METHODS FOR ISOLATING MICROVESICLES AND EXTRACTING NUCLEIC ACIDS FROM BIOLOGICAL SAMPLES

RELATED APPLICATIONS

[0001] This application claims priority to and the benefit of U.S. Provisional Application No. 62/022,538, filed July 9, 2014, U.S. Provisional Application No. 62/079,763, filed November 14, 2014, and U.S. Provisional Application No. 62/166,890, filed May 27, 2015, the contents of each of which are hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0002] The invention provides novel methods and kits for isolating nucleic acids from biological samples, including cell-free DNA and/or cell-free DNA and nucleic acids including at least RNA from microvesicles, and for extracting nucleic acids from the microvesicles and/or from the biological samples.

BACKGROUND

[0003] Membrane vesicles that are shed by cells are referred collectively as microvesicles. Microvesicles from various cell sources have been extensively studied with respect to protein and lipid content. Recently, microvesicles have been found to also contain both DNA and RNA, including genomic DNA, cDNA, mitochondrial DNA, microRNA (miRNA), and messenger RNA (mRNA).

[0004] Due to the genetic and proteomic information contained in microvesicles shed by cells, current research is directed at utilizing microvesicles to gain further insight into the status of these cells, for example, disease state or predisposition for a disease. In addition, current research is also directed at utilizing cell-free DNA to gain further insight into the status of cells.

[0005] Accordingly, there is a need for methods of isolating cell-free DNA and for isolating microvesicles from biological samples and methods of extracting high quality nucleic acids for accurate diagnosis of medical conditions and diseases.

SUMMARY

[0006] The present disclosure provides methods for isolation of cell-free DNA ("cfDNA," also known as circulating DNA) and/or for the combined isolation of cfDNA and nucleic acids including at least the RNA from microvesicles from a sample by capturing the DNA, DNA and

surface is an anion exchanger with quaternary amines. In some embodiments, the capture surface is a Q membrane, which is a positively charged membrane and is an anion exchanger with quaternary amines. For example, the Q membrane is functionalized with quaternary ammonium, $R\text{-}CH_2\text{-}N^+(CH_3)_3$. In some embodiments, the membrane has a pore size that is at least 3 μm.

[0011] Purification of the sample, including the microvesicle fraction, is performed using ion exchange techniques. In some embodiments, the ion exchange technique is a technique selected from those shown in the working examples provided herein.

[0012] In some embodiments, the agent used for on-membrane lysis is a phenol-based reagent. In some embodiments, the lysis reagent is a guanidinium-based reagent. In some embodiments, the lysis reagent is a high salt based buffer. In some embodiments, the lysis reagent is QIAzol. In some embodiments, the lysis reagent is a phenol-based lysis reagent, e.g., QIAzol, and it is used at a volume of about 700 ul.

[0013] The present disclosure provides a method for extracting nucleic acids from a biological sample comprises (a) providing a biological sample; (b) contacting the biological sample with a capture surface under conditions sufficient to retain the microvesicle fraction on or in the capture surface; (c) lysing the microvesicle fraction while the microvesicles are on or in the capture surface; and (d) extracting the nucleic acids from the microvesicle fraction. Alternatively, the method for extracting nucleic acids from the biological sample further comprises eluting the microvesicle fraction from the capture surface after step (b), collecting the eluted microvesicle fraction, and extracting the nucleic acids from the eluted microvesicle fraction. Optionally, the eluted microvesicle fraction can be concentrated by a spin concentrator to obtain a concentrated microvesicle fraction, and the nucleic acids are subsequently extracted from the concentrated microvesicle fraction.

[0014] The present disclosure also provides a method for extracting nucleic acids from a biological sample comprises (a) providing a biological sample; (b) contacting the biological sample with a capture surface under conditions sufficient to retain the microvesicle fraction on or in the capture surface; and (c) eluting the microvesicle fraction while the microvesicles are on or in the capture surface. The eluted microvesicle fraction can then be processed for further analysis. Optionally, the eluted microvesicle fraction can be concentrated by a spin concentrator to obtain a concentrated microvesicle fraction. In some embodiments, the nucleic acids are subsequently extracted from the concentrated microvesicle fraction.

[0014a] In one aspect of the present disclosure, there is provided a method for extracting DNA and RNA from a biological sample comprising:

(a) providing a biological sample;

(b) contacting the biological sample with a capture surface under conditions sufficient to retain cell-free DNA and microvesicles from the biological sample on or in the capture surface, wherein the capture surface comprises three membranes that are positively charged and functionalized with quaternary ammonium $R\text{-}CH_2\text{-}N^+(CH_3)_3$;

(c) contacting the capture surface with a phenol-based lysis reagent comprising phenol and guanidinium while cell-free DNA and the microvesicles are on or in the capture surface, thereby releasing the DNA and RNA from the sample and producing a homogenate;

(d) adding chloroform to the homogenate; and

(e) extracting the DNA, the RNA, or both the DNA and RNA from the homogenate.

[0014b] In another aspect of the present disclosure, there is provided a method for extracting DNA and RNA from a biological sample comprising:

(a) providing a biological sample;

(b) contacting the biological sample with a capture surface under conditions sufficient to retain cell-free DNA and microvesicles from the biological sample on or in the capture surface, wherein the capture surface comprises one or more beads that are positively charged and functionalized with quaternary ammonium $R\text{-}CH_2\text{-}N^+(CH_3)_3$;

(c) contacting the capture surface with a phenol-based lysis reagent comprising phenol and guanidinium while cell-free DNA and the microvesicles are on or in the capture surface, thereby releasing the DNA and RNA from the sample and producing a homogenate;

(d) adding chloroform to the homogenate; and

(e) extracting the DNA, the RNA, or both the DNA and RNA from the homogenate.

[0015] In some embodiments, the capture surface is a membrane. In one embodiment, the membrane comprises regenerated cellulose. For example, the membrane has a pore size at least 1 μm, such as for example, in a range between 2-5 μm. In some embodiments, the membrane has a pore size in a range between 3-5 μm. In some embodiments, the membrane comprises polyethersulfone (PES).

[0016] In some embodiments, the membrane is charged. In some embodiments, the membrane is positively charged. In some embodiments, the membrane is negatively charged.

[0017] In some embodiments, the membrane is functionalized. For example, the membrane is functionalized with quaternary ammonium $R\text{-}CH_2\text{-}N^+(CH_3)_3$.

[0018] In one embodiment, the capture surface comprises more than one membrane. In some embodiments, the capture surface comprises at least two membranes, wherein each membrane is adjacently next to the other membrane(s). In some embodiments, the capture surface comprises at least three membranes, wherein each of the three membranes is directly adjacent to one another. In some embodiments, the capture surface comprises at least four membranes, wherein each of the four membranes is directly adjacent to one another.

[0019] In some embodiments, the capture surface is a bead. For example, the bead is magnetic. Alternatively, the bead is non-magnetic. In yet another embodiment, the bead is functionalized with an affinity ligand.

[0020] In some embodiments, the capture surface is a slurry of polymer(s). In some embodiments, the slurry of polymer(s) is shaped into a bead.

[0021] In some embodiments, the biological sample is plasma. In some embodiments, the biological sample is serum. In some embodiments, the biological sample is urine. In some embodiments, the biological sample is cerebrospinal fluid. In some embodiments, the biological sample is cell culture supernatant.

[0022] In some embodiments, the method described herein further comprises contacting the biological sample with a loading buffer. The loading buffer is in the range of pH 4-8. In one embodiment, the loading buffer has a neutral pH.

[0023] The methods described herein provide for the extraction of nucleic acids from microvesicles. Preferably, the extracted nucleic acids are DNA and/or DNA and RNA. The extracted RNA may comprise messenger RNA, ribosomal RNA, transfer RNA, or small RNAs such as microRNAs, or any combination thereof.

[0024] Various nucleic acid sequencing techniques are used to detect and analyze nucleic acids such as cell free DNA and/or RNA extracted from the microvesicle fraction from biological samples. Analysis of nucleic acids such as cell free DNA and/or nucleic acids extracted from microvesicles for diagnostic purposes has wide-ranging implications due to the non-invasive nature in which microvesicles can be easily collected. Use of microvesicle analysis in place of invasive tissue biopsies will positively impact patient welfare, improve the ability to conduct longitudinal disease monitoring, and improve the ability to obtain expression profiles even when tissue cells are not easily accessible (e.g., in ovarian or brain cancer patients).

[0025] In some embodiments, the present disclosure is directed to compositions and methods for providing an in-process control for nucleic acid sequencing techniques, including, for example, next-generation sequencing (NGS) assays, to detect low-frequency sequence variants. These controls provide a number of technical advantages.

[0026] The biological sample is a bodily fluid. The bodily fluids can be fluids isolated from anywhere in the body of the subject, preferably a peripheral location, including but not limited to, for example, blood, plasma, serum, urine, sputum, spinal fluid, cerebrospinal fluid, pleural fluid, nipple aspirates, lymph fluid, fluid of the respiratory, intestinal, and genitourinary tracts, tear fluid, saliva, breast milk, fluid from the lymphatic system, semen, cerebrospinal fluid, intra-organ system fluid, ascitic fluid, tumor cyst fluid, amniotic fluid and combinations thereof. For example, the bodily fluid is urine, blood, serum, or cerebrospinal fluid.

[0027] In any of the foregoing methods, the nucleic acids are DNA and/or DNA and RNA. Examples of RNA include messenger RNAs, transfer RNAs, ribosomal RNAs, small RNAs (non-protein-coding RNAs, non-messenger RNAs), microRNAs, piRNAs, exRNAs, snRNAs and snoRNAs.

[0028] In any of the foregoing methods, the nucleic acids are isolated from or otherwise derived from a sample, including RNA isolated from the microvesicle fraction of a sample.

[0029] In any of the foregoing methods, the nucleic acids are cell-free nucleic acids, also referred to herein as circulating nucleic acids. In some embodiments, the cell-free nucleic acids are DNA or RNA.

[0030] Various embodiments of the present disclosure will now be described in detail. It will be appreciated that modification of the details may be made without departing from the

scope of the present disclosure . Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

[0031] All patents, patent applications, and publications identified are expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the present disclosure. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representations as to the contents of these documents are based on the information available to the applicants and do not constitute any admission as to the correctness of the dates or contents of these documents.

BRIEF DESCRIPTION OF THE FIGURES

[0032] Figure 1 is a schematic demonstrating one embodiment of the RNA and DNA isolation protocol for isolating a microvesicle fraction, releasing the microvesicle nucleic acids, and extracting RNA and DNA using two separate protocols.

[0033] Figure 2 is a schematic demonstrating another embodiment of the RNA and DNA isolation protocol for isolating a microvesicle fraction, releasing the microvesicle nucleic acids, and extracting RNA and DNA using a single protocol.

[0034] Figure 3 is a graph showing the effect of chloroform concentration in phase separation for isolating microvesicle RNA and DNA in a single extraction, as demonstrated by detection of wild-type BRAF RNA and DNA.

[0035] Figure 4 is a graph showing the effect of chloroform concentration in phase separation for isolating microvesicle RNA and DNA in a single extraction, as demonstrated by detection of GAPDH RNA and DNA.

[0036] Figure 5 is a graph showing that the adjustment of pH in phase separation influences the DNA extraction and detection.

[0037] Figure 6 is a graph showing the effect of titration of sample volume of cerebrospinal fluid (CSF) on microvesicle RNA extraction and detection.

[0038] Figure 7 is a graph showing the comparison of detection of microvesicle RNA targets from ultracentrifugation and EXO60 isolation methods.

What is claimed is:

1. A method for extracting DNA and RNA from a biological sample comprising:
   (a) providing a biological sample;
   (b) contacting the biological sample with a capture surface under conditions sufficient to retain cell-free DNA and microvesicles from the biological sample on or in the capture surface, wherein the capture surface comprises three membranes that are positively charged and functionalized with quaternary ammonium $R\text{-}CH_2\text{-}N^+(CH_3)_3$;
   (c) contacting the capture surface with a phenol-based lysis reagent comprising phenol and guanidinium while cell-free DNA and the microvesicles are on or in the capture surface, thereby releasing the DNA and RNA from the sample and producing a homogenate;
   (d) adding chloroform to the homogenate; and
   (e) extracting the DNA, the RNA, or both the DNA and RNA from the homogenate.

2. The method of claim 1, wherein each of the membranes is an anion exchanger functionalized with quaternary ammonium $R\text{-}CH_2\text{-}N^+(CH_3)_3$.

3. The method of claim 1 or 2, wherein the membrane has a pore size of at least 3 μm.

4. The method of any one of claims 1 to 3, wherein said three membranes are directly adjacent to one another.

5. The method of any one of claims 1 to 4, wherein the three membranes are identical to each other.

6. The method of any one of claims 1 to 5, wherein each of the three membranes comprises regenerated cellulose.

7. The method of any one of claims 1 to 2, 4 to 6, wherein each of the three membranes has a pore size in a range between 2-5 μm.

8. A method for extracting DNA and RNA from a biological sample comprising:

(a) providing a biological sample;

(b) contacting the biological sample with a capture surface under conditions sufficient to retain cell-free DNA and microvesicles from the biological sample on or in the capture surface, wherein the capture surface comprises one or more beads that are positively charged and functionalized with quaternary ammonium $R\text{-}CH_2\text{-}N^+(CH_3)_3$;

(c) contacting the capture surface with a phenol-based lysis reagent comprising phenol and guanidinium while cell-free DNA and the microvesicles are on or in the capture surface, thereby releasing the DNA and RNA from the sample and producing a homogenate;

(d) adding chloroform to the homogenate; and

(e) extracting the DNA, the RNA, or both the DNA and RNA from the homogenate.

9. The method of claim 8, wherein the one or more beads are an anion exchanger functionalized with quaternary ammonium $R\text{-}CH_2\text{-}N^+(CH_3)_3$.

10. The method of any one of claims 1 to 9, wherein the biological sample is plasma or serum.

11. The method of any one of claims 1 to 10, wherein the biological sample is between 0.2 to 4 mL.

12. The method of any one of claims 1 to 11, wherein the biological sample is urine, cerebrospinal fluid or cell culture supernatant.

13. The method of any one of claims 1 to 12, wherein step (a) further comprises processing the biological sample by filtering the biological sample.

14. The method of claim 13, wherein the filtration is performed using a 0.8 μm filter.

15. The method of any one of claims 1 to 14, wherein step (b) further comprises a centrifugation step after contacting the biological sample with the capture surface.

16. The method of any one of claims 1 to 15, wherein step (b) further comprises washing the capture surface after contacting the biological sample with the capture surface.

17. The method of any one of claims 1 to 16, wherein step (c) further comprises a centrifugation step after contacting the capture surface with the phenol-based lysis reagent.

18. The method of any one of claims 1 to 17, wherein step (d) further comprises adding a control to the homogenate prior to adding chloroform to the homogenate.

19. The method of any one of claims 1 to 18, wherein the method further comprises step (f) ethanol conditioning of the extraction from step (e); step (g) binding of the ethanol-conditioned extraction to a silica column; and step (h) eluting the extraction from the silica column.