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Methods and apparatus for filtration

Abstract

The invention features methods and apparatus for concentrating a nucleic acid. Methods of the invention include providing an initial suspension of a nucleic acid and an initial liquid, contacting the initial suspension with a housing having a filter that does not pass the nucleic acid, pressurizing the housing to produce a filtrate and a nucleic acid retentate from the initial solution, and detecting the volume of the nucleic acid retentate. Apparatus of the invention include a chamber configured to hold a filter housing containing a nucleic acid suspension, a pressure source to filter the suspension, and a detector to depressurize the housing upon detecting the volume reaching a predetermined threshold. The methods and apparatus described herein are useful in filtering, concentrating, and reconstituting nucleic acids, such as mRNA, in processes such as complete buffer replacement.

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Background/Summary

BACKGROUND OF THE INVENTION

(1) Nucleic acids, such as ribonucleic acid (RNA), are being explored as therapeutic and diagnostic agents. As a result, efficient methods for processing nucleic acids are of particular interest.

(2) Dialysis, diafiltration, and ultrafiltration can be used for processing proteins and nucleic acids. Dialysis is used for clean-up of nucleic acids for removal of low molecular weight contaminants, such as salts. However, conventional dialysis methods are performed in batches, require large amount of samples, take several hours, and incur significant sample loss. When only a small amount of sample is available, sample loss can become even more pronounced. As an alternative, ultrafiltration is based on forced separation of molecules according to size using a semipermeable membrane of a defined range of pore sizes. Ultrafiltration can be used in protein and nucleic acid purification for concentrating protein and nucleic acid molecules, changing the composition of a buffer solution, and removing low-molecular-weight solutes from these sample solutions. This technique is routinely applied in small laboratory experiments through the use of centrifugal filtration. However, centrifuge-based methods of ultrafiltration are labor intensive and time-consuming.

(3) Thus, there remains a need for fully automated and time efficient methods and apparatus for removing contaminants from nucleic acid suspensions.

SUMMARY OF INVENTION

(4) The present invention provides methods and apparatus for filtering and concentrating preparations of nucleic acid (e.g., RNA, e.g., mRNA).

(5) In one aspect, the present invention provides methods of concentrating a nucleic acid by providing an initial suspension of the nucleic acid and an initial liquid; contacting the initial suspension with a housing having a filter that does not pass the nucleic acid; pressurizing the housing to produce a filtrate and a nucleic acid retentate from the initial suspension; and detecting the volume of the nucleic acid retentate. When the volume of retentate reaches a predetermined threshold, the housing depressurizes and stops the process.

(6) In some embodiments, the method further includes injecting an additional liquid into the housing and repeating the pressurizing and detecting steps. The method includes increasing the pressure of the housing by injecting a gas (e.g., air, argon, N.sub.2, or CO.sub.2) into the housing. The gas can be injected, e.g., through a sealed cap. In some embodiments, the pressure of the gas within the filter housing is between about 2 bar and about 10 bar (e.g., between 2 bar and 10 bar, between 3 bar and 10 bar, between 4 bar and 9 bar, between 5 bar and 9 bar, between 6 bar and 8 bar, about 2 bar, about 3 bar, about 4 bar, about 5 bar, about 6 bar, about 7 bar, about 8 bar, about 9 bar, or about 10 bar).

(7) The method may further include detecting the liquid level of the nucleic acid retentate within the housing. Detecting can be by capacitive sensing (e.g., using a capacitive liquid level detector).

(8) In other embodiments, the additional liquid is different from the initial liquid. In some embodiments, the additional liquid is at least 9-fold (e.g., at least 9-fold, at least 10-fold, at least 11-fold, at least 12-fold, at least 13-fold, at least 14-fold, at least 15-fold, at least 16-fold, at least 17-fold, at least 18-fold, at least 19-fold, at least 20-fold, or greater) greater than the volume of the predetermined threshold. In other embodiments, the additional liquid has a volume about equal to the difference between that of the initial suspension and the predetermined threshold. The additional liquid can be a citrate buffer (e.g., sodium citrate, such as 2 mM sodium citrate).

(9) In some embodiments, the nucleic acid retentate has about a 10-fold greater, 11-fold greater, 12-fold greater, 13-fold greater, 14-fold greater, or 15-fold greater concentration of nucleic acid than the initial suspension.

(10) In some embodiments, the method achieves complete buffer replacement.

(11) The method may include a filter that has a molecular weight cutoff (MWCO) of at least 10,000 daltons (e.g., at least 10,000 daltons, at least 20,000 daltons, at least 30,000 daltons, at least 40,000 daltons, at least 50,000 daltons, at least 60,000 daltons, at least 70,000 daltons, at least 80,000 daltons, at least 90,000 daltons, at least 100,000 daltons, or greater).

(12) In some embodiments, the nucleic acid is ribonucleic acid (RNA, e.g., messenger RNA (mRNA)). The nucleic acid can be encapsulated, e.g., in lipid nanoparticles.

(13) In another aspect, the invention features an apparatus for concentrating nucleic acid including a chamber configured to hold a housing having a filter to hold an initial suspension of nucleic acid in contact with the filter; a pressure source configured to releasably engage and pressurize the housing to force liquid in the initial suspension through the filter to produce a filtrate and a nucleic acid retentate; and a detector configured to detect the volume of the nucleic acid retentate. The detector can be configured to depressurize the housing upon detecting the volume of the retentate reach a predetermined threshold.

(14) In some embodiments, the chamber is configured to receive the housing provided by a user and position the housing for engagement with the pressure source.

(15) In some embodiments, the pressure source is configured to inject a gas (e.g., air, argon, N.sub.2, or CO.sub.2) into the housing.

(16) The detector can be a liquid level detector (e.g., a capacitive liquid level detector).

(17) The apparatus may further include a liquid injector configured to inject an additional liquid (e.g., the same liquid or a different liquid from the initial liquid) into the housing. The liquid

injector may be in communication with the liquid level detector.
(18) In some embodiments, the liquid injector can be configured to inject the additional liquid into the housing upon detecting the volume of a nucleic acid retentate drop below a predetermined threshold.
(19) The invention also provides a filtered nucleic acid suspension produced by any of the preceding methods.

Description

BRIEF DESCRIPTION OF THE DRAWINGS
(1) FIG. **1** is a diagram of an apparatus including a chamber holding a housing including a filter, a pressure source, a liquid level detector, and a liquid injector.
DEFINITIONS
(2) As used herein, “pressure source” refers to an element that facilitates pressure transfer into a housing of the apparatus. A pressure source can be a line or valve that connects the housing to, e.g., an external compressed gas tank.
(3) As used herein, “depressurize,” “depressurizes,” and “depressurization” refer to the change in relative pressure of the housing versus the pressure in the space separated from the housing by the filter.
(4) The term “purified” when used in relation to a nucleic acid, refers to one that is separated from at least one contaminant or component.
(5) As used herein, a “contaminant” is any substance which makes another unfit, impure, or inferior. Thus, a purified nucleic acid (e.g., DNA and RNA) is present in a form or setting different from that in which it is found in nature, or a form or setting different from that which existed prior to subjecting it to a treatment or purification method.
(6) As used herein, the term “complete buffer replacement” refers to the process of exchanging at least 99% (by volume) of an initial buffer solution with a new buffer solution.
(7) As used herein, the term “concentrating” refers to a process of decreasing the volume of the solution occupied by a substance without substantially decreasing the quantity (e.g., number of molecules or mass) of the substance. The volume of a solution is decreased, for example, by removing a liquid portion of the solution and/or by removing contaminates from (e.g., sanitizing or sterilizing) the solution.
DETAILED DESCRIPTION
(8) The invention relates to methods and apparatus for filtering or concentrating a sample including a nucleic acid and nucleic acid suspensions filtered using the same. As an alternative to conventional dialysis, ultrafiltration provides greater efficiency in buffer exchange by enhancing filtration by force, e.g., by centrifugation, such that a retentate is isolated from the liquid and smaller soluble contaminates. The present invention provides an alternative method of ultrafiltration and/or diafiltration by providing a pressure gradient, produced other than by centrifugation, across the filter membrane. This pressure gradient drives the filtrate away from the nucleic acids retained in the pressurized housing by the filter membrane. The invention provides for automated cycles of pressurization and resuspension of the retentate, preventing the filter from drying out, which maintains the molecular integrity of the nucleic acid. Apparatus useful for performing these methods, in addition to filtered nucleic acid formulations, are also provided.
(9) Methods
(10) The present invention provides methods for concentrating a nucleic acid. The method includes providing an initial suspension including the nucleic acid and an initial liquid; contacting the initial suspension with a housing having a filter that does not pass the nucleic acid; pressurizing the housing to produce a filtrate and a nucleic acid retentate; and detecting the volume of the nucleic

acid retentate. When the volume of the retentate reaches a predetermined threshold, the housing depressurizes, thereby concentrating the nucleic acid. Each component of this method is described in detail below.

(11) Initial Suspension

(12) The initial suspension can be a crude suspension of nucleic acid in a sample (e.g., having an initial liquid containing impurities from, e.g., a biological sample) or a substantially pure suspension of nucleic acid, e.g., previously purified by affinity chromatography, wherein, e.g., the suspension is to be resuspended in a different buffer, e.g., sodium citrate. The term suspension includes liquids wherein the nucleic acid is formally suspended, dissolved, or a combination thereof. The liquid of the initial suspension is herein referred to as the initial liquid.

(13) The initial suspension may include (e.g., as part of the initial liquid) tris(hydroxymethyl)aminomethane (Tris), e.g., at a concentration of about 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM, 0.7 mM, 0.8 mM, 0.9 mM, 1.0 mM, 1.1 mM, 1.2 mM, 1.3 mM, 1.4 mM, 1.5 mM, 1.6 mM, 1.7 mM, 1.8 mM, 1.9 mM, 2.0 mM, 2.1 mM, 2.2 mM, 2.3 mM, 2.4 mM, 2.5 mM, 2.6 mM, 2.7 mM, 2.8 mM, 2.9 mM, 3.0 mM, 3.5 mM, 4.5 mM, 5.0 mM, 5.5 mM, 6.0 mM, 6.5 mM, 7.0 mM, 7.5 mM, 8.0 mM, 8.5 mM, 9.0 mM, 9.5 mM, 10 mM, 15 mM, 20 mM, or greater.

(14) Additionally or alternatively, the initial suspension may also include a chelating agent. Exemplary chelating agents include ethylenediaminetetraacetic acid (EDTA), citric acid monohydrate, disodium edetate, dipotassium edetate, edetic acid, fumaric acid, malic acid, phosphoric acid, sodium edetate, tartaric acid, and/or trisodium edetate. In some embodiments, the initial suspension includes a chelating agent (e.g., EDTA) at a concentration of about 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM, 0.7 mM, 0.8 mM, 0.9 mM, 1.0 mM, 1.1 mM, 1.2 mM, 1.3 mM, 1.4 mM, 1.5 mM, 1.6 mM, 1.7 mM, 1.8 mM, 1.9 mM, 2.0 mM, 2.1 mM, 2.2 mM, 2.3 mM, 2.4 mM, 2.5 mM, 2.6 mM, 2.7 mM, 2.8 mM, 2.9 mM, 3.0 mM, 3.5 mM, 4.5 mM, 5.0 mM, 5.5 mM, 6.0 mM, 6.5 mM, 7.0 mM, 7.5 mM, 8.0 mM, 8.5 mM, 9.0 mM, 9.5 mM, 10 mM, 15 mM, 20 mM, or greater.

(15) Exemplary buffering agents that may be included as part of the initial liquid include, but are not limited to, citrate buffer solutions, acetate buffer solutions, phosphate buffer solutions, ammonium chloride, calcium carbonate, calcium chloride, calcium citrate, calcium glubionate, calcium gluceptate, calcium gluconate, d-gluconic acid, calcium glycerophosphate, calcium lactate, propanoic acid, calcium levulinate, pentanoic acid, dibasic calcium phosphate, phosphoric acid, tribasic calcium phosphate, calcium hydroxide phosphate, potassium acetate, potassium chloride, potassium gluconate, potassium mixtures, dibasic potassium phosphate, monobasic potassium phosphate, potassium phosphate mixtures, sodium acetate, sodium bicarbonate, sodium chloride, sodium citrate, sodium lactate, dibasic sodium phosphate, monobasic sodium phosphate, sodium phosphate mixtures, tromethamine, magnesium hydroxide, aluminum hydroxide, alginic acid, pyrogen-free water, isotonic saline, and/or combinations thereof.

(16) The initial suspension may include one or more salts (e.g., a sodium salt, a potassium salt, a magnesium salt, a lithium salt, a calcium salt, a manganese salt, a cesium salt, an ammonium salt, or an alkylammonium salt, e.g., NaCl, KCl, MgCl.sub.2, Ca.sup.2+, MnCl.sub.2, and/or LiCl).

(17) In some embodiments, the buffering agent and/or salt may be at a concentration of about 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM, 0.7 mM, 0.8 mM, 0.9 mM, 1.0 mM, 1.1 mM, 1.2 mM, 1.3 mM, 1.4 mM, 1.5 mM, 1.6 mM, 1.7 mM, 1.8 mM, 1.9 mM, 2.0 mM, 2.1 mM, 2.2 mM, 2.3 mM, 2.4 mM, 2.5 mM, 2.6 mM, 2.7 mM, 2.8 mM, 2.9 mM, 3.0 mM, 3.5 mM, 4.5 mM, 5.0 mM, 5.5 mM, 6.0 mM, 6.5 mM, 7.0 mM, 7.5 mM, 8.0 mM, 8.5 mM, 9.0 mM, 9.5 mM, 10 mM, 15 mM, 20 mM, or greater.

(18) In some embodiments, the nucleic acid of the initial suspension is at a concentration from about 0.01 mg/ml to about 5 mg/ml (e.g., about 0.01 mg/ml, about 0.05 mg/ml, about 0.1 mg/ml, about 0.2 mg/ml, about 0.3 mg/ml, about 0.4 mg/ml, about 0.5 mg/ml, about 0.6 mg/ml, about 0.7

mg/ml, about 0.8 mg/ml, about 0.9 mg/ml, about 1.0 mg/ml, about 1.1 mg/ml, about 1.2 mg/ml, about 1.3 mg/ml, about 1.4 mg/ml, about 1.5 mg/ml, about 1.6 mg/ml, about 1.7 mg/ml, about 1.8 mg/ml, about 1.9 mg/ml, about 2.0 mg/ml, about 2.5 mg/ml, about 3.0 mg/ml, about 3.5 mg/ml, about 4.0 mg/ml, or about 5.0 mg/ml).

(19) The initial suspension and/or the initial liquid may have a pH of from about 3.0 to about 8.0 (e.g., of about 3.0, about 3.1, about 3.2, about 3.3, about 3.4, about 3.5, about 3.6, about 3.7, about 3.8, about 3.9, about 4.0, about 4.1, about 4.2, about 4.3, about 4.4, about 4.5, about 4.6, about 4.7, about 4.8, about 4.9, about 5.0, about 5.1, about 5.2, about 5.3, about 5.4, about 5.5, about 5.6, about 5.7, about 5.8, about 5.9, about 6.0, about 6.1, about 6.2, about 6.3, about 6.4, about 6.5, about 6.6, about 6.7, about 6.8, about 6.9, about 7.0, about 7.1, about 7.2, about 7.3, about 7.4, about 7.5, about 7.6, about 7.7, about 7.9, or about 8.0).

(20) Particular examples of the initial liquid are 10 mM Tris, 1 mM EDTA, 0.5 M NaCl, 10 mM Tris, 1 mM EDTA, and 0.1 M NaCl.

(21) Nucleic Acids

(22) The initial suspension includes a nucleic acid (e.g., a homogeneous or heterogeneous suspension of nucleic acid, e.g., having the same or a variety of nucleic acid molecules).

(23) In addition to nucleic acid, samples to be purified using the methods and apparatus described herein may include, for example, a surfactant (e.g., sodium dodecyl sulfate), a buffer (e.g., a sodium acetate buffer), a chelating agent (e.g., EDTA), a solvent (e.g., chloroform, ethanol, and phenol), other types of RNA (e.g., ribosomal RNA (rRNA) and transfer RNA (tRNA)), adenosine triphosphate (ATP), an enzyme (e.g., *E. coli* Poly(A) Polymerase), or any other component. In some embodiments, the sample may include components relevant to in vitro transcription reactions. In some embodiments, the sample may include components relevant to polyadenylation reactions. In some embodiments, the sample may include other types of RNA (e.g., rRNA and tRNA), e.g., of an in-vitro transcription (IVT) process.

(24) Additionally or alternatively, nucleic acid used in the methods described herein includes nanoparticle-encapsulated nucleic acid (e.g., RNA, or mRNA encapsulated in nanoparticles (e.g., lipid nanoparticles or polymeric nanoparticles)). Nanoparticle encapsulated nucleic acid is known in the art and described, e.g., in US Publication Nos. 20130244278 and 20130244279, and in International Patent Publication No. WO 2000006120A1.

(25) Herein, in a nucleotide, nucleoside, or polynucleotide (such as the polynucleotides of the invention, e.g., mRNA molecule), the terms “alteration” or, as appropriate, “alternative” refer to alteration with respect to A, G, U or C ribonucleotides. Generally, herein, these terms are not intended to refer to the ribonucleotide alterations in naturally occurring 5′-terminal mRNA cap moieties.

(26) The alterations may be various distinct alterations. In some embodiments, where the polynucleotide is an mRNA, the coding region, the flanking regions and/or the terminal regions (e.g., a 3′-stabilizing region) may contain one, two, or more (optionally different) nucleoside or nucleotide alterations. In some embodiments, an alternative polynucleotide introduced to a cell may exhibit reduced degradation in the cell, as compared to an unaltered polynucleotide.

(27) The polynucleotides of the invention can include any useful alteration, such as to the nucleobase, the sugar, or the internucleoside linkage (e.g., to a linking phosphate/to a phosphodiester linkage/to the phosphodiester backbone). In certain embodiments, alterations (e.g., one or more alterations) are present in each of the nucleobase, the sugar, and the internucleoside linkage. Alterations according to the present invention may be alterations of ribonucleic acids (RNAs) to deoxyribonucleic acids (DNAs), e.g., the substitution of the 2′-OH of the ribofuranosyl ring to 2′-H, threose nucleic acids (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs), or hybrids thereof. Additional alterations are described herein.

(28) As described herein, in some embodiments, the polynucleotides of the invention do not

substantially induce an innate immune response of a cell into which the polynucleotide (e.g., mRNA) is introduced. Features of an induced innate immune response include 1) increased expression of pro-inflammatory cytokines, 2) activation of intracellular PRRs (RIG-I, MDA5, etc, and/or 3) termination or reduction in protein translation.

(29) The polynucleotides can optionally include other agents (e.g., RNAi-inducing agents, RNAi agents, siRNAs, shRNAs, miRNAs, antisense RNAs, ribozymes, catalytic DNA, tRNA, RNAs that induce triple helix formation, aptamers, and vectors). In some embodiments, the polynucleotides may include one or more messenger RNAs (mRNAs) having one or more alternative nucleoside or nucleotides (i.e., alternative mRNA molecules). Details for these polynucleotides follow.

(30) Nucleobase Alternatives

(31) The alternative nucleosides and nucleotides can include an alternative nucleobase. Examples of nucleobases found in RNA include, but are not limited to, adenine, guanine, cytosine, and uracil. Examples of nucleobases found in DNA include, but are not limited to, adenine, guanine, cytosine, and thymine. These nucleobases can be altered or wholly replaced to provide polynucleotide molecules having enhanced properties, e.g., increased stability such as resistance to nucleases.

(32) Alternative nucleotide base pairing encompasses not only the standard adenine-thymine, adenine-uracil, or guanine-cytosine base pairs, but also base pairs formed between nucleotides and/or alternative nucleotides including non-standard or alternative bases, wherein the arrangement of hydrogen bond donors and hydrogen bond acceptors permits hydrogen bonding between a non-standard base and a standard base or between two complementary non-standard base structures. One example of such non-standard base pairing is the base pairing between the alternative nucleotide inosine and adenine, cytosine, or uracil.

(33) In some embodiments, the nucleobase is an alternative uracil. Exemplary nucleobases and nucleosides having an alternative uracil include pseudouridine (ψ), pyridin-4-one ribonucleoside, 5-aza-uracil, 6-aza-uracil, 2-thio-5-aza-uracil, 2-thio-uracil (s.sup.2U), 4-thio-uracil (s.sup.4U), 4-thio-pseudouridine, 2-thio-pseudouridine, 5-hydroxy-uracil (ho.sup.5U), 5-aminoallyl-uracil, 5-halo-uracil (e.g., 5-iodo-uracil or 5-bromo-uracil), 3-methyl-uracil (m.sup.3U), 5-methoxy-uracil (mo.sup.5U), uracil 5-oxyacetic acid (cmo.sup.5U), uracil 5-oxyacetic acid methyl ester (mcmo.sup.5U), 5-carboxymethyl-uracil (cm.sup.5U), 1-carboxymethyl-pseudouridine, 5-carboxyhydroxymethyl-uracil (chm.sup.5U), 5-carboxyhydroxymethyl-uracil methyl ester (mchm.sup.5U), 5-methoxycarbonylmethyl-uracil (mcm.sup.5U), 5-methoxycarbonylmethyl-2-thio-uracil (mcm.sup.5s.sup.2U), 5-aminomethyl-2-thio-uracil (nm.sup.5s.sup.2U), 5-methylaminomethyl-uracil (mnm.sup.5U), 5-methylaminomethyl-2-thio-uracil (mnm.sup.5s.sup.2U), 5-methylaminomethyl-2-seleno-uracil (mnm.sup.5se.sup.2U), 5-carbamoylmethyl-uracil (ncm.sup.5U), 5-carboxymethylaminomethyl-uracil (cmnm.sup.5U), 5-carboxymethylaminomethyl-2-thio-uracil (cmnm.sup.5s.sup.2U), 5-propynyl-uracil, 1-propynyl-pseudouracil, 5-taurinomethyl-uracil (τm.sup.5U), 1-taurinomethyl-pseudouridine, 5-taurinomethyl-2-thio-uracil (τm.sup.5s.sup.2U), 1-taurinomethyl-4-thio-pseudouridine, 5-methyl-uracil (m.sup.5U, i.e., having the nucleobase deoxythymine), 1-methyl-pseudouridine (m.sup.1ψ), 5-methyl-2-thio-uracil (m.sup.5s.sup.2U), 1-methyl-4-thio-pseudouridine (m.sup.1s.sup.4ψ), 4-thio-1-methyl-pseudouridine, 3-methyl-pseudouridine (m.sup.3ψ), 2-thio-1-methyl-pseudouridine, 1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-1-deaza-pseudouridine, dihydrouracil (D), dihydropseudouridine, 5,6-dihydrouracil, 5-methyl-dihydrouracil (m.sup.5D), 2-thio-dihydrouracil, 2-thio-dihydropseudouridine, 2-methoxy-uracil, 2-methoxy-4-thio-uracil, 4-methoxy-pseudouridine, 4-methoxy-2-thio-pseudouridine, N1-methyl-pseudouridine, 3-(3-amino-3-carboxypropyl)uracil (acp.sup.3U), 1-methyl-3-(3-amino-3-carboxypropyl)pseudouridine (acp.sup.3 ψ), 5-(isopentenylaminomethyl)uracil (inm.sup.5U), 5-(isopentenylaminomethyl)-2-thio-uracil (inm.sup.5s.sup.2U), 5,2′-O-dimethyl-uridine (m.sup.5Um), 2-thio-2′-O-methyl-uridine (s.sup.2Um), 5-methoxycarbonylmethyl-2′-O-methyl-uridine (mcm.sup.5Um), 5-carbamoylmethyl-2′-O-methyl-uridine (ncm.sup.5Um), 5-carboxymethylaminomethyl-2′-O-methyl-uridine

(cmnm.sup.5Um), 3,2′-O-dimethyl-uridine (m.sup.3Um), and 5-(isopentenylaminomethyl)-2′-O-methyl-uridine (inm.sup.5Um), 1-thio-uracil, deoxythymidine, 5-(2-carbomethoxyvinyl)-uracil, 5-(carbamoylhydroxymethyl)-uracil, 5-carbamoylmethyl-2-thio-uracil, 5-carboxymethyl-2-thio-uracil, 5-cyanomethyl-uracil, 5-methoxy-2-thio-uracil, and 5-[3-(1-E-propenylamino)]uracil.

(34) In some embodiments, the nucleobase is an alternative cytosine. Exemplary nucleobases and nucleosides having an alternative cytosine include 5-aza-cytosine, 6-aza-cytosine, pseudoisocytidine, 3-methyl-cytosine (m3C), N.sub.4-acetyl-cytosine (ac4C), 5-formyl-cytosine (f5C), N.sub.4-methyl-cytosine (m4C), 5-methyl-cytosine (m5C), 5-halo-cytosine (e.g., 5-iodo-cytosine), 5-hydroxymethyl-cytosine (hm5C), 1-methyl-pseudoisocytidine, pyrrolo-cytosine, pyrrolo-pseudoisocytidine, 2-thio-cytosine (s2C), 2-thio-5-methyl-cytosine, 4-thio-pseudoisocytidine, 4-thio-1-methyl-pseudoisocytidine, 4-thio-1-methyl-1-deaza-pseudoisocytidine, 1-methyl-1-deaza-pseudoisocytidine, zebularine, 5-aza-zebularine, 5-methyl-zebularine, 5-aza-2-thio-zebularine, 2-thio-zebularine, 2-methoxy-cytosine, 2-methoxy-5-methyl-cytosine, 4-methoxy-pseudoisocytidine, 4-methoxy-1-methyl-pseudoisocytidine, lysidine (k2C), 5,2′-O-dimethyl-cytidine (m5Cm), N4-acetyl-2′-O-methyl-cytidine (ac4Cm), N4,2′-O-dimethyl-cytidine (m4Cm), 5-formyl-2′-O-methyl-cytidine (f5Cm), N4,N4,2′-O-trimethyl-cytidine (m42Cm), 1-thio-cytosine, 5-hydroxy-cytosine, 5-(3-azidopropyl)-cytosine, and 5-(2-azidoethyl)-cytosine.

(35) In some embodiments, the nucleobase is an alternative adenine. Exemplary nucleobases and nucleosides having an alternative adenine include 2-amino-purine, 2,6-diaminopurine, 2-amino-6-halo-purine (e.g., 2-amino-6-chloro-purine), 6-halo-purine (e.g., 6-chloro-purine), 2-amino-6-methyl-purine, 8-azido-adenine, 7-deaza-adenine, 7-deaza-8-aza-adenine, 7-deaza-2-amino-purine, 7-deaza-8-aza-2-amino-purine, 7-deaza-2,6-diaminopurine, 7-deaza-8-aza-2,6-diaminopurine, 1-methyl-adenine (m1A), 2-methyl-adenine (m2A), N6-methyl-adenine (m6A), 2-methylthio-N6-methyl-adenine (ms2m6A), N6-isopentenyl-adenine (i6A), 2-methylthio-N6-isopentenyl-adenine (ms2i6A), N6-(cis-hydroxyisopentenyl)adenine (io6A), 2-methylthio-N6-(cis-hydroxyisopentenyl)adenine (ms2io6A), N6-glycinylcarbamoyl-adenine (g6A), N6-threonylcarbamoyl-adenine (t6A), N6-methyl-N6-threonylcarbamoyl-adenine (m6t6A), 2-methylthio-N6-threonylcarbamoyl-adenine (ms2g6A), N6,N6-dimethyl-adenine (m62A), N6-hydroxynorvalylcarbamoyl-adenine (hn6A), 2-methylthio-N6-hydroxynorvalylcarbamoyl-adenine (ms2hn6A), N6-acetyl-adenine (ac6A), 7-methyl-adenine, 2-methylthio-adenine, 2-methoxy-adenine, N6,2′-O-dimethyl-adenosine (m6Am), N6,N6,2′-O-trimethyl-adenosine (m62Am), 1,2′-O-dimethyl-adenosine (m1Am), 2-amino-N6-methyl-purine, 1-thio-adenine, 8-azido-adenine, N6-(19-amino-pentaoxanonadecyl)-adenine, 2,8-dimethyl-adenine, N6-formyl-adenine, and N6-hydroxymethyl-adenine.

(36) In some embodiments, the nucleobase is an alternative guanine. Exemplary nucleobases and nucleosides having an alternative guanine include inosine (I), 1-methyl-inosine (m1I), wyosine (imG), methylwyosine (mimG), 4-demethyl-wyosine (imG-14), isowyosine (imG2), wybutosine (yW), peroxywybutosine (o2yW), hydroxywybutosine (OHyW), undermodified hydroxywybutosine (OHyW*), 7-deaza-guanine, queuosine (Q), epoxyqueuosine (oQ), galactosyl-queuosine (galQ), mannosyl-queuosine (manQ), 7-cyano-7-deaza-guanine (preQ0), 7-aminomethyl-7-deaza-guanine (preQ1), archaeosine (G+), 7-deaza-8-aza-guanine, 6-thio-guanine, 6-thio-7-deaza-guanine, 6-thio-7-deaza-8-aza-guanine, 7-methyl-guanine (m7G), 6-thio-7-methyl-guanine, 7-methyl-inosine, 6-methoxy-guanine, 1-methyl-guanine (m1G), N2-methyl-guanine (m2G), N2,N2-dimethyl-guanine (m22G), N2,7-dimethyl-guanine (m2,7G), N2, N2,7-dimethyl-guanine (m2,2,7G), 8-oxo-guanine, 7-methyl-8-oxo-guanine, 1-methyl-6-thio-guanine, N2-methyl-6-thio-guanine, N2,N2-dimethyl-6-thio-guanine, N2-methyl-2′-O-methyl-guanosine (m2Gm), N2,N2-dimethyl-2′-O-methyl-guanosine (m22Gm), 1-methyl-2′-O-methyl-guanosine (m1Gm), N2,7-dimethyl-2′-O-methyl-guanosine (m2,7Gm), 2′-O-methyl-inosine (Im), 1,2′-O-dimethyl-inosine (m1Im), 1-thio-guanine, and O-6-methyl-guanine.

(37) The alternative nucleobase of a nucleotide can be independently a purine, a pyrimidine, a

purine or pyrimidine analog. For example, the nucleobase can be an alternative to adenine, cytosine, guanine, uracil, or hypoxanthine. In another embodiment, the nucleobase can also include, for example, naturally-occurring and synthetic derivatives of a base, including pyrazolo[3,4-d]pyrimidines, 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo (e.g., 8-bromo), 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxy and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, deazaguanine, 7-deazaguanine, 3-deazaguanine, deazaadenine, 7-deazaadenine, 3-deazaadenine, pyrazolo[3,4-d]pyrimidine, imidazo[1,5-a]1,3,5 triazinones, 9-deazapurines, imidazo[4,5-d]pyrazines, thiazolo[4,5-d]pyrimidines, pyrazin-2-ones, 1,2,4-triazine, pyridazine; or 1,3,5 triazine. When the nucleotides are depicted using the shorthand A, G, C, T or U, each letter refers to the representative base and/or derivatives thereof, e.g., A includes adenine or adenine analogs, e.g., 7-deaza adenine).

(38) Alterations on the Sugar

(39) The alternative nucleosides and nucleotides, which may be incorporated into a polynucleotide of the invention (e.g., RNA or mRNA, as described herein), can be altered on the sugar of the nucleoside or nucleotide. In some embodiments, the alternative nucleosides or nucleotides include the structure:

(40) ##STR00001## wherein B.sup.1 is a nucleobase; each U and U′ is, independently, O, S, N(R.sup.U).sub.nu, or C(R.sup.U).sub.nu, wherein nu is 1 or 2 (e.g., 1 for N(R.sup.U).sub.nu and 2 for C(R.sup.U).sub.nu) and each R.sup.U is, independently, H, halo, or optionally substituted C.sub.1-C.sub.6 alkyl;

(41) each of R.sup.1, R.sup.1′, R.sup.1″, R.sup.2, R.sup.2′, R.sup.2″, R.sup.3, R.sup.4, and R.sup.5 is, independently, H, halo, hydroxy, thiol, optionally substituted C.sub.1-C.sub.6 alkyl, optionally substituted C.sub.2-C.sub.6 alkynyl, optionally substituted C.sub.1-C.sub.6 heteroalkyl, optionally substituted C.sub.2-C.sub.6 heteroalkenyl, optionally substituted C.sub.2-C.sub.6 heteroalkynyl, optionally substituted amino, azido, optionally substituted C.sub.6-C.sub.10 aryl; or R.sup.3 and/or R.sup.5 can join together with one of R.sup.1, R.sup.1′, R.sup.1″, R.sup.2, R.sup.2′, or R.sup.2″ to form together with the carbons to which they are attached an optionally substituted C.sub.3-C.sub.10 carbocycle or an optionally substituted C.sub.3-C.sub.9 heterocyclyl; each of m and n is independently, 0, 1, 2, 3, 4, or 5; each of Y.sup.1, Y.sup.2, and Y.sup.3, is, independently, O, S, Se, —NR.sup.N1—, optionally substituted C.sub.1-C.sub.6 alkylene, or optionally substituted C.sub.1-C.sub.6 heteroalkylene, wherein RN′ is H, optionally substituted C.sub.1-C.sub.6 alkyl, optionally substituted C.sub.2-C.sub.6 alkenyl, optionally substituted C.sub.2-C.sub.6 alkynyl, or optionally substituted C.sub.6-C.sub.10 aryl; and each Y.sup.4 is, independently, H, hydroxy, protected hydroxy, halo, thiol, boranyl, optionally substituted C.sub.1-C.sub.6 alkyl, optionally substituted C.sub.2-C.sub.6 alkenyl, optionally substituted C.sub.2-C.sub.6 alkynyl, optionally substituted C.sub.1-C.sub.6 heteroalkyl, optionally substituted C.sub.2-C.sub.6 heteroalkenyl, optionally substituted C.sub.2-C.sub.6 heteroalkynyl, or optionally substituted amino; and Y.sup.5 is O, S, Se, optionally substituted C.sub.1-C.sub.6 alkylene, or optionally substituted C.sub.1-C.sub.6 heteroalkylene; or a salt thereof.

(42) In some embodiments, the 2′-hydroxy group (OH) can be modified or replaced with a number of different substituents. Exemplary substitutions at the 2′-position include, but are not limited to, H, azido, halo (e.g., fluoro), optionally substituted C.sub.1-6 alkyl (e.g., methyl); optionally substituted C.sub.1-6 alkoxy (e.g., methoxy or ethoxy); optionally substituted C.sub.6-10 aryloxy; optionally substituted C.sub.3-8 cycloalkyl; optionally substituted C.sub.6-10 aryl-C.sub.1-6 alkoxy, optionally substituted C.sub.1-12 (heterocyclyl)oxy; a sugar (e.g., ribose, pentose, or any

described herein); a polyethyleneglycol (PEG), —O(CH.sub.2CH.sub.2O).sub.nCH.sub.2CH.sub.2OR, where R is H or optionally substituted alkyl, and n is an integer from 0 to 20 (e.g., from 0 to 4, from 0 to 8, from 0 to 10, from 0 to 16, from 1 to 4, from 1 to 8, from 1 to 10, from 1 to 16, from 1 to 20, from 2 to 4, from 2 to 8, from 2 to 10, from 2 to 16, from 2 to 20, from 4 to 8, from 4 to 10, from 4 to 16, and from 4 to 20); "locked" nucleic acids (LNA) in which the 2′-hydroxy is connected by a C.sub.1-6 alkylene or C.sub.1-6 heteroalkylene bridge to the 4′-carbon of the same ribose sugar, where exemplary bridges included methylene, propylene, ether, or amino bridges; aminoalkyl, as defined herein; aminoalkoxy, as defined herein; amino as defined herein; and amino acid, as defined herein.

(43) Generally, RNA includes the sugar group ribose, which is a 5-membered ring having an oxygen. Exemplary, non-limiting alternative nucleotides include replacement of the oxygen in ribose (e.g., with S, Se, or alkylene, such as methylene or ethylene); addition of a double bond (e.g., to replace ribose with cyclopentenyl or cyclohexenyl); ring contraction of ribose (e.g., to form a 4-membered ring of cyclobutane or oxetane); ring expansion of ribose (e.g., to form a 6- or 7-membered ring having an additional carbon or heteroatom, such as for anhydrohexitol, altritol, mannitol, cyclohexanyl, cyclohexenyl, and morpholino (that also has a phosphoramidate backbone)); multicyclic forms (e.g., tricyclo and "unlocked" forms, such as glycol nucleic acid (GNA) (e.g., R-GNA or S-GNA, where ribose is replaced by glycol units attached to phosphodiester bonds), threose nucleic acid (TNA, where ribose is replace with α-L-threofuranosyl-(3′.fwdarw.2′)), and peptide nucleic acid (PNA, where 2-amino-ethyl-glycine linkages replace the ribose and phosphodiester backbone).

(44) In some embodiments, the sugar group contains one or more carbons that possess the opposite stereochemical configuration of the corresponding carbon in ribose. Thus, a polynucleotide molecule can include nucleotides containing, e.g., arabinose or L-ribose, as the sugar.

(45) In some embodiments, the polynucleotide of the invention includes at least one nucleoside wherein the sugar is L-ribose, 2′-O-methyl-ribose, 2′-fluoro-ribose, arabinose, hexitol, an LNA, or a PNA.

(46) Alterations on the Internucleoside Linkage

(47) The alternative nucleotides, which may be incorporated into a polynucleotide of the invention, can be altered on the internucleoside linkage (e.g., phosphate backbone). Herein, in the context of the polynucleotide backbone, the phrases "phosphate" and "phosphodiester" are used interchangeably. Backbone phosphate groups can be altered by replacing one or more of the oxygen atoms with a different substituent.

(48) The alternative nucleotides can include the wholesale replacement of an unaltered phosphate moiety with another internucleoside linkage as described herein. Examples of alternative phosphate groups include, but are not limited to, phosphorothioate, phosphoroselenates, boranophosphates, boranophosphate esters, hydrogen phosphonates, phosphoramidates, phosphorodiamidates, alkyl or aryl phosphonates, and phosphotriesters. Phosphorodithioates have both non-linking oxygens replaced by sulfur. The phosphate linker can also be altered by the replacement of a linking oxygen with nitrogen (bridged phosphoramidates), sulfur (bridged phosphorothioates), and carbon (bridged methylene-phosphonates).

(49) The alternative nucleosides and nucleotides can include the replacement of one or more of the non-bridging oxygens with a borane moiety (BH.sub.3), sulfur (thio), methyl, ethyl, and/or methoxy. As a non-limiting example, two non-bridging oxygens at the same position (e.g., the alpha (α), beta (β) or gamma (γ) position) can be replaced with a sulfur (thio) and a methoxy.

(50) The replacement of one or more of the oxygen atoms at the a position of the phosphate moiety (e.g., α-thio phosphate) is provided to confer stability (such as against exonucleases and endonucleases) to RNA and DNA through the unnatural phosphorothioate backbone linkages. Phosphorothioate DNA and RNA have increased nuclease resistance and subsequently a longer half-life in a cellular environment.

(51) Other internucleoside linkages that may be employed according to the present invention, including internucleoside linkages which do not contain a phosphorous atom, are described herein.
(52) Filtration
(53) After preparation, the initial suspension is contacted with a filter, e.g., by transferring the initial suspension into a filter housing. A filter housing contains the nucleic acid throughout the duration of the filtration process (e.g., as part of the initial suspension and the retentate). The filter has an adequate pore size to prevent loss of nucleic acid from the housing. A housing may have any useful dimensions and geometry.
(54) In some embodiments, the filter housing is suitable to hold 0.1 to 200 ml, e.g., 0.1 to 5 ml, 10 to 30 ml, or 50 to 150 ml, or about 15 ml of solution. Alternatively, a filter housing can hold about 10 ml, about 20 ml, about 25 ml, about 30 ml, about 40 ml, about 50 ml, about 60 ml, about 70 ml, about 75 ml, about 80 ml, about 90 ml, about 100 ml of liquid, or more.
(55) Suitable housings include, but are not limited to, single-well containers having a horizontally oriented membrane (as to the direction of the filtration force) such as a CENTRICON® device available from Millipore Corporation of Billerica, Mass., single-well containers having a vertical or substantially vertically oriented membrane such as a ULTRAFREE device available from Millipore Corporation of Billerica, Mass., multiple membrane-containing single-well containers having a vertical or substantially vertically oriented membrane such as an AMICON® ULTRA™ device (e.g., Amicon 0.5, Amicon 2, Amicon 4, or Amicon 15) available from Millipore Corporation of Billerica, Mass, a VIVASPIN™ device (e.g., Vivaspin 20 or Vivaspin 100) available from GE Heathcare, or a multiwell plate such as a MULTISCREEN® plate or an ULTRACELL™ plate available from Millipore Corporation of Billerica, Mass.
(56) Filter membranes of the invention can be any material suitable for use with a nucleic acid suspension. Representative suitable ultrafiltration membranes include those formed from polyethersulfone, polysulfone, polyimide, polyvinylidenedifluoride (PVDF), cellulose acetate, or regenerated cellulose (e.g., low-binding regenerated cellulose) such as or Vira Pure™ cellulosic ultrafiltration membranes available from Millipore Corporation, Bedford, Mass., USA.
(57) The pore size of the filter is commonly defined by molecular weight cutoff (MWCO) and will depend on the size of the molecule to be filtered, molecular shape, electrical charge, sample concentration, sample composition, and operating conditions. Table 1 provides a general guide for selecting a MWCO for a filter of the invention according to the size of the nucleic acid.
(58) TABLE-US-00001 TABLE 1 Suitable MWCO filters according to nucleic acid size. Base Pairs Bases MWCO Double Strands (DS) Single Strands (SS) 1K 5-16 9-32 3K 16-32 32-65 5K 25-50 50-95 10K 50-145 95-285 30K 145-285 285-570 50K 240-475 475-950 100K 475-1,450 950-2,900 300K 1,450-2,900 2,900-5,700 1000K 4,800-9,500 >9,500
(59) In accordance with this invention, a nucleic acid suspension is filtered with a filtration membrane to selectively retain nucleic acid molecules while permitting passage of solvent and small molecule solutes (e.g., contaminants, e.g., buffering ions) therethrough. In some embodiments, the filter has a MWCO between about 10 and about 100 kilodaltons (kD) (e.g., at least 10,000 daltons (e.g., at least 10,000 daltons, at least 20,000 daltons, at least 30,000 daltons, at least 40,000 daltons, at least 50,000 daltons, at least 60,000 daltons, at least 70,000 daltons, at least 80,000 daltons, at least 90,000 daltons, at least 100,000 daltons). In some embodiments, the MWCO is greater than 100,000 daltons.
(60) In circumstances where nucleic acid is encapsulated in lipid nanoparticles, the guidelines given by Table 1 may underestimate the optimal molecular weight cutoff values, as encapsulation (e.g., lipid nanoparticle encapsulation) can enhance the effective size of the nucleic acid. Accordingly, it may be necessary to increase the pore size of the filter when filtering lipid nanoparticles according to the size and composition of the nanoparticles and the composition of the filtrate.
(61) The housing can be pressurized by sealing the housing and injecting a gas into the housing.

The gas can be supplied by an external tank of, e.g., compressed air, argon, N.sub.2, or CO.sub.2 and transferred into the housing according to known techniques (e.g., through a valve). In some embodiments, the gas is injected through a sealed cap.

(62) Filtration occurs in response to a housing pressure between about 1 bar and about 10 bar, e.g., between 1 bar and 10 bar, between 2 bar and 10 bar, between 3 bar and 9 bar, between 5 bar and 9 bar, between 6 bar and 8 bar, about 2 bar, about 3 bar, about 4 bar, about 5 bar, about 6 bar, about 7 bar, about 8 bar, about 9 bar, or about 10 bar). In some embodiments, the pressure of the gas within the filter housing is about 7 bar. A suitable pressure is high enough to efficiently filter the suspension without rupturing the filter membrane.

(63) When utilizing the conditions provided by any of the methods described herein, substantially complete nucleic acid retention (e.g., 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) can be attained over the course of filtering all or a portion of the initial liquid from the filter housing. The final volume of retentate, e.g., the threshold amount of nucleic acid suspension in the housing, can be set by the operator by, e.g., installing or configuring a liquid level detector accordingly (e.g., by attaching the liquid level sensor at a height aligned with the threshold volume).

(64) In some embodiments, the liquid level is a detected by change in capacitance across the filter housing, e.g., by a capacitive liquid level detector. Capacitive liquid level detectors do not require contact with the inside of the housing and are thus suitable for detecting solutions containing sensitive biological materials. Capacitive liquid level detectors suitable for use as part of the present methods are known in the art and described, e.g., in U.S. Pat. No. 5,017,909. A capacitive liquid level detector of the invention can have a liquid level sensing precision of between about 10 μm and about 500 μm (e.g., 10 μm, 20 μm, 30 μm, 40 μm, 50 μm, 60 μm, 70 μm, 80 μm, 90 μm, 100 μm, 200 μm, 300 μm, 400 μm, or 500 μm). The volume associated with a height of retentate can be configured by adjusting the width of the housing at that height.

(65) Methods of the invention feature automatic depressurization upon sensing a threshold level of retentate (e.g., a level corresponding a retentate volume between about 0.1 ml and 10 ml, e.g., about 0.1 ml, 0.2 ml, 0.3 ml, 0.4 ml, 0.5 ml, 0.6 ml, 0.7 ml, 0.8 ml, 0.9 ml, 1.0 ml, 1.1 ml, 1.2 ml, 1.3 ml, 1.4 ml, 1.5 ml, 1.6 ml, 1.7 ml, 1.8 ml, 1.9 ml, 2.0 ml, or greater). Switch mechanisms and circuitry for communication between a liquid level sensor and a pressure valve necessary for automatic depressurization are known in the art.

(66) After the filtration step, the threshold volume of nucleic acid suspension (i.e., the retentate) is more concentrated than the initial suspension. In some embodiments, the nucleic acid retentate has about a 2-fold greater, 3-fold greater, 4-fold greater, 5-fold greater, 6-fold greater, 7-fold greater, 8-fold greater, 9-fold greater, 10-fold greater, 11-fold greater, 12-fold greater, 13-fold greater, 14-fold greater, 15-fold greater, 16-fold greater, 17-fold greater, 18-fold greater, 19-fold greater, 20-fold greater or more concentration of nucleic acid than the initial suspension. In some embodiments, the nucleic acid retentate has about a 13-fold greater concentration of nucleic acid than the initial suspension.

(67) Refilling

(68) The invention features a method for sequential filtering and filling (e.g., with an additional liquid) to enable complete buffer replacement (e.g., wherein the additional liquid is different from the initial liquid). In accordance with this aspect of the invention, one or more refilling steps can be performed. Overall several sequential filtration steps may be required to yield a complete buffer replacement. In some embodiments, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more sequential filtrations may be required, depending on the concentration of nucleic acid and/or other components/contaminates of the initial suspension. In some embodiments, the process is repeated 6 times to achieve complete buffer replacement.

(69) Exemplary buffering agents that may be included as part of the additional liquid include, but are not limited to, citrate buffer solutions, acetate buffer solutions, phosphate buffer solutions,

ammonium chloride, calcium carbonate, calcium chloride, calcium citrate, calcium glubionate, calcium gluceptate, calcium gluconate, d-gluconic acid, calcium glycerophosphate, calcium lactate, propanoic acid, calcium levulinate, pentanoic acid, dibasic calcium phosphate, phosphoric acid, tribasic calcium phosphate, calcium hydroxide phosphate, potassium acetate, potassium chloride, potassium gluconate, potassium mixtures, dibasic potassium phosphate, monobasic potassium phosphate, potassium phosphate mixtures, sodium acetate, sodium bicarbonate, sodium chloride, sodium citrate, sodium lactate, dibasic sodium phosphate, monobasic sodium phosphate, sodium phosphate mixtures, tromethamine, magnesium hydroxide, aluminum hydroxide, alginic acid, pyrogen-free water, isotonic saline, and/or combinations thereof.

(70) In some embodiments, the buffering agent may be at a concentration of about 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM, 0.7 mM, 0.8 mM, 0.9 mM, 1.0 mM, 1.1 mM, 1.2 mM, 1.3 mM, 1.4 mM, 1.5 mM, 1.6 mM, 1.7 mM, 1.8 mM, 1.9 mM, 2.0 mM, 2.1 mM, 2.2 mM, 2.3 mM, 2.4 mM, 2.5 mM, 2.6 mM, 2.7 mM, 2.8 mM, 2.9 mM, 3.0 mM, 3.5 mM, 4.5 mM, 5.0 mM, 5.5 mM, 6.0 mM, 6.5 mM, 7.0 mM, 7.5 mM, 8.0 mM, 8.5 mM, 9.0 mM, 9.5 mM, 10 mM, 15 mM, 20 mM, or greater.

(71) In some embodiments, the additional liquid is at least 9-fold (e.g., at least 9-fold, at least 10-fold, at least 11-fold, at least 12-fold, at least 13-fold, at least 14-fold, at least 15-fold, at least 16-fold, at least 17-fold, at least 18-fold, at least 19-fold, at least 20-fold, or greater) the volume of the predetermined threshold of the retentate. For example, the volume of the additional liquid may be selected according to the volume of the filtrate to maintain an equivalent volume to be filtered as part of each sequential filtration. Accordingly, the additional liquid has a volume about equal to the difference between that of the initial suspension and the predetermined threshold. The additional liquid can be the buffer meant to replace the initial liquid (e.g., a citrate buffer, e.g., sodium citrate, e.g., 2.0 mM sodium citrate). Alternatively, the additional liquid may be adjusted during each sequential filtration step, e.g., as a step-wise progression towards the conditions of the final buffer.

(72) The filtrate can be discarded, repurposed, or passed through the filter one or more additional times according to any of the preceding methods.

(73) The sequential filtration and refilling steps of the present invention provide a fully automated process requiring little-to-no operator intervention. At the end of the process, the operator removes the filter housing containing the final nucleic acid suspended in the desired buffer at the desired concentration.

(74) Apparatus

(75) The present invention provides apparatus for concentrating nucleic acid, e.g., according to any of the preceding methods. An apparatus of the invention includes a chamber to hold a filter housing, a liquid level detector, a pressure source, and a pump. Furthermore, an apparatus may include a plurality (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more) of any one or more of the preceding elements, e.g., to filter samples in parallel. An exemplary apparatus for concentrating nucleic acid is shown holding a housing and a filtrate tube in FIG. **1**.

(76) The apparatus includes a chamber configured to hold a housing having a filter configured to hold an initial suspension of a nucleic acid in contact with the filter; a pressure source configured to releasably engage and pressurize the housing to force liquid in the initial suspension through the filter to produce a filtrate and a nucleic acid retentate; and a detector configured to detect the volume of the nucleic acid retentate and depressurize the housing upon detecting the volume reaching predetermined threshold.

(77) The chamber can be configured to receive the housing from the operator and position it appropriately for engagement with the pressure source. Optionally, the chamber can be part of a system to move the housing into contact with the pressure source (e.g., laterally moved from the front of the apparatus to the back). A pressure cap can be included as part of the apparatus to facilitate this positioning. For example, the pressure source can feed through the pressure cap, e.g., as an integrated element, and can be displaced with the pressure cap in an upward position when

the housing is being installed in the chamber. Upon installation of the housing, the pressure cap can be lowered into place over the housing to create an airtight seal around the housing. In this configuration, the pressure source is operatively connected to the inside of the housing through, e.g., a metal valve assembly or a structurally adequate tubing assembly.

(78) Pressure Sources

(79) The apparatus described herein include or are otherwise coupled to a pressure source (e.g., a compressed air source). In some embodiments, a separate apparatus may include the pressure supply. For example, a pressure source of the invention may be a valve assembly or conduit that connects with an external pressure source (e.g., a compressed air source).

(80) The pressure source may be a high pressure source. For example, the pressure source may facilitate air transfer from, e.g., an air compressor, a compressed gas tank, a house gas line, or the like. In some embodiments, the pressure source may be configured to provide an inert gas (e.g., air, argon, N.sub.2, or CO.sub.2) to the sample container. The pressure source may be fluidically connected to a port in contact with a source of inert gas (e.g., a pressurized chamber, house gas lines, or gas cylinder). A valve assembly and/or flow meter or other pressure gauge may be used to control the amount of inert gas inputted into the apparatus. In some embodiments, one or more components or regions of the apparatus are held at positive pressure, such that suction must be applied to draw additional gas into the one or more components or regions of the apparatus. In other embodiments, one or more components or regions of the apparatus are held at negative pressure, such that the addition of gas into the one or more components or regions of the apparatus requires metering, e.g., by the valve assembly. Upon suctioning or metering of gas into a region of the apparatus, the pump may facilitate the transfer of the gas to the sample container via, e.g., a fluidic connection to an opening in the sample container (e.g., tubing, such as polyethylene tubing). Gas may be removed from a sample container via an opening in the sample container (e.g., by removing a cap, lid, or cover forming a seal at an opening) or by suction provided by a pressure source.

(81) Alternatively, the pressure source may be a low pressure source. For example, the pressure source may be capable of providing vacuum and/or suction. Pressure sources may include one or more peristaltic pumps, syringe pumps, rotary pumps, momentum transfer pumps, diffusion pumps, scroll pumps, and/or diaphragm pumps. In some embodiments, the pressure source is a positive displacement or infusion pump such as a peristaltic pump or a syringe pump. In some embodiments, a low pressure source may include a house or central vacuum system. In other embodiments, a suction source of the invention may include a portable suction device. The pressure source may be of any useful size, pumping speed, and geometry and, if included as a component of the apparatus, may be disposed in any useful location.

(82) Liquid Level Detectors

(83) The apparatus includes one or more liquid level detectors to detect the level of the retentate and trigger depressurization of the housing upon reaching that level, as described above. The liquid level detector can be one sensor or a plurality, e.g., an array, of sensors. The liquid level detector can be a capacitive sensor, which can be installed on the apparatus at a position lateral to the filter, e.g., adjacent to the threshold liquid surface level, such that a liquid level drop below the threshold level triggers the sensor. Capacitive liquid level detectors are known in the art. Additionally or alternatively, other detectors or combinations of detectors can be used, including optical sensors, inductive sensors, and cameras.

(84) Liquid Injector

(85) In some embodiments, the apparatus further includes a liquid injector configured to inject an additional liquid into the housing. The liquid injector can be attached to a pump (e.g., a peristaltic pump, a syringe pump, a rotary pump, a momentum transfer pump, a diffusion pump, a scroll pump, or a diaphragm pump) configured to pump the additional liquid from a reservoir to the housing. For example, in some embodiments, the liquid injector is a syringe pump driven by a

stepper motor with a lead screw (e.g., Hamilton PSD6).
(86) The liquid injector can pump the additional liquid into the housing at any suitable rate. For example, the additional liquid can be injected at a rate suitable to sufficiently mix the nucleic acid suspension with the additional liquid. Further, the rate may be suitable to remove any adsorbed nucleic acid from the membrane fibers. Suitable rates of injection are about 0.1 milliliters per second (ml/s), about 0.2 ml/s, about 0.3 ml/s, about 0.4 ml/s, about 0.5 ml/s, about 1.0 ml/s, about 2.0 ml/s, about 3.0 ml/s, about 4.0 ml/s, about 5.0 ml/s, about 6.0 ml/s, about 7.0 ml/s, about 8.0 ml/s, about 9.0 ml/s, about 10 ml/s, about 15 ml/s, about 20 ml/s, about 25 ml/s, about 30 ml/s, about 35 ml/s, about 40 ml/s, about 45 ml/s, about 50 ml/s, or greater.
(87) In some embodiments, the liquid injector is in communication with the liquid level detector. In some embodiments, the liquid injector injects the additional liquid into the housing upon detecting a volume of a nucleic acid retentate below a predetermined threshold. In some embodiments, the liquid injector is configured to inject the additional liquid into the housing after depressurization.
(88) Reservoirs
(89) The apparatus described herein may include one or more reservoirs for housing a liquid. A reservoir may have any useful geometry and dimensions and be formed of any useful materials. In some embodiments, the reservoir is fluidically connected to or configured to be fluidically connected to the sample container via one or more portions of tubing. In some embodiments, a valve assembly, gauge, or flow meter is disposed between the sample container and the reservoir.
(90) The apparatus of the invention may include multiple reservoirs for housing different materials. These reservoirs may be of the same or different geometries, dimensions, and/or materials. For example, the apparatus may include a first reservoir for housing a first liquid (e.g., water, acetonitrile, acetone, or methanol) and a second reservoir for housing a second liquid, e.g., reagent, buffer, or eluting liquid. For example, a reservoir may be included as part of the invention to contain the additional liquid (e.g., a wash buffer). Additionally or alternatively, a reservoir may be a filtrate tube used to collect a filtrate. A reservoir may also be configured to collect and house waste materials.
(91) Valve Assemblies
(92) The apparatus described herein may include one or more valve assemblies. A valve assembly is a fluid control mechanism used to control the flow of fluids throughout the apparatus. The valve assembly includes a valve to stop, start, or throttle fluid flow. The valve assembly may include any useful valve type, including but not limited to a ball valve, diaphragm valve, and needle valve. In addition to a valve, the valve assembly may include components such as a casing, an electrical or mechanical actuation mechanism (e.g., an electric motor, a hydraulic or pneumatic component, and a solenoid) and associated components (e.g., electric connections and cables), sensors, fasteners (e.g., screws, bolts, clips, and clamps), and mechanical connectors.
(93) A valve assembly may be fluidically connected to one or more components of an apparatus, such as a housing, an injector, a reservoir, an outlet for collection or waste, an inlet for gas, or a pump. In some embodiments, the apparatus for concentrating a nucleic acid includes a sample container, a reservoir, an inlet for inert gas, and an outlet for collection or waste, any of which may be fluidically connected to a valve assembly. A pump (e.g., a peristaltic or syringe pump) may be used to facilitate fluid transfers between components of the apparatus.
(94) In some embodiments, the apparatus includes more than one valve assembly. For example, an apparatus may include two, three, four, five, or more valve assemblies.
(95) Other Components
(96) An apparatus for concentrating nucleic acid may include components in addition to those described in the preceding sections. For example, the apparatus may include one or more electrical components, cables, tubing, fasteners, connectors, containers for collection of waste or other materials, caps, lids, covers, temperature control (e.g., heating) elements, flow meters, computers, screens, casings, and housings.

(97) In some embodiments, the apparatus includes a temperature control element. A temperature control element may include a solvent bath (e.g., a water bath), an electric heater, heating tape, a thermocouple, a sensor, a jacket, insulation, or any other useful element. In some embodiments, the apparatus includes a temperature control element that surrounds all or a portion of a housing or reservoir. For example, the housing may be wrapped with a heating element such as a heating jacket. In other embodiments, a surface or portion thereof of a housing or a filtrate tube is exposed to a heating element. For instance, any reservoir of the device may be disposed on a heatable plate or other heating element. Tubing in the apparatus may also be in contact with a heating element such as heating tape or a heating jacket.
(98) In some embodiments, the apparatus includes one or more containers for collection of waste or other materials. These containers may be of any useful shape and dimensions and may be made of any useful materials. In some embodiments, containers for collection of waste or other materials are test tubes or vials. Containers for collection of waste or other materials may be capable of accepting any volume of liquid. In some embodiments, collection containers may be capable of accepting a volume of 1 or more ml (e.g., 1 ml, 2 ml, 5 ml, 10 ml, 15 ml, 20 ml, or more). Collection containers include at least one opening through which waste or other materials can be added and may include a cover, lid, or cap to block the opening while materials are not being added. If more than one collection container is present, the collection containers may be organized in an array, and/or a mechanism for transferring the collection containers between different areas of the apparatus may be provided. For example, a mechanical track, carousel, robotic gantry, or other mechanism may be used to position a first collection container such that it can collect materials from, e.g., the housing. After a volume of materials (e.g., waste) is added to the first collection container, the transfer mechanism can be used to move the first collection container to a different location and to position a second collection container such that it can collect materials from, e.g., the housing.
(99) Any or all of the components described as part of the apparatus of the invention can be in communication with one another or external elements through a software and/or hardware interface, according to configurations known in the art. Software and/or hardware can be configured to allow a user to choose whether or not to inject an additional liquid, e.g., to resuspend the retentate or recover a concentrated solution of nucleic acid. Additionally or alternatively, a software and/or hardware can actuate pressurization and/or depressurization, e.g., in response to a liquid level detector. Software and/or hardware useful as part of the apparatus is known in the art.
(100) Materials
(101) The apparatus described herein may comprise any useful materials. For example, the housing, filters, liquid level detector, liquid injector, tubing, pressure source, valve assembly, reservoirs, and other components may include and/or be formed from any useful polymer or plastic. Such materials may include, e.g., polystyrene, polypropylene, polyvinyl chloride, or combinations thereof. Polymers and/or plastics of the invention may be composite materials in which additives to the polymers and/or plastics, such as ceramics or particles, alter the mechanical properties.
(102) Elements of the invention may also include and/or be formed from glass. For example, an apparatus of the invention may include a reservoir made wholly or partially from glass.
(103) In some embodiments, the liquid level detector, liquid injector, tubing, pressure source, valve assembly, reservoirs, and other components may include and/or be formed from any useful metal or metal alloy, e.g., stainless steel or aluminum.
(104) Compositions
(105) The invention further provides a filtered nucleic acid suspension produced by methods and apparatus described herein. Such nucleic acid suspensions can be further processed according to known methods, e.g., into pharmaceutical compositions. For example, nucleic acid suspensions provided by the present invention can be mixed with pharmaceutically acceptable excipients or one or more additional active substances, e.g., therapeutically and/or prophylactically active substances.

General considerations in the formulation and/or manufacture of pharmaceutical agents may be found, for example, in Remington (The Science and Practice of Pharmacy, 22nd Edition, A. R. Gennaro, Lippincott, Williams & Wilkins, Baltimore, MD, 2012).

(106) Relative amounts of the active ingredient, the pharmaceutically acceptable excipient, and/or any additional ingredients in a pharmaceutical composition in accordance with the present disclosure may vary, depending upon the identity, size, and/or condition of the subject being treated and further depending upon the route by which the composition is to be administered. For example, the composition may comprise between 0.1% and 99% (w/w) of the active ingredient. By way of example, the composition may comprise between 0.1% and 100%, e.g., between 0.5 and 50%, between 1-30%, between 5-80%, at least 80% (w/w) active ingredient.

EXAMPLES

Example 1: Automated Concentration or Solvent Exchange Using an Apparatus of the Invention

(107) Operation of the apparatus begins with preparation of an initial nucleic acid suspension in each of six EMD Millipore Amicon 15 filter housings containing a 30,000 MWCO filter. 13.0 mL of a 100 μg/ml mRNA suspension is placed in a filter housing positioned within a 50 mL filtrate tube to receive the filtrate as it passes through the filter. Each housing and filtrate tube are loaded within a chamber to hold the housing in place within the apparatus.

(108) Next, the apparatus pulls the six housings under pressure caps, and the pressure caps lower to engage the housings with the pressure source. The pressure source is activated upon confirmation by a sensor and injects air into the housing, pressurizing the housing to 7 bar and forcing the liquid in the initial nucleic acid suspension, but not the mRNA molecules, through the filter and into the filtrate tube.

(109) Capacitive liquid level detectors are attached at the apparatus laterally at each of the filters. As the liquid level of the suspension in all six of the housings drops to a height corresponding to 1.0 mL or less, the capacitive liquid level sensors actuate depressurization of the housing by opening a valve.

(110) After depressurization, the pressure caps lift from the housings, and the housings are ejected from the apparatus to complete the nucleic acid concentration protocol. If complete solvent exchange is required, the housings remain in the system, and a pump refills the filter housing by adding 12.5 mL of 2 mM sodium citrate. The process is automatically repeated five additional times for a total of six cycles, at which point the osmolality of the nucleic acid suspension has stabilized and buffer replacement is complete. After completion of six cycles, the pressure caps lift from the housings, and the housings are ejected from the system.

OTHER EMBODIMENTS

(111) It is to be understood that while the present disclosure has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the present disclosure, which is defined by the scope of the appended claims. Other aspects, advantages, and alterations are within the scope of the following claims.

Claims

1. An apparatus for concentrating nucleic acid, the apparatus comprising: a) a chamber comprising a housing having a filter configured to hold an initial suspension comprising nucleic acid in contact with the filter; b) a pressure source configured to releasably engage and pressurize the housing to force liquid in the initial suspension through the filter to produce a filtrate and a nucleic acid retentate; c) a pressure cap configured to engage the housing with the pressure source; d) a detector configured to detect the volume of the nucleic acid retentate and depressurize the housing upon detecting the volume reaching a predetermined threshold; e) a liquid injector configured to inject an additional liquid into the housing; f) a pump that is connected to the liquid injector and a liquid reservoir; and g) a processor that: i) is in communication with the detector; ii) causes the pressure

source to depressurize and lift the pressure cap from the housing upon receiving a signal from the detector indicating a reduction in the volume of the nucleic acid retentate beneath a predetermined threshold; and iii) causes the pump to automatically inject additional liquid from the liquid reservoir, through the injector, and into the housing upon receiving the signal from the detector indicating the reduction in the volume of the nucleic acid retentate beneath the predetermined threshold and upon the pressure cap being lifted.

2. The apparatus of claim 1, wherein the chamber is configured to receive the housing provided by a user and position the housing for engagement with the pressure source.

3. The apparatus of claim 1, wherein the pressure source is configured to inject a gas into the housing.

4. The apparatus of claim 1, wherein the detector is a capacitive liquid level detector.

5. The apparatus of claim 1, wherein the liquid injector is configured to inject the additional liquid into the housing after depressurization of the housing.

6. The apparatus of claim 1, wherein the liquid injector adds the additional liquid into the housing at a rate suitable to sufficiently mix the suspension with the additional liquid.

7. The apparatus of claim 1, wherein the liquid injector adds the additional liquid at a rate suitable to remove adsorbed nucleic acid from membrane fibers of the filter.

8. The apparatus of claim 1, wherein the liquid injector is attached to a pump.