SPECIAL ISSUE: EXTRACELLULAR RNA COMMUNICATION CONSORTIUM

Meeting report: discussions and preliminary findings on extracellular RNA measurement methods from laboratories in the NIH Extracellular RNA Communication Consortium

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Extracellular RNAs (exRNAs) have been identified in all tested biofluids and have been associated with a variety of extracellular vesicles, ribonucleoprotein complexes and lipoprotein complexes. Much of the interest in exRNAs lies in the fact that they may serve as signalling molecules between cells, their potential to serve as

biomarkers for prediction and diagnosis of disease and the possibility that exRNAs or the extracellular particles that carry them might be used for therapeutic purposes. Among the most significant bottlenecks to progress in this field is the lack of robust and standardized methods for collection and processing of biofluids, separation of different types of exRNA-containing particles and isolation and analysis of exRNAs. The Sample and Assay Standards Working Group of the Extracellular RNA Communication Consortium is a group of laboratories funded by the U.S. National Institutes of Health to develop such methods. In our first joint endeavour, we held a series of conference calls and in-person meetings to survey the methods used among our members, placed them in the context of the current literature and used our findings to identify areas in which the identification of robust methodologies would promote rapid advancements in the exRNA field.

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Investigators in the Extracellular RNA Communication Consortium are probing a variety of scientific questions, such as elucidating mechanisms responsible for the biogenesis of RNA-containing extracellular particles, their uptake and potential function in recipient cells; discovering extracellular RNA (exRNA) biomarkers for various diseases and developing exRNA-based therapeutics. Although a majority of the projects within the Extracellular RNA Communication Consortium involve the characterization of exRNAs, they have diverse goals and methods for exRNA and extracellular particle isolation and analysis.

For example, many groups, particularly those focused on biomarkers, require methods that give highly robust results from samples obtained using a clinically feasible collection and processing platform. The biomarker groups may also prefer isolation methods that are more comprehensive rather than those that select for specific types of particles and specific exRNAs, reasoning that these latter manoeuvers might result in loss of potential biomarkers. For other groups, such as those focused on understanding the biogenesis of a specific type of particle, optimization of yield and purity and bioactivity of that particle are the driving forces. These varying priorities cannot always be satisfied at the same time. For example, some protocols optimized to give high yields of RNA may result in low bioactivity or reduced RNA diversity. To discuss these issues, this report will focus on the basic challenges as identified by Extracellular RNA Communication Consortium members. It will emphasize the need to develop validated methods for sample collection, processing, RNA isolation and next-generation sequencing (NGS) library construction for exRNA analysis. We recognize that there are a number of other pertinent topics that will not be addressed here. Some of these have been addressed by existing organizations, and others we intend to cover in future reports. In terms of priorities that are being addressed by other organizations, the International Society on Thrombosis and Haemostasis has worked extensively on standardization of detection of vesicles by flow cytometry (1). The European Network on Microvesicles and Exosomes in Health and Disease, supported by the European Cooperation in Science and Technology framework, has a working group focused on guidelines for nomenclature and analysis. ISEV has published 2 position papers on publication standards (2) and analysis of extracellular vesicle (EV) RNA and bioinformatics (3). Finally, the External RNA Controls Consortium has developed spike-in control mixes for both long and small RNAs (4).

Context

The discovery of exRNAs in biofluids has sparked considerable interest in their use as disease-specific diagnostic and prognostic biomarkers. However, the discovery of specific exRNAs as "disease-specific" reporters depends on the notion that differential profiles of exRNAs across different samples is predominantly reflective of changes related to the disease process, rather than differences arising from the use of different exRNA isolation or analysis methods. Therefore, the discovery of robust disease-specific exRNAs as clinically relevant biomarkers depends on standardized techniques for sample processing and exRNA measurement that minimize variability across technical replicates and across different measurement sites.

The development of standard techniques for exRNA isolation and analysis has been challenging because of the large number of interacting biological and experimental variables. First and foremost, exRNAs are present in variety of compartments that have diverse biophysical properties. These compartments include EVs (5,6), lipoproteins (7) and ribonucleoprotein particles (8). Different methods for isolating exRNAs could preferentially enrich for exRNAs present in certain compartments, thereby

introducing intentional or unintentional bias in the discovery process. Second, exRNAs have been found in every tested biofluid. Variables involved in biofluid sample collection and processing include biofluid type, use of preservatives, collection container material, holding time and temperature and any centrifugation or filtration methods used, each of which may influence the quantity, quality and type of RNAs isolated. Third, bias introduced during NGS library construction can be substantial (9–14). Fourth, exRNAs are generally present at low concentrations in biofluids, which makes it challenging to obtain sufficient RNA for downstream assays, particularly for NGS-based assays. Most recent studies suggest RNA yields of 20–50 ng/ml of total plasma (15) and ≤ 5 ng of EV-associated RNA per ml of biofluid, both of which are significantly less than the 1 µg of RNA typically used in NGS analysis of RNA isolated from tissue. The low amounts of input RNA required for NGS library construction can lead to a higher incidence of adaptor dimer by-products (15), as well as potential sampling errors, resulting in high variability in the measurement of low abundance RNAs. Finally, inhibitors present in biofluids or in collection methods may confound molecular assays, including both qRT-PCR and NGS-based methods.

In the following sections, we will give a brief overview of the relevant literature, followed by a description of preliminary data shared by members of the Extracellular RNA Communication Consortium. We will conclude with potential future directions. In future collaborative studies, we aim to develop a clearer understanding of common sources of biological and experimental variability. We also strive to identify robust and standardized methods for sample collection and processing, RNA isolation and exRNA analysis. Such techniques will promote the success of efforts to discover exRNA biomarkers, to understand exRNA biology and to develop therapeutic approaches using exRNAs.

Methodology for sample collection and isolation of EVs and exRNAs

As part of an effort to address key topics in the field, the Extracellular RNA Communication Consortium has set up several working groups directed at the challenges associated with the isolation and analysis of exRNAs. Of these, the Sample and Assay Standards Working Group (SAS WG) was tasked with assessing the current state of the art for methods for biofluid sample collection, exRNA isolation and exRNA analysis. To this end, the SAS WG surveyed the protocols currently employed by the 19 laboratories in the Extracellular RNA Communication Consortium.

Biofluid collection and processing methods

Witwer et al. (16) comprehensively reviewed the numerous published reports demonstrating that EVs and exRNAs can be found in a wide variety of biofluids and showing that sample collection and processing methods can affect results of downstream assays. One of the most significant variables for sample collection is the choice of anticoagulant used for plasma samples, as it has been noted that they can influence both the number of EVs in plasma and the performance of downstream assays.

The International Society on Thrombosis and Haemostasis has been active in developing standardized methods for measurement of microparticle counts, particularly in plasma (17). A recent study used these methods, recognizing that the formation of EVs in vitro after sample collection could confound accurate measurement of extracellular biomarkers, to compare the levels of EVs in plasma collected in acid-citrate-dextrose (ACD, Becton-Dickinson, Franklin Lakes, NJ, USA), heparin (Becton-Dickinson), citrate (Becton-Dickinson), citrate-theophylline-adenosine-dipyridamole (Becton-Dickinson) and citrate phosphate dextrose adenine (Greiner Bio-One International, Kremsmunster, Austria) tubes (18). EVs were measured by flow cytometry and the ZYMUPHEN assay (Aniara, Westchester, Ohio, USA). This study found that the presence of citrate was correlated with a lower EV count, postulating that this might be due to chelation of calcium by citrate. The study also found that the dextrose in ACD tubes appears to inhibit in vitro vesiculation, compared to tubes containing citrate only. In a separate study, the Breakefield group has observed that heparin binds to EVs and blocks their transfer between cells (19).

Outside the exRNA literature, it has been appreciated that the most commonly used anticoagulants, heparin and citrate, interfere with PCR (20,21). The Witwer et al. review discusses this point, mentioning the use of the alternative anticoagulants EDTA and NaF/KOx. The authors recommend collecting samples in multiple tube types, because different downstream assays will likely show different sensitivities to specific additives (16).

The most common biofluids studied by members of the Extracellular RNA Communication Consortium were serum and plasma, but there were groups that evaluated cerebral spinal fluid (CSF), cell culture supernatant, saliva and bile. Six of the groups studying serum or plasma, as well as the groups studying CSF, used biofluid samples collected using methods that did not include a robust cell removal step (such as high-speed centrifugation or membrane filtration) prior to freezing for long-term storage. Two groups studying serum and plasma performed a cell removal step prior to freezing, either by centrifugation at $14,000 \times g$ or filtration through a 0.8 micron membrane filter. All groups completed initial sample processing within 2 hours of collection with long-term storage of samples in 1–2 mL aliquots at $-80^\circ C$.

The Skog group has tested the yields of several micro-RNAs (miRNAs) and long RNAs as measured by qPCR. They noted that, for plasma collection, heparin strongly interfered with downstream qPCR. Likewise, specimens

collected in citrate (Becton-Dickinson, NJ, USA) or plasma preparation tubes (PPT, containing both dipotassium EDTA as the anticoagulant and a gel polymer that forms a barrier between the plasma and cellular material after centrifugation, Becton-Dickinson, NJ, USA) showed overall higher C_T values corresponding to lower RNA concentrations as compared to specimens collected in plain EDTA tubes. None of the Consortium groups reported using NaF/KOx as an anticoagulant, as it is not a commonly used additive for clinical lab draws. Although the effects of the different anticoagulants on downstream assays are important considerations when choosing a sample collection tube type, it can be difficult to predict the future uses of banked samples, and it is often not feasible to collect in multiple tube types from the same patient. Many Consortium groups are using EDTA as the anticoagulant for plasma sample collection for projects in which qPCR and/or NGS are anticipated to be the downstream readouts. We appreciate that a comparison of in vitro vesiculation in ACD versus EDTA has yet to be performed.

The major rationale for methods that include a robust cell removal step is that they increase the likelihood that the extracted RNAs will represent exRNAs as opposed to intracellular RNAs (i.e. RNAs that are from cells that are intact in the source biofluid, but are lysed during sample processing or storage). However, these methods require processing of fresh samples using specialized protocols, and therefore they cannot be applied to previously banked biofluid samples. Moreover, concerns have been raised in discussions among Extracellular RNA Communication Consortium members regarding potential loss of larger EVs with membrane filtration or centrifugation speeds greater than $2,000 \times g$. At this time, there is no clear consensus on this point, other than the need to record in detail the sample collection methodology used.

Experience from the Wong group, which studies salivary exRNAs, reveals the need for customized processing methods for specific biofluids. In these experiments, RNA extracted from saliva that had been centrifuged at $2,600 \times g$ contained significant levels of rRNA. Because intact rRNAs have been shown to be largely absent from RNA extracted from EVs (6,22), the investigators were concerned that there might be excessive cell lysis. However, subsequent centrifugation at $10,000 \times g$ for 5 minutes showed that all of the intact rRNAs detectable by Bioanalyzer (Agilent, Santa Clara, CA, USA) were found in the pellet; this observation, combined with the migration of the rRNA peaks (which was more rapid than typically seen for eukaryotic 28S and 18S rRNAs) supported the notion that these rRNAs were of bacterial origin. This conclusion was further confirmed by NGS analysis of these samples, in which only 6–14% of reads mapped to the human genome and 60–70% of reads mapped to the microbiome, with the majority of sequences representing bacterial rRNAs. These findings demonstrate that rRNA contamination in salivary exRNA samples, which would be presumed to be of intracellular origin in most biofluids, was likely due to the high bacterial load in saliva.

Special considerations when using cell culture supernatants

EVs can be isolated from cell culture supernatants, and the ability to experimentally manipulate the cells and produce large volumes of supernatant make these attractive systems to use for study of the biogenesis and functions of EVs. However, there are specific variables that must be taken into account in these studies. For example, if the cell culture medium is supplemented with foetal bovine serum, the serum should be depleted of endogenous EVs. This task is typically done by ultracentrifugation for prolonged periods of time or by immunoaffinity methods using antibodies raised to antigens commonly found on the surfaces of bovine EVs. Because these methods do not fully deplete the serum of EVs and exRNAs (23), it is preferable to use serum-free media formulations when possible. Both the Breakefield and Patel groups have observed that cell density can affect the release of EVs into the cell culture supernatant, with increasing density associated with decreased release. A possible explanation for this phenomenon is that EVs can be transferred from cell to cell, and a higher density of cells favours cell-to-cell trafficking over release into the supernatant.

EV and particle enrichment methods

We note that a discussion of nomenclature for EVs and other extracellular particles remains an area of active debate that is outside the scope of this report. Although defining subtypes of EVs based on the mechanism of biogenesis is an attractive method, such a scheme cannot be easily applied given the dearth of knowledge about these processes (24). One convention that has been adopted is to name the vesicles based on the source of the biofluid. Terms such as epididimosomes, argosomes, prominino-somes, prostasomes, dexosomes, texosomes, archaeosomes and oncosomes have all been used in this regard (25). Another classification scheme involves definition by size of the EV. This classification scheme is built on the observation that EVs derived from distinct biogenic mechanism often differ by size (26,27). The term "exosomes" has been used to refer to EVs ~ 40–100 nm in size, whereas the term "microvesicles" is typically used to refer to larger EVs of ~ 100–$1,000$ nm (26). We recognize that these size cut-offs are somewhat arbitrary (24) and that EVs defined solely by size-based nomenclatures are likely to be heterogeneous in molecular composition (28). Moreover, EVs isolated using one method are likely to differ from those isolated using another (29). The available data is rather preliminary for establishing prescriptive standards for EV definition or isolation (24,25,29). As a result, it is important for investigators to specify the operational definition of the EVs under investigation and the methods used for EV isolation

and assessment of EV purity and size, to enable meaningful comparison of results among different studies.

Two recent studies have compared the performance of different EV isolation methods. Kalra et al. compared differential ultracentrifugation, EpCAM affinity purification and OptiPrep (Sigma-Aldrich, St.Louis, MO, USA) density gradient ultracentrifugation on plasma samples. They used mass spectrometry, detection of the exosome marker TSG101 and electron microscopy to evaluate the yield and purity of their preparations (30). The report concluded that density gradient ultracentrifugation gave the best results, but that quicker and less laborious methods would be valuable for biomarker studies.

Van Deun et al. applied 4 EV isolation protocols to conditioned medium: differential ultracentrifugation, OptiPrep (Sigma-Aldrich) density gradient ultracentrifugation, ExoQuick (System Biosciences, Mountain View, CA, USA) precipitation, and Total Exosome Isolation (Life Technologies, Carlsbad, CA, USA) precipitation. They evaluated the purity and RNA content of the resulting EVs by assessing particle count and morphology by nanoparticle tracking analysis and electron microscopy, enrichment of CD63 and other exosomal marker proteins, presence of contaminating AGO2 and gene expression microarray (Agilent) (31). The report concluded that, although density gradient ultracentrifugation was the most time- and labour-intensive method, it produced the highest yield and purity EVs, as well as the highest complexity RNA content.

Extracellular RNA Communication Consortium groups have used a variety of methods to enrich for EVs. These methods can be divided into 5 classes: precipitation, filtration, gel filtration, affinity purification and differential ultracentrifugation.

Precipitation-based methods
Three groups reported using a kit-based precipitation technique [e.g. ExoQuick (System Biosciences) and the Total Exosome Isolation Reagent (Life Technologies)]. These techniques precipitate EVs of various sizes, as well as ribonucleoprotein complexes, from biofluids. The kits use polyethylene glycol (PEG)/sodium chloride (NaCl)-based methods initially developed for precipitation of macromolecular complexes, including virus particles. It is believed that the PEG polymers sterically exclude the macromolecular complexes from a portion of the solvent volume, increasing the effective concentration of the particles to their solubility limit (32). It has been noted by Extracellular RNA Communication Consortium members that EVs isolated using these methods display different light-scattering properties and particle sizes (as measured using the NanoSight instrument (Malvern Instruments, Malvern, UK)) and lower bioactivity compared to those isolated by ultracentrifugation (see below, "Differential centrifugation-based methods").

These methods also sediment ribonucleoprotein and lipoprotein complexes (33), which have themselves been reported to carry small RNAs, including miRNAs (7). Consequently, unintended co-purification of vesicular and non-vesicular macromolecular structures could potentially complicate subsequent analyses.

Membrane filtration-based methods
Two Consortium groups reported using sequential fractionation of cell culture supernatant through syringe filters with successively smaller pore sizes. According to these groups, this approach had a number of potential advantages over other methods for EV purification: (a) improved homogeneity in the size of vesicles obtained; (b) avoidance of high g-forces involved in ultracentrifugation-based approaches (34,35); (c) higher yield of EVs and exRNAs; (d) avoidance of precipitants that might interfere with downstream applications, in contrast to PEG/NaCl precipitation; (e) no need for specialized large equipment, compared to ultracentrifugation.

However, the method used by the 2 Consortium groups was quite time-consuming, as filtration through membranes with small pore sizes (~20 nm) was slow and the filters sometimes clogged. In addition, there are 2 significant concerns regarding the filtration approach, which need to be further studied: the potential disruption of EVs due to shear forces as they pass through the filters and the co-purification of non-vesicular particles and protein aggregates. It is possible that these effects can be mitigated (e.g. by adjusting the chemical composition, pore size, uniformity of pore size and structure of the filters or by controlling the pressure applied during filtration), but such studies have not yet been performed.

Gel filtration-based methods
The basis of gel filtration chromatography is that molecules pass through a bed of porous beads, with the speed of passage determined by molecular size and shape, as well as interactions between the beads, buffers and molecules. Overall, large molecules do not enter the pores and therefore move through the column quickly; small molecules readily enter the pores and pass through the column more slowly. Beads of different chemical compositions that affect their hydrophobicity/hydrophilicity, compatibility with different buffers and resistance to different solvents are available in a variety of pore sizes.

Gel filtration has been used extensively to purify nucleic acids, peptides, proteins, lipids and viruses. Methods using Sepharose 2B beads (Sigma-Aldrich, St. Louise, MO, USA) to purify EVs for RNA and proteomic profiling (36) and for separating platelet-derived vesicles greater than 70 nm in diameter from HDL and protein (37) have been reported. None of the Extracellular RNA Communication Consortium groups used gel filtration chromatography (also referred to as "size exclusion chromatography") as their primary modality for EV or non-vesicular particle

isolation. However, it has been reported in the literature that gel filtration results in EV co-isolation with high-density lipoprotein (HDL) and low-density lipoprotein (LDL) particles (38). The overall consensus in the Extracellular RNA Communication Consortium at this point is that further studies are required to fully characterize the effects of different variables (particularly bead type and buffer composition) on fractionation of different EV and non-vesicular particle types.

Affinity purification-based methods
None of the Extracellular RNA Communication Consortium groups reported using affinity-based methods for purification of EVs routinely, although some groups had experience with this approach (39). The groups focusing on purification of exRNA-containing lipoprotein complexes used it on a regular basis (see below, "Isolation of exRNA-containing lipoproteins"). In this method, biofluids are passed over solid phase substrates coupled to antibodies or other molecules that bind to specific antigens on the surfaces of the extracellular particles. This strategy has been used to isolate extracellular particles that express disease-specific antigens. For example, purification of epithelial tumour-derived EVs from plasma can be achieved by selection for EVs carrying tumour-specific (e.g. PSMA) or epithelial (e.g. EpCAM) markers using magnetic bead sorting (40,41). However, the specificity and yield from this approach is highly dependent on the differential expression of specific markers on subpopulations of EVs, the type and performance of the antibodies used and the type and surface size of the magnetic beads used. Also, non-vesicular RNAs may be physically entrapped by this purification method. Thus, optimization of this method is critically needed to assess whether enrichment of disease-specific circulating EVs will be useful for exRNA biomarker discovery. Microfluidics devices have also been used to capture EVs by antibody affinity (39). A combination of different antibodies has been shown effective in enriching for normal EVs, and a specific cocktail of antibodies could be used to enrich for EVs from a specific disease, e.g. cancer.

Differential centrifugation-based methods
Sixteen of the Extracellular RNA Communication Consortium groups reported using a version of the classic differential centrifugation approach to collect EVs (42–44). Typically, there is a low-speed centrifugation step (300–3,000 × g for 5–30 minutes) to remove cells and cell debris, a high-speed centrifugation step (10,000–20,000 × g for 30 minutes) to remove larger classes of EVs and then an ultracentrifugation step (100,000–167,000 × g for 1–18 hours) to collect the exosome pellet for analysis. Sedimentation efficiencies vary with rotor type (k factor) and fluid viscosity, accounting for the range in speeds and durations of ultracentrifugation (45–47).

The differential centrifugation approach is supported by more than a decade of research in the exosome/microvesicle field, which was preceded by more than 50 years of research in the field of virology. This methodology has been demonstrated in many publications to enrich for many of the major sources of exRNAs, including different types of EVs, as well as endogenous viruses and some protein aggregates. However, differential centrifugation has several drawbacks. In practical terms, it requires extensive processing of samples and is difficult to scale up, particularly in terms of numbers of samples. The populations of vesicles/particles pelleted by the high-speed centrifugation (10,000–20,000 × g) and ultracentrifugation steps (>100,000 × g) are heterogeneous in size (35), although this can be reduced with a pre-filtration step to remove the largest vesicles (42). It has not been established how efficiently non-vesicular exRNAs (such as exRNAs associated with ribonucleoprotein or lipoprotein complexes) are pelleted by the ultracentrifugation step. In addition, it has been noted by some members of the Extracellular RNA Communication Consortium that prolonged ultracentrifugation appears to alter the morphology of EVs. Given that the yield of exRNA using differential centrifugation is markedly lower than when using the other methods discussed above and that differential centrifugation requires specialized large equipment, many groups will prefer to use a more facile method, unless differential centrifugation offers a substantial benefit for their particular experimental system. Finally, the differential centrifugation approach can be difficult to standardize. One reason for this is the variation in ultracentrifugation speed and duration seen in commonly used protocols. Moreover, even when the g-force and duration parameters are held constant, results can differ with sample volume and viscosity, rotor type (e.g. fixed angle vs. swing-out), the dimensions and chemical composition of centrifuge tubes and instrument model (45).

It has been found that density gradient ultracentrifugation (DGUC) performed after standard ultracentrifugation (48,49) or after an alternative EV concentration method (31) can result in higher purity and better uniformity of size of EVs. However, the only groups in the Consortium who reported using it regularly were the groups focusing on lipoproteins (see below, "Isolation of exRNA-containing lipoproteins").

Isolation of exRNA-containing lipoproteins
In addition to EVs, exRNA is also transported by lipoproteins in plasma (7). The most abundant lipoproteins are very low-density lipoproteins (VLDL), LDL, and HDL. As their names suggest, lipoproteins are classified based on their density – VLDL (0.94–1.006 g/mL), LDL (1.006–1.063 g/mL) and HDL (1.063–1.21 g/mL). Apolipoprotein B (apoB) containing VLDL (approximately 60 nm in diameter) is synthesized and secreted by the liver.

It undergoes remodelling in circulation due to lipase hydrolysis of VLDL triglycerides. This process produces smaller but denser LDL (25 nm in diameter). Conversely, apolipoprotein A-I (apoA-I), the structure-function protein in HDL (7–12 nm in diameter), is secreted by the liver and small intestines in a nascent lipid-poor form that is quickly lipidated with cholesterol and phospholipids by ATP-binding cassette transporter A1 (50). As such, VLDL and LDL are identified as apoB particles and HDL is defined by apoA-I, and thus affinity chromatography can be used to isolate distinct lipoprotein classes. Both apoB and apoA-I particles transport and deliver miRNAs to recipient cells (7).

Historically, the most common method for isolation of lipoproteins was density gradient ultracentrifugation (DGUC), using solutions of salts, such as potassium bromide, to set up the gradient. By using distinct density layers, each lipoprotein class can be separated by ultracentrifugation. This process requires multiple 24 h runs to sequentially isolate VLDL, LDL and finally HDL; it then requires extensive dialysis to remove the salts from the density buffers. The main benefit of DGUC is the high yield of lipoproteins that can be obtained (e.g. ∼1 mg of total HDL protein per 1 mL of plasma). The disadvantages of DGUC are that it involves exposures to high gravitational forces and high salt buffers; it is time-consuming, and exosomes (1.10–1.21 g/mL) have a similar density to HDL and can be co-purified with HDL from plasma samples (51).

Fast protein liquid chromatography (FPLC) with a size-exclusion gel filtration column can cleanly separate HDL from exosomes due to the relatively large difference in size (approximately 10 nm in diameter for HDL and 40–100 nm for exosomes). Given the limited input volume (∼1 mL), FPLC is often used downstream of DGUC. A disadvantage of FPLC is that other macro-protein complexes of similar size can co-fractionate with lipoproteins (e.g. antibodies). At this time, these potentially contaminating co-fractionated complexes or proteins have not been found to carry miRNAs or other RNA species, and so this may not be as important an issue for exRNA studies.

Another method for isolation of lipoproteins is immunoaffinity purification using antibodies raised against apoB or apoA-I. Micro-spin columns can be loaded with Sepharose 4B beads conjugated to anti-apoB or anti-apoA-I antibodies (Academy Bio-medical Co., Houston, TX, USA) to purify VLDL + LDL or HDL, respectively. This method only requires a few hours, but is limited by the binding capacity of the columns, which results in low yields of total lipoprotein (∼100 µg of total HDL protein per mL plasma). Compared to DGUC and FPLC, immunoaffinity purification is the least specific method, due to non-specific interactions. The main advantage of this method is that it can rapidly isolate lipoproteins from small sample volumes.

The best electron-microscopy-based approach for isolation of highly pure HDL from plasma is to perform DGUC followed by FPLC and to collect HDL fractions based on the distribution of total cholesterol. Because LDL and VLDL are similar in size to exosomes, FPLC alone results in cross-contamination of these particles; therefore, DGUC followed by FPLC is also a sound strategy for separation of apoB-containing particles from exosomes. It is important to appreciate that the ultracentrifugation step of the differential centrifugation strategy for isolation of EVs (see below, "Differential centrifugation-based methods") will also co-pellet both LDL and HDL.

In summary, DGUC, FPLC and immunoaffinity methods can all be used to isolate miRNA carrying VLDL, LDL and HDL. Each method has its own benefits and limitations, and the use of multiple methods in tandem is a useful strategy for improving the purity of the final material.

Summary of EV and particle enrichment methods
Based on the discussions held in the SAS WG, it is clear that a wide range of methods are used by members of the Extracellular RNA Communication Consortium. It is also apparent that there is a lack of consensus regarding either the preferred methodology or accepted experimental parameters for the enrichment of EVs or other exRNA-containing particles from biofluids. Systematic studies comparing the exRNA content and bioactivity of EVs isolated using different approaches are starting to be reported. Such studies are essential for promoting progress in this field. To further address these issues, 2 subgroups have been formed under the umbrella of the SAS WG: the Vesicle Isolation and Function Subgroup and the Non-Vesicular or Lipoprotein-Associated Subgroup.

RNA isolation methods
Efforts to determine optimal methods for isolation and analysis of exRNA from biofluids have been initiated by several investigators over the past 3 years (52–60). Eldh et al. (52) compared RNA yield (by spectrophotometry and qPCR), purity (by spectrophotometry) and size distribution (by Bioanalyzer (Agilent)) of exRNAs isolated from cultured cell media using several different kits and methods, noting the best results with the Exiqon miRCURY kit (Vedbaek, Denmark) and adequate results with the miRNeasy (Qiagen, Venlo, Limburg) and mirVana (Life Technologies, Carlsbad, CA, USA) kits for small RNAs.

McAlexander et al. (53) also tested different commercially available RNA isolation kits to determine the optimal method for improving yield of extracellular miRNAs (assayed by qRT-PCR for synthetic miRNA spike-ins as well as endogenous miRNAs). The Exiqon miRCURY biofluids kit was determined to have the best performance

among the tested methods for isolation of miRNA from platelet-poor plasma and CSF in this study.

Sedlackova et al. compared the miRCURY RNA isolation kit (Exiqon) and the Circulating Nucleic Acid kit (Qiagen, Valencia, CA, USA) on plasma samples from pregnant women and concluded that the miRCURY kit was superior for isolation of both miRNAs and DNA based on qPCR of miR-15 and miR-451 for miRNAs, and *AR* and *DYS14* for DNA (54).

Kroh et al. compared the miRNeasy (Qiagen) and the mirVana PARIS (Life Technologies) kits on serum and plasma and found that the miRNeasy (Qiagen) kit using a $10 \times$ volume of the TRIzol reagent (Life Technologies) had a yield $2-3 \times$ that of the mirVana (Life Technologies) kit (55).

Monleau et al. used serum and compared the miRNeasy mini (Qiagen), plasma/serum circulating RNA purification (Norgen Biotek, Ontario, Canada) and Nucleospin miRNA plasma (Macherey-Nagel, Duren, Germany) kits, using the TaqMan low-density array for miRNA (Life Technologies) as a readout. They concluded that the Nucleospin kit resulted in a higher number of detected miRNAs (56).

Moret et al. isolated miRNAs from serum using the mirVana PARIS (Life Technologies), TRIzol LS (Life Technologies) and miRNeasy serum/plasma (Qiagen) kits using different amounts of spike-in control RNA and using NanoDrop, Bioanalyzer (Agilent) and the Affymetrix miRNA 3.0 microarray (Affymetrix, Santa Clara, CA, USA) as the readout. The results focused on a comparison of the quantification, size distribution and microarray results for the miRNeasy (Qiagen) method with different amounts of spike-in RNA. Moret et al. concluded that using a 10-fold lower amount of spike-in than that recommended by the manufacturer gave the best yield and sensitivity on the microarray. An in-depth comparison of results for the 3 purification methods was not shown, but the authors stated that methods that require organic extraction, such as TRIzol LS (Life Technologies), should be avoided (57).

The types of RNA isolation methods used by laboratories in the Extracellular RNA Communication Consortium varied widely. The large majority of methods used solutions containing guanidinium isothiocyanate (GITC) for disruption of EVs and other exRNA-containing particles. However, the methods differed at 2 subsequent steps: (a) whether they include a phenol/chloroform extraction [e.g. TRIzol (Life Technologies), miRNeasy (Qiagen) and mirVana (Life Technologies)] or not [e.g. miRCURY Biofluids (Exiqon), Plasma/Serum Circulating and Exosomal RNA Purification (Norgen Biotek) and Direct-Zol (Zymo, Irvine, CA, USA)]; and (b) whether the exRNA is concentrated using alcohol precipitation (e.g. the standard TRIzol protocol) or a spin column (nearly all of the other methods).

Comparisons between different exRNA isolation kits

Several groups in the Extracellular RNA Communication Consortium have performed pilot studies comparing 2–6 RNA isolation kits. Here, we will discuss preliminary results from 3 of these groups for illustrative purposes only, to show the challenges encountered when attempting to draw general conclusions from studies performed in different laboratories. We wish to emphasize that a large multicentre comparison has not been done; we do not intend for readers to base decisions on the choice of RNA isolation method for their studies on the results presented here alone.

Three groups each compared RNA isolation from plasma and/or serum using 3 different commercial kits. There was little overlap in the kits used by the groups.

The Gandhi group isolated RNA from frozen serum and plasma using 3 kits: the miRNeasy Mini Kit (Qiagen) with 0.2 ml input volume, the Circulating RNA Isolation Kit (Norgen Biotek) with 1 ml input volume and the Exosome RNA Isolation Kit (Norgen Biotek) with 1 ml input volume. The RNA samples were eluted in 50 or 100 µl and quantified using the NanoDrop (Nanodrop). They were further analysed using the nCounter miRNA Expression assay (nanoString, Seattle, WA, USA), which interrogates 800 miRNAs. After obtaining the results, it was learned from the manufacturer that the 2 Norgen kits are the same kit marketed under 2 different names; therefore, the samples isolated using these 2 kits can be considered as replicates. As expected, the yield and performance of the RNA samples isolated using these 2 kits were very similar. Although the yield of RNA was lower for the Norgen kits than for the miRNeasy kit, the number of detectable miRNAs as assessed by the NanoString assay was higher for the Norgen kits. It also appeared that the RNA yield for plasma and serum samples was similar, but the number of detectable miRNAs was higher for the plasma samples than the serum samples. Overall, the overlaps in the sets of genes detected in the different samples was quite good.

The Freedman group isolated RNA from 0.2 ml plasma samples using 3 different commercial RNA isolation kits: the miRCURY RNA Isolation Kit for biofluids from Exiqon, the TaqMan miRNA ABC Purification Kit from Life Technologies and the miRNeasy Serum/Plasma Kit from Qiagen. All input plasma volumes and RNA elution volumes were held constant across the 3 kits, and the isolated RNA samples were analysed using 90 qRT-PCR miRNA Assays (miScript miRNA Assays from Qiagen) run on Dynamic Arrays on the BioMark System (Fluidigm, South San Francisco, CA, USA). In this set of experiments, the TaqMan ABC Kit showed the lowest total exRNA yield, but it also showed the lowest C_T and standard deviation values (and therefore the highest measured quantity) for detected miRNAs, indicating that exRNA yield cannot be used as a definitive arbiter

of the performance of an RNA isolation kit. The major limitation of the Taqman ABC kit is that it is a targeted kit, unlike the other kits used in this study, only isolating selected or targeted miRNAs; thus it does not allow for discovery of novel RNAs.

The Patel group isolated RNA from 0.5 ml fresh or frozen serum and fresh or frozen plasma using 3 different kits: the Plasma/Serum Circulating and Exosomal RNA Purification Mini Kit (Norgen Biotek); the SeraMir Exosome RNA Purification Kit (System Biosciences); and the Total Exosome Isolation and Total Exosome RNA Isolation Kit (Life Technologies). The RNA yield was measured using NanoDrop (Nanodrop) and the Bioanalyzer RNA Pico Kit (Agilent). With NanoDrop (Nanodrop), the RNA yields were comparable for serum and plasma samples and for fresh and frozen samples.

Each of these 3 groups used a different set of assays for evaluating the yield of RNA (a panel of qRT-PCR assays; NanoDrop and NanoString; NanoDrop and Bioanalyzer (Agilent)). There was minimal overlap in the RNA isolation kits used. For these reasons, it is not possible to draw strong conclusions regarding the relative performance of the kits across groups, even though they used the same biofluid types.

A fourth group, the Wong group, compared RNA yield from 0.5 ml saliva samples using 6 RNA isolation methods: (a) an organic extraction method (TRIzol LS); (b) 3 spin filter-based methods [QIAamp Viral (Qiagen), NucleoSpin (Clontech, Mountain View, CA, USA) and mirVana (Life Technologies)]; and (c) 2 methods combining organic extraction and spin filter clean-up [miRNeasy micro (Qiagen) and Quick-RNA micro (Zymo)]. The purified RNA samples were treated with DNase and then precipitated and resuspended in 10 μl RNase-free water. The quantity and size distributions of the resulting RNA samples were assessed using the RiboGreen reagent and the Bioanalyzer (Agilent), respectively, with the best yields from the NucleoSpin and miRNeasy micro kits. The minimal overlap in the RNA isolation and analysis methods used and the use of a different biofluid type by this group prevents useful comparisons between this and the other 3 studies.

Thus, despite the many studies that have been performed thus far, we believe that a large-scale multicentre effort, with NGS as the final readout, would be useful. It will be challenging to reach a consensus method, because all methods have their advantages and disadvantages. It is important to appreciate that, when comparing different methods, one needs to control for a wide number of variables. For data to be comparable across labs and methods, the different labs will need to work from a standardized sample set, as the efficiency and performance of different methods will vary, depending on biological differences between samples, such as the amount of AGO2-bound miRNA or cell-free DNA (cfDNA), which are isolated at different efficiencies by different methods.

For any experiment aimed at comparing 2 or more methods of RNA isolation, it is important to report and control for variables, some of which are listed below:

a. Sample collection and processing variables.
   1) Sample type, including viscosity (e.g. serum, plasma, platelet-poor plasma, urine, dilution in PBS or other buffer, etc.).
   2) Collection tube type and any additives.
   3) Sample holding time and temperature for the samples prior to processing.
   4) Cell removal steps (centrifugation parameters; type and pore size of filters used).
   5) Frozen storage before or after processing.
b. Possibility that sample processing steps might remove exRNA-containing EVs or protein or lipoprotein complexes. For example, removing the platelets from plasma is often done by centrifugation, but the protocols used are not standardized, with some protocols including fairly high g-force centrifugation steps that will also pellet a fraction of the EVs. Thus, analysis of the resulting supernatant can underestimate the exRNA content.
c. Differential co-purification of cfDNA by different methods and differential susceptibility of each RNA quantification and analysis method to contaminating DNA (see below, "Special considerations for exRNA quantification").
d. Different methods will isolate exRNAs associated with the various exRNA-containing vesicles/particles (such as EVs and AGO2-containing ribonucleoprotein complexes) with different efficiencies. It is not well understood for the large majority of protocols what the co-purification rates are for these entities.
e. Variability in yield and complexity of exRNA populations due to differences in sample input. This type of variability can be problem for input sample volumes at both the high and the low end. For example, the yield of exRNA can plateau with input sample volumes greater than 1 ml, but low input volumes can result in sampling error for low abundance RNAs. These effects have not been systematically characterized.
f. The compatibility of the method with samples collected in a clinically feasible workflow.
g. The ability to easily standardize and adopt the method across multiple labs.
h. The efficiency of capture of RNAs of both high and low molecular weight.

When evaluating the yield and performance of RNA, it is helpful to use the method the operator is eventually planning to use for the downstream analysis of the target (i.e. if qRT-PCR is the analytical method that will be used, a qRT-PCR based evaluation of RNA yield/reproducibility should be used rather than Bioanalyzer (Agilent), Qubit

(Life Technologies), nanoparticle tracking analysis or other method, because each method has a bias and may not give the same result).

For interlab comparisons or validations of different methods, we believe that it is important to include standardized samples, spike-ins and analysis reagents in the study design. To address these and other related issues, the SAS WG has formed the RNA Isolation Subgroup.

Special considerations for exRNA quantification
Quantification of exRNAs is particularly challenging, given that they are typically present at low concentrations and have a wide range of lengths (~ 15 nt to thousands of nucleotides), with a prominent population of small RNAs (<200 nts). It is important to keep in mind that different measurement techniques will yield very different total amounts of RNA. Furthermore, it is important to consider the characteristics of each quantification method, in terms of the limit of detection, dynamic range and specificity for nucleic acid type, so the most accurate method can be used for the expected yield of RNA (61).

The lower limit for the NanoDrop is 2 ng/µl (with increased variability in measurements at concentrations below 5 ng/µl) and the upper limit is 3,000 ng/µl. In many cases, it is probably not the best choice for exRNA quantification due to the low yields of exRNA from most biofluids; in addition, it is a spectrophotometric assay and thus detects DNA and protein in addition to RNA.

The Qubit RNA Assay (Life Technologies, also available for microplate format assays as the Quant-iT RNA Assay Kit and the Quant-iT RNA BR Assay Kit, Life Technologies) is highly specific for RNA. However, the lower limit at which it can confidently quantify RNA is 5 ng in a 200 µl assay volume; typically, purified exRNA samples do not contain enough RNA to be quantified using this method. The RiboGreen reagent (available as the Quant-iT RiboGreen RNA Assay Kit and Reagent, Life Technologies) has a lower limit of detection of 200 pg in a 200 µl assay volume, but binds to DNA as effectively as it binds to RNA. The use of the standards to generate standard curves is essential for both the RiboGreen (Life Technologies) and Qubit (Life Technologies) quantification methods, as differences may appear between quantifications done on different days. Assays adapted for a multi-well plate format are very useful, utilizing a plate reader to both increase the throughput of the assay and minimize technical variability in the assay between samples.

Although all of these methods can be used to quantify RNA, only the Bioanalyzer (Agilent), which has a lower range limit of 50 pg/µl for the RNA Pico and Small RNA Kits (Agilent), can evaluate the size distribution of the RNA molecules. However, this method is not as reproducible in terms of RNA quantification as the Qubit (Life Technologies) or RiboGreen-based assays (Life Technologies) and does not distinguish between RNA

and DNA. The Bioanalyzer (Agilent) methods are also affected by impurities that can quench the fluorescent signal. Variability in the height of the internal marker peak, an uneven baseline and an imperfect size standard ladder are indicators that there may be factors present that compromise the accuracy of Bioanalyzer (Agilent) quantification.

qRT-PCR is another valuable quantification method that can be performed using a standard curve to allow for absolute quantification of selected transcripts. Especially while an RNA isolation protocol is being optimized, qRT-PCR experiments should include a "no reverse transcriptase" control to detect DNA contamination and a spike-in positive control to evaluate for the present of reverse transcriptase inhibitors.

An additional concern that may impact the choice of measurement technique is potential contamination associated with the different RNA isolation methods, which can significantly influence RNA measurements due to the low concentration of RNA molecules in many exRNA samples. Guanidinium salts and organic solvents are used in many RNA isolation methods, and carryover of these will distort NanoDrop (Nanodrop) measurements. Guanidinium salts and ethanol also interfere with the Ribo-Green assay. GlycoBlue (Life Technologies), which is frequently employed as a carrier for RNA precipitation, is not compatible with the Qubit RNA assay (Life Technologies).

Thus, the measured total RNA yield will vary based on the quantification method used. We conclude that it is necessary, for comparisons across experiments and between groups, to select a consistent RNA quantification method to ensure reproducible results. In choosing a quantification method, it is important to consider the sensitivity, nucleic acid specificity and reproducibility of each method. For many groups, practical considerations, such as availability of required equipment, cost and time needed to run the assays, will also be factors in this choice.

Other variables in RNA isolation
Further complicating comparisons of different RNA isolation methods is the fact that many of the kit-based methods include optional Proteinase K (PK) and DNase digestion steps. In addition, several groups have explored using phosphorylation of exRNA samples to improve small RNA sequencing library yield.

Proteinase K treatment. Within the Extracellular RNA Communication Consortium, there has been significant disagreement about the impact of PK digestion on yield and quality of exRNA. PK may enable more effective dissociation of exRNAs from protein complexes; at the same time, it is possible that this dissociation of exRNAs from protein complexes may make them more vulnerable to RNases. To explore these possibilities, the Das lab performed small RNA-seq on exRNAs purified from the

same plasma sample without PK, with PK added prior to GITC and with PK added in the GITC buffer. In this experiment, addition of PK following addition of GITC buffer to the sample resulted in an increase in RNA yield compared to samples with no PK or PK added prior to GITC and a modest increase in the number of miRNA species detected. The increase in the number of miRNA species detected was largely attributable to an increase in detection of lower abundance miRNA species. Additional experiments using multiple replicates of samples from multiple individuals must be performed for confirmation. In addition, it is possible that the balance of release versus degradation may differ significantly according to the biofluid type (e.g. the proteins present, proportion of exRNAs tightly associated with protein-containing complexes and concentration and types of RNases present), and this issue is also in need of systematic study.

DNase treatment. There has been concern in the Extracellular RNA Communication Consortium regarding potential contamination of exRNA preparations with cfDNAs, which could interfere with accurate quantification of the overall exRNA concentration using spectrophotometric or fluorescent nucleic acid binding dye-based methods. Methods that do not effectively remove DNA can be easily remedied using a DNase treatment step. Some investigators worry that preparations of DNase could be contaminated with RNases (despite the availability of certified preparations of RNase-free DNase from several manufacturers). Therefore, they use a DNase step selectively when planning to use a downstream assay that would be confounded by contaminating DNA. Methods that do not include an organic extraction tend to be more prone to significant extracellular DNA contamination, as phenol/chloroform extraction performed in acidic conditions causes RNA to partition in the aqueous phase with DNA in the organic phase. For some downstream assays, co-purified DNA can result in inaccurate measurements of exRNA yield. It can also be the source of false signals, because even trace levels of DNA may be detected by sensitive assays such as qRT-PCR and some NGS library preparation methods. However, it should be kept in mind that exDNA may also have functions and merit as biomarkers.

The Laurent lab performed a preliminary study using serum samples from 2 patients. The researchers isolated RNA from each of the samples using the Exiqon biofluids kit and treating them with either no nuclease, RNase, DNase or both RNase and DNase. They used RiboGreen for total RNA quantification, the RNA Pico Bioanalyzer (Agilent) to examine nucleic acid size distribution and qRT-PCR to quantify a specific miRNA, miR-486. It was observed that the Bioanalyzer (Agilent) tracings for the exRNA samples without any nuclease treatment included several high molecular weight peaks that disappeared with

DNase digestion. qRT-PCR (using the same amount of input RNA according to the RiboGreen assay) confirmed that the C_T values for miR-486 for the DNase-treated samples were about 1 C_T lower than for the non-treated samples. This finding is consistent with the conclusion that DNA contamination in the non-treated samples was causing the RiboGreen quantification to overestimate the RNA concentration by about 2-fold. As expected, RNase treatment resulted in a ~ 20-fold decrease in the quantity of miR-486.

RNA phosphorylation. Several Extracellular RNA Communication Consortium groups perform RNA phosphorylation of the isolated exRNAs prior to next-generation library construction when using methods that incorporate adaptor sequences by RNA–RNA ligation. The rationale for this manoeuver is that degraded RNAs fragments, or RNAs that have undergone base hydrolysis, frequently do not have the 5′ phosphate necessary for 5′ adaptor ligation, or they have a 3′ phosphate, which prevents 3′ adaptor ligation. In this situation, 5′ phosphorylation and 3′ dephosphorylation of the input RNA with T4 polynucleotide kinase (T4 PNK) can improve library yields.

Several of the groups in the Consortium have noted that exRNA isolated using the miRCURY Biofluids kit produced low library yields of small RNA. The Das group showed that while the yield of RNA using the miRCURY biofluids kit was equivalent to other kits, depending on type of library preparation method chosen, the RNA isolated using this method required the use of RNA phosphorylation with T4 PNK to yield usable libraries for RNA-seq. The Laurent group reasoned that because T4 PNK displays significant sequence bias, it would be reasonable to use OptiKinase (Affymetrix), a modified form of T4 PNK that does not display this bias (62). However, OptiKinase (Affymetrix) does not have 3′ dephosphorylation activity (62), so they used a mixture of T4 PNK and OptiKinase (Affymetrix) to treat exRNA isolated using the miRCURY Biofluids kit prior to library construction using the NEBNext Small RNA Library Prep Set (New England Biolabs, Ipswich, MA, USA). They found that the non-phosphorylated samples yielded a significant fraction of shorter products corresponding to adaptor dimers. At the same time, for the samples treated with T4 PNK and OptiKinase (Affymetrix), the lengths of nearly all of the products were consistent with adaptors attached to miRNA or piRNA inserts, and the overall yield of products of the desired size was approximately 20-fold higher. However, when the libraries made from phosphorylated and non-phosphorylated RNA samples were sequenced, researchers found that the phosphorylated samples showed a shift in the distribution of reads. They noted a markedly increased percentage of reads mapping to rRNA sequences and concomitant decreases

in the percentages of reads mapping to non-rRNA transcripts, including miRNA, tRNA and piRNA sequences. These results highlight the need to complete the entire NGS analysis process for evaluation of the effects of upstream experimental variables, rather than depending on commonly used proxy measures of library yield (such as bioanalyzer (Agilent) tracings, nucleic acid quantification, or qPCR) for evaluation of library quality.

Downstream evaluation of EVs and exRNAs

The downstream evaluation of EVs and exRNAs in biofluids can be divided into 2 categories: assessment of biological function of EVs and molecular analysis of purified exRNAs.

Assays of biological function

Assays for evaluation of the biological function of EVs are inherently difficult to standardize, as they depend on the biological system and the specific function under investigation. Major questions posed by Consortium members in this area include the following:

a. Do EVs from cell culture supernatants have similar function to those from the corresponding cells in vivo? The answer to this question may vary according to cell culture conditions. There may be a variety of EV subpopulations, with some subpopulations that are shared between in vitro and in vivo cells, but others that may be released in vitro in response to specific stimuli that are encountered in the cell culture milieu. For example, the Breakefield group observed that cells release more EVs when they are less confluent, and live imaging has suggested a high exchange rate of EVs among cells. This observation is important when trying to isolate EVs from cells in vitro, as higher numbers of cells do not necessarily correspond to higher numbers of EVs. This correlation has been tested on a limited number of cell types and may not be representative of other cell types.

b. What is the impact of different experimental manipulations on EV function (e.g. filtration, high-speed centrifugation and ultracentrifugation)? This is an area of investigation that has not been systematically addressed.

c. Are there in vitro or in vivo functional assays that are generally useful? Different methods have been used to label EVs to track their fate in culture and in vivo (63). The usual method is to employ a lipophilic fluorescent lipid dye (e.g. PKH67 (Sigma-Aldrich) or XenoLight DiR (PerkinElmer, Waltham, MA, USA)) that intercalates within the vesicle membrane. This method can be a very effective way of tracking vesicle uptake over relatively short time periods (hours). Caveats are that the unbound dye residues can form

precipitates that register as nanoparticles, and that the half-lives of these dyes are much longer than the vesicles, with the dyes being able to intercalate into other membranes in cells. Others have labelled vesicle membranes with fluorescent membrane proteins fused to myristoylation and/or palmitoylation peptide signals (64) or to EV membrane proteins, such as CD63 or CD81 (65,66). In vivo distribution studies of EVs administered intravenously report different half-lives depending on the labels used, with some dyes showing long retention times [24 hours (67,68), a membrane-bound luciferase label giving a 2-phase exponential decay half-life of approximately 30–60 minutes to 23 hours (69) and vesicles labelled with biotinylated lectins having a 2-minute half-life (70)].

Molecular analysis

The repertoire of exRNA released by various cells and detected in body fluids is as yet incompletely defined. A number of low-, medium-, and high-throughput profiling platforms are available for characterization of exRNAs, including qRT-PCR, NanoString, microarrays and NGS. The choice of methodology for a particular project should depend on its specific goals and experimental settings, but it should be kept in mind that different expression profiling technologies will introduce various sources of bias, which will hamper interstudy comparisons and integrative analysis of the resulting datasets.

Cross-platform comparisons

Comparisons across different analysis approaches have identified some of the important types of systematic bias. In the most comprehensive study to date focused on miRNA expression platforms, Mestdagh et al. compared 12 commercial platforms available from 9 major vendors (71). This miRQC study included hybridization, qRT-PCR and NGS-based profiling of human tissues and serum RNA, as well as of a set of positive and negative control samples. Assessment of several performance metrics, such as specificity, required input, sensitivity, titration response, accuracy and reproducibility, enabled identification of strengths and weaknesses for each particular method. As expected, superior sensitivity, critically important for low-input exRNA samples, was observed for qPCR-based platforms. Of note, the average validation rate for differentially expressed miRNAs was ~55% between any 2 platforms, indicating a need for validation experiments using an alternative platform. These results suggest that similar rigorously designed comparative studies evaluating profiling technologies for other protein-coding and non-coding RNA species, especially in low-input conditions, are critically needed.

NGS library preparation methods

It is well appreciated that using NGS for gene expression profiling in different tissues has allowed investigators to

examine cellular processes in health and disease in un-precedented detail. NGS offers the possibility of discovery of disease-specific exRNA biomarkers, including coding RNAs, short and long non-coding RNAs and novel RNAs that have not previously been mapped to the human genome. At the same time, the use of NGS for exRNA discovery involves several unique challenges. Burgos et al. (72,73) were able to successfully profile exRNA from plasma and CSF after optimizing exRNA isolation using several commercially available kits. The use of the mirVana PARIS (Life Technologies) isolation kit followed by the Illumina TruSeq small RNA library preparation protocol (Illumina, Inc., San Diego, CA, USA) produced approximately 37% of reads mapping to miRNA for plasma and 27% mapping to miRNA for CSF, with low variability between technical replicates. Similarly, Williams et al. (15) obtained ∼2 million mappable reads/sample (no mapping rate reported) from small RNA sequencing of exRNAs from plasma. Tsui et al. (74) obtained ∼3–12 million analysable reads/sample (no mapping rate reported), and Koh et al. (75) obtained ∼16 million mappable reads/sample (mapping rate of ∼80%) from plasma from long RNA-seq data. Other groups have used NGS to profile small exRNAs in exosomal and non-exosomal fractions from blood (76) and urine (77). The Wang group has compared results from 3 small RNA sequencing library preparation protocols (78). Although the results of these studies indicate the feasibility of using NGS to characterize exRNAs from a variety of biofluids, they are difficult to compare to each other, given differences in biofluid type and methods for RNA isolation and NGS library construction.

There is strong consensus in the Consortium that standardized and robust methods for next-generation library construction from exRNA samples for both small and long RNA sequencing, and a systematic characterization of biases would be extremely valuable. There are a number of papers comparing cDNA synthesis and NGS library preparation kits (13,78–88). From these published reports, which include studies with bulk, low-input, single-cell and degraded cellular RNA samples, it is apparent that different methods vary greatly in ease of use, robust library preparation for very low input samples, handling of ribosomal RNA in the input sample (i.e. depletion vs. avoidance) and options for acquiring strand-specific data. Careful consideration of the results from these published comparisons can help with selection of library construction methods for exRNAs. However, unique features of exRNAs (e.g. variability in the types of RNAs present, which can differ among biofluids and vesicle/particle types) make it difficult to extrapolate results obtained using cellular RNA samples to exRNA samples. Some exRNA-specific studies have been performed by a few Extracellular RNA Communication Consortium member labs, but large-scale, rigorous studies of yield,

variances and sequence-specific biases specifically for NGS of exRNAs have yet to be performed.

The Das group assessed 3 different library preparation methods [NEBNext Small RNA Library Prep Kit (New England Biolabs), ScriptSeq RNA-Seq Library Preparation Kit (Illumina) and the SMARTer Universal Low Input RNA Kit (Clontech)] on RNA isolated using the miRCURY biofluids kit (Exiqon) from the same plasma sample. These 3 different libraries gave very different mapped reads, with the NEBNext Small RNA Library Prep Kit (New England Biolabs) yielding the highest number of detected miRNAs and the SMARTer Universal Low Input RNA Library Preparation Kit yielding the highest number of detected long RNAs in terms of genes with >0 counts. Although there was some overlap between these different methods, there was a substantial fraction of identified transcripts that were unique to each method. Interestingly, the correlations in reads for the most abundant miRNA species among different library preparation methods appeared to be reasonable, with most of the variances being noted for the lower abundance miRNA species. These results are perhaps not surprising, given that the NEBNext Small RNA Library Prep Kit (New England Biolabs) is designed specifically for sequencing of small RNAs, while the SMARTer Universal Low Input RNA Kit and the ScriptSeq RNA-Seq Library Preparation Kit are designed for sequencing of long RNAs. The SMARTer kit uses a template switching approach, which may result in higher efficiency reverse transcription and better performance in long RNA sequencing compared to the ScriptSeq kit.

The Wong group at UCLA used 2 commercially available kits [the NEBNext Small RNA Library Prep Kit (New England Biolabs) and the NEBNext Ultra Directional RNA Library Prep Kit (New England Biolabs) with pretreatment with RNase R (Illumina)]. Synthetic spike-in RNAs were added to the total RNA samples purified using the TRIzol LS method from saliva samples to serve as internal standards to evaluate library efficiency, reproducibility, to normalize data across different samples and to calculate absolute RNA abundance. The resulting data were mapped to miRBase, piRNABank, RFam, the Human Oral Microbiome Database, UCSC KnownGenes, Gencode and Noncode. These studies have provided an initial assessment of the exRNA landscape of human saliva; they highlight the unusual abundance of piRNAs, as well as the first description of circular RNA, in saliva (89).

The Van Keuren-Jensen laboratory tested 3 whole transcriptome amplification kits (to make and amplify double-stranded cDNA) and 3 library construction kits (to ligate adaptors and amplify the final library). The 3 cDNA synthesis and amplification kits were (a) the Ovation RNA-Seq System V2 (NuGEN, San Carlos, CA, USA), (b) the Ovation RNA-Seq FFPE System (Nu-GEN; the researchers reasoned that this kit might

provide better coverage of exRNAs, which have a large percentage of small and potentially fragmented RNAs) and (c) the SMARTer Universal Low Input RNA Kit (Clontech). The 3 library construction kits were (a) the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs), (b) the Ovation Ultralow Library Systems (NuGEN) and (c) the KAPA Library Amplification Kit (Kapa Biosystems, Wilmington, MA, USA). The starting material was 5 ng RNA isolated from 2 pooled plasma samples using the mirVana PARIS kit (Life Technologies). For the Ovation FFPE kit, 2 pools with 100 ng were tested to see if increasing the input RNA changed the number of detected RNAs dramatically. Each pool was tested using all 9 combinations of cDNA synthesis/amplification and library construction kit. For every sample, >95% of all reads could be mapped using STAR (90), with 52–94% of mapped reads mapping uniquely. In most cases, the reads that mapped to more than one location in the genome were quite short. For the cDNA synthesis kits, the Ovation FFPE and SMARTer cDNA and amplification kits performed the best (with unique mapping rates >70% and comparable library complexity). All 3 of the library construction kits appeared to give similar results. In order to decrease the percentage of reads mapping to rRNAs, researchers also performed additional limited testing of ribo-depletion using the Ribo-Zero Gold kit (Illumina), with modifications recommended by Clontech for samples with low starting amounts of RNA. However, extensive additional testing is warranted.

The state of the field for RNA-Seq is changing rapidly, with new methods (91,92), new kits and new versions of existing kits becoming available all the time. The SAS WG has formed an RNA-seq subgroup to utilize the preliminary results from member groups to design future systematic studies that can be performed in a controlled manner across several labs to ensure generalizability of results.

Systematic bias in small RNA sequencing library preparation methods

Sequence-specific bias has been well recognized as a problem. There have been a handful of published studies measuring the same RNA sample using different commercial protocols and comparing the resulting read numbers for the same miRNA (79–81) or using synthetic miRNAs to systematically compare results using different mutant ligases and ligation conditions (80,81,93). It is now clear that that the difference between the numbers of reads for the same miRNA between 2 protocols can be as much as 100-fold (80). Recently, a number of issues related to sequence-specific bias were reviewed (82), and several suggestions were made to mitigate the bias of measurements using RNA-seq (81,83,93).

The Galas group has begun systematically investigating the effects of sequence specificity on the read frequencies of synthetic oligonucleotides using different library construction protocols. The approach has been to use very large sets of small synthetic ribo-oligonucleotides (e.g. 65,000 different 23-mers) to study the effects of sequence and protocol differences. The conclusions from these results are in agreement with previous results in that there is a difference of approximately 4 orders of magnitude between the highest and the lowest of the read frequencies resulting from libraries constructed from equimolar populations of ribo-oligonucleotides. The Galas group has found that the distributions of frequencies are quite similar between different protocols [e.g. TruSeq Small RNA Preparation Kit (Illumina) and NEBNext Small RNA Sequencing Library Preparation Kit (New England Biolabs)], but that the representations of individual ribo-oligonucleotides can vary significantly within these distributions. Important questions that have arisen are how much of the sequence is needed to predict the read frequency and whether the 5′ and 3′ terminal sequences act independently in determining to overall read frequency. These preliminary results suggest that additional systematic studies are needed in order to both optimize the library construction protocols and to construct procedures for computational inference of molecular concentrations from NGS data. The research community will need these kinds of systematic studies to fully characterize the sources of bias, to mitigate bias in protocols and to allow corrections to the read frequencies in determining the best estimates of the actual specific RNA concentrations in the original RNA sample.

Data normalization

Due to technical variations in sample processing and exRNA extraction and analysis, proper normalization is critical for consistent detection of true biological differences between samples. For qRT-PCR and NGS, it would be very useful if endogenous reference RNAs were identified. Exogenous spike-in synthesized miRNAs such as *C. elegans* miR-39/54/238 may be helpful, but are not sufficient, because they lack the capacity to normalize biological and pathological variations. For NGS, other normalization approaches (e.g. global scaling, distribution-based normalization or RPKM) can be applied, but it is as yet unclear what the optimal approach will be for exRNA data. To identify endogenous controls, it will be essential to perform a systematic survey of total exRNA profiles by RNA sequencing in large populations with a wide variety of health conditions. For any given test exRNA that one would wish to interrogate, the optimal endogenous control RNA would be of the same RNA biotype, highly conserved across species, similar in abundance to the test exRNA and stably and universally expressed regardless of technical or biological variance (94).

For circulating miRNA quantification, miR-16-5p has been used as internal reference control (95). However, serum or plasma prepared from haemolysed blood specimens may contain levels of miR-16-5p that are increased by 20–30 fold (96), diminishing the utility of miR-16-5p as an endogenous control. Liang Wang from the Patel project has performed studies aimed at identification of novel internal controls for exRNA quantification (78,97). In these experiments, plasma-derived exRNA transcriptome profiles from 192 individuals with various health conditions were generated; an analysis pipeline including the Bestkeeper (98) and Normfinder (99) algorithms was used to estimate the most stable transcripts among highly abundant exRNAs. This analysis revealed several exRNA candidates that were relatively stable across these individuals regardless of age, gender and health conditions. The most notable candidates include miR-30a-5p and miR-30e-5p for miRNA (100), PIR35469 and PIR61647 for piRNA, and RN7SK for lncRNA. This preliminary study suggests that it may be possible, at least in plasma, to identify a set of exRNAs with relatively stable abundances, which may be used as internal reference standards for exRNA quantification.

Conclusions

In this report, we have outlined the range of methods used by members of the Extracellular RNA Communication Consortium for biofluid collection and processing, EV/particle enrichment, exRNA isolation and exRNA analysis. We have attempted to show how our collective experience has identified key variables involved in these processes. We hope that our findings will inform future studies aimed at developing standardized approaches that will allow reproducible results to be obtained between experiments and across groups. We appreciate that there will never be a "one size fits all" solution, as different methods will always have different strengths and weaknesses depending on the biological problem at hand. However, we believe that the development of a robust set of standardized methods that are well characterized and validated for the most common biofluids and downstream assays would be a valuable starting point for all future studies. As a new and growing area of research, the exRNA field is simultaneously faced with unique challenges and unprecedented opportunities. The challenges include the low exRNA concentrations present in most biofluids and the variety of particles carrying exRNAs, many of which co-purify in commonly used isolation methods and are as yet poorly characterized. On the other hand, new methods are being developed and refined at a rapid rate for purification of different particles based on size, density and protein composition, as well as for RNA isolation and analysis. The availability of increasingly cost effective NGS-based analyses has been and will continue to lead to rapid advancements in our knowledge. We also hope that close collaborations and other interactions among research groups, promoted by the Extracellular RNA Communication Consortium and other organizations in the field, such as the International Society for EVs and the American Society for Exosomes and Microvesicles, will further accelerate progress in this field.

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