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Overview of Protocols for Studying Extracellular RNA and Extracellular Vesicles

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Abstract

Understanding the role of extracellular RNA (exRNA) has emerged as an exciting avenue for biomarker, therapeutic, as well as basic cell-cell communication applications and discoveries. Multiple protocols, kits, and procedures have been developed in the last decade to allow fractionation as well as isolation of subpopulations of macromolecules of interest found in biofluids. Here, we introduce the protocols decision tree developed by the Extracellular RNA Communication Consortium and available on their website (exRNA portal), and compare all methods currently available to the exRNA field and report pros and cons for each platform.

Keywords

Extracellular RNA; Exosomes; Biomarkers; Ribonuclear particles; Kits; Protocols

The Extracellular RNA Communication Consortium (ERCC) has spent the last several years developing protocols for the isolation and characterization of extracellular RNA (exRNA). The main goal of this work has been to ensure that the protocols are robust enough to minimize variation in methodology and results across labs. The amount of exRNA found in biofluids is very low, limited by both our isolation and detection/sequencing technologies. Therefore, it is vital to further progress this kind of cross-validation work in order to better understand the natural function of exRNAs and their applications as disease biomarkers and therapeutic agents.

For researchers who have just begun studying extracellular RNA, including the extracellular vesicles in which they are often encapsulated by, the ERCC has developed a protocols decision tree to help select the appropriate set of protocols for isolating EVs and exRNA from the biofluids of interest. The protocols decision tree [1] is available on the ERCC's website, the exRNA Portal [2]. While some of the protocols in the decision tree have been developed by ERCC researchers, many of the protocols consist of previously published methods that have been modified by ERCC members during the course of their experiments and are being periodically updated. In particular, the decision tree makes reference mostly to protocols published on *Nature's* Protocol Exchange (NPE) repository. Initial versions were

released on NPE in December 2015, and a second version of most protocols were release on NPE in June 2017. The major difference between the first and second versions of the protocols is an increase in the initial amount of biofluid processed, from 200 to 500 μ L. We found that the larger input volume led to higher levels of RNA isolation and therefore more uniformity in results across labs. The decision tree will continue to be updated with new protocol versions as they become available.

Here, we introduce the ERCC protocols decision tree and discuss some of the nuanced differences between classes of EV isolation methods, highlighting methods that have existed in the field for some time.

There are several methods and commercial kits available to isolate extracellular vesicles (EVs) and extracellular RNA (exRNA) from human biofluids. Multiple studies have reported on a variety of these methods, but to date there is not one method or kit that suits all studies. The type of biofluid, the sample volume, and the fraction of exRNAs of interest are some of the criteria used to determine what method should be used for a particular study. Here, we have compiled a list of methods and kits most widely reported in literature for isolation of EVs, exRNAs, or other components of biofluids. Broadly speaking, all methods can be classified into five categories (*see* Table 1), the most widely used being ultracentrifugation.

Drawbacks of ultracentrifugation include the need for expensive instrumentation (ultracentrifuges and rotors) and the belief that the EV population after ultracentrifugation will be contaminated with cell-free DNA and proteins. Depending on the speed and time of ultracentrifugation, some ribonucleoprotein (RNP) and lipoprotein (LPP) complexes may also sediment. After ultra-centrifugation, some researchers further purify the EV population using density gradients or size exclusion chromatography. Sucrose gradients have been used widely for several years but are being replaced more recently by iodixanol (OptiPrep) gradients. This is because some groups have reported that sucrose may inhibit the biological effects of EVs, while EVs prepared with OptiPrep better retain their biological activity.

Size exclusion chromatography is also widely used and is suitable for fractionation of sedimented EVs, as well as unprocessed biofluids. Recently, Izon introduced a commercial kit to speed up this method, with relatively good results. Size exclusion chromatography yields a very clean population of EVs with the drawback being loss of EVs during the multistep purification process.

The first commercial EV isolation kit (ExoquickTM) was launched on the market about 7 years ago, and is based on the principle of polyethylene glycol/sodium chloride precipitation, which has long been used for concentration of viruses. Since that time, several other kits using a precipitation strategy were launched by other manufacturers. Each of these kits (SBI, Life Technologies, Norgen Biotek, and Exiqon) has slightly different proprietary approaches to EV precipitation. Drawbacks to EV precipitation kits include coprecipitation of other unwanted molecules found in the biofluids and the difficulty of isolating EVs from large volumes of starting material. Ultrafiltration (e.g., using Millipore Amicon filters) is often used by researchers to concentrate large volumes, either before or after EV purification.

Filtration-based methods which isolate specific size ranges of EVs can also be performed using commercially available devices. In addition, several kits and protocols for affinity purification have been developed by biotechnology companies and academic research laboratories. Antibody-based affinity methods (ExoCap, Microfluids, μ NMR) and heparin-coated agarose or magnetic beads have been shown to bind subpopulations of EVs from cell culture media, plasma, and serum efficiently. Another affinity kit, the ME^{TE} kit, includes a proprietary peptide that, according to the manufacturer, binds to heat-shock proteins found on the surface of the plasma membrane, suggesting a possible method to enrich EVs with high levels of heat-shock proteins. All of these methods yield a pure sample of EVs and can be scaled up, although scale-up costs can be significant, particularly in the case of antibody-based methods. These methods are limited to isolating a subset of EVs that express a specific antigen. For researchers who are interested in targeting a specific population of EVs that displays one of these antigens, this may be a good option. However, at this point, for most cases, the biology is unclear on the diversity of EVs released by cells. Therefore, one antibody, or a pool of three to four anti-bodies, may not isolate all relevant EVs present in the sample.

ExoRNeasy isolates EVs based on their affinity to a proprietary membrane. ERCC members using this kit have reported that it can efficiently separate EVs (they bind to the membrane with high affinity) from other exRNA-containing particles, such as RNP complexes, which can be collected in the flow-through. Thus, this kit offers the extra advantage of efficiently separating EVs from RNP complexes. The exoRNeasy/exoEasy kit can yield a pure EV population from a volume as low as 200 L or as high as 4 mL of plasma or serum with one loading per column, and up to 100 mL of cultured media per column by loading the column 3–4 times. Larger volumes require loading multiple filters.

Two kits available on the market are based on a two-step procedure where the sample is first either filtered (e.g., PureExo) or concentrated (e.g., Exo-Spin) and then resuspended and allowed to bind to proprietary beads. Intact EVs are then eluted in phosphate-buffered saline (PBS) and can be used for a variety of down-stream assays. These kits do not offer feasible approaches to scale up to larger volumes.

Many other combinations and variations on these methods have also been reported in literature, and this list is not meant to comprehensively encompass all reports in the field. It is a simple overview of the major classes of EV and RNA isolation methods currently available.

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Table 1

Methods of extracellular vesicle purification and extracellular RNA isolation

EV isolation		Manufacturer	Application	Reference
Ultracentrifugation	Step 1. 300–3000 ×g 5–30 min	The most widely used protocol	Removes cells	[3]
	Step 2. 10,000–20,000 ×g for 30 min ^a		Removes debris	
	Step 3. 100,000–167,000 ×g for 1–18 h		Pellets EVs	
Sample purification	Sucrose gradient	Lab-developed method	All biofluids	[3]
	OptiPrep gradient	Sigma-Aldrich	All biofluids	[4]
	Size exclusion chromatography	Lab-developed method	All biofluids	[5]
	qEV	Izon	All biofluids	[4]
	Exo Quick™	System Biosciences	Serum, plasma, cell culture media	[6]
Precipitation	Total exosome isolation	Life Technologies Inc.	Serum, plasma, cell culture media	[7]
	Exosome RNA isolation	Norgen Biotek Corp.	Serum, plasma, urine	[8]
	miRCURY™ exosome kit	Exiqon	Serum, plasma, urine, CSF, cell culture media	[9]
Filtration	Sequential fractionation using different size filters	Krichevsky Lab ¹	Cell culture media	[10]
	ExoMir™	BIOO Scientific	All biofluids	[11]
Affinity purification	ExoCap™ (CD9, CD63, CD81, EpCAM)	JSR Micro	Serum, plasma, cell culture media	MBL International
	Microfluidics Immunoaffinity	Irimia Lab	Serum, plasma	[12]
	Micro nuclear magnetic resonance (μNMR)	Weissleder Lab	Serum, ascites	[13]
	Heparin beads	Lab-developed method	Serum, plasma, cell culture media	[14]
	exoRNeasy	Qiagen	Serum, plasma, cell culture media	[15]
	PureExo ^R ^b	101Bio	All biofluids	[16]
	Exo-spin™ ^c	Cell Guidance System	Serum, plasma	[4]
	ME ^{TE} exosome kit	New England Peptide	Serum, plasma	[17]

^aThis step is omitted by several research groups

^bFiltration first, then affinity to proprietary matrix

^cPrecipitation first, then affinity to proprietary matrix