based on fractional read counts was used to generate frequency profiles which were visualized as heat maps (Fig. 5). Calibrator heatmaps and ratios of set2: set1 calibrator reads helped to detect samples with less than expected RNA content, indicative of low-input RNA content in the biofluid, inefficient RNA isolation, or damage of RNA spike-ins by RNases.
- 10 Unsupervised clustering of calibrator RNAs tended to cluster samples according to the 3' adapters used for barcoding in sRNA-based cDNA library generation (Fig. 6a). This is not unexpected since the same calibrator sets were added to all samples and it was observed earlier, that the group of 24 adapters cause a bias in ligation towards, favoring associations of certain miRNAs and calibrator molecules over others, which can result in differences greater than one
- 15 order of magnitude. Clustering of calibrator RNAs was not driven by batches, suggesting that sample processing and library generation did not strongly influence RNA composition. Some samples of batch 58 cluster together but the clustering is driven by the absence of two less abundant calibrator sequences due to overall lower read coverage in these samples.

- Unsupervised clustering of merged miRNAs from plasma based on twelve individual samples of
- 20 eight different individuals taken at different timepoints over the course of two months revealed distinctive groups of individuals (Fig. 6b): an isolated branch of the dendrogram contains a cluster of all twelve samples of individual P12; similarly 8 out of 12 samples from individual P14 are localized on another branch, which is not as clearly separated since four samples of two other individuals are located here. On a third branch, eight samples from another individual, P5,
- 25 are co-localized but their distance to other samples is less, indicated by additional members on this branch. For the rest of the samples, clustering does not strictly discriminate individuals, but many samples of the same source are co-localized. For those the isolated groups of P12 and P14, no obvious batch or adapter effects on clustering are observed, although within these groups samples generated using the same 3' adapter tend to be located next to each other in the
- 30 dendrograms. For with very similar miRNA composition, those processed using the same 3'

barcoding adapter frequently cluster together, suggesting that adapter bias takes over when no distinctive miRNA pattern exists which separates groups of samples. In addition to clustering of individuals, each gender appears to be enriched on separate sides of the dendrograms, without being completely separated. Strong influence of timepoint was not observed when the sample was taken onto clustering.

Using miRNA annotations for human viruses, two bk virus (bkv) miRNAs B1-5p and B1-3p in urine of kidney transplant patients were detected. Initial infection of bkV is asymptomatic and leads to persistence at a low level in the urinary tract and kidneys in 82% of normal healthy controls. In individuals treated with immunosuppressants, however, virus activity can increase and lead to the renal failure. Differential expression analysis shows that bkV-derived miRNAs B1-5p and B1-3p show respective 61-fold and 91-fold differences in abundance when comparing the population that tested virus positive using either an antibody or an rt-pcr-based assay with those that did not (Fig. 7c). All antibody-positive samples show normalized read counts of at least 10000 for B1-5p (Fig. 7b), and they are located on branch 1 of a dendrogram (Fig. 7c), which shows very high levels of both bkV miRNA B1-5p and B1-3p in a heatmap generated by unsupervised clustering. The first to third quantile of the pcr-positive samples, range from 25 to 1250 normalized read counts for B1-5p, Fig. 7b, others are in the range of the previously observed antibody positive samples, and most of the PCR-positive samples (25 of 34) are located on the first two branches of the dendrogram (Fig 7a). In contrast, the first to third quantile of samples tested virus negative show counts from 0 to 15 normalized reads counts (Fig. 7b). Hence, bkV B1-5p read abundance data for quantiles 1 to 3 clearly separates the three populations: virus negatives, pcr positives and antibody positives. Based on this data, a lower threshold of 25 normalized reads could be used to discriminate virus-negative from virus-positive samples. However, in population of the samples tested virus negative by pcr or antibody, several additional samples are above this threshold (3rd quantile, Fig 7b), suggesting that this miRNA-based detection method bkV allows to potentially find more samples with detectable bkV abundance than the other two tests. It is definitely more sensitive than the antibody-based test, which could only detect very high bkV levels.

Summary

Sequencing data was used for differential analysis of miRNAs abundance, in order to identify and study markers for disease subcategories and disease-associated conditions. Using miRNA sequence data from biofluids, it was possible to identify key components discriminating biofluid types, discriminate biological replicates of individuals from each other, and identify non-human RNAs.

Table 1a

Biofluid type	Total reads	Adapter reads	Calibrators used		Additional technical categories			Remaining reads
			set 1	set 2	set 3 calibrators	long calibrators	Markers	
Plasma, n=96								
median	2,836,362	573,748	5,937	12,792	7	1	67	2,009,283
min	146,768	43,942	305	991	0	0	3	89,015
max	19,671,378	15,579,690	23,503	81,127	51	45	1,100	16,605,906
Serum n=72								
median	2,729,874	621,591	5,207	12,821	5	1	91	1,749,152
min	91,780	15,564	214	723	0	0	0	59,160
max	31,993,329	11,876,862	124,488	193,438	186	33	2,274	26,489,291
Urine n=282								
median	1,394,313	13,069	1,215	15,566	5	2	50	1,355,370
min	16,402	43	5	355	0	0	0	14,058
max	58,464,218	400,371	8,000	117,434	513	2,964	22,389	58,443,672

Table 1b

Sample ID	Biofl. type	Total reads	Adapter reads	Calibrators used		Additional technical categories			Remaining reads
				set 1	set 2	set 3 calibrators	long calibrators	markers	
55-1	plasma	3,480,235	276,752	13,476	19,153	14	3	22	3,170,815
55-2	plasma	6,788,399	286,368	9,048	14,456	19	11	97	6,478,400
55-3	plasma	4,701,381	745,142	16,377	21,330	31	0	10	3,918,491
55-4	plasma	2,615,874	622,209	7,641	19,053	10	3	12	1,968,946
55-5	plasma	5,177,172	433,290	14,116	15,225	18	1	9	4,714,513
55-6	plasma	15,297,676	13,214,331	4,558	12,871	6	4	102	2,065,804
55-7	plasma	5,554,418	2,347,770	5,458	14,577	11	0	30	3,186,571
55-8	plasma	8,701,539	4,111,760	11,739	19,670	20	8	40	4,558,302
55-9	plasma	8,967,447	760,179	12,806	26,109	27	1	247	8,168,078
55-10	plasma	2,634,070	513,744	6,965	11,627	9	0	1,100	2,100,425
55-11	plasma	910,423	189,606	1,563	6,752	4	0	271	712,227
55-12	plasma	2,825,090	303,509	4,756	10,495	1	1	76	2,506,252
55-13	plasma	1,547,443	270,663	3,904	7,495	2	0	10	1,265,369
55-14	plasma	2,052,360	278,194	4,227	9,487	3	1	238	1,760,210
55-15	plasma	3,307,533	1,236,764	5,498	13,252	4	1	385	2,051,819
55-16	plasma	4,186,024	302,221	6,837	10,780	7	0	24	3,868,155
55-17	plasma	7,828,175	820,077	6,148	15,575	22	4	127	7,086,222
55-18	plasma	7,043,690	1,561,968	7,180	16,834	8	1	248	5,457,453
55-19	plasma	721,727	165,466	2,266	7,533	1	0	19	546,442
55-20	plasma	2,153,807	688,293	5,681	11,558	7	3	487	1,447,780
55-21	plasma	4,706,986	1,694,196	7,222	15,670	12	2	68	2,988,816
55-22	plasma	3,614,675	778,077	8,592	10,266	18	4	311	2,815,407
55-23	plasma	1,634,450	470,186	4,592	9,116	2	0	32	1,150,522
55-24	plasma	3,361,674	758,370	7,703	12,089	0	1	106	2,583,405
56-1	plasma	3,658,072	182,933	7,317	19,597	10	1	10	3,448,204
56-2	plasma	1,928,208	178,159	8,581	15,902	10	0	82	1,725,474
56-3	plasma	3,719,716	490,606	9,370	21,304	20	2	10	3,198,406
56-4	plasma	5,998,569	526,641	12,053	21,520	18	0	16	5,436,321
56-5	plasma	3,653,221	361,362	9,035	18,200	5	0	13	3,264,606
56-6	plasma	17,685,847	15,579,690	5,878	17,749	8	5	124	2,082,393
56-7	plasma	4,055,848	2,201,664	6,020	19,462	12	1	7	1,828,482
56-8	plasma	7,206,722	3,220,090	12,547	22,279	12	17	30	3,951,747
56-9	plasma	10,342,616	540,681	12,706	25,228	29	0	251	9,763,521
56-10	plasma	1,780,654	459,721	4,991	13,819	2	1	1,097	1,309,023
56-11	plasma	644,156	241,207	1,533	9,821	3	0	477	391,115
56-12	plasma	2,256,680	410,209	5,523	15,693	7	0	113	1,825,135
56-13	plasma	1,718,766	254,993	3,645	9,740	3	2	14	1,450,371
56-14	plasma	2,424,987	314,025	5,655	12,594	13	2	307	2,092,191
56-15	plasma	4,044,496	1,000,422	10,511	13,658	16	1	444	3,019,444
56-16	plasma	1,332,640	311,397	6,985	13,180	7	2	22	1,001,067
56-17	plasma	4,539,925	605,412	7,408	17,423	27	0	137	3,909,520
56-18	plasma	4,189,108	1,536,072	9,604	16,071	5	0	288	2,627,068
56-19	plasma	1,102,759	107,312	4,035	9,363	2	0	24	982,023
56-20	plasma	3,202,638	679,413	5,058	12,921	5	2	537	2,504,704
56-21	plasma	2,474,244	1,148,067	4,567	15,800	8	1	64	1,305,737
56-22	plasma	2,328,442	688,343	6,284	12,685	15	8	369	1,622,738
56-23	plasma	2,227,740	429,271	6,914	12,275	16	0	47	1,777,217
56-24	plasma	2,118,075	777,348	6,627	14,477	6	3	87	1,319,327
57-1	plasma	3,754,381	550,480	9,009	24,854	15	4	47	3,169,972
57-2	plasma	3,825,111	751,521	12,605	24,410	40	2	215	3,038,318
57-3	plasma	5,249,098	1,780,587	15,966	35,375	35	0	32	3,417,103

57-4	plasma	2,847,833	663,271	5,131	17,012	0	0	4	2,162,215
57-5	plasma	3,310,293	625,223	7,714	16,091	0	9	40	2,669,218
57-6	plasma	9,383,724	8,334,026	2,067	6,195	6	1	52	1,041,377
57-7	plasma	2,725,931	1,529,341	2,969	10,370	0	0	31	1,183,220
57-8	plasma	11,637,602	7,314,452	12,192	30,604	34	45	58	4,280,217
57-9	plasma	14,446,860	1,634,235	23,503	37,836	50	4	301	12,750,931
57-10	plasma	1,388,412	454,441	2,785	8,690	6	0	668	921,822
57-11	plasma	379,083	104,910	831	3,919	0	0	160	269,263
57-12	plasma	1,222,703	256,074	2,926	8,199	0	0	80	855,414
57-13	plasma	618,688	219,660	1,702	4,866	0	0	12	592,448
57-14	plasma	1,446,507	258,940	2,538	7,372	3	0	116	1,177,538
57-15	plasma	3,292,635	1,341,654	4,896	12,712	10	0	483	1,932,880
57-16	plasma	1,918,498	378,900	5,206	10,941	6	0	50	1,523,395
57-17	plasma	4,302,159	1,623,985	9,777	29,565	10	0	246	2,639,576
57-18	plasma	3,855,593	1,300,827	8,784	18,053	2	0	230	2,527,697
57-19	plasma	647,739	166,942	1,840	4,616	0	0	4	474,337
57-20	plasma	2,254,526	661,096	6,810	11,253	8	0	366	1,574,993
57-21	plasma	9,124,100	3,769,896	14,407	22,309	9	3	80	5,317,396
57-22	plasma	2,964,377	766,354	6,590	8,393	14	2	243	2,182,781
57-23	plasma	1,540,630	465,935	3,822	6,270	2	0	22	1,064,579
57-24	plasma	1,664,638	521,506	5,996	8,665	0	0	46	1,128,425
58-1	plasma	3,281,733	552,226	11,811	20,030	19	1	42	2,697,602
58-2	plasma	10,550,723	1,547,565	13,275	45,268	15	14	247	8,944,339
58-3	plasma	13,813,847	3,679,954	19,097	62,476	33	10	20	10,052,257
58-4	plasma	959,246	308,008	1,662	4,462	6	0	3	645,105
58-5	plasma	1,932,477	595,267	4,025	12,494	0	0	5	1,220,686
58-6	plasma	2,283,870	2,109,494	449	1,145	2	0	25	172,755
58-7	plasma	1,014,480	634,665	577	2,623	0	0	3	377,212
58-8	plasma	19,374,106	11,672,840	16,134	28,562	15	13	29	7,656,513
58-9	plasma	13,377,454	3,589,806	18,239	81,127	35	14	680	9,687,351
58-10	plasma	527,663	183,836	802	2,160	1	1	139	340,724
58-11	plasma	148,768	56,302	305	1,095	0	0	51	89,015
58-12	plasma	294,610	97,239	442	1,917	3	3	66	194,940
58-13	plasma	226,722	87,358	466	1,076	0	6	7	137,809
58-14	plasma	388,000	122,718	1,292	2,277	0	0	31	261,682
58-15	plasma	2,030,586	922,255	1,444	3,993	7	4	121	1,102,762
58-16	plasma	867,511	305,907	3,009	5,702	5	0	13	552,875
58-17	plasma	19,871,378	2,998,901	22,496	43,632	51	5	387	16,605,906
58-18	plasma	4,117,902	1,361,699	5,105	12,116	0	2	167	2,738,793
58-19	plasma	190,747	43,942	506	991	1	0	5	145,302
58-20	plasma	1,075,290	515,733	1,831	4,047	10	0	230	553,439
58-21	plasma	10,651,760	5,037,385	11,006	17,486	13	10	122	5,585,738
58-22	plasma	908,174	376,695	1,587	2,363	6	0	79	525,424
58-23	plasma	542,596	183,785	1,243	1,760	0	1	6	355,803
58-24	plasma	611,175	254,086	1,400	2,372	0	0	8	353,307

Table 1c

Sample ID	Biofi. type	Total reads	Adapter reads	Calibrators used		Additional technical categories			Remaining reads
				set 1	set 2	set 3 calibrators	long calibrators	markers	
60-1	serum	5,423,727	552,503	23,863	35,480	42	9	43	4,811,785
60-2	serum	3,874,826	640,663	16,633	31,008	28	0	213	3,186,281
60-3	serum	8,011,747	1,399,992	32,232	40,578	32	1	54	6,538,858
60-4	serum	2,617,479	779,824	7,576	24,608	5	0	2	1,805,464
60-5	serum	3,137,594	668,919	12,058	25,037	22	2	28	2,431,528
60-6	serum	14,810,063	11,876,882	4,225	11,760	5	3	79	2,917,129
60-7	serum	4,448,773	2,043,563	8,066	15,670	21	9	13	2,381,431
60-8	serum	12,254,009	6,814,433	18,904	38,496	38	31	58	5,382,049
60-9	serum	7,117,242	1,311,196	27,276	50,281	64	3	569	5,727,843
60-10	serum	3,289,009	602,519	8,330	14,324	9	1	1,655	2,662,171
60-11	serum	395,500	137,970	993	5,506	1	0	416	250,614
60-12	serum	2,758,768	366,256	4,283	13,555	6	1	131	2,372,536
60-13	serum	1,280,885	269,506	3,868	7,306	8	3	12	1,000,182
60-14	serum	3,322,775	345,350	5,869	10,175	6	0	327	2,961,028
60-15	serum	5,557,662	1,502,029	13,718	16,992	14	3	663	4,024,243
60-16	serum	2,061,639	467,869	7,670	14,320	3	0	766	1,571,011
60-17	serum	7,476,782	1,682,134	16,988	33,363	21	6	303	5,743,967
60-18	serum	3,511,017	1,396,721	10,884	20,737	9	2	448	2,082,216
60-19	serum	876,094	229,714	2,863	7,589	5	0	12	635,901
60-20	serum	2,137,789	821,427	6,751	15,130	8	2	849	1,293,622
60-21	serum	8,535,154	3,356,964	13,041	25,447	11	10	125	5,139,556
60-22	serum	2,192,690	897,357	4,241	11,250	1	0	546	1,279,295
60-23	serum	1,417,074	324,087	3,622	5,491	1	0	49	1,083,824
60-24	serum	1,490,551	593,634	4,381	10,228	0	0	89	882,209
61-1	serum	11,892,582	648,952	20,565	30,959	23	0	28	11,192,055
61-2	serum	7,377,723	1,045,074	17,610	38,249	29	6	216	6,276,539
61-3	serum	6,907,068	2,321,210	29,255	50,756	49	7	31	4,505,760
61-4	serum	1,722,665	598,363	5,025	12,702	1	0	2	1,106,572
61-5	serum	2,165,371	665,887	7,981	18,468	13	2	28	1,472,992
61-6	serum	6,035,649	5,383,412	1,588	3,814	0	3	25	646,807
61-7	serum	1,846,704	1,297,342	2,190	7,377	7	0	11	539,777
61-8	serum	14,305,092	8,544,610	14,957	36,374	20	33	83	4,709,015
61-9	serum	14,602,645	1,846,575	45,687	49,422	80	3	647	12,660,231
61-10	serum	1,102,824	239,842	3,239	4,414	3	1	587	854,738
61-11	serum	984,090	106,401	1,354	3,580	0	0	245	872,530
61-12	serum	1,538,514	222,916	2,157	6,583	0	0	93	1,306,755
61-13	serum	667,800	229,388	1,859	4,629	12	2	7	431,893
61-14	serum	1,778,043	254,006	2,465	6,566	1	0	164	1,512,841
61-15	serum	3,019,794	1,833,351	2,893	14,431	5	0	458	1,168,656
61-16	serum	1,673,000	481,634	5,787	12,939	1	0	257	1,172,382
61-17	serum	8,766,826	2,686,663	19,401	46,831	39	8	564	6,013,320
61-18	serum	5,619,093	1,859,648	15,491	22,715	6	0	433	3,720,800
61-19	serum	486,160	141,958	1,588	3,575	0	0	7	338,032
61-20	serum	3,688,737	830,638	4,889	11,227	4	1	704	2,841,274
61-21	serum	9,522,524	5,914,103	14,800	32,095	22	13	253	3,561,238
61-22	serum	3,596,075	870,715	4,505	7,431	4	2	316	2,713,102
61-23	serum	4,307,325	475,344	4,892	5,431	11	0	34	3,821,613
61-24	serum	1,454,298	582,143	5,388	8,031	2	0	60	858,674
62-1	serum	2,965,724	439,920	12,450	22,981	19	3	52	2,490,299
62-2	serum	22,595,674	2,065,094	67,096	93,593	41	1	611	20,369,238
62-3	serum	26,246,626	5,043,593	81,598	144,746	93	4	3	20,976,589

62-4	serum	669,333	161,226	815	3,590	0	0	0	503,702
62-5	serum	2,702,979	653,874	13,359	21,494	5	0	44	2,014,203
62-6	serum	581,218	511,146	214	723	0	0	13	69,122
62-7	serum	549,284	265,784	579	2,490	1	0	12	280,398
62-8	serum	17,433,143	8,699,859	35,342	40,155	117	23	80	8,657,567
62-9	serum	31,993,329	5,183,635	124,488	193,438	186	17	2,274	26,489,291
62-10	serum	242,983	63,396	783	1,769	1	0	112	176,922
62-11	serum	91,780	31,473	314	793	0	0	40	59,160
62-12	serum	233,269	57,837	922	1,664	0	12	163	172,671
62-13	serum	141,192	43,077	255	899	0	0	41	96,920
62-14	serum	280,623	76,432	908	2,254	0	0	63	200,966
62-15	serum	672,666	373,931	1,328	3,089	0	0	162	294,156
62-16	serum	2,069,230	238,603	4,508	6,586	0	1	29	1,819,503
62-17	serum	18,849,878	3,095,532	46,976	86,005	88	26	737	15,620,514
62-18	serum	2,923,166	1,537,521	8,313	16,205	0	0	178	1,980,949
62-19	serum	103,062	15,564	318	911	0	0	14	86,257
62-20	serum	1,991,205	292,512	2,605	3,099	0	0	150	1,692,839
62-21	serum	5,388,478	2,105,268	18,079	14,838	0	0	96	3,250,197
62-22	serum	325,387	124,573	1,057	1,535	0	1	142	198,079
62-23	serum	255,045	62,339	1,118	1,375	11	0	27	190,175
62-24	serum	356,354	160,885	1,251	2,190	0	0	31	191,997

Table 1d

Sample ID	Biofl. type	Total reads	Adapter reads	Calibrators used		Additional technical categories			Remaining reads
				set 1	set 2	set 3 calibrators	long calibrators	markers	
13-1	urine	334,123	5,626	18	33,353	0	0	246	294,880
13-2	urine	27,777,856	4,111	4,436	20,042	0	2	181	27,749,084
13-3	urine	2,469,348	5,405	3,414	31,940	45	8	109	2,428,427
13-4	urine	345,574	4,447	26	774	0	0	43	340,284
13-5	urine	1,734,912	9,084	4,170	26,133	10	10	25	1,695,480
13-6	urine	12,712,093	94,915	2,012	22,192	9	7	188	12,592,770
13-7	urine	2,336,677	14,539	3,819	36,199	7	1	477	2,281,635
13-8	urine	2,311,228	13,052	3,721	26,909	28	0	90	2,267,428
13-9	urine	5,197,598	8,414	4,961	35,734	33	3	126	5,148,327
13-10	urine	3,891,968	4,641	2,354	20,378	0	1	90	3,864,504
13-11	urine	732,172	2,963	1,308	14,296	0	0	73	713,532
13-12	urine	4,462,281	3,681	3,083	25,205	46	2	259	4,430,005
13-13	urine	2,601,643	4,428	3,924	25,633	34	13	71	2,567,540
13-14	urine	1,528,149	4,710	3,057	30,661	4	1	302	1,489,414
13-15	urine	1,984,533	6,364	3,016	28,709	20	0	1,025	1,945,399
13-16	urine	1,928,102	4,249	3,497	32,129	1	0	149	1,888,077
13-17	urine	5,002,424	5,872	5,533	44,731	48	9	120	4,946,111
13-18	urine	16,107,771	7,739	8,000	29,098	21	2,964	83	16,059,866
13-19	urine	2,568,669	5,329	3,742	21,308	20	7	40	2,538,223
13-20	urine	1,411,543	6,056	3,389	23,238	32	9	53	1,378,766
13-21	urine	2,814,440	18,131	5,169	31,123	1	5	193	2,759,818
13-22	urine	31,478,462	7,144	3,868	30,795	12	277	300	31,436,066
13-23	urine	2,959,662	7,998	4,458	33,242	16	15	55	2,913,878
13-24	urine	6,516,725	8,015	3,567	24,072	0	16	142	6,480,913
14-1	urine	4,557,634	6,163	4,335	42,572	30	9	351	4,504,174
14-2	urine	1,374,041	4,873	5,839	30,553	17	0	215	1,332,544
14-3	urine	2,238,983	6,114	4,654	45,992	5	0	169	2,182,049
14-4	urine	1,360,455	3,346	2,494	47,392	11	4	10	1,307,198
14-5	urine	2,577,426	7,206	3,727	32,417	4	3	86	2,533,983
14-6	urine	26,255,476	11,247	2,347	26,235	2	20	223	26,215,402
14-7	urine	1,928,466	6,397	903	29,906	10	2	682	1,890,566
14-8	urine	2,039,139	15,655	5,102	40,350	34	23	95	1,977,880
14-9	urine	2,641,023	9,829	5,201	48,660	104	11	207	2,577,011
14-10	urine	1,763,016	4,689	2,991	25,981	5	3	155	1,729,192
14-11	urine	1,821,160	1,419	1,149	12,225	0	3	37	1,806,327
14-12	urine	5,001,164	3,894	4,347	29,426	14	1	250	4,963,232
14-13	urine	5,968,859	2,439	2,219	14,975	17	0	149	5,949,060
14-14	urine	1,393,871	3,625	2,098	18,974	31	22	247	1,368,874
14-15	urine	2,451,043	4,561	895	20,360	14	3	1,177	2,424,033
14-16	urine	7,415,337	4,241	2,278	25,964	18	245	210	7,382,381
14-17	urine	26,558,639	4,612	2,818	27,468	46	14	44	26,523,637
14-18	urine	2,850,346	3,627	4,184	19,337	46	1	71	2,823,080
14-19	urine	3,072,699	4,538	442	6,405	2	347	60	3,060,905
14-20	urine	2,622,696	3,895	3,032	19,053	2	5	100	2,596,609
14-21	urine	6,583,452	12,534	3,827	25,843	8	18	122	6,541,100
14-22	urine	18,126,633	4,433	1,230	20,473	11	0	121	18,100,365
14-23	urine	1,584,650	5,117	1,418	18,629	8	3	35	1,559,440
14-24	urine	1,599,114	3,236	1,250	12,998	0	6	110	1,581,514
15-1	urine	438,252	2,143	786	12,475	9	6	27	422,806
15-2	urine	685,328	1,626	1,308	6,591	0	1	23	675,779

15-3	urine	1,693,337	2,843	1,994	13,841	9	0	20	1,674,630
15-4	urine	2,044,790	1,788	1,388	14,663	1	20	1	2,026,929
15-5	urine	209,652	3,449	119	11,535	6	0	6	194,537
15-6	urine	556,921	16,946	1,351	10,142	5	3	28	528,446
15-7	urine	1,548,184	5,586	622	12,117	11	2	26	1,529,820
15-8	urine	58,237,017	10,271	1,847	10,791	11	4	21	58,214,072
15-9	urine	737,615	4,244	1,550	15,228	8	0	20	716,565
15-11	urine	5,311,871	2,907	773	8,180	6	0	14	5,299,991
15-11	urine	18,204,157	1,247	701	5,824	3	0	7	18,196,375
15-12	urine	552,120	1,892	1,003	7,983	8	0	38	541,196
15-13	urine	182,161	1,531	588	5,530	1	0	10	174,401
15-14	urine	454,446	1,961	916	7,562	5	0	33	443,969
15-15	urine	4,589,998	2,486	763	5,427	4	1	103	4,581,214
15-16	urine	1,949,547	1,897	821	6,232	4	1	19	1,940,573
15-17	urine	392,515	2,182	784	6,733	6	2	16	382,792
15-18	urine	480,561	2,313	1,002	5,750	6	2	19	471,469
15-19	urine	311,087	1,981	831	6,009	0	3	9	302,254
15-20	urine	731,074	2,047	775	4,834	0	0	13	723,405
15-21	urine	603,413	6,258	1,013	5,362	3	3	33	590,741
15-22	urine	719,800	2,944	913	6,222	2	0	44	709,675
15-23	urine	624,924	2,743	809	7,227	6	3	8	614,128
15-24	urine	2,294,059	1,987	545	5,446	2	1	16	2,286,062
16-1	urine	365,997	3,587	702	12,659	20	0	53	368,976
16-2	urine	951,614	2,714	687	9,522	1	0	57	938,633
16-3	urine	586,070	3,215	883	12,199	1	0	14	569,756
16-4	urine	58,464,216	2,899	1,644	15,994	3	0	6	58,443,672
16-5	urine	477,937	4,315	845	10,682	2	0	6	462,087
16-6	urine	654,740	17,482	927	10,805	3	2	34	625,487
16-7	urine	528,923	4,923	1,102	12,608	4	0	79	510,207
16-8	urine	628,615	6,659	889	11,654	8	0	18	609,187
16-9	urine	2,876,388	4,655	942	15,239	13	0	40	2,855,499
16-10	urine	1,196,321	3,503	760	8,213	6	0	40	1,183,799
16-11	urine	41,009	1,666	5	355	0	0	7	38,976
16-12	urine	580,434	3,522	555	7,087	4	0	61	569,205
16-13	urine	784,604	2,977	1,229	10,516	5	0	59	769,818
16-14	urine	861,160	3,924	1,201	16,798	9	0	89	839,139
16-15	urine	720,560	3,887	1,630	14,964	4	0	227	699,868
16-16	urine	4,756,128	3,472	812	12,134	15	3	57	4,739,635
16-17	urine	1,058,414	3,679	1,211	19,428	24	0	35	1,034,037
16-18	urine	4,018,366	4,406	2,078	15,611	17	0	28	3,996,226
16-19	urine	50,942,549	4,206	1,278	13,464	9	0	8	50,923,562
16-20	urine	854,496	3,420	1,463	11,035	6	0	37	838,535
16-21	urine	815,581	7,843	1,756	16,502	9	1	27	789,443
16-22	urine	1,150,745	4,096	1,692	18,411	10	4	148	1,126,388
16-23	urine	578,258	4,927	1,373	22,451	3	0	22	549,482
16-24	urine	1,533,721	4,458	1,495	19,090	4	1	25	1,508,646
17-1	urine	1,572,004	13,203	1,278	12,540	5	74	565	1,544,339
17-2	urine	1,023,690	13,221	1,033	8,297	4	5	474	1,000,656
17-3	urine	4,218,905	21,118	1,658	15,154	5	0	304	4,180,666
17-4	urine	1,498,569	17,021	2,027	17,561	5	2	2	1,461,931
17-5	urine	1,752,742	20,792	1,024	14,882	0	4	119	1,715,921
17-6	urine	412,777	43,148	859	15,632	1	3	309	352,625
17-7	urine	741,872	55,182	1,126	16,274	1	9	2,076	665,204
17-8	urine	2,823,150	108,708	1,606	15,201	11	8	143	2,497,473
17-9	urine	627,078	27,998	1,504	22,463	208	3	232	574,670

17-10	urine	561,010	21,108	764	9,177	0	0	240	529,721
17-11	urine	373,069	6,187	431	7,250	0	0	50	359,151
17-12	urine	822,180	9,285	969	10,309	13	2	356	801,246
17-13	urine	417,151	14,830	411	5,076	26	5	130	396,673
17-14	urine	1,728,063	10,183	935	9,051	59	2	328	1,707,505
17-15	urine	354,717	25,722	691	8,658	7	2	2,427	317,210
17-16	urine	1,084,291	14,229	1,208	10,446	3	2	236	1,058,167
17-17	urine	1,010,769	19,470	1,145	12,628	7	3	10	977,506
17-18	urine	696,150	13,156	1,070	6,365	2	4	35	675,518
17-19	urine	835,330	11,633	580	5,128	2	6	16	817,965
17-20	urine	759,980	11,500	898	5,263	2	1	23	742,293
17-21	urine	836,429	68,799	773	8,248	1	0	37	758,571
17-22	urine	937,639	28,581	863	7,888	4	6	92	900,205
17-23	urine	674,944	23,158	605	6,222	2	132	3	644,822
17-24	urine	506,243	10,012	613	6,287	1	0	18	489,312
19-1	urine	2,137,035	29,677	4,110	98,257	60	2	127	2,004,802
19-2	urine	4,923,933	21,395	7,481	96,740	29	14	120	4,798,154
19-3	urine	3,499,569	33,369	3,121	55,262	0	1	55	3,407,761
19-4	urine	3,780,535	36,174	2,141	52,188	12	1	7	3,690,012
19-5	urine	10,777,669	33,111	2,098	23,755	2	3	18	10,718,682
19-6	urine	2,750,288	338,678	1,151	20,699	4	28	40	2,389,688
19-7	urine	1,265,325	51,446	2,174	43,539	9	13	92	1,168,052
19-8	urine	1,968,567	85,091	2,235	116,335	14	25	64	1,764,803
19-9	urine	1,463,868	38,187	548	11,795	0	3	21	1,413,314
19-10	urine	1,709,054	15,025	1,474	21,845	3	0	76	1,670,631
19-11	urine	577,470	3,693	850	11,373	0	0	21	561,533
19-12	urine	818,023	17,768	1,050	23,314	6	2	90	775,793
19-13	urine	718,722	7,008	341	5,590	0	0	6	705,777
19-14	urine	448,563	13,684	330	9,399	0	0	50	425,100
19-15	urine	2,975,843	25,536	1,917	55,933	5	16	1,349	2,891,087
19-16	urine	920,436	19,628	2,267	48,211	7	6	24	850,293
19-17	urine	15,369,840	42,632	6,196	117,434	25	21	62	15,203,470
19-18	urine	11,384,262	130,274	3,130	51,636	53	15	65	11,199,089
19-19	urine	822,515	5,158	680	12,749	0	0	113	803,815
19-20	urine	2,201,254	25,804	3,038	27,462	14	0	130	2,144,806
19-21	urine	1,608,337	103,053	456	11,136	0	3	268	1,493,421
19-22	urine	1,355,294	40,053	622	9,377	0	22	245	1,304,975
19-23	urine	650,459	18,681	1,358	16,303	18	6	1	614,092
19-24	urine	1,326,836	34,333	1,430	24,843	0	9	8	1,266,213
20-1	urine	1,403,802	12,961	955	18,145	6	1	1,349	1,370,385
20-2	urine	1,296,775	7,736	846	12,917	0	3	1,214	1,274,059
20-3	urine	2,930,141	12,643	1,897	23,758	11	2	964	2,890,866
20-4	urine	593,064	17,352	921	25,003	13	2	8	549,765
20-5	urine	2,237,457	15,236	1,492	23,439	0	4	417	2,196,869
20-6	urine	2,410,747	400,371	712	18,772	7	47	736	1,990,102
20-7	urine	16,494,162	52,295	1,352	27,601	0	10	5,557	16,407,347
20-8	urine	1,848,861	29,935	1,675	26,288	0	0	354	1,790,609
20-9	urine	2,420,407	12,459	1,773	33,787	513	1	869	2,371,005
20-10	urine	1,678,558	12,473	904	13,899	4	0	602	1,650,676
20-11	urine	2,701,726	4,845	214	8,239	0	0	184	2,688,244
20-12	urine	864,079	14,928	334	6,488	0	0	1,130	841,199
20-13	urine	423,340	9,886	538	9,505	74	0	243	403,094
20-14	urine	577,143	16,102	558	19,440	132	0	737	540,174
20-15	urine	1,394,754	14,754	1,127	20,490	14	0	6,513	1,351,856
20-16	urine	1,503,611	14,028	786	19,398	0	0	874	1,488,525

20-17	urine	990,225	16,972	1,053	25,944	22	0	12	946,222
20-18	urine	1,463,350	67,432	1,462	16,461	4	3	7	1,377,961
20-19	urine	842,099	8,719	847	12,126	6	0	32	820,368
20-20	urine	547,726	22,344	975	14,097	0	4	51	510,255
20-21	urine	1,539,010	25,568	1,187	17,547	9	1	68	1,494,630
20-22	urine	844,803	21,804	748	17,422	0	1	133	803,895
20-23	urine	927,119	20,075	1,262	17,451	11	7	19	888,294
20-24	urine	1,065,749	43,117	1,648	15,733	0	0	42	1,005,209
21-1	urine	572,455	13,277	888	26,174	0	0	78	532,038
21-2	urine	900,027	13,062	2,623	29,090	5	1	43	855,203
21-3	urine	5,064,989	16,063	3,445	26,801	7	1	51	5,018,621
21-4	urine	1,522,071	10,067	1,488	23,698	0	1	0	1,486,617
21-5	urine	552,248	15,026	647	15,694	0	0	13	520,869
21-6	urine	1,030,050	265,607	912	14,853	0	17	57	740,744
21-7	urine	612,044	23,889	1,323	16,718	16	0	80	570,018
21-8	urine	1,059,216	62,722	1,490	27,064	32	53	63	967,792
21-9	urine	2,138,006	17,348	3,582	41,151	1	3	90	2,075,831
21-10	urine	886,235	11,639	778	14,665	0	0	103	869,050
21-11	urine	514,692	3,744	444	5,653	0	1	30	504,820
21-12	urine	3,574,972	6,159	1,368	11,795	0	1	61	3,555,588
21-13	urine	2,063,272	6,248	800	8,510	0	0	63	2,047,651
21-14	urine	974,055	9,721	1,445	19,221	8	0	192	943,468
21-15	urine	1,790,976	20,111	2,175	19,227	0	2	331	1,749,130
21-16	urine	874,430	10,904	1,664	23,179	5	0	53	836,625
21-17	urine	20,644,475	18,513	2,439	45,486	20	47	70	20,777,900
21-18	urine	197,134	11,002	968	4,747	15	0	43	180,959
21-19	urine	465,880	7,140	652	11,807	0	5	72	446,204
21-20	urine	1,060,378	16,003	1,796	16,263	0	2	195	1,026,118
21-21	urine	2,214,533	50,175	2,699	31,629	23	16	395	2,129,596
21-22	urine	899,059	18,034	1,389	15,084	0	0	325	864,227
21-23	urine	1,341,901	14,236	1,219	13,050	0	4	15	1,313,377
21-24	urine	3,231,522	21,862	1,333	16,395	6	76	15	3,191,835
22-1	urine	1,117,157	37,488	1,881	15,338	7	6	24	1,062,431
22-2	urine	973,372	35,568	554	13,155	6	0	23	904,066
22-3	urine	2,136,881	37,015	1,940	15,520	12	3	14	2,082,377
22-4	urine	367,714	26,311	75	1,692	2	9	0	339,623
22-5	urine	760,225	41,452	1,282	15,285	1	11	4	702,210
22-6	urine	3,092,179	153,426	1,101	15,236	7	23	37	2,922,347
22-7	urine	1,255,400	46,153	1,617	16,882	3	5	40	1,190,700
22-8	urine	1,294,795	72,791	1,496	16,491	15	8	42	1,203,952
22-9	urine	1,087,030	39,353	1,793	21,310	8	3	28	1,024,535
22-10	urine	674,936	35,003	795	12,721	0	4	30	626,385
22-11	urine	1,137,520	17,310	713	8,167	0	5	17	1,111,308
22-12	urine	3,922,484	22,765	1,480	13,727	3	6	45	3,884,458
22-13	urine	960,174	33,637	1,793	27,322	8	5	20	897,388
22-14	urine	905,691	34,230	772	6,240	2	6	48	864,351
22-15	urine	2,763,777	42,460	988	29,846	6	31	146	2,690,620
22-16	urine	4,396,900	35,325	3,074	35,542	11	143	43	4,322,762
22-17	urine	16,161,986	37,125	1,968	49,862	25	11	8	16,072,998
22-18	urine	484,449	41,429	301	7,027	0	2	5	435,685
22-19	urine	821,431	33,115	182	4,082	0	2	14	784,026
22-20	urine	2,837,329	34,688	517	11,110	7	32	41	2,790,934
22-21	urine	1,907,081	63,273	3,203	35,339	13	4	59	1,805,170
22-22	urine	1,380,840	36,014	3,250	34,090	8	7	213	1,307,258
22-23	urine	6,865,156	39,472	3,261	28,984	14	8	18	6,793,399

22-24	urine	2,934,655	53,489	3,445	34,311	15	7	45	2,843,363
23-1	urine	361,276	15,359	1,004	14,547	4	2	53	330,307
23-2	urine	1,007,364	16,119	1,032	11,840	7	2	43	978,321
23-3	urine	338,733	14,415	172	2,844	0	0	31	321,271
23-4	urine	817,070	16,097	1,748	15,939	9	2	14	783,261
23-5	urine	1,137,184	20,261	1,601	13,281	8	11	13	1,102,009
23-6	urine	1,167,481	46,631	951	10,272	0	5	25	1,109,597
23-7	urine	1,284,768	32,449	1,219	13,823	0	12	59	1,237,206
23-8	urine	1,553,145	87,660	533	12,500	0	10	27	1,452,215
23-9	urine	1,545,171	25,905	1,384	16,301	5	2	27	1,501,547
23-10	urine	1,224,094	18,000	942	9,698	0	0	32	1,195,422
23-11	urine	762,552	9,011	277	8,048	0	1	5	745,210
23-12	urine	699,557	10,836	608	14,734	0	9	28	673,342
23-13	urine	1,402,904	20,706	2,332	20,937	12	1	32	1,358,884
23-14	urine	2,256,763	13,434	2,813	33,014	23	25	99	2,207,355
23-15	urine	402,530	30,933	404	5,855	0	0	228	365,110
23-16	urine	849,544	13,076	1,282	32,794	27	13	30	802,322
23-17	urine	607,061	25,153	493	15,124	3	0	8	566,280
23-18	urine	27,867,147	17,091	2,448	18,955	3	1,157	32	27,827,461
23-19	urine	1,726,524	18,117	2,263	26,052	2	9	104	1,679,977
23-20	urine	471,773	13,962	161	7,710	6	3	135	449,796
23-21	urine	1,934,071	72,102	3,682	33,463	32	10	276	1,824,506
23-22	urine	12,816,186	27,415	2,595	29,049	21	2	301	12,756,803
23-23	urine	459,640	30,118	156	3,278	0	10	13	426,065
23-24	urine	429,597	11,165	420	3,580	0	0	15	414,417
24-1	urine	8,137,350	36,183	1,833	20,461	6	4	28	8,078,775
24-2	urine	879,786	29,181	150	2,500	0	2	11	847,942
24-3	urine	1,957,954	41,249	2,663	20,502	5	11	6	1,893,518
24-4	urine	2,943,043	35,288	544	22,858	14	3	0	2,884,336
24-5	urine	4,727,977	39,618	3,640	17,604	3	2	6	4,667,104
24-6	urine	1,504,581	48,263	1,022	19,609	7	10	24	1,435,646
24-7	urine	5,590,070	53,465	2,208	20,309	11	1	20	5,514,056
24-8	urine	27,500,349	107,938	2,574	23,586	24	15	20	27,366,192
24-9	urine	7,230,779	46,622	2,408	29,486	25	2	6	7,152,230
24-10	urine	1,533,529	31,579	295	8,597	3	1	9	1,493,045
24-11	urine	4,098,516	10,537	635	5,192	1	4	12	4,082,135
24-12	urine	8,750,763	24,921	2,532	16,065	2	3	50	8,707,190
24-13	urine	7,887,031	12,369	813	5,353	0	0	9	7,868,487
24-14	urine	12,328,070	11,694	414	6,471	9	0	38	12,309,444
24-15	urine	1,550,305	18,654	606	7,340	10	1	132	1,523,562
24-16	urine	892,921	9,655	662	7,130	5	1	15	875,453
24-17	urine	1,640,256	5,462	515	4,552	5	8	3	1,629,711
24-18	urine	489,034	8,058	500	3,959	2	0	7	478,508
24-19	urine	5,116,504	6,758	296	2,771	0	2	52	5,106,625
24-20	urine	2,772,122	10,894	795	5,662	8	1	102	2,754,660
24-21	urine	10,895,346	22,091	985	6,324	5	31	261	10,865,649
24-22	urine	1,308,442	15,345	952	8,208	1	3	198	1,283,735
24-23	urine	1,184,667	13,676	658	5,776	2	0	2	1,164,553
24-24	urine	404,394	7,132	165	4,601	0	1	9	393,086
32-16	urine	1,863,823	10,830	2,242	30,025	0	0	8,662	1,812,064
32-17	urine	3,339,843	20,698	923	20,796	2	0	44	3,297,380
32-18	urine	12,477,265	42,482	2,283	17,745	2	0	111	12,414,642
32-19	urine	939,776	9,958	1,138	13,071	0	0	253	915,356
32-20	urine	4,368,840	10,759	2,350	15,964	0	0	352	4,339,395
32-21	urine	2,015,273	50,989	1,789	26,347	0	1	1,594	1,934,573

32-22	urine	3,320,913	24,153	1,918	53,739	16	20	22,389	3,218,678
32-23	urine	608,595	19,896	514	15,629	0	0	208	572,348
32-24	urine	1,134,140	21,079	859	16,751	0	1	354	1,095,096
37-13	urine	16,402	910	36	1,394	0	0	4	14,058
37-14	urine	81,435	415	203	2,720	3	0	20	78,074
37-15	urine	518,710	1,174	921	5,167	0	0	76	511,372
37-16	urine	131,687	596	730	4,816	0	0	11	125,534
37-17	urine	632,135	967	3,051	27,731	17	0	15	600,354
37-18	urine	640,658	8,529	1,555	6,076	3	0	17	624,478
37-19	urine	300,018	43	92	921	0	0	3	298,958
37-20	urine	224,479	1,118	378	3,253	0	0	26	219,704
37-21	urine	1,140,298	2,445	789	11,441	6	0	38	1,125,579

Table 1 Overview of RNA annotation categories of sRNA data from Biofluids a Summary table listing total read counts, quality control and other technical annotations as well as remaining reads. b, c, d, per-sample lists of a for plasma, serum, and urine.

Table 2a

Biofluid	Total reads*	Mapped to hg19	miRNA	rRNA	tRNA	scRNA	Other ncRNA	mRNA	Unmapped to hg19
Plasma n=96									
median	2,009,283	84.5%	75.2%	2.3%	0.3%	2.5%	0.7%	2.7%	15.5%
min	89,015	36.5%	23.5%	1.0%	0.2%	0.8%	0.3%	0.8%	5.0%
max	16,605,906	95.0%	90.8%	4.9%	0.6%	7.1%	1.7%	22.2%	63.5%
Serum n=72									
median	1,749,152	75.0%	44.8%	8.3%	4.7%	6.2%	1.4%	4.0%	25.0%
min	59,160	37.4%	16.3%	2.9%	1.6%	1.7%	0.7%	1.0%	6.1%
max	26,489,291	93.9%	78.7%	16.4%	15.3%	17.1%	2.2%	19.5%	62.6%
Urine n=282									
median	1,355,370	54.3%	15.7%	5.8%	9.4%	1.1%	1.4%	6.1%	45.7%
min	14,058	1.9%	0.0%	0.1%	0.2%	0.0%	0.1%	0.2%	1.7%
max	58,443,672	98.3%	85.4%	32.6%	94.0%	37.3%	7.9%	27.9%	98.1%

Table 2b

Sample ID	Samp. Type	Sample statistics		ncRNA annotation					mRNA	Unmapped to hg19
		total reads*	Mapped to hg19	miRNA	rRNA	tRNA	scRNA	Other ncRNA		
55-1	plasma	3,170,615	85.3%	77.0%	2.8%	0.4%	2.7%	0.6%	1.6%	14.7%
55-2	plasma	6,478,400	36.5%	23.5%	1.5%	0.5%	2.0%	1.2%	7.9%	63.5%
55-3	plasma	3,918,491	85.0%	77.0%	1.8%	0.4%	3.5%	0.6%	1.7%	15.0%
55-4	plasma	1,966,946	80.5%	71.7%	2.5%	0.4%	2.5%	0.6%	2.9%	19.4%
55-5	plasma	4,714,513	91.3%	85.0%	1.3%	0.3%	3.4%	0.4%	1.0%	8.7%
55-6	plasma	2,065,804	73.9%	51.8%	2.4%	0.3%	2.0%	0.7%	16.5%	26.2%
55-7	plasma	3,166,571	66.8%	80.0%	1.6%	0.2%	2.1%	0.6%	2.3%	13.2%
55-8	plasma	4,558,302	83.0%	71.7%	2.9%	0.5%	4.7%	0.7%	2.5%	17.0%
55-9	plasma	8,168,078	88.9%	81.1%	1.9%	0.3%	3.1%	0.9%	1.7%	11.1%
55-10	plasma	2,100,425	84.3%	75.3%	2.5%	0.6%	3.1%	0.7%	2.1%	15.7%
55-11	plasma	712,227	86.9%	76.5%	1.9%	0.3%	6.1%	0.6%	1.6%	13.1%
55-12	plasma	2,506,252	60.0%	49.3%	1.7%	0.4%	2.3%	0.9%	5.4%	40.0%
55-13	plasma	1,265,369	87.8%	77.0%	2.8%	0.4%	5.5%	0.6%	1.5%	12.2%
55-14	plasma	1,760,210	64.9%	55.4%	2.7%	0.4%	1.2%	0.9%	4.4%	35.1%
55-15	plasma	2,051,619	82.5%	70.3%	3.6%	0.4%	5.1%	0.6%	2.3%	17.5%
55-16	plasma	3,866,155	92.2%	84.3%	1.6%	0.3%	4.0%	0.5%	1.3%	7.8%
55-17	plasma	7,086,222	93.6%	89.3%	1.1%	0.2%	1.5%	0.4%	1.0%	6.4%
55-18	plasma	5,457,453	91.3%	84.1%	2.7%	0.3%	2.3%	0.5%	1.4%	8.7%
55-19	plasma	546,442	66.3%	56.6%	1.8%	0.3%	2.5%	0.8%	4.2%	33.7%
55-20	plasma	1,447,780	76.7%	66.5%	2.6%	0.4%	3.2%	0.9%	2.9%	23.3%
55-21	plasma	2,989,816	84.8%	78.0%	1.6%	0.3%	2.6%	0.5%	1.8%	15.2%
55-22	plasma	2,815,407	89.4%	81.6%	1.9%	0.3%	3.4%	0.6%	1.5%	10.6%
55-23	plasma	1,150,522	81.6%	70.4%	2.7%	0.4%	5.1%	0.7%	2.4%	18.4%
55-24	plasma	2,583,405	81.6%	75.9%	2.0%	0.3%	0.9%	0.5%	2.0%	18.4%
56-1	plasma	3,448,204	87.6%	81.7%	1.9%	0.3%	1.7%	0.6%	1.5%	12.4%
56-2	plasma	1,725,474	82.6%	72.3%	2.4%	0.3%	4.9%	0.6%	2.1%	17.4%
56-3	plasma	3,198,406	88.1%	81.8%	1.6%	0.3%	2.6%	0.4%	1.4%	11.9%
56-4	plasma	5,438,321	93.5%	88.0%	1.8%	0.3%	1.8%	0.5%	1.3%	6.5%
56-5	plasma	3,264,606	87.1%	81.3%	1.4%	0.4%	2.0%	0.4%	1.5%	12.9%
56-6	plasma	2,082,393	65.4%	36.7%	2.0%	0.3%	1.5%	0.7%	22.2%	34.6%
56-7	plasma	1,828,482	77.7%	67.5%	2.3%	0.3%	3.3%	0.7%	3.6%	22.3%
56-8	plasma	3,951,747	87.0%	79.6%	1.6%	0.2%	3.0%	0.5%	2.0%	13.0%
56-9	plasma	9,763,521	95.0%	90.8%	1.1%	0.2%	1.7%	0.5%	0.8%	5.0%
56-10	plasma	1,309,023	79.5%	70.6%	1.9%	0.3%	3.4%	0.6%	2.7%	20.5%
56-11	plasma	391,115	72.2%	58.2%	2.7%	0.4%	6.4%	0.9%	3.7%	27.8%
56-12	plasma	1,825,135	80.1%	70.5%	2.7%	0.4%	3.0%	0.7%	2.8%	19.9%
56-13	plasma	1,450,371	85.7%	77.7%	2.7%	0.2%	2.6%	0.5%	2.0%	14.3%
56-14	plasma	2,092,191	88.6%	83.8%	1.4%	0.2%	1.3%	0.4%	1.4%	11.4%
56-15	plasma	3,019,444	88.4%	78.1%	2.3%	0.3%	5.4%	0.6%	1.8%	11.6%
56-16	plasma	1,001,067	77.9%	67.2%	2.6%	0.4%	4.0%	0.7%	2.8%	22.1%
56-17	plasma	3,909,520	92.8%	88.5%	1.0%	0.2%	1.5%	0.3%	1.2%	7.2%
56-18	plasma	2,627,068	71.5%	61.6%	2.4%	0.3%	2.1%	0.7%	4.3%	28.5%
56-19	plasma	982,023	81.4%	72.3%	2.1%	0.3%	3.5%	0.7%	2.5%	18.6%
56-20	plasma	2,504,704	82.1%	74.6%	2.0%	0.3%	1.7%	0.8%	2.7%	17.9%
56-21	plasma	1,305,737	72.8%	62.1%	3.5%	0.3%	2.7%	0.7%	3.4%	27.2%
56-22	plasma	1,622,738	72.4%	63.8%	1.6%	0.3%	2.0%	0.7%	3.9%	27.6%
56-23	plasma	1,777,217	80.2%	72.3%	2.5%	0.4%	4.9%	0.7%	2.5%	16.8%
56-24	plasma	1,319,327	79.8%	72.6%	2.5%	0.3%	1.1%	0.7%	2.7%	20.2%
57-1	plasma	3,169,972	75.8%	64.2%	3.0%	0.5%	2.4%	1.0%	4.7%	24.2%
57-2	plasma	3,036,318	83.8%	71.5%	2.9%	0.4%	5.1%	0.9%	3.1%	16.2%
57-3	plasma	3,417,103	72.6%	58.4%	2.8%	0.5%	3.2%	1.2%	6.5%	27.4%
57-4	plasma	2,162,215	68.4%	79.2%	2.6%	0.3%	2.5%	0.8%	2.9%	11.6%
57-5	plasma	2,669,216	85.5%	78.7%	1.5%	0.2%	2.1%	0.6%	2.4%	14.5%
57-6	plasma	1,041,377	73.7%	51.7%	1.6%	0.2%	1.0%	0.7%	18.5%	26.3%
57-7	plasma	1,183,220	84.8%	75.0%	2.1%	0.3%	2.8%	0.7%	3.7%	15.4%
57-8	plasma	4,280,217	80.9%	69.8%	2.6%	0.4%	2.6%	0.9%	4.5%	19.1%

57-9	plasma	12,750,901	93.0%	87.4%	1.1%	0.2%	2.0%	0.8%	1.4%	7.0%
57-10	plasma	921,622	85.3%	75.8%	2.8%	0.3%	2.9%	0.7%	2.8%	14.7%
57-11	plasma	269,263	82.1%	67.3%	2.9%	0.4%	7.1%	0.9%	3.5%	17.9%
57-12	plasma	955,414	84.4%	74.2%	2.6%	0.3%	3.3%	0.7%	3.3%	15.6%
57-13	plasma	592,448	76.5%	60.8%	9.3%	0.5%	4.5%	1.7%	5.8%	23.5%
57-14	plasma	1,177,538	83.9%	75.6%	2.1%	0.3%	1.5%	0.8%	3.7%	16.1%
57-15	plasma	1,932,880	85.6%	74.2%	3.3%	0.3%	3.5%	0.8%	3.4%	14.4%
57-16	plasma	1,523,395	87.2%	77.9%	2.0%	0.3%	3.7%	0.7%	2.6%	12.8%
57-17	plasma	2,638,576	77.7%	67.0%	2.6%	0.4%	2.2%	0.8%	4.6%	22.3%
57-18	plasma	2,527,697	86.1%	76.6%	2.6%	0.3%	3.0%	0.6%	3.1%	13.9%
57-19	plasma	474,337	81.7%	71.0%	2.7%	0.4%	2.7%	1.0%	4.0%	18.3%
57-20	plasma	1,574,993	82.1%	71.0%	3.1%	0.5%	2.5%	1.1%	3.9%	17.9%
57-21	plasma	5,317,396	83.1%	74.2%	1.8%	0.3%	2.1%	0.8%	3.9%	16.9%
57-22	plasma	2,182,781	90.3%	82.8%	2.7%	0.4%	1.8%	0.7%	1.9%	9.7%
57-23	plasma	1,064,579	86.5%	78.9%	2.7%	0.3%	3.3%	0.7%	2.5%	11.5%
57-24	plasma	1,128,425	86.6%	78.8%	2.8%	0.4%	1.4%	0.8%	2.6%	13.2%
58-1	plasma	2,697,602	68.9%	55.2%	2.4%	0.6%	2.7%	1.2%	6.8%	31.1%
58-2	plasma	8,944,339	91.3%	84.7%	2.1%	0.3%	2.6%	0.6%	1.6%	8.1%
58-3	plasma	10,052,257	87.6%	81.6%	1.7%	0.3%	1.1%	0.7%	2.2%	12.4%
58-4	plasma	645,105	92.4%	86.4%	1.7%	0.2%	1.7%	0.5%	1.9%	7.6%
58-5	plasma	1,220,596	73.4%	61.7%	2.9%	0.6%	1.9%	1.1%	5.2%	26.6%
58-6	plasma	172,755	72.6%	49.0%	1.9%	0.2%	0.8%	0.6%	20.1%	27.4%
58-7	plasma	377,212	53.8%	39.4%	1.8%	0.4%	1.5%	1.5%	9.4%	48.2%
58-8	plasma	7,656,513	91.9%	85.7%	1.7%	0.2%	1.8%	0.5%	1.9%	9.1%
58-9	plasma	9,687,351	88.5%	81.0%	1.4%	0.2%	2.5%	1.3%	2.1%	11.5%
58-10	plasma	940,724	85.7%	74.6%	4.9%	0.4%	2.3%	0.8%	2.8%	14.3%
58-11	plasma	89,015	87.7%	76.4%	2.0%	0.4%	5.6%	0.8%	2.5%	12.3%
58-12	plasma	194,940	82.6%	73.0%	2.7%	0.2%	2.5%	0.7%	3.4%	17.4%
58-13	plasma	137,609	87.1%	77.8%	2.2%	0.3%	2.7%	0.8%	3.2%	12.9%
58-14	plasma	261,682	85.6%	77.8%	2.5%	0.3%	1.7%	0.7%	2.6%	14.4%
58-15	plasma	1,102,782	92.9%	86.7%	2.3%	0.2%	1.3%	0.8%	1.8%	7.1%
58-16	plasma	552,875	83.6%	71.7%	2.6%	0.3%	4.7%	0.9%	3.3%	16.4%
58-17	plasma	16,605,906	93.2%	85.3%	1.6%	0.3%	3.7%	0.6%	1.7%	6.8%
58-18	plasma	2,738,793	89.4%	81.7%	2.4%	0.2%	1.9%	0.6%	2.5%	10.6%
58-19	plasma	145,302	88.8%	81.5%	2.2%	0.3%	1.3%	0.8%	2.7%	11.2%
58-20	plasma	553,439	80.3%	74.7%	2.4%	0.2%	1.6%	0.9%	3.5%	16.7%
58-21	plasma	5,585,738	77.6%	68.5%	1.3%	0.3%	1.3%	0.9%	5.5%	23.2%
58-22	plasma	525,424	74.1%	65.7%	1.6%	0.5%	1.0%	0.8%	4.5%	25.9%
58-23	plasma	355,003	88.3%	80.7%	2.1%	0.3%	1.9%	0.7%	2.6%	11.7%
58-24	plasma	353,307	85.3%	78.5%	2.1%	0.2%	1.1%	0.6%	2.7%	14.7%

Table 2c

Sample ID	Samp. Type	Sample statistics		ncRNA annotation					mRNA	Unmapped to hg19
		Total reads*	Mapped to hg19	miRNA	rRNA	tRNA	scRNA	Other ncRNA		
60-1	serum	4,911,785	77.4%	39.4%	6.8%	15.3%	10.6%	1.7%	9.5%	22.6%
60-2	serum	3,186,281	68.9%	39.9%	8.8%	7.3%	7.3%	1.2%	4.3%	31.1%
60-3	serum	6,538,858	76.1%	43.7%	10.5%	9.6%	7.1%	1.5%	3.7%	23.9%
60-4	serum	1,805,464	72.1%	36.7%	12.3%	11.2%	6.1%	1.5%	4.4%	27.9%
60-5	serum	2,431,528	68.9%	35.9%	8.8%	11.9%	6.8%	1.5%	3.9%	31.1%
60-6	serum	2,917,129	81.0%	61.1%	2.9%	2.3%	3.7%	1.1%	9.8%	19.0%
60-7	serum	2,381,431	79.0%	46.0%	10.3%	8.9%	8.4%	1.7%	5.7%	21.0%
60-8	serum	5,382,049	77.7%	44.3%	10.1%	12.2%	6.4%	1.1%	3.7%	22.3%
60-9	serum	5,727,843	76.1%	43.5%	7.2%	10.9%	9.0%	2.2%	3.3%	23.9%
60-10	serum	2,662,171	59.6%	33.3%	8.0%	5.6%	4.8%	1.4%	6.2%	40.4%
60-11	serum	250,614	84.2%	31.7%	8.9%	4.7%	13.2%	1.4%	4.2%	35.8%
60-12	serum	2,372,536	88.4%	71.8%	4.3%	2.9%	6.4%	1.0%	2.0%	11.6%
60-13	serum	1,000,182	78.3%	53.0%	8.5%	3.8%	8.1%	1.3%	3.7%	21.7%
60-14	serum	2,961,028	90.6%	76.1%	6.5%	3.0%	2.6%	0.9%	1.6%	9.4%
60-15	serum	4,024,243	49.3%	26.3%	5.4%	4.1%	4.6%	1.4%	7.4%	50.7%
60-16	serum	1,571,011	75.7%	47.2%	6.9%	5.4%	11.0%	2.0%	3.3%	24.3%
60-17	serum	5,743,967	77.5%	49.0%	3.6%	6.5%	12.6%	1.9%	3.9%	22.5%
60-18	serum	2,062,216	71.6%	38.5%	16.3%	6.5%	4.5%	1.1%	4.7%	28.4%
60-19	serum	635,901	72.1%	41.9%	11.3%	7.1%	6.1%	1.7%	3.9%	27.9%
60-20	serum	1,293,622	63.2%	33.8%	13.0%	4.1%	4.3%	1.8%	6.3%	36.8%
60-21	serum	5,139,556	44.1%	22.0%	5.7%	2.2%	1.9%	1.7%	10.5%	55.9%
60-22	serum	1,279,295	70.7%	41.3%	13.1%	6.2%	3.4%	1.5%	5.1%	29.3%
60-23	serum	1,083,824	55.9%	25.0%	10.1%	5.3%	4.3%	1.9%	9.3%	44.1%
60-24	serum	682,209	52.4%	30.2%	8.2%	2.4%	1.9%	1.6%	8.0%	47.6%
61-1	serum	11,192,055	90.5%	70.6%	4.4%	7.3%	5.7%	0.9%	1.6%	9.5%
61-2	serum	6,276,539	91.8%	48.3%	6.4%	12.6%	10.2%	1.4%	2.9%	18.2%
61-3	serum	4,505,760	68.8%	34.7%	10.1%	11.4%	6.8%	1.5%	4.3%	31.2%
61-4	serum	1,106,572	79.7%	44.2%	12.4%	10.9%	6.8%	1.4%	4.0%	20.3%
61-5	serum	1,472,992	71.6%	39.5%	10.0%	10.3%	6.3%	1.5%	4.0%	28.4%
61-6	serum	646,807	67.6%	29.7%	9.3%	5.0%	2.9%	1.2%	19.5%	32.4%
61-7	serum	539,777	59.4%	34.6%	6.1%	2.3%	7.5%	1.5%	7.3%	40.6%
61-8	serum	4,703,015	48.6%	27.1%	6.3%	3.3%	3.6%	1.3%	7.1%	51.4%
61-9	serum	12,660,231	87.9%	53.3%	6.0%	12.6%	12.2%	1.8%	2.0%	12.1%
61-10	serum	854,738	81.0%	48.9%	11.6%	6.5%	9.8%	1.4%	2.8%	19.0%
61-11	serum	872,530	50.4%	22.7%	3.1%	2.7%	13.0%	1.7%	7.3%	49.6%
61-12	serum	1,306,755	87.1%	70.0%	5.0%	2.0%	6.6%	1.1%	2.3%	12.9%
61-13	serum	431,893	79.3%	48.5%	9.7%	4.4%	12.3%	1.3%	3.1%	20.7%
61-14	serum	1,512,841	89.9%	67.8%	11.5%	4.0%	3.6%	1.1%	1.8%	10.1%
61-15	serum	1,168,656	71.2%	44.9%	5.8%	4.3%	10.5%	1.6%	4.2%	28.8%
61-16	serum	1,172,382	71.6%	33.9%	11.5%	9.4%	10.2%	1.6%	5.0%	28.4%
61-17	serum	6,013,320	77.6%	48.5%	8.6%	8.7%	9.7%	1.4%	3.6%	22.4%
61-18	serum	3,720,800	69.1%	39.3%	7.6%	7.4%	8.3%	1.1%	5.3%	30.9%
61-19	serum	339,032	74.9%	45.4%	8.9%	7.4%	7.5%	1.7%	4.0%	25.1%
61-20	serum	2,841,274	88.7%	71.4%	6.9%	3.6%	3.4%	1.1%	2.2%	11.3%
61-21	serum	3,561,238	68.2%	38.1%	13.6%	5.0%	5.1%	1.6%	4.8%	31.8%
61-22	serum	2,713,102	43.1%	24.4%	3.2%	1.9%	2.1%	1.7%	9.7%	56.9%
61-23	serum	3,821,613	93.9%	78.7%	6.5%	2.7%	4.2%	0.7%	1.0%	6.1%
61-24	serum	858,674	74.1%	50.2%	12.6%	3.4%	2.2%	1.3%	4.5%	25.9%
62-1	serum	2,490,299	74.3%	43.9%	8.8%	4.5%	10.7%	2.0%	4.4%	25.7%
62-2	serum	20,369,238	76.3%	38.6%	8.6%	15.2%	8.8%	1.4%	3.6%	23.7%
62-3	serum	20,976,589	77.7%	51.0%	7.0%	5.0%	9.3%	1.8%	9.5%	22.3%
62-4	serum	503,702	88.6%	72.7%	4.3%	1.9%	6.1%	1.3%	2.3%	11.4%
62-5	serum	2,014,203	72.8%	44.7%	7.9%	7.5%	7.2%	1.8%	3.7%	27.2%
62-6	serum	63,122	58.1%	26.8%	6.9%	4.9%	2.6%	1.3%	15.6%	41.9%
62-7	serum	280,398	83.1%	68.9%	4.5%	1.6%	4.1%	1.2%	2.8%	16.9%
62-8	serum	8,657,567	79.8%	44.9%	16.4%	7.7%	5.6%	1.4%	9.8%	20.2%

62-9	serum	26,488,291	90.7%	54.5%	8.1%	4.7%	8.4%	2.0%	3.0%	19.3%
62-10	serum	175,922	51.1%	41.6%	6.8%	1.6%	3.0%	1.2%	5.1%	38.9%
62-11	serum	59,160	80.1%	48.7%	5.8%	2.7%	17.1%	2.2%	3.5%	19.9%
62-12	serum	172,571	72.5%	43.5%	13.7%	3.7%	4.5%	1.5%	5.5%	27.5%
62-13	serum	95,920	74.4%	52.3%	8.2%	1.6%	6.2%	1.3%	4.8%	25.6%
62-14	serum	200,966	78.3%	47.6%	13.3%	6.4%	5.5%	1.6%	3.9%	21.7%
62-15	serum	294,156	71.2%	48.6%	9.8%	3.3%	3.4%	1.3%	4.8%	28.8%
62-16	serum	1,819,503	37.4%	16.3%	4.2%	2.3%	3.4%	1.6%	9.6%	62.6%
62-17	serum	15,620,514	85.5%	58.8%	5.1%	4.3%	13.0%	1.6%	2.6%	14.5%
62-18	serum	1,360,949	73.6%	40.7%	11.6%	4.5%	10.6%	1.3%	5.0%	26.4%
62-19	serum	86,257	91.1%	63.0%	6.8%	2.9%	3.7%	1.3%	3.3%	18.9%
62-20	serum	1,692,639	93.5%	77.9%	6.8%	2.1%	3.8%	1.3%	1.8%	6.5%
62-21	serum	3,250,197	79.5%	52.4%	12.2%	6.5%	3.7%	1.4%	3.4%	20.5%
62-22	serum	198,079	75.2%	51.3%	10.7%	3.7%	3.5%	1.7%	4.3%	24.8%
62-23	serum	190,175	78.2%	51.3%	13.6%	3.6%	4.6%	1.9%	3.3%	21.8%
62-24	serum	191,997	69.9%	47.2%	13.1%	2.1%	1.7%	1.0%	4.8%	30.1%

Table 2d

Sample ID	Sample Type	Sample statistics		ncRNA annotation					mRNA	Unmapped to HG19
		Total reads*	Mapped to HG19	miRNA	rRNA	IRNA	scRNA	Other ncRNA		
13-1	urine	294,880	35.8%	8.0%	7.1%	2.4%	0.5%	2.4%	15.9%	64.2%
13-2	urine	27,749,064	93.5%	71.8%	10.0%	1.4%	7.2%	1.4%	1.7%	6.5%
13-3	urine	2,428,427	64.2%	11.5%	8.9%	33.0%	0.7%	1.9%	8.4%	35.8%
13-4	urine	340,284	79.0%	16.4%	32.6%	9.1%	0.7%	7.8%	12.3%	21.0%
13-5	urine	1,695,460	54.0%	24.5%	8.6%	6.0%	1.2%	2.1%	11.7%	46.0%
13-6	urine	12,592,770	76.1%	14.6%	19.1%	28.5%	2.7%	1.6%	9.5%	23.9%
13-7	urine	2,281,635	38.6%	11.0%	7.2%	3.4%	3.2%	1.8%	12.0%	81.4%
13-8	urine	2,267,428	65.1%	28.0%	6.1%	15.9%	3.3%	2.0%	7.8%	34.9%
13-9	urine	5,148,327	58.7%	30.4%	8.0%	7.6%	2.3%	2.6%	8.0%	41.3%
13-10	urine	3,664,504	90.5%	41.6%	10.4%	11.1%	6.7%	5.1%	5.6%	19.5%
13-11	urine	713,532	49.0%	16.7%	7.7%	6.4%	2.3%	2.8%	13.1%	51.0%
13-12	urine	4,430,005	76.7%	7.3%	9.7%	51.9%	0.6%	1.8%	5.4%	23.3%
13-13	urine	2,567,540	74.4%	37.3%	8.7%	16.8%	1.1%	1.5%	9.0%	25.6%
13-14	urine	1,489,414	51.0%	14.8%	8.5%	13.7%	0.7%	2.9%	10.3%	49.0%
13-15	urine	1,945,399	61.2%	25.2%	10.0%	11.0%	3.9%	1.8%	9.4%	38.8%
13-16	urine	1,888,077	64.4%	36.8%	7.6%	9.7%	1.5%	1.4%	7.3%	35.6%
13-17	urine	4,948,111	53.9%	21.6%	8.7%	4.9%	7.7%	1.8%	9.2%	46.1%
13-18	urine	16,059,866	57.9%	27.2%	6.5%	13.2%	1.5%	2.6%	6.9%	42.1%
13-19	urine	2,536,223	50.5%	27.8%	4.1%	5.3%	2.5%	1.6%	6.7%	49.5%
13-20	urine	1,378,766	53.9%	17.2%	6.8%	16.4%	1.4%	2.1%	10.0%	46.1%
13-21	urine	2,759,618	36.1%	10.9%	7.6%	2.7%	0.9%	1.8%	12.4%	63.9%
13-22	urine	31,436,066	92.7%	54.3%	12.5%	8.0%	8.0%	4.8%	5.1%	7.3%
13-23	urine	2,913,878	65.5%	24.0%	10.8%	16.5%	1.7%	2.0%	10.5%	34.5%
13-24	urine	6,480,913	82.0%	23.5%	16.6%	35.0%	1.3%	1.4%	4.1%	18.0%
14-1	urine	4,504,174	43.2%	10.1%	6.2%	12.2%	1.2%	2.4%	11.1%	56.8%
14-2	urine	1,332,544	53.0%	18.6%	6.5%	16.6%	1.3%	1.6%	8.3%	47.0%
14-3	urine	2,182,049	49.1%	31.4%	3.5%	4.9%	0.9%	1.5%	6.8%	50.9%
14-4	urine	1,307,196	61.5%	10.9%	15.1%	14.2%	9.0%	4.6%	7.7%	38.5%
14-5	urine	2,533,983	56.4%	28.6%	3.9%	12.0%	2.1%	1.7%	8.1%	43.6%
14-6	urine	26,215,402	93.6%	38.6%	10.5%	20.0%	14.4%	7.9%	2.1%	6.4%
14-7	urine	1,890,566	68.1%	49.0%	5.0%	4.7%	1.3%	1.2%	6.9%	31.9%
14-8	urine	1,977,680	67.9%	37.7%	9.3%	7.2%	6.3%	1.5%	5.9%	32.1%
14-9	urine	2,577,011	56.7%	22.6%	7.1%	10.9%	3.5%	4.7%	7.9%	43.3%
14-10	urine	1,729,192	63.5%	12.4%	10.6%	26.4%	5.3%	2.0%	6.8%	36.5%
14-11	urine	1,806,327	88.2%	3.8%	5.9%	72.5%	1.3%	1.6%	3.1%	11.8%
14-12	urine	4,963,232	69.5%	10.8%	4.6%	44.7%	3.1%	1.3%	5.0%	30.5%
14-13	urine	5,949,060	75.5%	11.1%	8.9%	48.6%	1.4%	1.4%	4.2%	24.5%
14-14	urine	1,368,874	53.4%	29.4%	5.5%	8.8%	0.9%	1.5%	7.3%	46.6%
14-15	urine	2,424,033	68.0%	10.4%	8.6%	38.7%	3.1%	1.6%	5.7%	32.0%
14-16	urine	7,382,381	18.9%	2.7%	2.1%	1.5%	1.0%	1.6%	10.0%	81.1%
14-17	urine	26,523,637	94.8%	2.3%	4.3%	84.4%	0.7%	1.3%	1.8%	5.2%
14-18	urine	2,823,080	77.6%	49.7%	7.5%	10.9%	2.9%	1.4%	4.8%	22.4%
14-19	urine	3,060,905	19.6%	2.8%	2.1%	1.7%	0.3%	1.2%	11.6%	80.4%
14-20	urine	2,596,609	58.4%	37.0%	5.7%	3.3%	3.7%	1.8%	6.9%	41.6%
14-21	urine	6,541,100	62.6%	29.1%	9.9%	9.1%	4.6%	1.3%	6.5%	37.4%
14-22	urine	18,100,365	95.8%	71.9%	6.1%	9.2%	6.0%	0.9%	1.8%	4.2%
14-23	urine	1,559,440	47.2%	16.7%	12.4%	2.7%	4.1%	1.9%	9.4%	52.8%
14-24	urine	1,581,514	49.4%	27.9%	5.7%	5.8%	1.3%	1.4%	7.2%	50.6%
15-1	urine	422,806	26.9%	7.4%	5.4%	3.8%	1.6%	2.0%	6.6%	73.1%
15-2	urine	675,779	75.9%	45.1%	6.8%	15.4%	4.1%	1.5%	3.0%	24.1%
15-3	urine	1,674,630	39.3%	11.3%	2.7%	7.4%	0.5%	1.3%	16.1%	60.7%
15-4	urine	2,026,929	26.6%	2.7%	3.0%	14.5%	0.6%	0.9%	4.9%	73.4%
15-5	urine	194,537	41.2%	21.4%	4.0%	6.7%	1.4%	1.3%	6.6%	58.8%
15-6	urine	528,446	50.4%	21.7%	5.1%	11.2%	3.3%	2.0%	7.1%	49.6%
15-7	urine	1,529,820	12.5%	1.7%	1.4%	4.1%	0.3%	0.6%	4.3%	87.5%
15-8	urine	58,214,072	98.3%	1.3%	1.5%	94.0%	0.2%	1.0%	0.4%	1.7%
15-9	urine	716,565	63.7%	27.6%	5.1%	21.7%	3.3%	2.2%	3.8%	36.3%

15-10	urine	5,299,991	95.2%	1.2%	3.5%	89.3%	0.3%	1.1%	0.8%	4.8%
15-11	urine	18,196,375	97.9%	0.7%	3.0%	92.0%	0.5%	1.1%	0.6%	2.1%
15-12	urine	541,196	52.5%	10.4%	5.8%	27.7%	2.1%	2.1%	4.4%	47.5%
15-13	urine	174,401	41.3%	10.7%	4.9%	13.5%	3.5%	1.6%	7.0%	58.7%
15-14	urine	443,969	23.9%	8.2%	2.5%	6.0%	0.4%	1.0%	6.0%	76.1%
15-15	urine	4,581,214	94.8%	3.1%	4.2%	84.6%	0.8%	1.1%	1.1%	5.2%
15-16	urine	1,940,573	16.8%	7.5%	0.8%	1.0%	0.9%	0.7%	5.7%	83.2%
15-17	urine	382,782	56.4%	22.5%	8.7%	9.5%	6.8%	3.0%	5.9%	43.6%
15-18	urine	471,469	61.8%	34.5%	6.2%	9.2%	5.6%	1.7%	4.5%	38.2%
15-19	urine	302,254	32.2%	13.0%	3.3%	7.0%	1.3%	1.2%	6.4%	67.8%
15-20	urine	723,405	64.5%	45.8%	4.2%	6.6%	2.9%	1.4%	3.6%	35.5%
15-21	urine	590,741	59.5%	35.4%	4.2%	8.4%	4.6%	1.4%	4.4%	40.5%
15-22	urine	709,675	77.7%	34.7%	13.4%	22.0%	3.8%	1.2%	2.6%	22.3%
15-23	urine	614,128	37.4%	16.8%	3.0%	7.1%	3.8%	1.1%	5.6%	62.6%
15-24	urine	2,286,062	89.8%	69.9%	5.6%	8.2%	3.0%	1.0%	1.9%	10.2%
16-1	urine	368,976	30.7%	5.9%	5.6%	13.1%	1.4%	1.5%	3.2%	69.3%
16-2	urine	936,603	73.7%	4.4%	2.7%	59.4%	4.9%	0.8%	1.4%	26.3%
16-3	urine	569,758	41.7%	25.0%	5.9%	4.8%	1.6%	1.1%	3.3%	58.3%
16-4	urine	58,443,672	3.5%	0.6%	0.1%	0.2%	0.0%	0.2%	2.9%	96.5%
16-5	urine	462,087	22.9%	5.2%	4.9%	8.2%	0.4%	0.6%	3.5%	77.1%
16-6	urine	625,487	55.2%	13.3%	12.0%	15.8%	8.3%	0.9%	4.9%	44.8%
16-7	urine	510,207	40.5%	24.2%	5.0%	5.6%	1.4%	0.8%	3.5%	59.5%
16-8	urine	609,187	42.4%	4.6%	11.0%	20.7%	2.5%	0.8%	2.8%	57.6%
16-9	urine	2,855,489	90.4%	1.2%	2.6%	84.0%	0.6%	1.2%	0.7%	9.6%
16-10	urine	1,183,799	69.1%	16.5%	9.1%	37.5%	3.5%	0.8%	1.7%	30.9%
16-11	urine	38,976	54.1%	6.5%	5.5%	37.0%	0.7%	0.8%	3.7%	45.9%
16-12	urine	569,205	50.9%	17.3%	7.2%	19.2%	4.3%	0.6%	2.2%	49.1%
16-13	urine	769,818	63.2%	14.4%	8.4%	32.5%	4.7%	0.9%	2.3%	36.8%
16-14	urine	839,139	32.2%	10.0%	7.6%	9.9%	1.1%	0.7%	2.8%	67.8%
16-15	urine	699,868	49.8%	20.6%	5.5%	16.7%	2.8%	1.1%	3.2%	50.2%
16-16	urine	4,739,635	16.2%	5.6%	6.3%	1.8%	0.5%	0.3%	1.8%	83.8%
16-17	urine	1,034,037	34.7%	20.0%	4.1%	5.5%	1.4%	0.6%	3.0%	65.3%
16-18	urine	3,996,225	67.9%	41.1%	7.1%	16.3%	1.4%	0.8%	1.2%	32.1%
16-19	urine	50,923,562	1.9%	0.2%	0.1%	0.6%	0.1%	0.1%	0.9%	98.1%
16-20	urine	838,535	20.5%	7.5%	2.9%	6.2%	0.7%	0.6%	2.6%	79.5%
16-21	urine	789,443	36.6%	21.5%	4.1%	4.9%	2.4%	0.6%	3.2%	63.4%
16-22	urine	1,126,386	40.1%	15.8%	9.1%	9.7%	2.1%	0.7%	2.7%	59.9%
16-23	urine	549,482	35.4%	12.7%	4.2%	12.6%	1.8%	0.6%	3.4%	64.6%
16-24	urine	1,508,648	36.8%	16.0%	5.4%	8.7%	3.0%	1.0%	2.7%	63.2%
17-1	urine	1,544,339	44.3%	15.0%	6.7%	6.9%	1.2%	2.2%	11.2%	55.7%
17-2	urine	1,000,656	76.2%	40.8%	12.7%	11.0%	2.4%	2.1%	7.2%	23.8%
17-3	urine	4,180,666	91.6%	20.7%	5.5%	61.1%	0.7%	1.3%	2.4%	8.4%
17-4	urine	1,461,931	42.2%	15.5%	5.7%	5.2%	0.9%	2.2%	12.8%	57.8%
17-5	urine	1,715,921	63.1%	13.8%	16.2%	22.9%	1.1%	1.9%	7.2%	36.9%
17-6	urine	352,625	37.4%	5.8%	5.2%	6.6%	0.5%	2.6%	16.6%	62.6%
17-7	urine	665,204	30.4%	7.8%	3.9%	2.8%	0.2%	2.2%	13.5%	69.6%
17-8	urine	2,497,473	70.3%	3.4%	6.7%	52.0%	0.3%	1.5%	6.4%	29.7%
17-9	urine	574,670	46.8%	12.9%	7.3%	6.6%	1.0%	4.1%	14.9%	53.2%
17-10	urine	529,721	52.6%	24.0%	6.4%	9.2%	0.5%	2.1%	10.5%	47.4%
17-11	urine	359,151	69.2%	37.1%	9.2%	11.4%	1.5%	2.5%	7.6%	30.8%
17-12	urine	801,246	76.6%	18.1%	9.6%	35.9%	1.9%	3.0%	8.1%	23.4%
17-13	urine	396,673	69.2%	42.2%	6.8%	9.5%	1.4%	1.6%	7.7%	30.8%
17-14	urine	1,707,505	87.3%	2.2%	2.4%	78.2%	0.1%	1.3%	3.1%	12.7%
17-15	urine	317,210	38.9%	14.7%	5.2%	3.0%	0.2%	2.2%	13.5%	61.1%
17-16	urine	1,058,167	52.9%	28.1%	5.9%	8.4%	0.7%	1.7%	8.1%	47.1%
17-17	urine	977,506	64.3%	42.8%	3.4%	4.6%	1.7%	2.0%	9.8%	35.7%
17-18	urine	675,518	61.4%	31.3%	7.6%	10.0%	0.8%	2.3%	9.4%	38.6%
17-19	urine	817,965	77.6%	44.4%	8.3%	14.2%	3.6%	1.9%	5.3%	22.4%
17-20	urine	742,293	53.9%	28.7%	6.8%	5.5%	1.1%	1.8%	10.0%	46.1%
17-21	urine	758,571	30.1%	2.7%	2.9%	8.4%	0.3%	1.9%	13.9%	69.9%
17-22	urine	900,205	68.0%	35.5%	13.7%	7.9%	1.0%	1.9%	8.1%	32.0%
17-23	urine	644,822	46.5%	21.4%	3.4%	5.8%	0.9%	2.4%	12.6%	53.5%

17-24	urine	489,312	53.4%	31.4%	4.3%	7.7%	0.2%	1.5%	8.4%	46.6%
19-1	urine	2,004,802	22.0%	7.3%	4.5%	3.8%	0.5%	0.8%	5.0%	78.0%
19-2	urine	4,796,154	25.9%	13.6%	3.8%	3.4%	0.4%	0.8%	9.9%	74.1%
19-3	urine	3,407,761	40.3%	20.4%	5.3%	6.6%	3.8%	0.9%	3.4%	59.7%
19-4	urine	3,690,012	69.1%	47.1%	4.7%	6.1%	6.8%	1.6%	2.9%	30.9%
19-5	urine	10,718,682	32.2%	3.3%	13.2%	9.0%	0.8%	1.0%	4.9%	67.8%
19-6	urine	2,389,688	58.5%	3.6%	3.8%	33.5%	0.7%	1.4%	15.4%	41.5%
19-7	urine	1,168,052	27.7%	9.6%	5.6%	3.0%	0.9%	0.9%	7.7%	72.3%
19-8	urine	1,764,803	13.8%	2.1%	2.8%	1.8%	0.1%	0.7%	6.2%	86.2%
19-9	urine	1,413,314	34.8%	11.4%	8.3%	4.5%	1.0%	2.0%	7.6%	65.2%
19-10	urine	1,670,631	62.7%	43.5%	6.3%	5.6%	3.1%	0.3%	6.3%	37.3%
19-11	urine	561,533	57.6%	34.0%	5.4%	6.2%	6.6%	1.2%	4.2%	42.4%
19-12	urine	775,793	45.5%	23.4%	5.8%	5.9%	3.5%	1.2%	5.5%	54.5%
19-13	urine	705,777	31.4%	2.0%	10.6%	8.2%	1.1%	1.1%	6.4%	68.6%
19-14	urine	425,100	49.9%	9.8%	13.8%	16.9%	0.7%	1.3%	7.4%	50.1%
19-15	urine	2,891,087	37.9%	2.0%	3.7%	25.8%	0.4%	0.9%	5.1%	62.1%
19-16	urine	850,293	25.7%	7.4%	4.0%	6.3%	0.7%	0.8%	6.5%	74.3%
19-17	urine	15,203,470	79.1%	5.7%	4.7%	62.3%	3.0%	1.2%	2.0%	20.9%
19-18	urine	11,199,089	36.5%	1.2%	4.5%	25.8%	0.5%	0.7%	3.7%	63.5%
19-19	urine	803,815	41.7%	16.3%	4.1%	13.2%	1.3%	1.1%	5.6%	58.3%
19-20	urine	2,144,806	31.1%	8.0%	4.2%	10.3%	1.7%	1.1%	5.7%	68.9%
19-21	urine	1,493,421	50.3%	13.1%	19.7%	6.3%	1.6%	1.2%	6.3%	49.7%
19-22	urine	1,304,975	67.5%	38.5%	11.9%	5.9%	3.5%	1.5%	6.2%	32.5%
19-23	urine	614,092	31.7%	14.1%	3.6%	4.8%	0.9%	1.0%	7.4%	68.3%
19-24	urine	1,266,213	51.4%	29.8%	6.0%	5.4%	2.7%	1.6%	5.9%	48.6%
20-1	urine	1,370,385	71.7%	11.9%	13.1%	40.0%	1.4%	1.8%	9.6%	28.3%
20-2	urine	1,274,059	59.4%	45.1%	5.3%	3.7%	2.3%	0.7%	2.2%	40.6%
20-3	urine	2,890,866	37.7%	20.8%	3.7%	2.0%	8.1%	0.9%	2.3%	62.3%
20-4	urine	549,765	38.8%	13.6%	10.3%	7.2%	0.9%	1.4%	5.5%	61.2%
20-5	urine	2,196,869	70.5%	4.4%	5.4%	57.5%	0.4%	1.0%	1.8%	29.5%
20-6	urine	1,990,102	42.6%	6.3%	3.4%	9.1%	0.5%	1.4%	21.9%	57.4%
20-7	urine	16,407,347	94.9%	3.1%	2.6%	66.8%	0.5%	1.0%	0.5%	5.1%
20-8	urine	1,790,609	72.9%	10.1%	8.9%	48.8%	0.9%	1.3%	2.9%	27.1%
20-9	urine	2,371,005	41.2%	27.3%	6.5%	2.7%	0.8%	1.5%	2.5%	58.8%
20-10	urine	1,650,676	55.8%	36.7%	7.0%	7.0%	2.1%	0.6%	2.5%	44.2%
20-11	urine	2,668,244	94.9%	67.9%	14.5%	5.3%	5.6%	0.6%	1.1%	5.1%
20-12	urine	641,199	72.0%	35.4%	17.1%	9.2%	5.5%	1.5%	3.4%	28.0%
20-13	urine	403,094	46.6%	21.1%	10.5%	8.0%	1.4%	1.0%	4.5%	53.4%
20-14	urine	540,174	38.6%	12.7%	10.9%	8.8%	0.6%	0.8%	4.7%	61.4%
20-15	urine	1,351,856	65.9%	35.1%	6.4%	14.9%	5.1%	1.4%	3.1%	34.1%
20-16	urine	1,466,525	16.9%	6.1%	3.9%	1.2%	0.2%	0.6%	4.9%	83.1%
20-17	urine	946,222	34.8%	17.3%	4.2%	7.7%	1.5%	0.7%	3.5%	65.2%
20-18	urine	1,377,981	54.4%	35.1%	5.1%	5.1%	1.6%	1.3%	6.1%	45.6%
20-19	urine	820,369	43.0%	31.8%	3.3%	2.9%	1.6%	0.5%	2.9%	57.0%
20-20	urine	510,255	38.5%	23.6%	3.4%	3.3%	1.3%	1.0%	5.9%	61.5%
20-21	urine	1,494,630	75.5%	47.8%	6.6%	7.4%	10.3%	0.9%	2.6%	24.5%
20-22	urine	603,895	40.1%	20.7%	4.0%	4.0%	5.6%	1.1%	4.8%	59.9%
20-23	urine	866,294	16.8%	5.9%	2.0%	2.6%	1.6%	0.7%	4.1%	83.2%
20-24	urine	1,005,209	47.6%	24.9%	7.0%	6.2%	1.4%	1.1%	6.9%	52.4%
21-1	urine	592,038	29.6%	5.0%	6.5%	1.7%	0.1%	2.1%	14.3%	70.4%
21-2	urine	855,203	39.4%	11.0%	7.4%	5.7%	0.3%	1.9%	13.1%	60.6%
21-3	urine	5,018,621	53.2%	22.9%	11.0%	8.6%	1.3%	2.0%	7.5%	46.8%
21-4	urine	1,486,817	72.7%	4.0%	6.2%	54.3%	0.3%	1.7%	6.1%	27.3%
21-5	urine	520,869	40.8%	19.8%	4.6%	3.9%	0.4%	1.6%	10.6%	59.2%
21-6	urine	748,744	49.0%	10.6%	4.1%	3.1%	0.8%	2.5%	27.9%	51.0%
21-7	urine	570,018	45.9%	21.8%	6.8%	3.8%	0.9%	1.6%	11.2%	54.1%
21-8	urine	967,792	35.6%	5.2%	8.8%	7.9%	0.4%	1.6%	11.7%	64.4%
21-9	urine	2,075,831	68.3%	25.8%	15.1%	13.4%	2.8%	3.1%	8.0%	31.7%
21-10	urine	858,050	74.2%	17.3%	31.6%	14.0%	1.0%	2.1%	6.1%	25.8%
21-11	urine	504,820	81.1%	5.1%	14.3%	51.4%	1.1%	2.2%	6.9%	18.9%
21-12	urine	3,555,588	89.6%	5.2%	12.1%	65.7%	0.6%	1.9%	4.0%	10.4%
21-13	urine	2,047,651	83.7%	5.0%	24.6%	44.7%	0.7%	1.8%	6.9%	16.3%

21-14	uane	943,468	76.4%	7.2%	7.5%	53.2%	0.1%	1.9%	6.4%	23.6%
21-15	uane	1,749,130	60.2%	8.9%	5.6%	36.2%	0.2%	1.5%	7.8%	39.8%
21-16	uane	839,525	32.6%	7.6%	6.8%	2.3%	0.2%	1.8%	13.8%	67.4%
21-17	uane	20,777,900	93.0%	1.0%	2.9%	85.6%	0.0%	1.4%	2.0%	7.0%
21-18	uane	180,959	42.6%	16.3%	6.0%	4.7%	0.3%	1.8%	13.7%	57.2%
21-19	uane	446,204	31.5%	10.1%	4.0%	2.5%	0.3%	1.9%	12.9%	68.4%
21-20	uane	1,026,119	37.8%	15.3%	6.8%	2.0%	0.3%	1.8%	11.6%	62.2%
21-21	uane	2,129,596	45.7%	17.6%	12.0%	3.8%	0.8%	1.6%	10.0%	54.3%
21-22	uane	664,227	49.8%	25.9%	6.3%	3.7%	0.7%	1.8%	11.5%	50.2%
21-23	uane	1,313,377	71.1%	49.2%	7.2%	5.8%	1.2%	1.7%	6.0%	28.9%
21-24	uane	3,191,835	22.4%	1.6%	2.3%	5.4%	0.1%	1.6%	11.2%	77.6%
22-1	uane	1,082,491	39.8%	14.7%	5.3%	5.4%	0.9%	1.5%	10.9%	60.2%
22-2	uane	924,066	24.8%	5.0%	5.0%	1.4%	0.9%	1.3%	11.2%	75.2%
22-3	uane	2,082,377	66.2%	3.4%	6.6%	47.0%	0.3%	1.5%	7.4%	33.8%
22-4	uane	339,625	36.7%	7.1%	4.7%	11.7%	0.2%	1.1%	11.9%	63.3%
22-5	uane	702,210	32.8%	8.6%	4.4%	4.9%	1.0%	1.3%	12.5%	67.2%
22-6	uane	2,922,347	66.7%	1.3%	3.6%	50.2%	0.1%	1.3%	10.2%	33.3%
22-7	uane	1,190,700	46.9%	8.8%	5.3%	20.6%	0.7%	1.5%	10.0%	53.1%
22-8	uane	1,203,952	51.6%	18.4%	6.4%	15.7%	0.4%	2.2%	8.8%	48.2%
22-9	uane	1,024,595	41.3%	20.4%	5.0%	1.9%	0.3%	2.6%	11.0%	58.7%
22-10	uane	626,385	38.2%	6.9%	5.6%	12.7%	0.4%	1.4%	11.3%	61.8%
22-11	uane	1,111,308	74.2%	9.6%	5.9%	49.8%	1.2%	1.5%	6.1%	25.8%
22-12	uane	3,884,458	88.3%	3.6%	5.2%	74.6%	0.5%	1.2%	3.2%	11.7%
22-13	uane	897,389	36.3%	8.4%	5.6%	6.7%	1.7%	1.5%	12.3%	63.7%
22-14	uane	864,391	45.5%	28.0%	2.6%	4.6%	0.7%	1.2%	8.4%	54.5%
22-15	uane	2,690,620	61.6%	17.3%	7.8%	26.5%	0.5%	1.3%	8.3%	38.2%
22-16	uane	4,322,762	39.6%	19.0%	9.6%	4.2%	1.7%	1.7%	9.5%	60.2%
22-17	uane	16,072,989	92.7%	2.7%	4.0%	83.0%	0.1%	1.0%	1.9%	7.3%
22-18	uane	435,685	29.3%	8.1%	3.0%	3.0%	0.8%	1.3%	13.2%	70.7%
22-19	uane	784,026	61.1%	24.5%	5.7%	14.2%	7.5%	1.3%	8.0%	38.9%
22-20	uane	2,790,934	72.1%	11.3%	6.7%	43.5%	0.6%	2.1%	7.9%	27.9%
22-21	uane	1,605,170	53.5%	5.2%	7.5%	26.2%	0.4%	1.8%	12.5%	46.5%
22-22	uane	1,307,258	50.2%	23.7%	6.1%	4.8%	4.0%	1.4%	10.1%	49.8%
22-23	uane	8,793,399	83.6%	19.4%	7.8%	12.7%	37.3%	1.8%	4.7%	16.4%
22-24	uane	2,843,363	64.9%	16.1%	10.3%	24.7%	0.3%	2.0%	10.9%	35.7%
23-1	uane	330,307	45.5%	7.2%	9.5%	9.0%	0.3%	2.6%	16.8%	54.5%
23-2	uane	978,321	71.0%	5.9%	10.2%	42.2%	0.3%	2.3%	10.1%	29.0%
23-3	uane	321,271	67.8%	21.5%	20.0%	5.3%	0.4%	2.7%	17.9%	32.2%
23-4	uane	783,261	60.2%	23.7%	9.4%	12.0%	0.7%	2.4%	11.9%	39.8%
23-5	uane	1,102,009	72.5%	33.6%	15.8%	11.5%	1.0%	2.0%	8.6%	27.5%
23-6	uane	1,109,597	53.1%	20.7%	5.6%	6.0%	1.8%	3.0%	16.0%	46.9%
23-7	uane	1,237,206	39.7%	8.1%	2.8%	2.2%	0.3%	2.3%	18.0%	66.3%
23-8	uane	1,452,215	79.7%	4.4%	11.5%	53.6%	0.1%	1.8%	8.3%	20.3%
23-9	uane	1,501,547	80.9%	30.3%	8.6%	32.2%	0.7%	2.8%	8.2%	19.1%
23-10	uane	1,195,422	73.2%	22.8%	10.3%	28.0%	0.5%	2.2%	9.5%	26.8%
23-11	uane	745,210	85.9%	4.7%	12.3%	60.2%	0.5%	2.0%	6.3%	14.1%
23-12	uane	673,342	64.7%	33.7%	7.7%	10.1%	0.9%	2.0%	10.4%	35.9%
23-13	uane	1,358,884	71.2%	36.3%	10.3%	9.7%	0.8%	2.3%	11.8%	28.8%
23-14	uane	2,207,355	65.3%	21.7%	7.7%	23.3%	0.3%	2.0%	10.3%	34.7%
23-15	uane	365,110	55.0%	32.2%	4.8%	3.4%	0.7%	1.8%	12.1%	45.0%
23-16	uane	902,322	56.0%	31.5%	5.6%	2.7%	0.6%	2.1%	13.4%	44.0%
23-17	uane	566,290	59.2%	24.8%	5.9%	6.0%	0.4%	2.9%	17.1%	40.8%
23-18	uane	27,827,461	59.8%	9.9%	7.6%	29.4%	0.3%	1.8%	10.9%	40.2%
23-19	uane	1,679,977	43.5%	14.0%	8.3%	6.0%	0.2%	1.9%	13.0%	56.5%
23-20	uane	449,796	72.5%	22.1%	8.9%	28.5%	0.3%	2.0%	10.6%	27.5%
23-21	uane	1,824,506	55.1%	21.4%	6.2%	7.6%	0.7%	2.6%	16.6%	44.9%
23-22	uane	12,756,803	94.4%	85.4%	2.8%	2.3%	0.6%	0.6%	2.5%	5.6%
23-23	uane	426,065	62.3%	25.7%	13.7%	6.5%	0.4%	2.1%	13.8%	37.7%
23-24	uane	414,417	67.8%	21.5%	9.5%	21.7%	0.3%	2.7%	12.1%	32.2%
24-1	uane	8,078,775	83.3%	0.9%	2.0%	78.0%	0.4%	0.9%	1.1%	16.7%
24-2	uane	847,942	49.1%	6.6%	1.5%	35.4%	0.3%	0.7%	3.5%	51.9%
24-3	uane	1,893,518	36.0%	10.2%	5.5%	9.4%	6.0%	1.0%	3.9%	64.0%

24-4	urine	2,884,336	74.5%	5.8%	2.9%	59.4%	3.2%	1.1%	2.1%	25.5%
24-5	urine	4,667,104	48.0%	9.0%	4.4%	29.2%	1.4%	1.0%	3.0%	52.0%
24-6	urine	1,435,646	47.5%	6.2%	5.0%	30.2%	1.5%	0.9%	3.7%	52.5%
24-7	urine	5,514,056	78.3%	5.9%	3.4%	62.5%	3.1%	1.1%	2.5%	21.7%
24-8	urine	27,366,192	53.8%	3.8%	5.0%	35.4%	6.8%	0.9%	1.8%	46.2%
24-9	urine	7,152,230	61.3%	43.3%	9.5%	19.7%	5.4%	1.2%	1.8%	18.7%
24-10	urine	1,493,046	32.4%	17.2%	3.7%	5.0%	2.9%	1.0%	2.6%	67.6%
24-11	urine	4,082,135	42.4%	19.8%	3.9%	5.4%	9.4%	1.5%	2.4%	57.6%
24-12	urine	6,707,190	86.3%	4.6%	5.4%	71.4%	2.9%	1.1%	1.0%	13.7%
24-13	urine	7,868,487	93.3%	2.2%	3.5%	84.6%	0.6%	1.1%	1.3%	6.7%
24-14	urine	12,309,444	96.3%	0.5%	0.9%	93.4%	0.1%	1.2%	0.2%	3.7%
24-15	urine	1,523,562	68.0%	19.8%	4.9%	38.1%	2.4%	1.0%	2.7%	32.0%
24-16	urine	875,453	61.7%	9.9%	6.8%	39.1%	2.5%	0.9%	2.5%	38.3%
24-17	urine	1,629,711	40.9%	20.7%	2.8%	9.3%	3.6%	1.1%	3.4%	59.1%
24-18	urine	478,508	48.5%	9.0%	7.6%	25.2%	2.2%	1.0%	3.6%	51.5%
24-19	urine	5,106,625	94.0%	1.4%	1.3%	89.4%	0.5%	1.0%	0.4%	6.0%
24-20	urine	2,754,660	85.2%	3.5%	2.6%	76.3%	0.7%	1.0%	1.1%	14.8%
24-21	urine	10,865,649	34.0%	22.5%	1.5%	2.1%	3.1%	0.9%	3.9%	66.0%
24-22	urine	1,283,735	62.9%	12.8%	14.3%	26.7%	3.5%	1.0%	2.6%	37.1%
24-23	urine	1,164,553	66.3%	4.6%	4.0%	52.1%	2.1%	1.1%	2.4%	33.7%
24-24	urine	393,086	27.0%	4.0%	4.0%	13.0%	0.3%	0.8%	4.3%	73.0%
32-16	urine	1,812,064	67.8%	32.7%	10.3%	12.5%	2.0%	2.5%	7.8%	32.2%
32-17	urine	3,297,380	79.1%	2.9%	4.6%	65.4%	0.4%	1.1%	9.9%	21.9%
32-18	urine	12,414,642	96.5%	3.6%	2.8%	86.1%	2.1%	1.2%	0.8%	3.5%
32-19	urine	915,356	34.3%	13.7%	2.8%	5.4%	4.5%	1.0%	6.8%	65.7%
32-20	urine	4,339,395	92.4%	2.1%	3.5%	84.1%	0.3%	1.1%	1.3%	7.6%
32-21	urine	1,934,573	39.8%	15.0%	4.2%	4.5%	7.6%	1.7%	6.8%	60.2%
32-22	urine	3,218,678	92.2%	65.7%	1.9%	7.4%	0.5%	2.0%	4.9%	17.6%
32-23	urine	672,348	51.5%	22.0%	6.3%	10.0%	4.0%	1.7%	8.5%	49.5%
32-24	urine	1,095,096	26.1%	6.6%	3.9%	5.4%	0.2%	1.2%	8.7%	73.9%
37-13	urine	14,058	26.2%	9.0%	4.3%	4.7%	1.2%	1.1%	6.0%	73.8%
37-14	urine	76,074	57.2%	27.7%	4.7%	19.3%	1.1%	0.9%	3.5%	42.8%
37-15	urine	511,372	77.2%	51.9%	7.7%	4.8%	9.2%	1.4%	2.3%	22.8%
37-16	urine	125,534	51.9%	21.9%	3.4%	21.2%	1.2%	0.7%	3.4%	48.1%
37-17	urine	600,354	38.8%	14.5%	6.0%	11.8%	0.8%	0.9%	4.8%	61.2%
37-18	urine	624,478	82.3%	17.7%	5.0%	54.2%	2.4%	1.0%	2.0%	17.7%
37-19	urine	298,959	92.0%	81.3%	3.4%	1.9%	3.8%	0.5%	1.0%	8.0%
37-20	urine	219,704	94.1%	17.4%	4.5%	5.4%	0.7%	0.9%	5.2%	65.9%
37-21	urine	1,125,579	75.7%	23.8%	5.1%	40.8%	2.0%	1.3%	2.6%	24.3%

Table 2 Overview of sRNA composition from biofluids. a Summary table listing prevalent HG19-derived sRNA categories and mRNA, as well as remaining HG19-unmapped reads. b, c, d per-sample lists of a for plasma, serum, and urine. Data listed here was generated excluding RNA reads in technical annotation categories (see Table 1).

Table 3

13-6 / Acute Cell Ref	Total reads	Un. reads (unassigned)	Reads: un. per total		
	12,712,093	2,981,620	23.5%		
Subset	Contigs	Contig-aligned reads, counts			min. log(E-value)
		individual	per all cont.	per un.reads	
All generated in sample	353	343,052	100.0%	11.5%	
Mapped to unique biological orders	133	202,026	56.2%	6.8%	
Mapped contigs, listed by orders					
Eukaryotes	71	149,542	41.6%	5.0%	-26.7
Firmicutes	17	40,641	11.3%	1.4%	-12.2
α -Proteobacteria	14	4,717	1.3%	0.2%	-6.7
CFB group bacteria	16	3,742	1.0%	0.1%	-8.3
Σ Additional orders, < 5 contigs	15	3,384	0.9%	0.1%	
Total	133	202,026	56.2%	6.8%	
Mapped contigs, listed by genera, (species)					
Amoeba (vermamoeba vermiformis: 48)	66	149,950	40.6%	4.9%	-26.7
Lactobacillus sp.	15	39,540	11.0%	1.3%	-12.2
Prevotella (dentalis: 3)	8	2,173	0.6%	0.1%	-6.7
Σ Additional genera, < 2 contigs	12	3,450	1.0%	0.1%	
Σ Ambiguous genus assignments	32	10,913	3.0%	0.4%	
Total	133	202,026	56.2%	6.8%	

14-16, Normal	Total reads	Un. reads (unassigned)	Reads: un. per total		
	7,415,337	5,975,790	80.6%		
Subset	Σ Contigs	Contig-aligned individual read counts			min. log(E-value)
		absolute	/ all cont.	/ un.reads	
All generated in sample	465	3,123,215	100.0%	52.3%	
Mapped to unique biological orders	207	2,374,179	76.0%	39.7%	
Mapped contigs, by orders					
Firmicutes	155	2,318,169	74.2%	38.8%	-51.7
Ascomycetes	20	14,845	0.5%	0.2%	-20.0
High GC Gram+	9	1,817	0.1%	0.0%	-8.0
Bacteria	8	35,513	1.1%	0.6%	-27.2
Mycoplasmata	5	1,463	0.0%	0.0%	-7.4
Σ Additional orders, < 5 contigs	10	2,372	0.1%	0.0%	
Total	207	2,374,179	76.0%	39.7%	
Mapped contigs, by genera, (species)					
Candida (albicans: 6)	12	5,775	0.2%	0.1%	-20.0
Lactobacillus (Delbrueckii: 6)	140	2,145,551	68.7%	35.9%	-51.7
Micrococcus luteus	5	1,391	0.0%	0.0%	-8.0
Ureaplasma sp.	5	1,463	0.0%	0.0%	-7.4
Σ Additional genera, < 2 contigs	13	61830	20.0%	12.9%	
Σ Ambiguous genus assignments	32	158,169	5.1%	2.6%	
Total	207	2,374,179	76.0%	39.7%	

15-16, Normal	Total reads	Un. reads (unassigned)	Reads: un. per total		
	4,756,128	3,560,757	83.5%		
Subset	Σ Contigs	Contig-aligned individual read counts			min. log(E-value)
		absolute	/ all cont.	/ un.reads	
All generated in sample	658	2,090,441	100.0%	52.8%	
Mapped to unique biological orders	301	1,089,357	52.1%	27.5%	
Mapped contigs, by orders					
High GC Gram+	104	577,883	27.6%	14.6%	-9.4
B-proteobacteria	41	147,278	7.0%	3.7%	-14.0
Eudicots	33	129,981	6.2%	3.3%	-79.0
Firmicutes	26	29,073	1.4%	0.7%	-8.0
Fusobacteria	24	77,873	3.7%	2.0%	-9.0
α -Proteobacteria	19	14,731	0.7%	0.4%	-10.5
CFB group bacteria	17	11,720	0.6%	0.3%	-8.0
Bacteria	11	4,930	0.2%	0.1%	-8.0
γ -Proteobacteria	10	11,291	0.5%	0.3%	-8.0
Σ Additional orders, < 5 contigs	16	84,597	4.0%	2.1%	
Total	301	1,089,357	52.1%	27.5%	
Mapped contigs, by genera, (species)					
<i>Propionibacterium propionicum</i>	17	223,577	11.2%	5.9%	-9.4
<i>Corynebacterium</i>	16	12,975	0.6%	0.3%	-8.5
<i>Streptococcus</i>	16	20,600	1.0%	0.5%	-8.0
<i>Leptotrichia (buccalis: 3)</i>	13	37,220	1.8%	0.9%	-8.5
<i>Actinomyces (meyer: 2)</i>	12	25,209	1.2%	0.6%	-24.6
<i>Neisseria (elongata: 2)</i>	10	24,419	1.2%	0.6%	-12.2
<i>Candidatus Saccharibacteria</i>	7	2,512	0.1%	0.1%	-8.0
<i>Actinobaculum schaalii</i>	4	2,175	0.1%	0.1%	-6.7
<i>Anacardium occidentale</i>	4	43,174	2.1%	1.1%	-79.0
<i>Capnocytophaga</i>	2	1,578	0.1%	0.0%	-7.7
<i>Dechloromonas aromatica</i>	2	510	0.0%	0.0%	-8.5
<i>Oerriosea agamarum</i>	2	851	0.0%	0.0%	-8.0
Σ Additional genera, < 2 contigs	63	230,404	11.0%	5.8%	
Σ Ambiguous genus assignments	133	454,154	21.7%	11.5%	
Total	301	1,089,357	52.1%	27.5%	

16-4, normal	Total reads	Un. reads (unassigned)	Reads: un. per total		
	58,464,218	58,383,746	96.5%		
Subset	Σ Contigs	Contig-aligned individual read counts			min. log(E-value)
		absolute	/ all cont.	/ un.reads	
All generated in sample	1019	37,635,015	100.0%	66.7%	
Mapped to unique biological orders	402	28,716,054	76.3%	49.1%	
Mapped contigs, by orders					
Enterobacteria	365	28,602,751	76.0%	48.9%	-125.7
Ascomycetes	17	7,189	0.0%	0.0%	-9.7
Σ Additional orders, < 5 contigs	20	106,114	0.3%	0.2%	
Total	402	28,716,054	76.3%	49.1%	
Mapped contigs, by genera, (species)					
Klebsiellae (pneumoniae: 53)	165	1,494,642	4.0%	2.6%	-114.0
Escherichia coli	3	1,343,196	3.6%	2.3%	-10.7
Salmonella enterica	3	239,819	0.6%	0.5%	-28.5
Arsenophonus endosymbiont	3	775	0.0%	0.0%	-7.4
Fusaria (graminearum: 2)	3	706	0.0%	0.0%	-8.2
Σ Additional genera, < 2 contigs	28	7,539,933	20.0%	12.9%	
12/27/2015 12/27/2015	197	18,036,983	47.9%	30.9%	
Total	402	28,716,054	76.3%	49.1%	

14-17 / Normal, N/A	Total reads	Un. reads (unassigned)	Reads: un. per total		
	26,558,639	1,358,873	5.1%		
Subset	Contigs	Contig-aligned reads, counts			min. log(E-value)
		individual	per all cont.	per un.reads	
All generated in sample	243	192,514	100.0%	14.2%	
Mapped to unique biological orders	83	88,050	45.7%	6.5%	
Mapped contigs, listed by orders					
Firmicutes	40	64,653	33.7%	4.8%	-17.2
High GC Gram+	14	8,212	4.3%	0.6%	-6.5
A-proteobacteria	8	2,633	1.4%	0.2%	-6.0
Enterobacteria	6	2,145	1.1%	0.2%	-8.3
Σ Additional orders, < 5 contigs	15	10,207	5.3%	0.8%	
Total	83	88,050	45.7%	6.5%	
Mapped contigs, listed by genera, (species)					
Lactobacillus sp.	30	35,384	20.5%	2.9%	-14.4
Micrococcus luteus	4	1,426	0.7%	0.1%	-6.5
Σ Additional genera, < 2 contigs	16	15058	7.8%	1.1%	
Σ Ambiguous genus assignments	33	32,172	16.7%	2.4%	
Total	83	88,040	45.7%	6.5%	

16-19, Acute cell rejection		Total reads	Un. reads (unassigned)	Reads: un. per total	
		50,942,549	49,939,886	98.0%	
Subset	Σ Contigs	Config-aligned individual read counts			min. log(E-value)
		absolute	/all cont.	/un.reads	
All generated in sample	2477	41,897,796	100.0%	66.7%	
Mapped to unique biological orders	833	26,784,825	63.9%	53.6%	
Mapped contigs, by orders					
High GC Gram+	251	12,703,700	30.3%	24.9%	-26.0
β-Proteobacteria	171	7,740,803	18.5%	15.2%	-41.0
Fusobacteria	98	1,672,841	4.0%	3.3%	-15.3
Firmicutes	88	612,197	1.5%	1.2%	-10.2
Bacteria	70	210,344	0.5%	0.4%	-22.0
OFB group bacteria	68	174,318	0.4%	0.3%	-10.7
γ-Proteobacteria	32	756,939	1.8%	1.5%	-13.7
Eudicots	17	22,303	0.1%	0.0%	-81.3
α-Proteobacteria	13	38,074	0.1%	0.1%	-8.5
ε-Proteobacteria	6	3,629	0.0%	0.0%	-8.5
Σ Additional orders, < 5 contigs	19	2,849,677	6.8%	5.6%	
Total	833	26,784,825	63.9%	53.6%	
Mapped contigs, by genera, (species)					
Neisseria sp. (lactamica: 6) (sicca: 6)	95	1,720,335	4.1%	3.4%	-41.0
Leptotrichia sp. (Succalis: 15)	74	1,447,087	3.5%	2.8%	-15.3
Propionibacterium propionicum	51	6,533,131	15.6%	12.8%	-26.0
Streptococcus sp.	49	376,263	0.9%	0.7%	-10.2
Actinomyces sp.	35	802,110	1.9%	1.6%	-10.2
Candidatus Saccharibacteria sp.	27	55,254	0.1%	0.1%	-10.2
Corynebacterium sp.	26	296,066	0.7%	0.6%	-12.0
Capnocytophaga sp.	13	43,209	0.1%	0.1%	-9.5
Actinobaculum schaalii	10	1,339,942	3.2%	2.6%	-10.2
Fusobacterium nucleatum	10	11,273	0.0%	0.0%	-10.2
Veillonella parvula	9	17,279	0.0%	0.0%	-8.5
Cardiobacterium hominis	8	5,358	0.0%	0.0%	-13.7
Prevotella sp. (melaninogenica: 3)	6	4,519	0.0%	0.0%	-8.0
Dichelobacter nodosus	5	82,562	0.2%	0.2%	-8.5
Mesorhizobium sp.	4	653	0.0%	0.0%	-5.5
Rothia sp.	4	16,653	0.0%	0.0%	-8.5
Anacardium occidentale	3	4,071	0.0%	0.0%	-81.3
Campylobacter sp.	3	651	0.0%	0.0%	-7.5
Porphyromonas gingivalis	3	22,698	0.1%	0.0%	-7.0
Tsukamurella paurometabola	3	144,033	0.3%	0.3%	-9.2
Acetohalobium arabaticum	2	111,686	0.3%	0.2%	-8.0
Desulfococcus oleovorans	2	2,706,849	6.5%	5.3%	-8.0
Σ Additional genera, < 2 contigs	117	1,799,970	8.2%	12.9%	
Σ Ambiguous genus assignments	274	9,243,133	18.1%	18.1%	
Total	833	26,784,825	63.9%	53.6%	

Table 3 Identification of xenobiotic RNA sources in selected urine samples

Urine samples with at least two million total reads and more than 80% hg19-unmapped reads, 14-16, 16-4, 16-16, 16-19, and two randomly selected samples 14-17 (5.1% unmapped reads) and 13-16 9 were included for this analysis. From each selected samples unmapped sequences larger than 25 nucleotides and represented by at least 30 individual reads were used to generate

contigs using the SeqMan (DNASTAR, v 12.3.1.4) Using the parameters: match size: 14, min. match percentage: 97, match spacing: 75, gap penalty: 0, gap length penalty: 0.7, max. mismatch end bases: 2. Only contigs with consensus sequences which were at least 30 nt longer and based on at least two unique unmapped sequences. Generated contigs were considered and used as queries for NR database at the National Center of Biotechnology information (NCBI) using the BLASTN program. Numbers of the total contigs generated and those unambiguous assigned to a taxonomic order are listed. For both subsets contributing unmapped total reads used in contig generation are listed and related to all sequences used in contig generation (/ all cont.) and to the entire unmapped read counts (/un. reads). For taxonomic evaluation only top score returned target matches were considered irrespective of their number of occurrences within a target sequence or genome, if the E-value for that matched hit was at least 10^{-5} Or lower. For assignments of order, genus and species to a contig, taxonomic obtained had to be unambiguous: Only if for any given contig a set of equivalent matches comprised entirely of organisms from the same biological order, that order was assigned. In the same manner, family and species information was assigned, if returned top matches showed no ambiguity.

The resulting taxonomic assignments sorted the number of supporting contigs and the number of participating sequences are listed in the lower two tables, sorted by identified orders and genera, respectively. Species information was included if specific; in cases where several but not all contigs could be unambiguously assigned up to the species level or where evidence for the presence of at least two different species within one genus was found, species information is listed in parenthesis together with the number of supporting contigs.

CLAIMS

1. A method for isolating RNA from a biological fluid, said method comprising:
 - a. contacting the biological fluid with a denaturing solution comprising at least one of reducing agent and detergent to form a biological fluid mixture;
 - 5 b. contacting the biological fluid mixture with a protease to form a protease treated biological fluid mixture;
 - c. contacting the protease treated biological fluid mixture with an organic extraction solution, forming a solution having an aqueous phase containing the RNA and an organic phase;
 - 10 d. binding the RNA to a silica based solid phase by contacting the aqueous phase with said silica based solid phase;
 - e. contacting the silica based solid phase with a first wash solution comprising alcohol, chaotropic agent, and reducing agent; and
 - 15 f. eluting the RNA from the silica based solid phase comprising contacting the silica based solid phase with an aqueous solution to provide isolated RNA.
2. The method of claim 1, wherein said biological fluid is selected from the group consisting of urine, serum, and plasma.
3. The method of claim 1, wherein said biological fluid is homogenized tissue.
4. The method of claim 1, wherein said protease is proteinase K.
- 20 5. The method of claim 1, wherein said detergent is an anionic detergent.
6. The method of claim 5, wherein said anionic detergent is SDS.
7. The method of claim 6, wherein said SDS is present at a concentration of at least 2% (w/v), at least 3% (w/v), at least 4% (w/v), or at least 5% (w/v).
8. The method of claim 1, wherein said chaotropic agent is selected from the group
25 consisting of guanidine and urea.

9. The method of claim 1, wherein said reducing agent is selected from the group consisting of DTT, BME, and TCEP.
10. The method of claim 1, wherein said denaturing solution is heated to at least 50 °C, at least 55 °C, at least 60 °C, or at least 65 °C.
- 5 11. The method of claim 1, wherein said RNA is between 5-500 nucleotides, 5-250 nucleotides, 5-100 nucleotides, or 5-50 nucleotides.
12. The method of claim 1, wherein said organic extraction solution further comprises at least one of chaotropic agent and phenol.
- 10 13. The method of claim 1, wherein said organic extraction solution further comprises chloroform.
14. The method of claim 1, wherein chloroform is contacted with the biological fluid after the biological fluid has contacted the organic extraction solution.
15. The method of claim 1, wherein said reducing agent of steps (d) and (e) is independently selected from the group consisting of DTT, BME, and TCEP.
- 15 16. The method of claim 15, wherein said reducing agent is TCEP.
17. The method of claim 16, wherein said TCEP has a concentration between 1mM and 20 mM, between 1 mM and 10 mM, or between 1 and 5 mM.
18. The method of claim 1, wherein said chaotropic agent is selected from the group consisting of guanidine and urea.
- 20 19. The method of claim 1, wherein said alcohol of steps (d) and (e) is independently selected from the group consisting of methanol, ethanol, butanol, and propanol.
20. The method of claim 1, wherein said method further comprises at least one alcohol wash.
21. The method of claim 1, wherein said aqueous solution comprises at least one of water, buffer, and nucleic acid preservative.

22. A kit for isolating RNA from a biological fluid comprising:

- a. a denaturing solution;
- b. an organic extraction solution;
- c. a nucleic acid binding solution; and
- 5 d. a first wash solution comprising alcohol, chaotropic agent, and reducing agent.

23. The kit according to claim 23, wherein said kit further comprises a silica based solid phase.

24. The kit according to claim 24, wherein said kit further comprises an aqueous elution solution.

10 25. The method according to claim 1, further comprising:

- a. contacting the organic phase of step (c) with a DNA extraction solution containing a buffer and chaotropic agent to form a second solution having an second organic phase and second aqueous phase;
- b. binding the DNA to a silica based solid phase by contacting the second aqueous phase with a silica based solid phase;
- 15 c. contacting the silica based solid phase having bound DNA with a first wash solution comprising alcohol, chaotropic agent, and reducing agent; and
- d. eluting the DNA from the silica based solid phase comprising contacting the silica based solid phase with an aqueous solution.

20 26. A method for isolating DNA from a biological fluid, said method comprising:

- a. contacting the biological fluid with a denaturing solution comprising at least one of reducing agent and detergent to form a biological fluid mixture;
- b. contacting the biological fluid mixture with a protease to form a protease treated biological fluid mixture;

- c. contacting the protease treated biological fluid mixture with an organic extraction solution, forming a solution having a first aqueous phase containing RNA and an organic phase containing DNA;
- d. contacting the organic phase with a DNA extraction solution containing a buffer and a chaotropic agent to form a second solution having a second aqueous phase containing DNA;
- e. binding the DNA to a silica based solid phase by contacting the second aqueous phase with said silica based solid phase;
- f. contacting the silica based solid phase with a first wash solution comprising alcohol, chaotropic agent, and reducing agent; and
- g. eluting the DNA from the silica based solid phase comprising contacting the silica based solid phase with an aqueous solution to isolate DNA.

27. The method according to claim 1, wherein the silica based solid phase is further contacted with a second wash solution comprising alcohol and reducing agent.

28. The method according to claim 27, wherein the second wash solution further comprises a chaotropic agent.

29. The method according to claim 28, wherein said chaotropic agent is present in an amount equal to or less than the chaotropic agent in the first wash solution.

30. The method according to claim 29, wherein said chaotropic agent is selected from the group consisting of guanidine and urea.

31. The method according to claim 1, wherein the aqueous phase of step (c) is contacted with a nucleic acid binding solution comprising an alcohol and a chaotropic agent.

32. The method according to claim 31, wherein the alcohol is isopropanol and the chaotropic agent is GITC.

33. The method according to claim 31, wherein the nucleic acid binding solution further comprises at least one salt, and a reducing agent.

34. The method according to claim 25, wherein the second aqueous phase is contacted with a nucleic acid binding solution containing alcohol, at least one salt, a chaotropic agent, and a reducing agent.

5 35. The method according to claim 26, wherein the second aqueous phase of step (d) is contacted with a nucleic acid binding solution containing alcohol, at least one salt, a chaotropic agent, and a reducing agent.

36. The method according to claim 26, wherein the DNA extraction solution of step (d) comprises at least 3M chaotropic agent.

10 37. The method according to claim 34, wherein DNA extraction solution comprises isopropanol, MgCl_2 , CaCl_2 , guanidine, and TCEP.

38. The kit according to claim 22, wherein the nucleic acid binding solution comprises an alcohol, at least one salt, a chaotropic agent, and a reducing agent.

39. The kit according to claim 22, further comprising a second wash solution comprising an alcohol and a reducing agent.

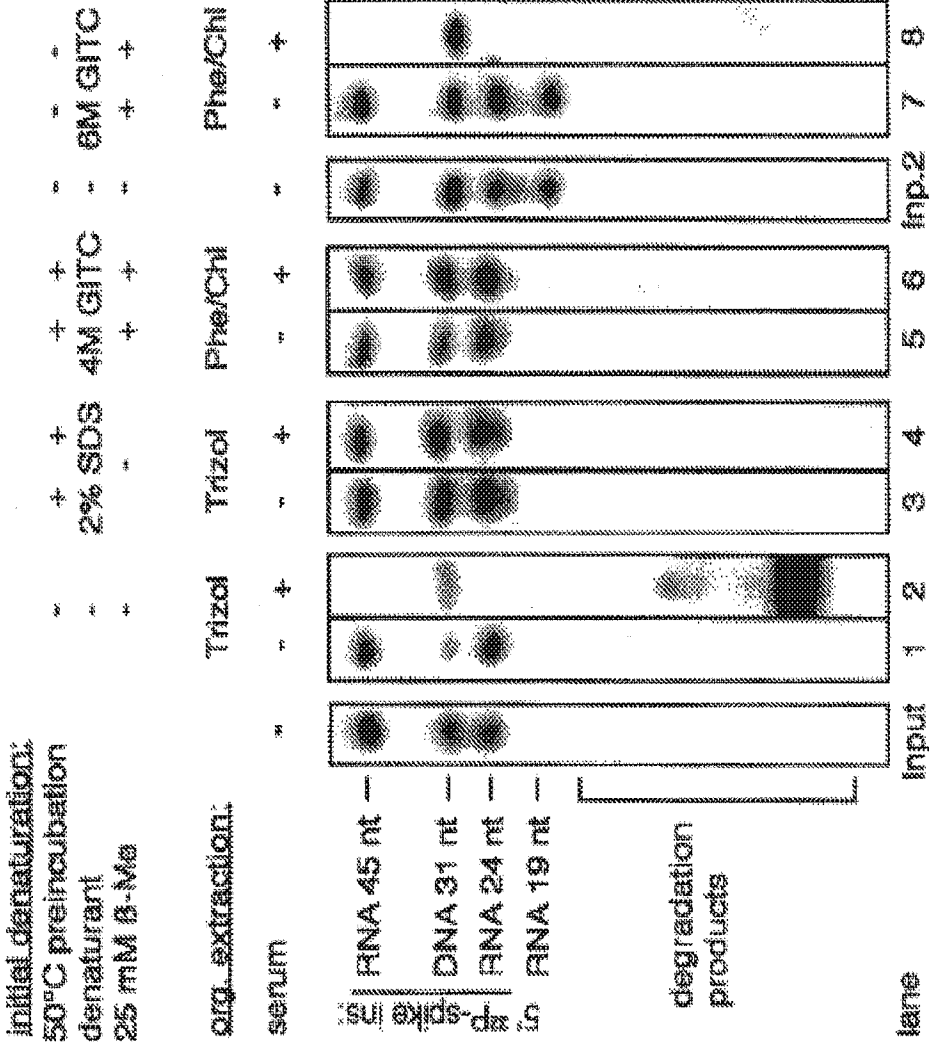
15 40. The kit according to claim 22, further comprising a protease.

41. The kit according to claim 22, further comprising a silica based solid phase in the form of a column.

20

25

FIGURE 1 A



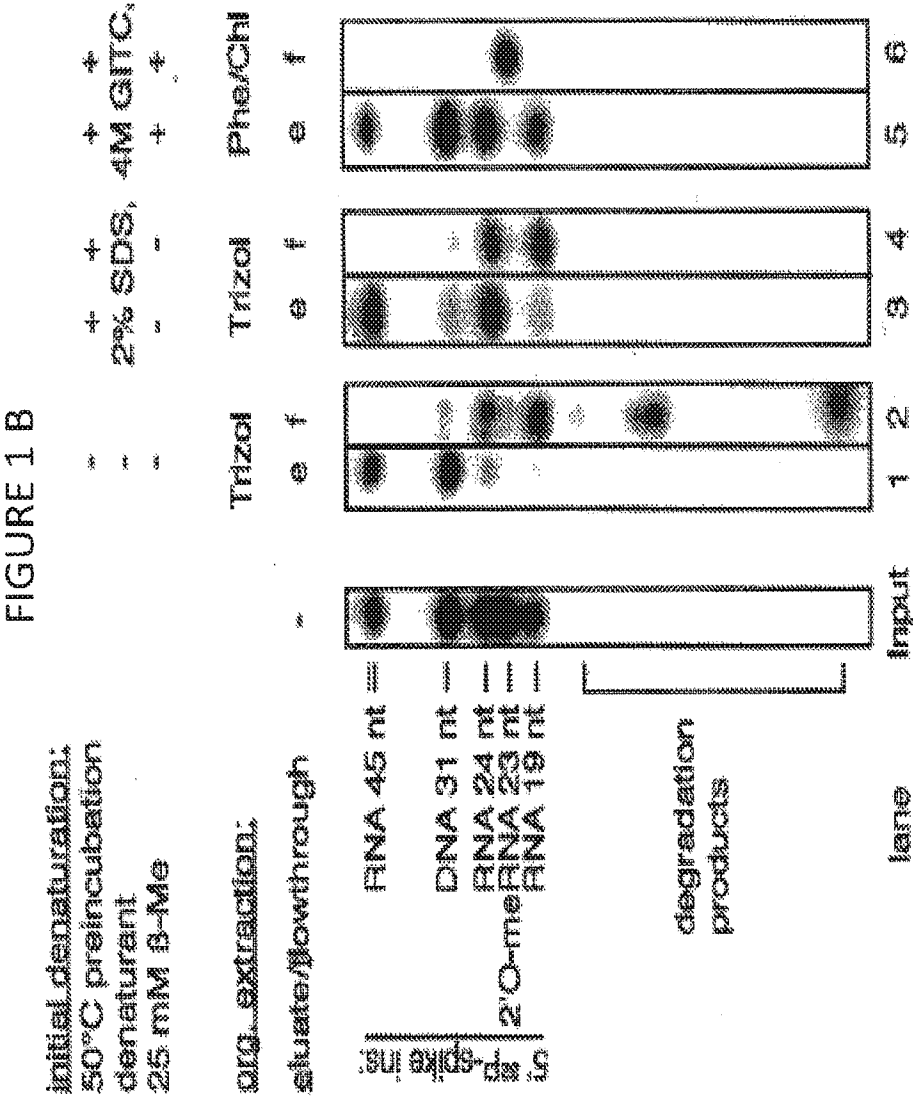


FIGURE 2 A
Manual

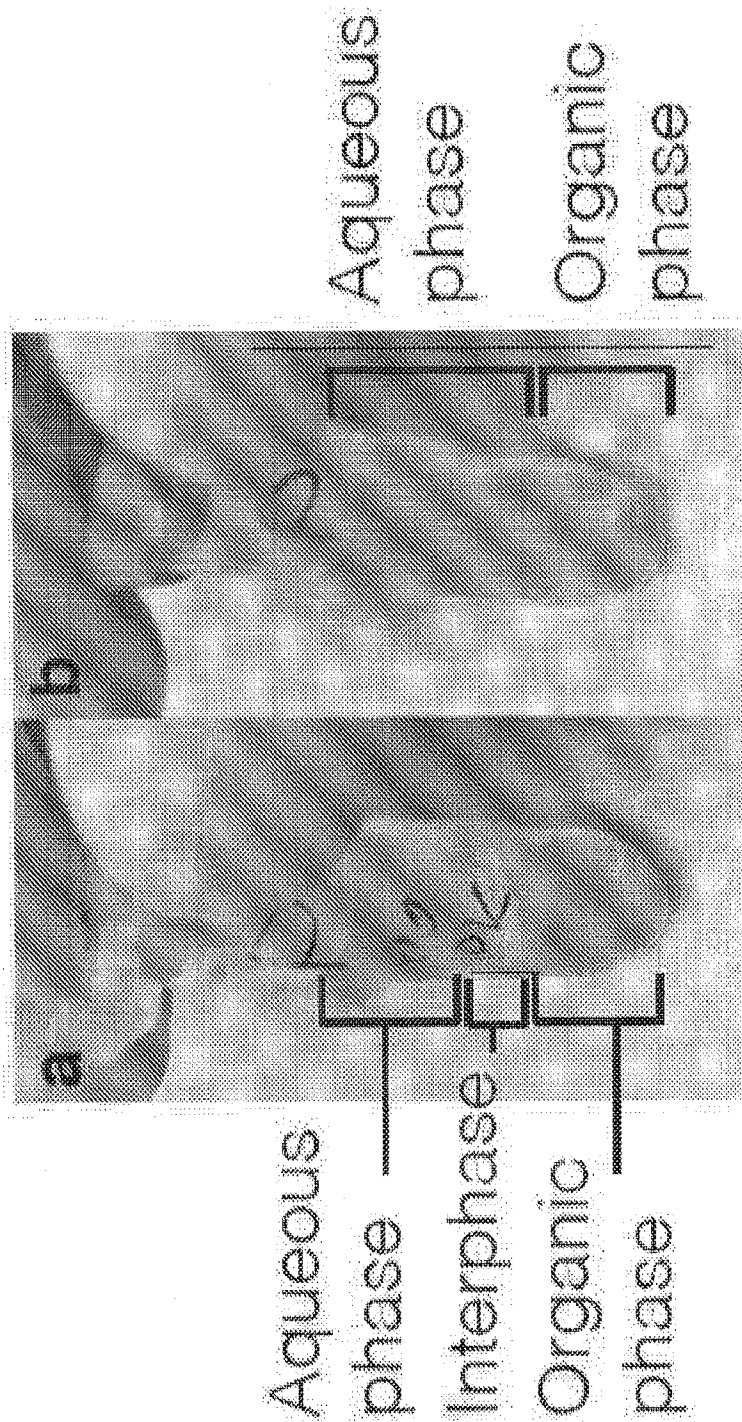
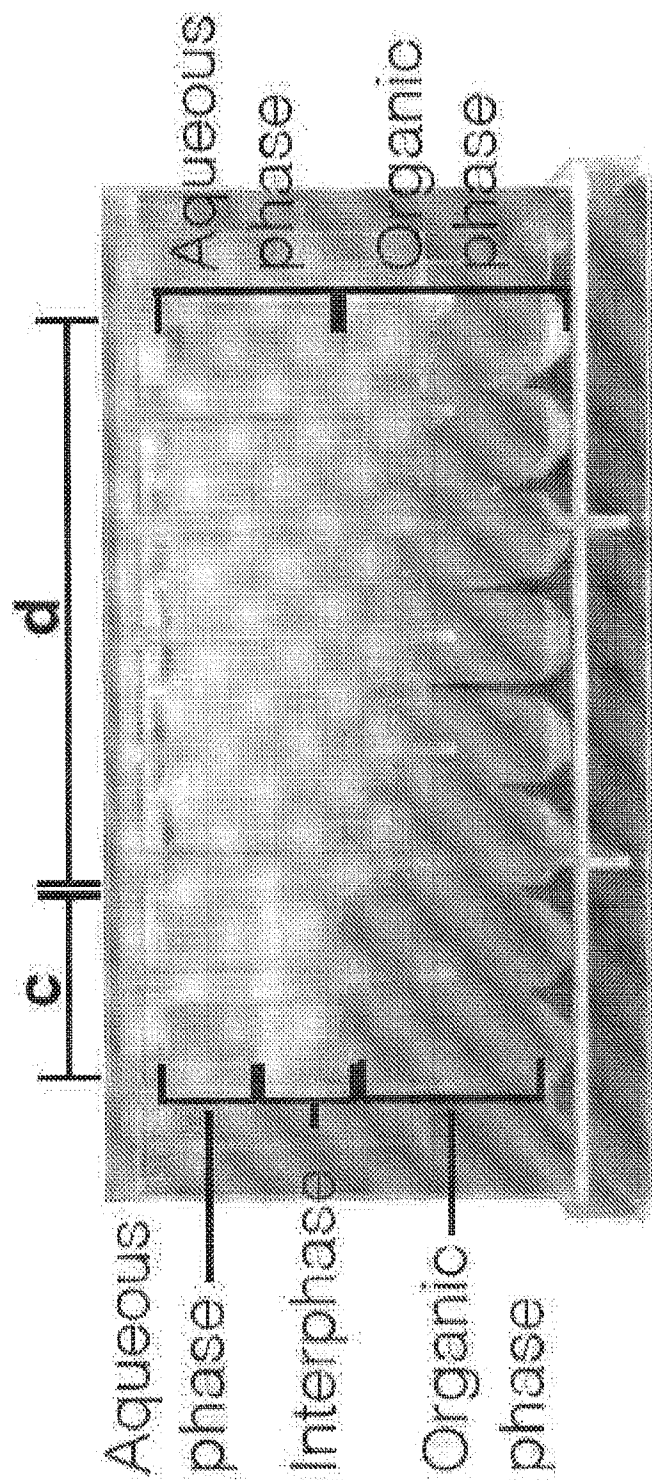


FIGURE 2 B

Fully automated



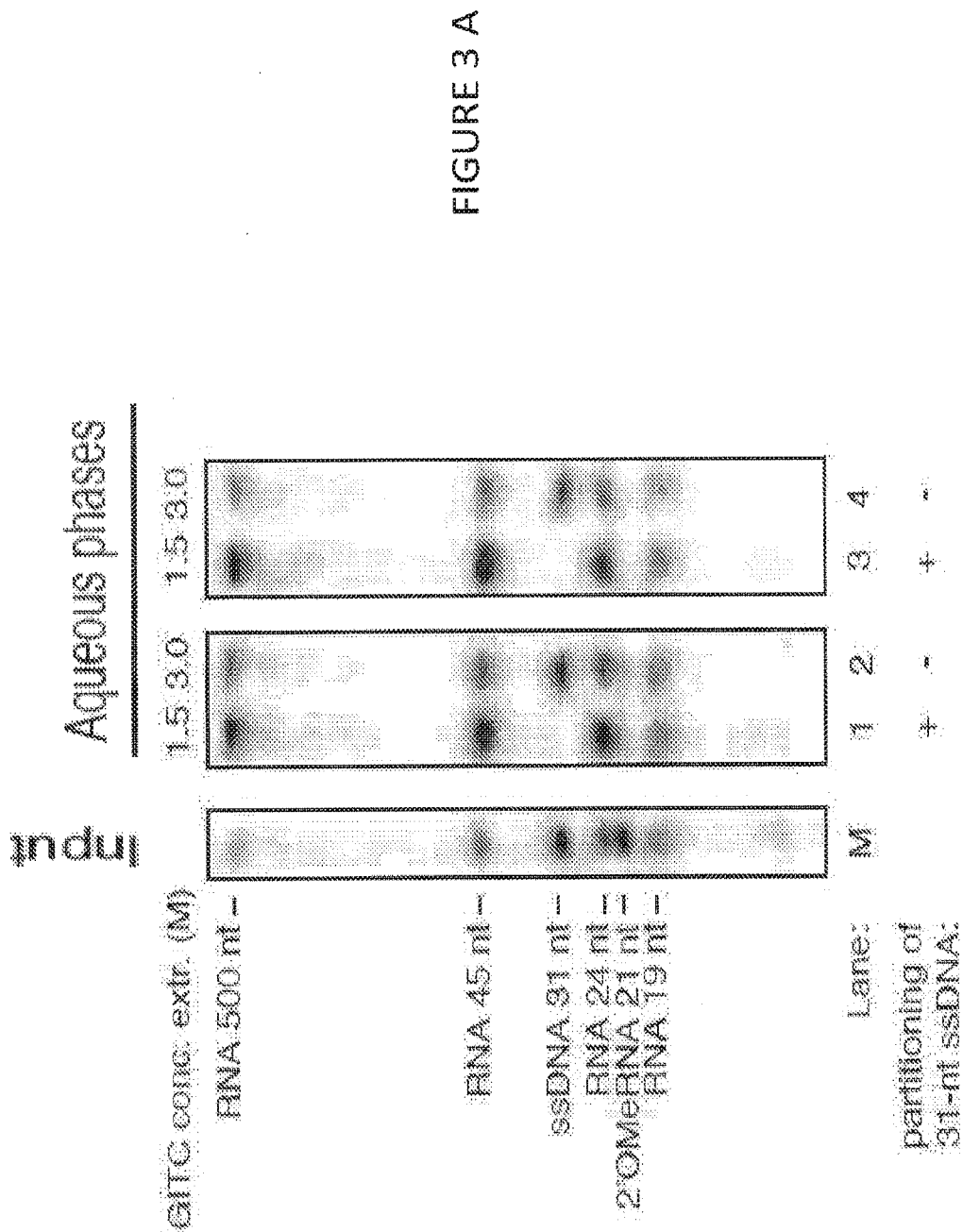
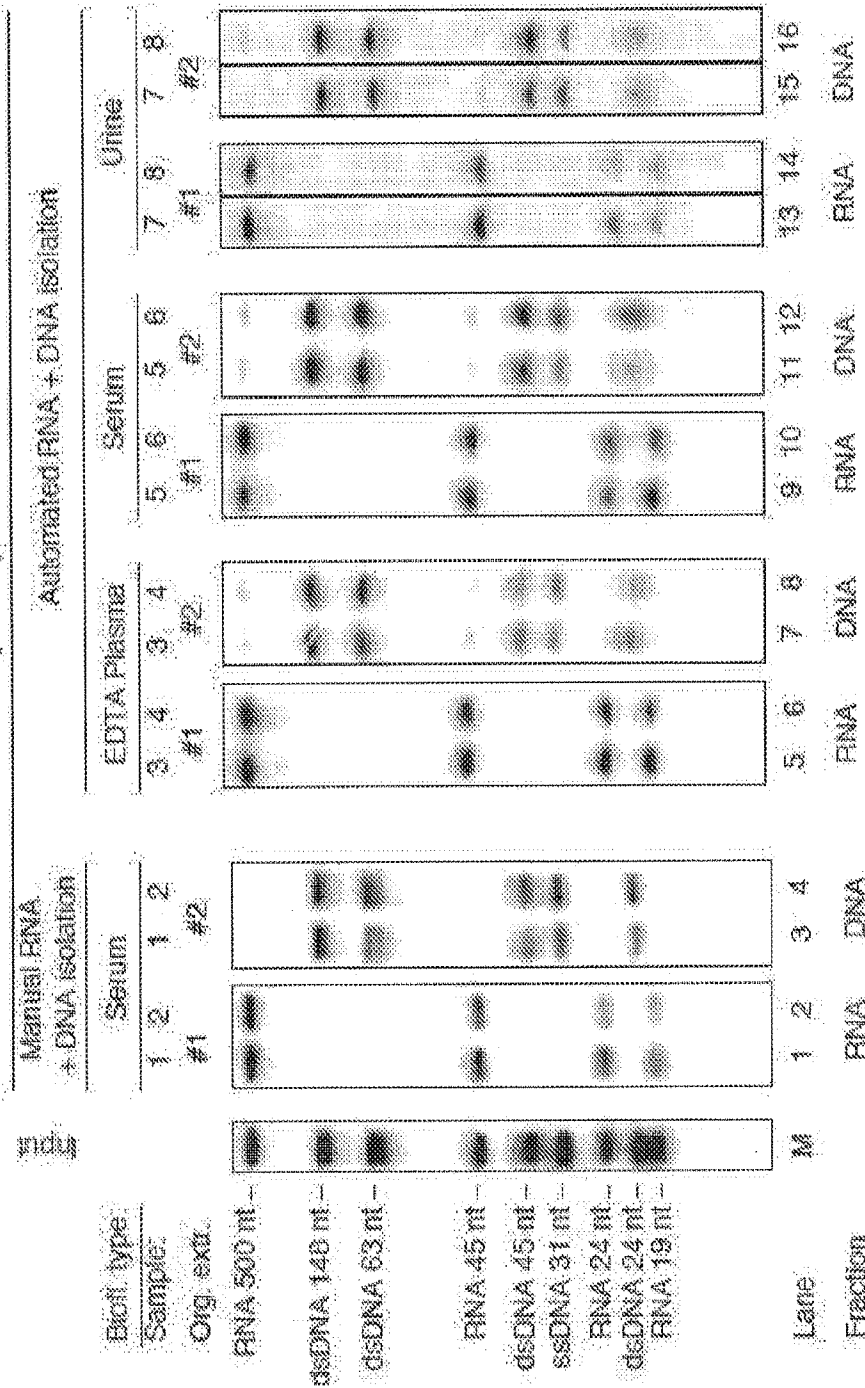


FIGURE 3 B
Aqueous phases



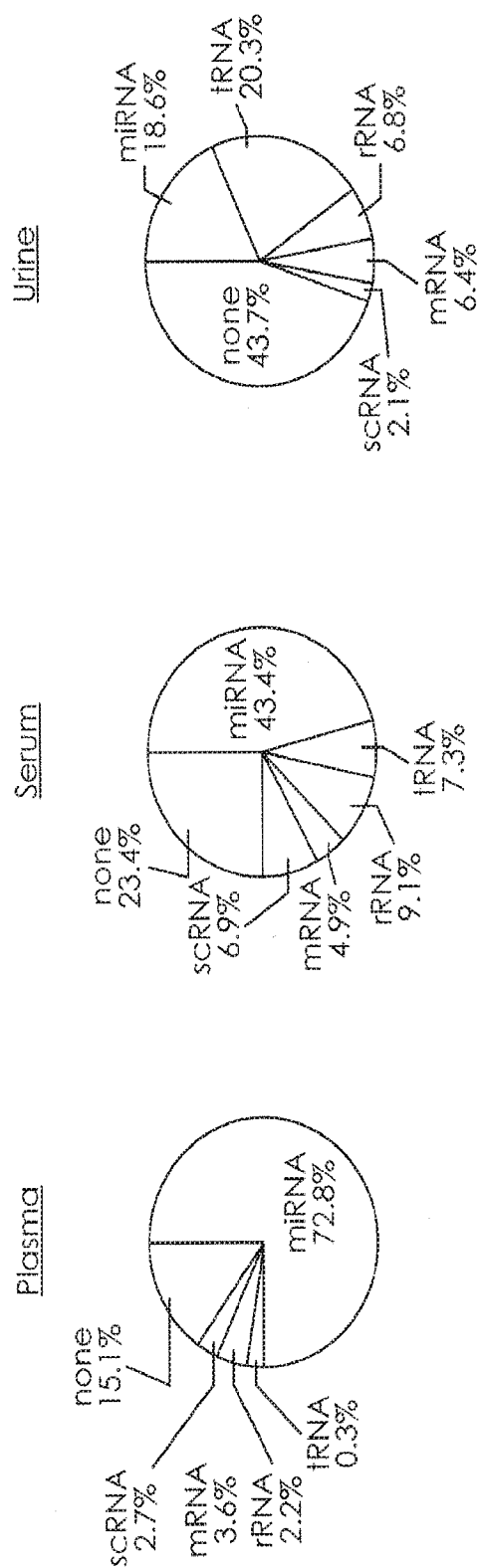
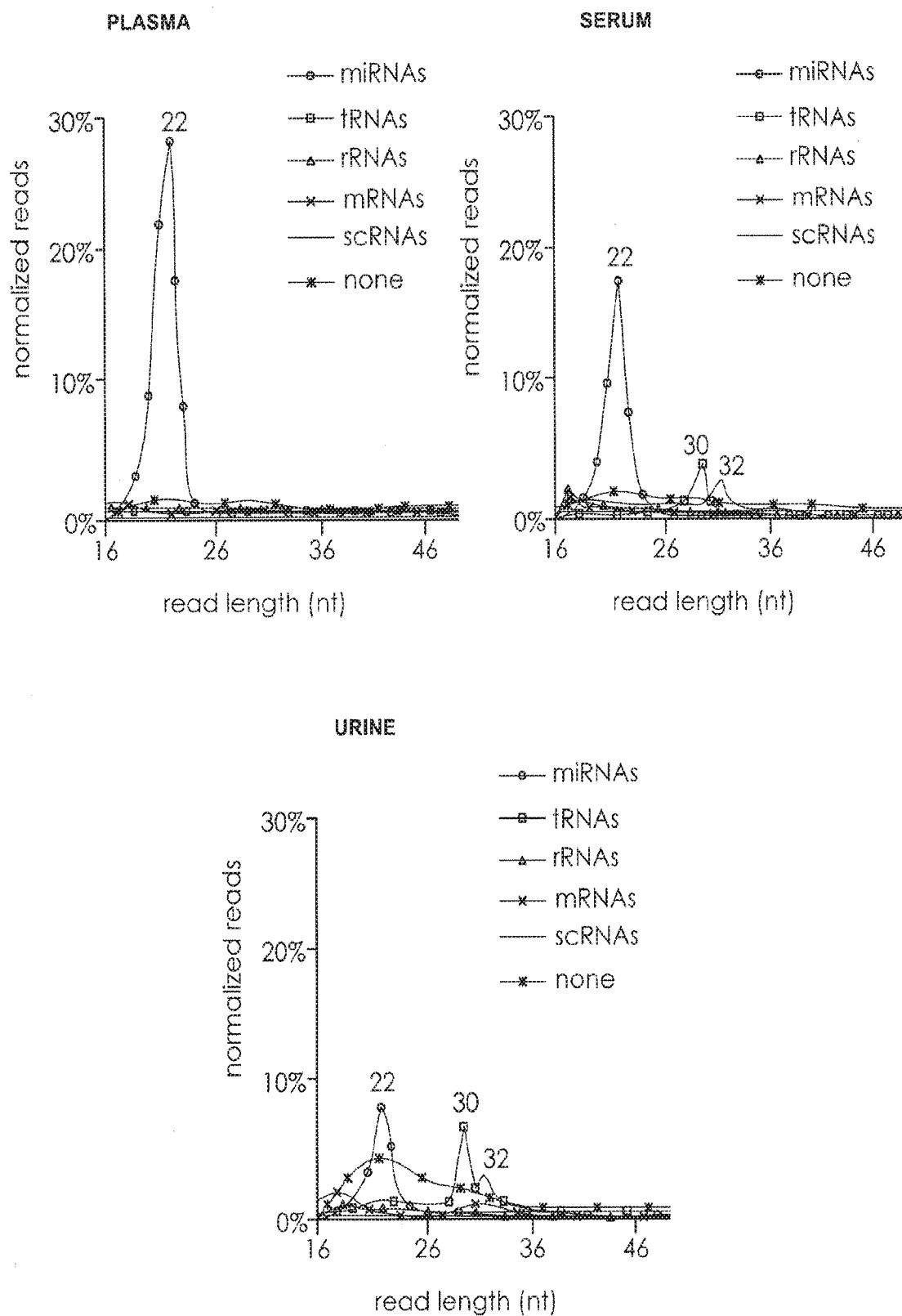
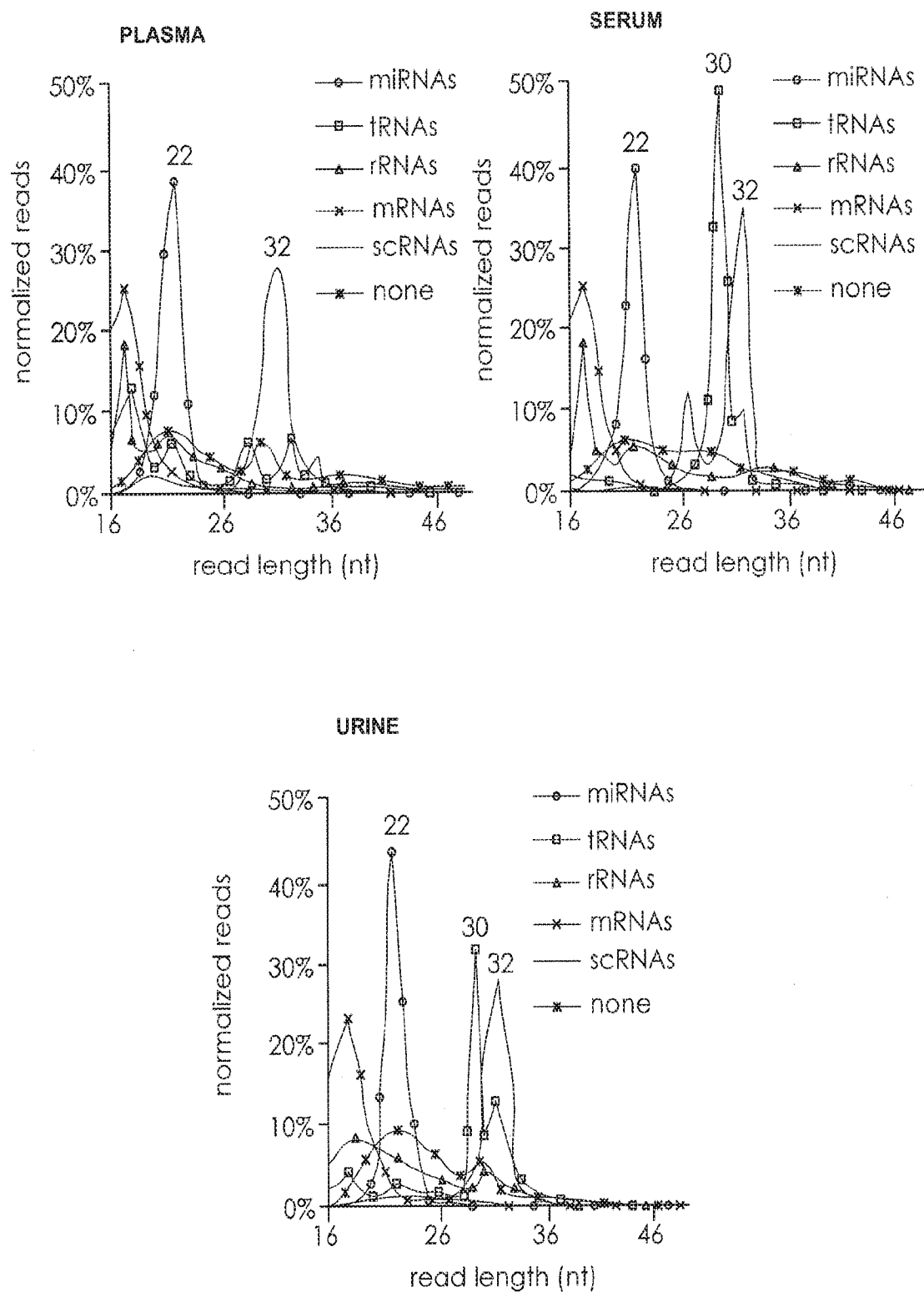


FIG. 4A

**FIG. 4B**

**FIG. 4C**

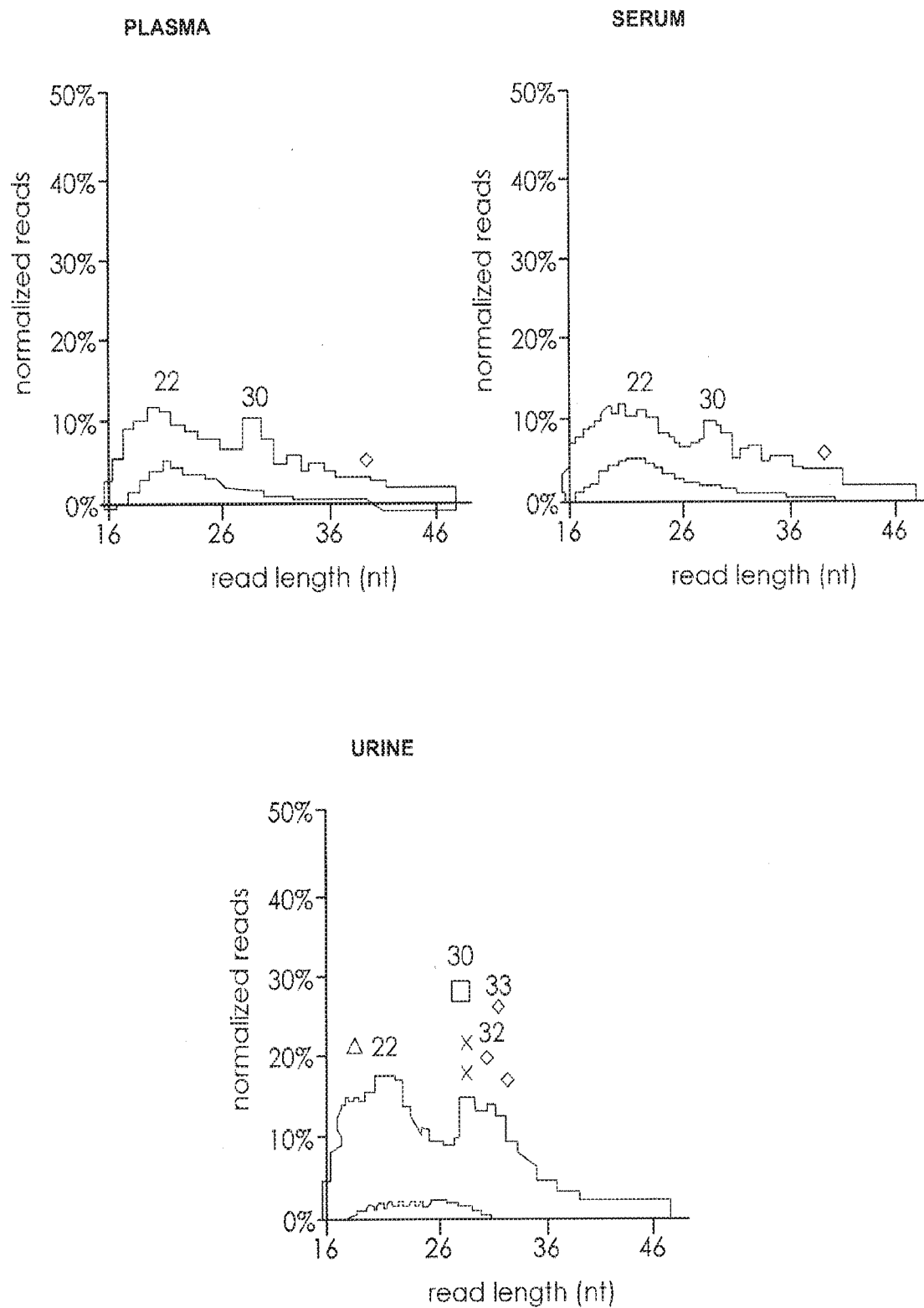
**FIG. 4D**

FIGURE 5 A

Plasma

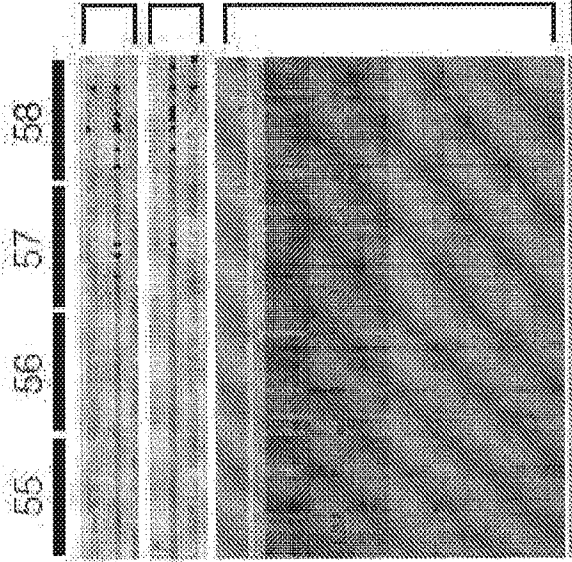


FIGURE 5 B

Serum

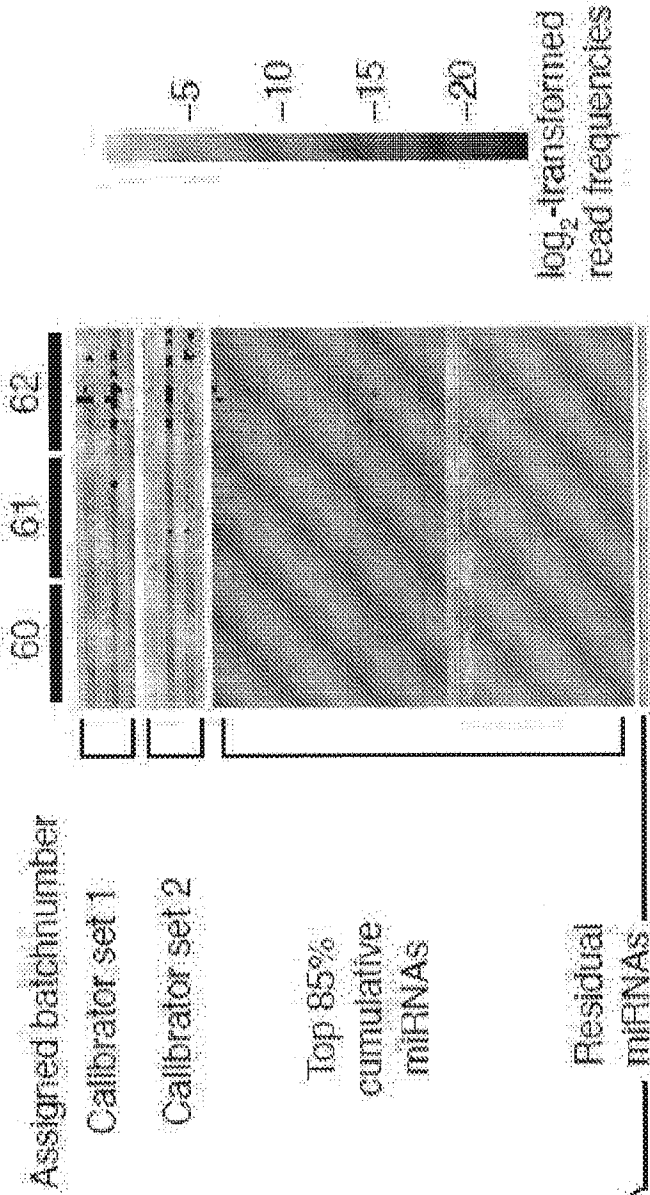


FIGURE 5 C

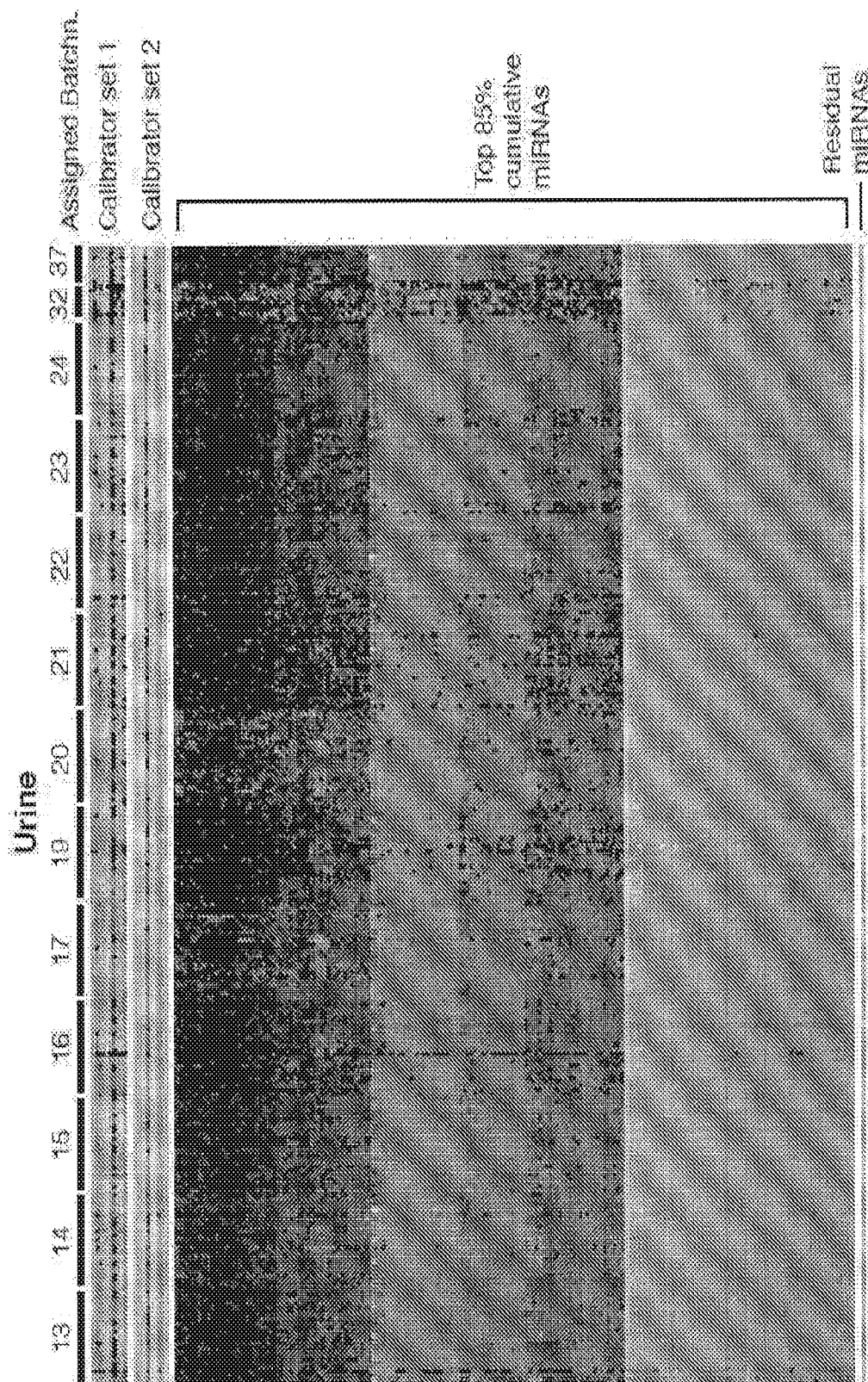
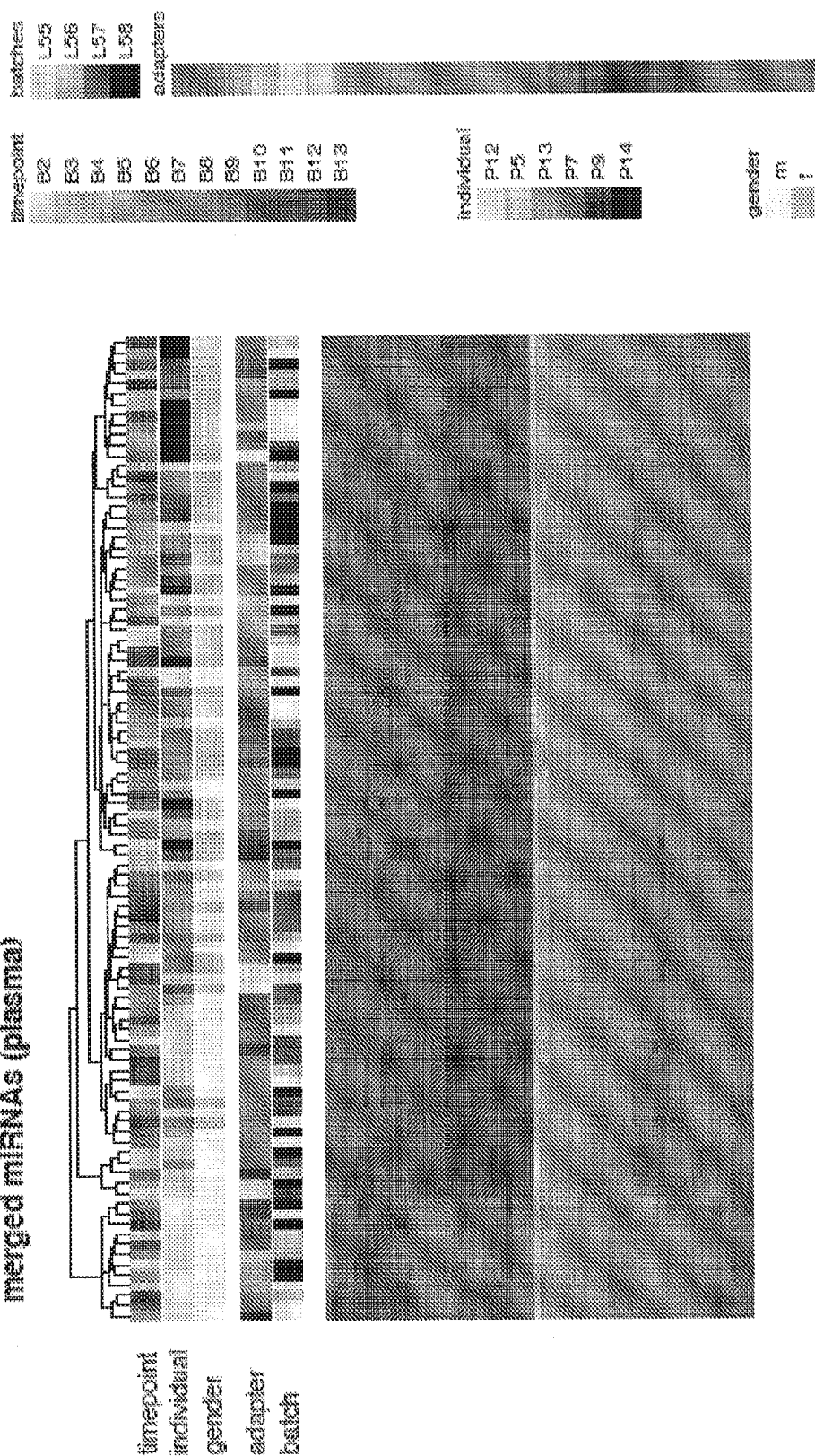


FIGURE 6 A

merged miRNAs (plasma)



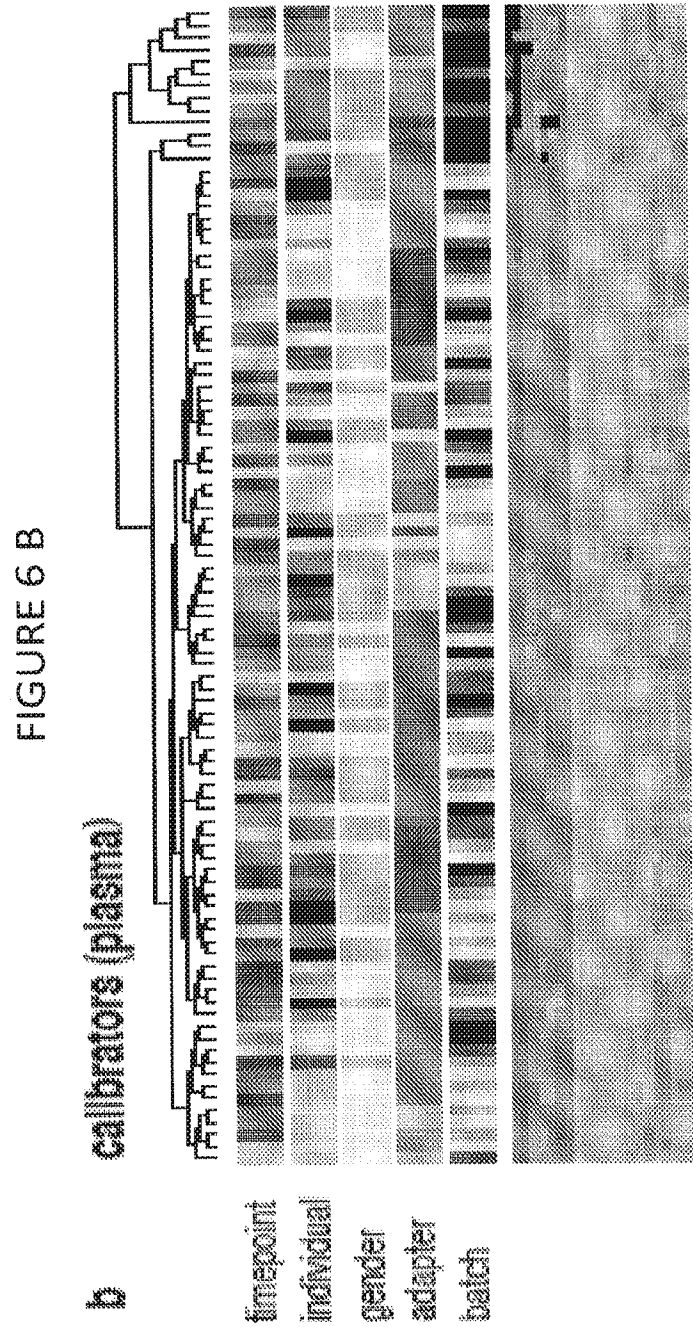
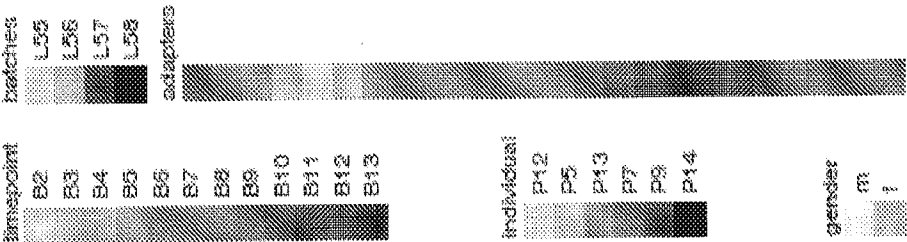


FIGURE 7 B

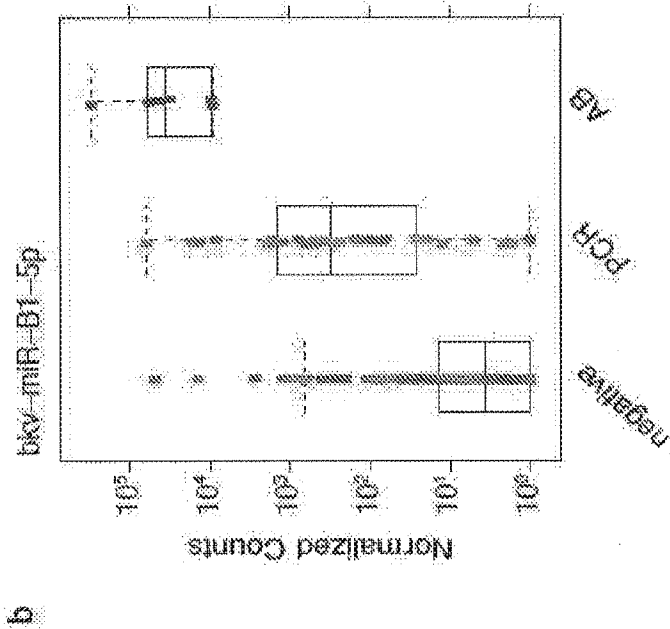


FIGURE 7 A

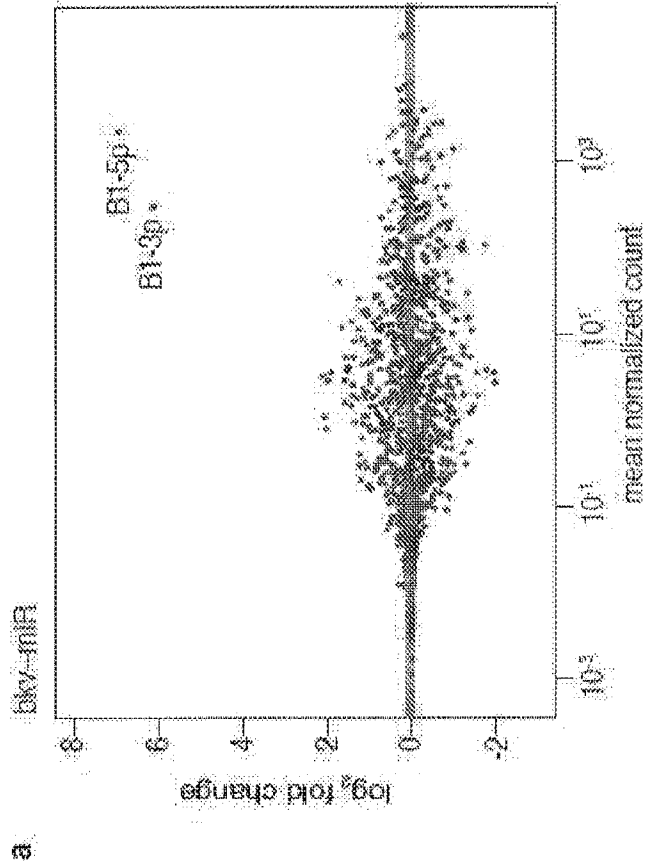
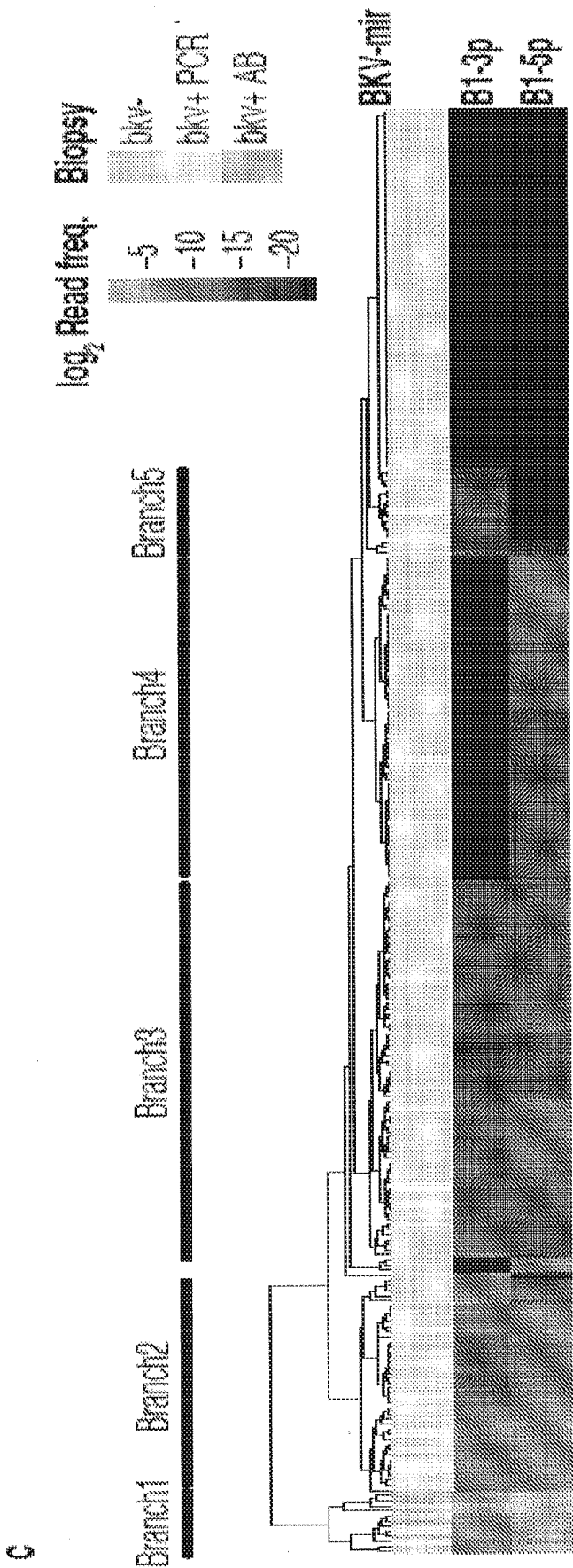


FIGURE 7 C



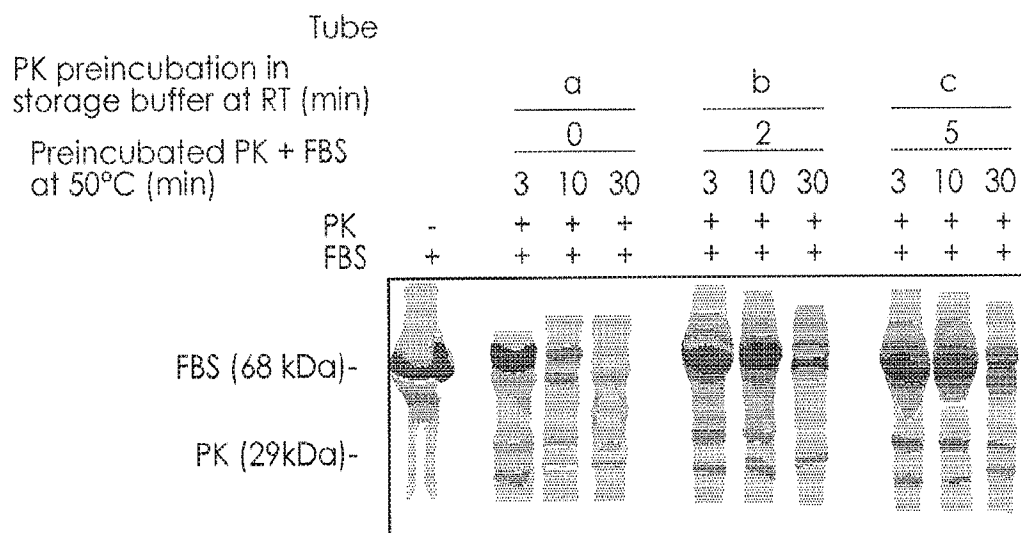
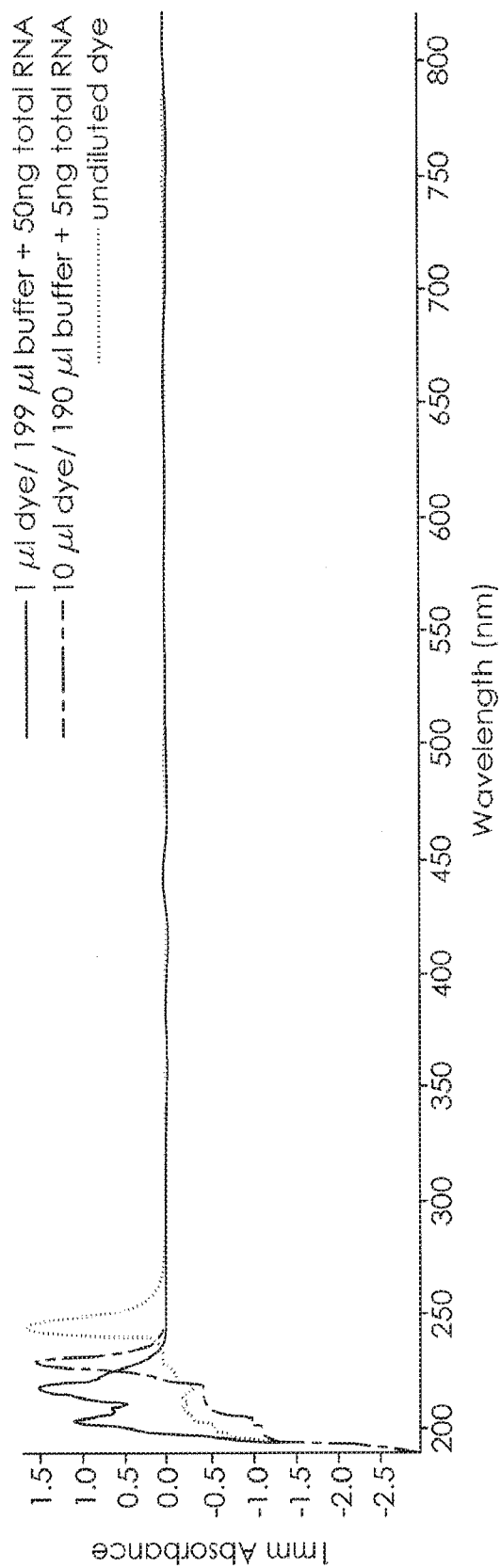


FIG. 8

**FIG. 9**

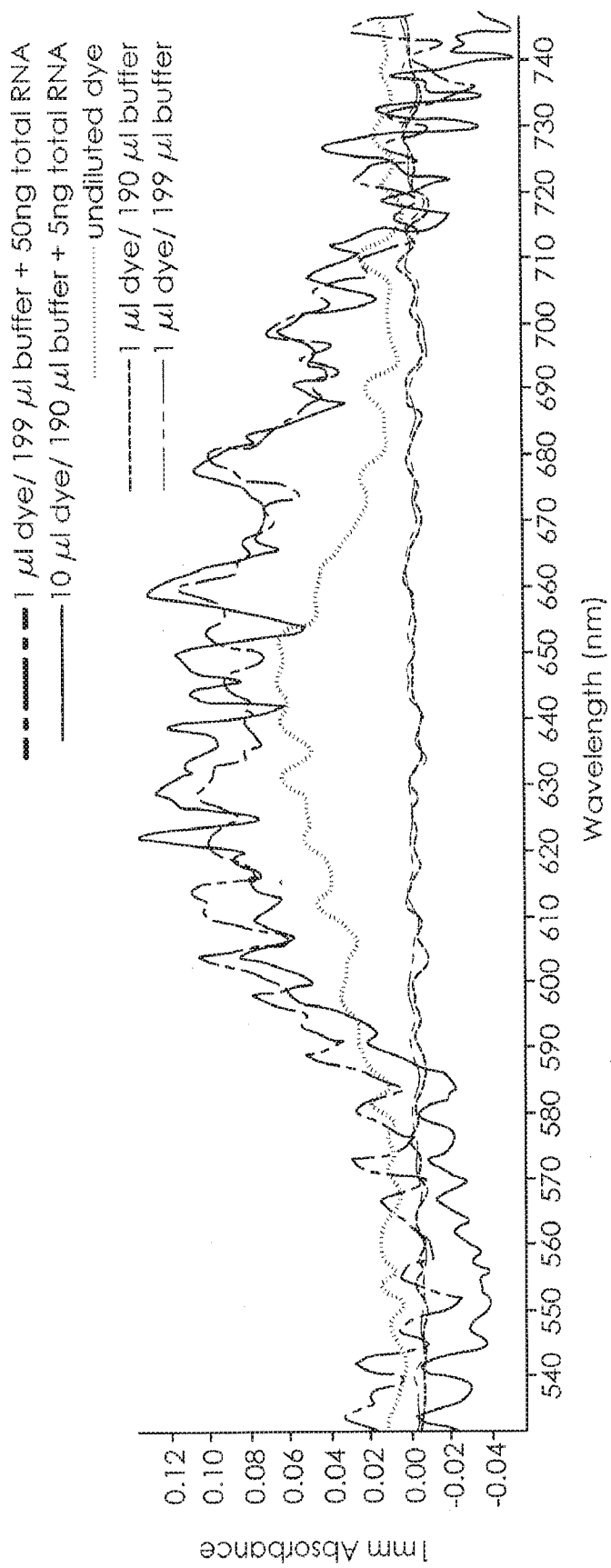
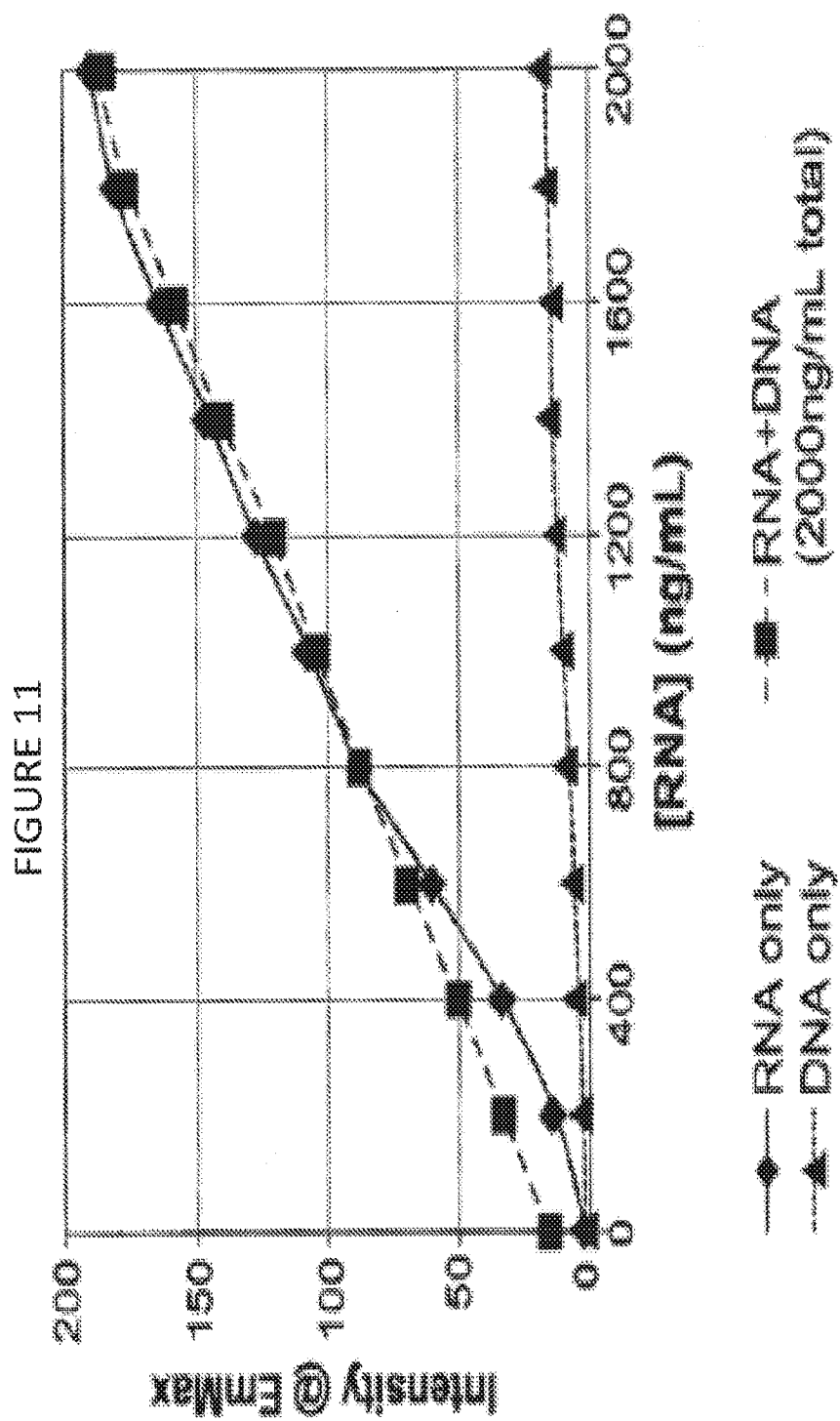


FIG. 10



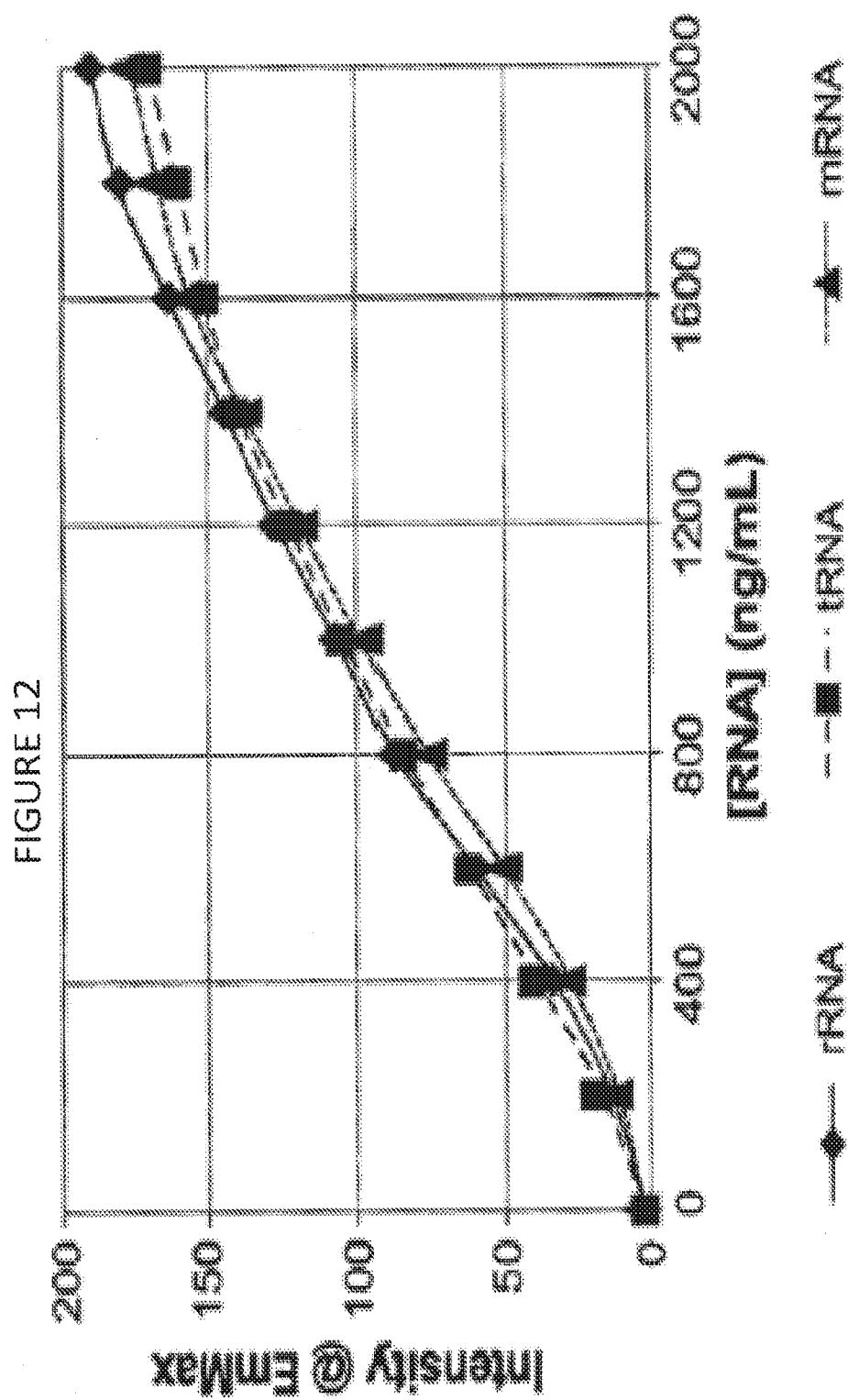


FIGURE 13

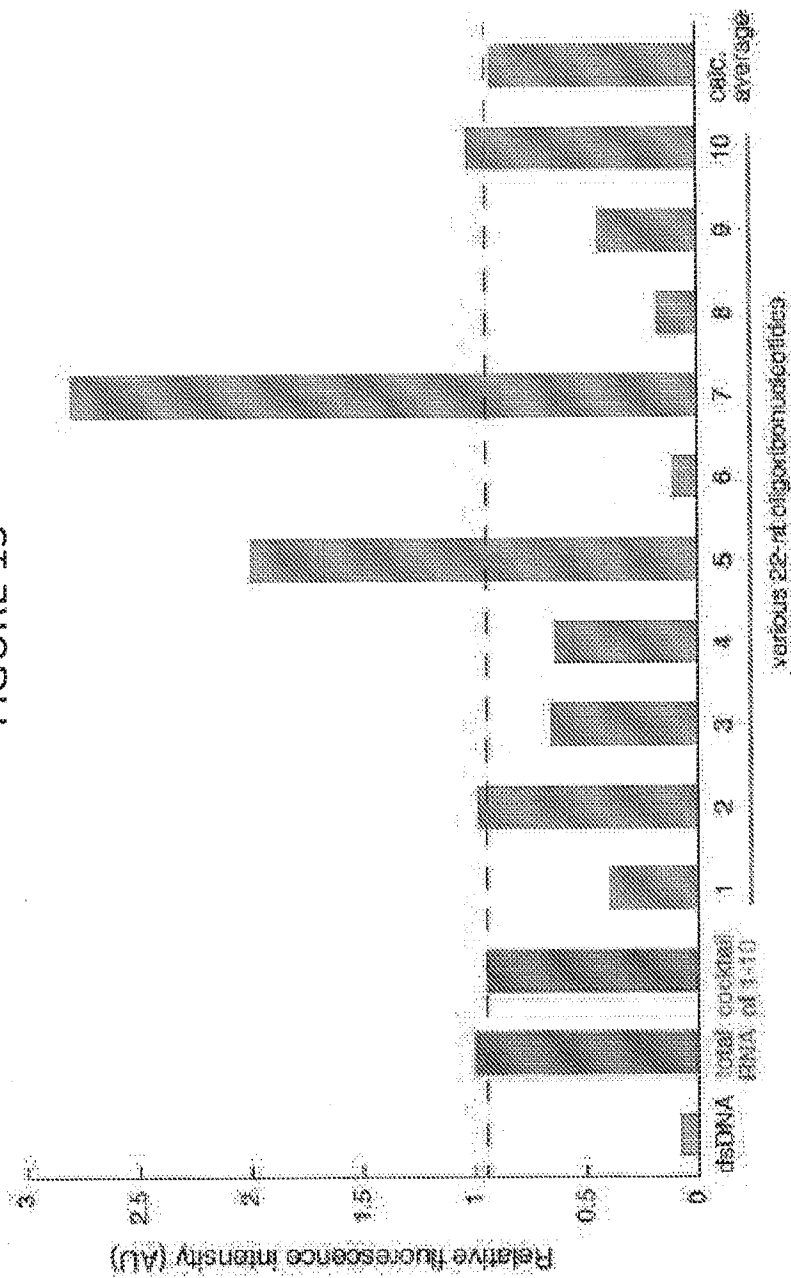


FIGURE 14

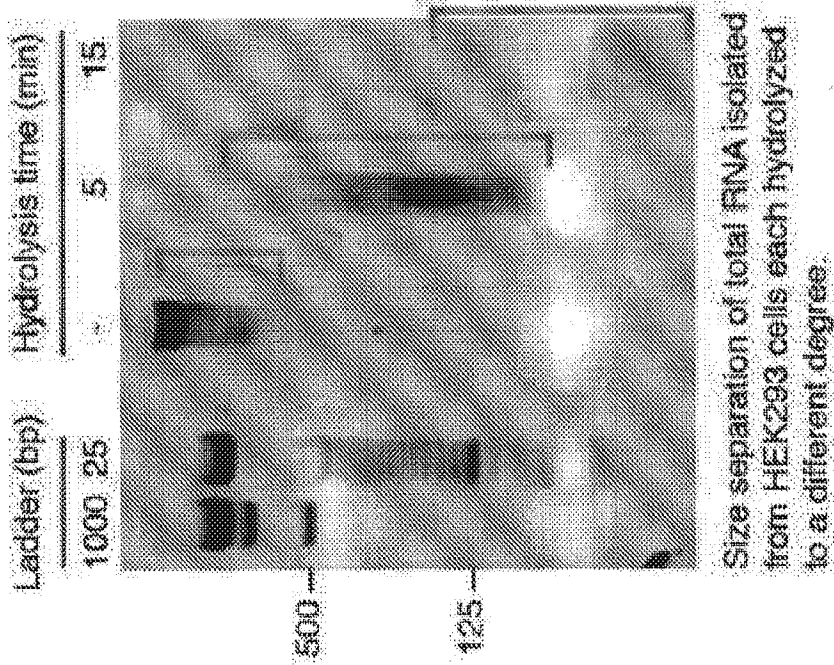
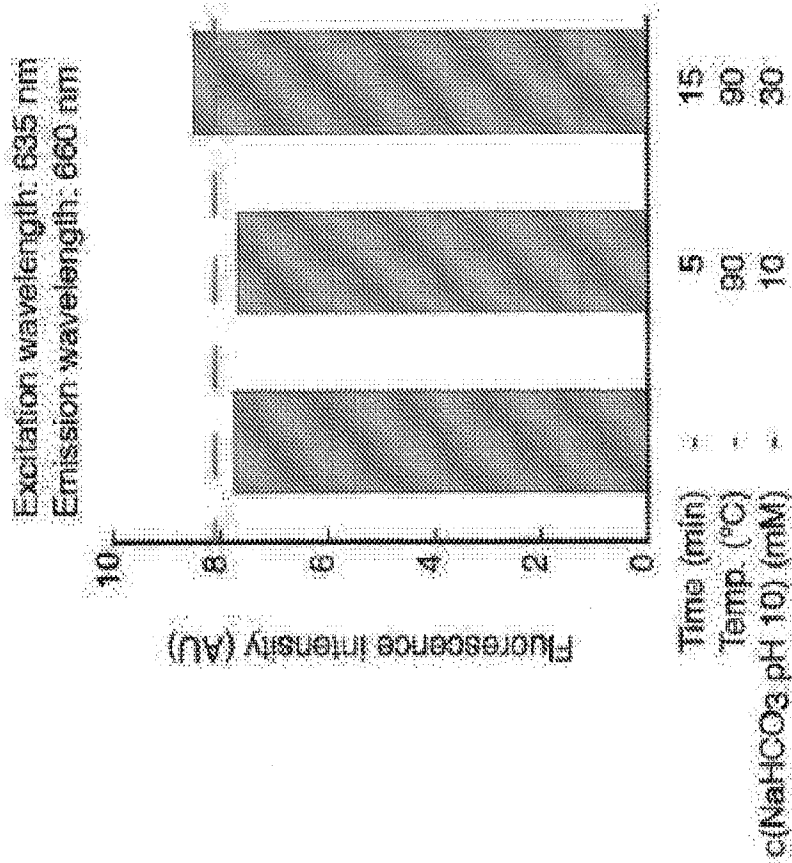
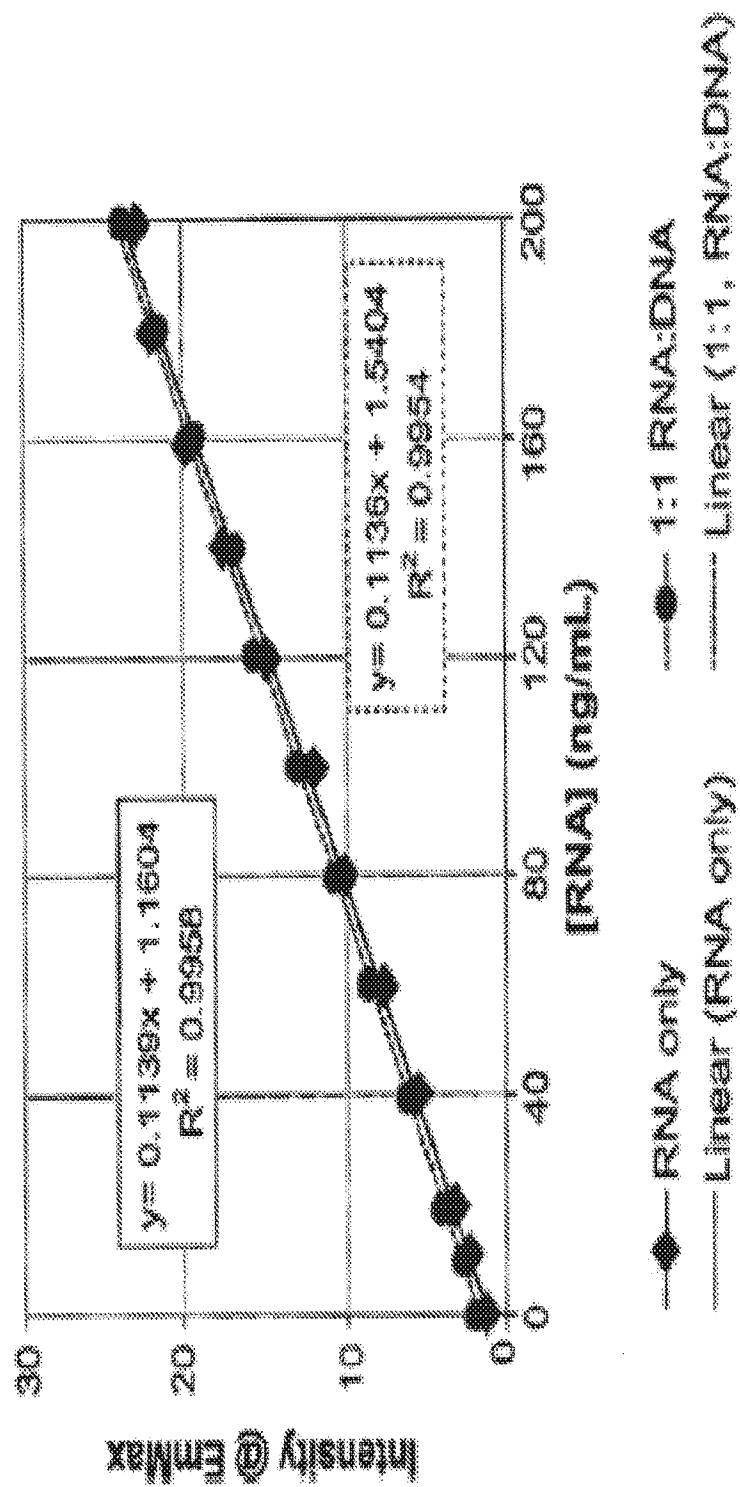
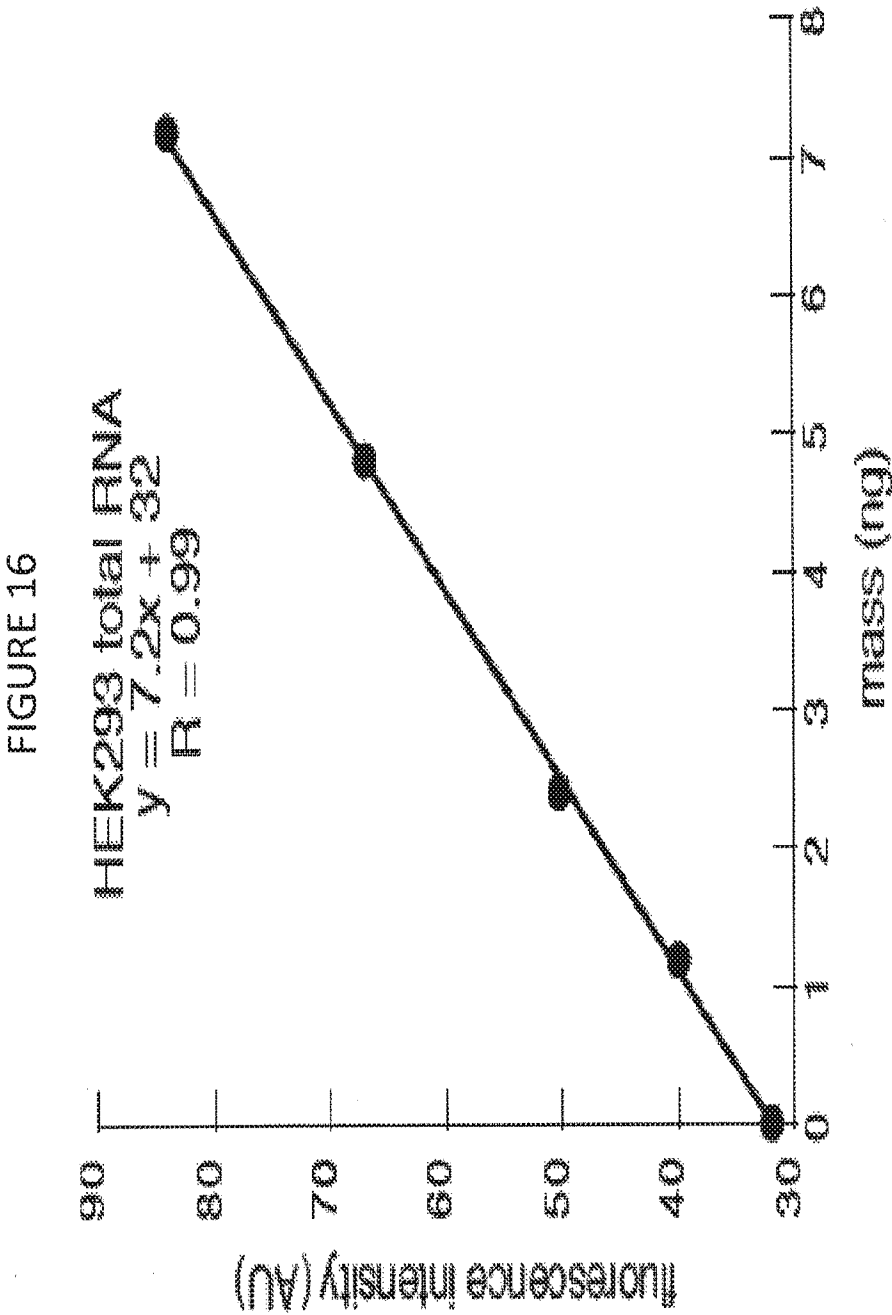


FIGURE 15





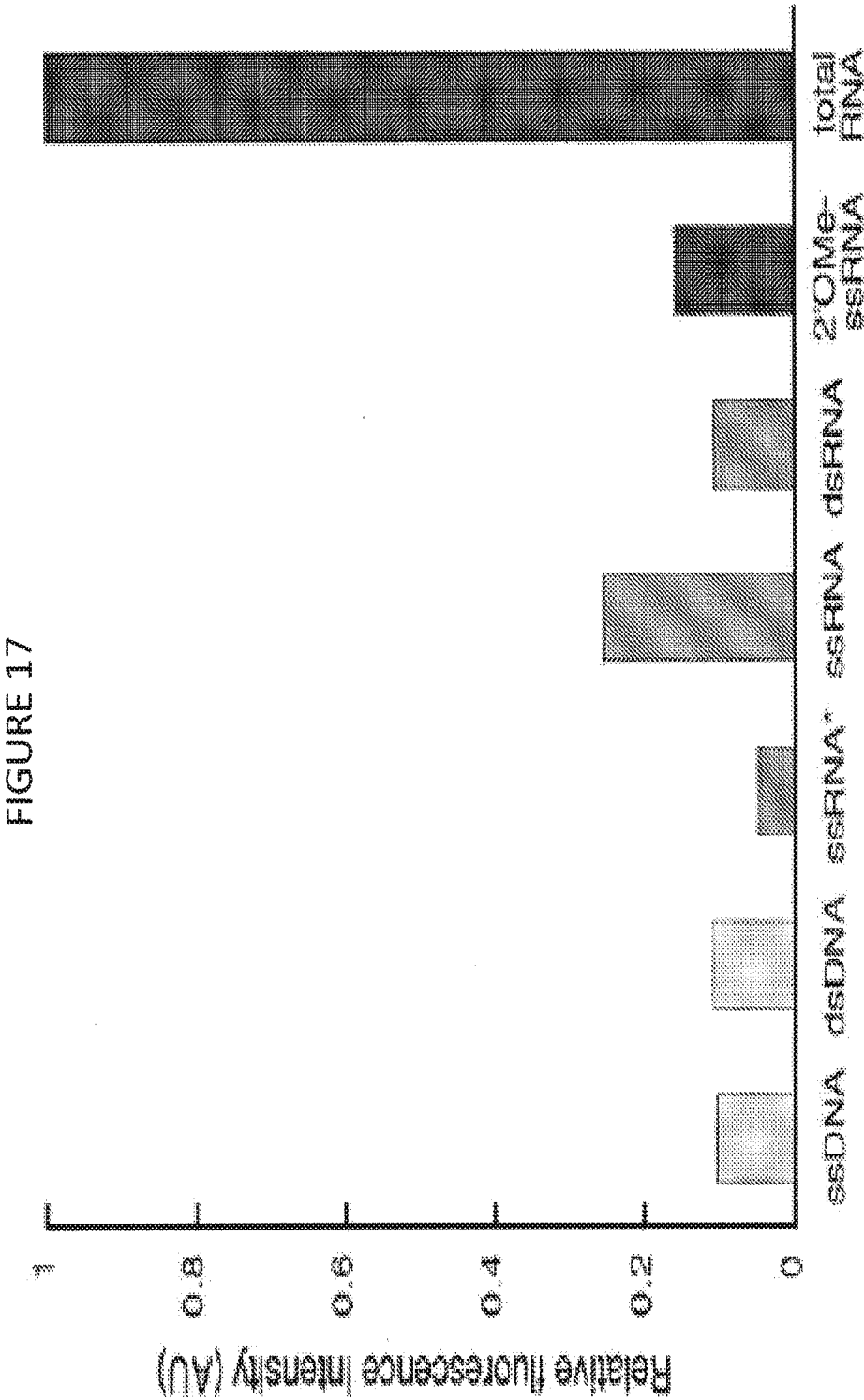


FIGURE 18

