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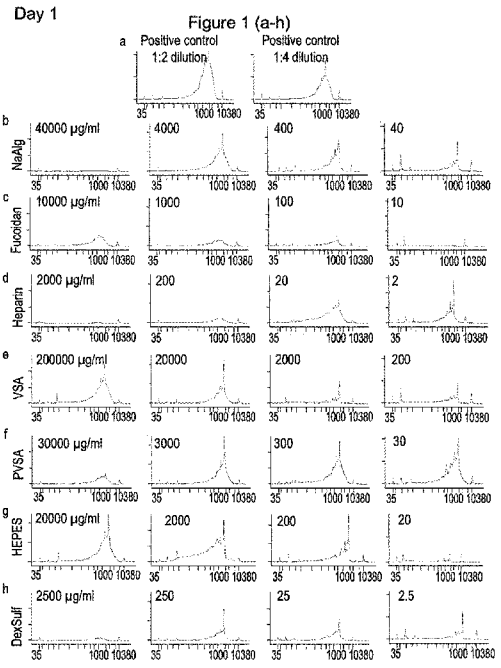
(54) Title: DRY STORAGE

(57) Abstract: There is provided devices and methods for storage of biological material comprising RNA, wherein stability of RNA is increased.



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Dry storage

FIELD

The present invention relates to devices and methods for effective storage of biological samples, hereunder dry storage of RNA samples, preventing RNA degradation.

BACKGROUND

Biomedical research and biotechnology rely on polymeric nucleic acids. Yet during their storage and use, nucleic acids encounter nucleases that degrade nucleic acid. For example, human skin is an abundant source of nucleases that can be transferred accidentally to surfaces and solutions, and biological samples analysed for nucleic acid content are themselves generally a source of them. A ribonuclease (commonly abbreviated RNase), is a type of nuclease that catalyzes the degradation of RNA into smaller components.

Messenger RNA (mRNA) serves as the critical conduit for genetic information, carrying the information that is ultimately utilized from protein synthesis by the cell. The mRNA profiles of biological specimens, be they tissue, liquid biopsies, or cell cultures, contain vital information reflective of the specimens' conditions, as do profiles of other types of RNA. Today, extracted RNA can be subjected to RNA-sequencing library preparation followed by high-throughput sequencing, allowing efficient and global characterization of the complex RNA profile contained in a biological sample. However, RNA is notoriously labile and prone to degradation by ubiquitous ribonucleases (RNases) present in the environment and within biological samples themselves. Thus, RNA degradation needs to be prevented during sample collection, storage,

and in the following RNA detection procedure. Most commonly, for the storage of biological RNA samples, the samples are preserved in liquid form through cryopreservation, typically requiring storage in specialized freezers at -80°C.

Ribonuclease inhibitor (RI) is a large 50 kDa protein present in the cytosol of mammalian cells. RI forms extremely tight complexes with certain RNases and controls the activity of RNases. Inhibitors of ribonuclease are useful in a variety of molecular biology applications where RNase contamination is a potential problem. Examples of these applications include mRNA isolation and purification, storage, reverse transcription of mRNA, RNA Sequencing (RNA-seq), and *in situ* RNA-sequencing. One solution is the pre-treatment of samples and solutions with diethylpyrocarbonate (DEPC), which is effective for ribonuclease inhibition. However, DEPC and other similar chemicals are known carcinogens and require caution and training for their use. These chemicals also react quite readily with amine, thiol, and alcohol groups so some solutions (e.g., primary amine containing compounds such as Tris) cannot be treated with DEPC at all. Finally, DEPC must be inactivated by autoclaving post-treatment, but DEPC residues may still interfere with downstream enzymatic reactions such as reverse transcription (RT) and polymerase chain reaction (PCR).

The capture of intact RNA is a requisite of RNA-sequencing (RNA-seq) methods to accurately record the transcriptome of the analyzed sample material. Any RNA-protective agent added to the RNA sample must be directly compatible with downstream reaction steps (such as reverse transcription and PCR), otherwise it needs to be removed from the RNA sample e.g., by an additional RNA purification step.

Thus, the use of *in vitro* synthesized biological RNase inhibitors (i.e. recombinant RI proteins) is a nearly universal feature during cell-lysis

and storage as well as reverse transcription in RNA-seq protocols. However, the use of recombinant inhibitors is inconvenient due to its relatively high cost fraction to the library, but also due to its degradability; which may introduce batch variation in library yield and quality due to production lot, storage time, and temperature conditions for the inhibitor. Thus, there is a need for ribonuclease inhibitors for applications which require capture of intact RNA. If thermostable RNase inhibitors could be identified, this would enable new and simplified workflows, and may increase reproducibility and throughput of RNA-seq applications. Importantly, to satisfactorily replace recombinant RNase inhibitors in RNAseq library preparation, such ribonuclease inhibitors must not only be capable of preserving cellular RNA in the lysis buffer but should be fully compatible with each of the following library preparation steps that are universal for all RNA analysis of relevance, including reverse transcription and amplification by PCR, without introducing base errors or reducing sensitivity to detect RNA species in contained in the analysed sample material.

DETAILED DESCRIPTION

Protein-based RIs are considered specific for RNase whereas chemicals with RNase inhibitory do in general also affect or inhibit other enzymes which are critical in the molecular biology application, such as reverse transcriptase and DNA polymerase. The inventors have found that certain chemicals are suitable for use in applications where inhibition of RNase activity is desirable such that certain specific concentrations do not negatively affect other enzymes.

Although there are potentially many chemical substances or treatments that may in principle inhibit RNase activity, these are generally expected to also negatively affect RNA-seq library yield as well as the

quality and error rate in the final sequencing library. Thus, from a chemical compound being a potent RNase inhibitor it does not follow that the agent is also suitable RNase inhibitor in RNA-seq.

In its broadest aspect, the present invention relates to a method of using chemical RNase inhibitors alone or in combinations inhibitors in devices for storage of biological samples comprising RNA as well as to such devices. The devices and methods may be used in applications where inhibition of RNase activity is desired, including in RNA sequencing and storing of biological samples before such sequencing.

The present invention provides devices for dry storage of biological RNA samples, conserving RNA from degradation. In specific embodiments, fibrous or synthetic sheet materials are pre-treated with sodium alginate (NaAlg), heparin, fucoidan, vinyl sulfonic acid (VSA), polyvinyl sulfonic acid (PVSA), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), and/or dextran sulfate (DexSulf), which the inventors demonstrate prevents degradation of RNA in dry-storage devices. This provides a solution to a multitude of issues associated with conventional RNA-storage methods, such as cryopreservation or storage of RNA in bone-dry crystalline form. The ability to store RNA in a dry, stabilized state, on a absorbent material, at ambient temperatures or below without compromising its integrity provides a multiple advantage in fields of molecular biology, biomedicine, and biotechnology.

Practically, dry storage of RNA offers several benefits over traditional liquid cryopreservation.

The inventors have surprisingly found that recombinant biological RNase inhibitors widely used in RNA-seq library preparation and other use of RNA can be replaced by a sulfonated polymer, a sulfonated monomer, or a carboxylated polymer, supplied in defined concentration

ranges, yielding bulk RNA-seq and scRNAseq libraries of equal or superior quality at virtually no cost for RNA inhibition. Moreover, the thermostability of chemical RNase inhibition means it need not be supplemented twice during RNA collection and cDNA library preparation (i.e. in the cell lysis or collection step and in the reverse transcription step), which enables new and simplified workflows, increasing reproducibility and throughput of RNA-seq. For, example, stable premade sample collection buffers can be made, frozen, thawed, and kept at room-temperature for extended periods of time, or even subjected to high-temperature conditions before use.

These findings are particularly surprising since, for example, an exemplary agent of the invention, poly(vinylsulfonic acid) (PVSA), was known to be a strong inhibitor of catalysis by RNA polymerase and reverse transcriptase (Chambon *et al.*, 1967; Althaus *et al.*, 1992). The inventors identified that surprisingly low titres of PVSA form a concentration range (optimally 0.1-120 µg/mL in lysis buffer) in which PVSA can effectively replace normally used recombinant RI in RNAseq library preparation without negatively affecting the quality of the RNAseq library. Importantly, this application as well as the workable concentration range is contrasting to a study utilizing PVSA in cell-free protein translation at >40-fold higher concentration with decoupling of in vitro transcription and translation by a purification step (Earl 2018 Bioengineering PMID: 28662363).

As it appears from the above disclosure, other chemicals may also be suitable for use as RNase inhibitors.

Combined use of two or more RNase inhibitors is also contemplated.

The present invention provides devices for dry storage of biological samples, conserving RNA from degradation and use of such devices. In specific embodiments, an absorbent sheet material is pre-treated with

one of the following agents: polyvinyl sulfonic acid (PVSA), vinyl sulfonic acid (VSA), sodium alginate (NaAlg), dextran sulfate (DexSulf), fucoidan, heparin, and/or 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), which the inventors demonstrate prevents degradation of RNA in the storage device. This provides a solution to a multitude of issues associated with conventional RNA-storage methods, such as cryopreservation or storage of RNA in bone-dry crystalline form. The ability to store RNA in a dry, stabilized state, at ambient temperatures without compromising its integrity provides a multiple advantage in fields of molecular biology, biomedicine, and biotechnology.

Practically, dry storage of RNA offers several benefits over traditional liquid cryopreservation, including but not limited to:

Rapid Sample Collection: a sample, optionally in the form of a lysate may be applied to a storage device according to the invention without the need for additional steps such as preparing storage buffers and/or cooling or freezing the collected sample or samples.

Space Efficiency: The compact nature of the dry storage medium maximizes space utilization, enabling a greater number of samples to be stored in a given physical volume compared to cryogenic vials in freezers.

Cost-Effective Storage: Eliminating the need for ultra-low temperature storage equipment reduces energy consumption and operational costs. The present invention obviates the need for continuous power supply and expensive refrigerants, making RNA storage more cost-effective and sustainable.

Ease of Transportation: Dry storage vastly simplifies the handling and transportation of RNA samples. Samples can be shipped at ambient

temperatures without the risk of thawing, which is a critical vulnerability of cryopreserved samples during transit.

Rapid Sample Preparation: Dry storage potentially allows for more rapid reconstitution and preparation of RNA samples for downstream applications, as the rewarming and thawing steps inherent to cryopreservation are circumvented.

Safety and Accessibility: The dry storage method does not require the handling of hazardous materials such as liquid nitrogen, enhancing safety in laboratory settings. It also facilitates wider accessibility to RNA storage and transportation, including in resource-limited settings that lack specialized cryopreservation facilities.

Reduced Risk of Contamination: By providing a sealed, desiccated environment, the present invention minimizes the risk of sample cross-contamination that can occur with liquid storage systems.

Time range for storage: The dry storage device and method provides for storage at ambient temparature and below for extended periods while maintaining RNA quality. Thus, samples may be stored for 1, 2, 3, 4, 5, 6, 7 days, or more, such as ten days, two weeks, three weeks, one month, two months, three months, four months, five months, six months, seven months, eight months, nine months, ten months, eleven months, a year, or two years, with an acceptable quality and yield.

The described invention fulfills the need for a robust method of RNA sample preservation that is practical, economical, and conducive to the widespread storage and distribution of RNA samples. It leverages the stabilization of RNA in a desiccated state achieved by chemical compounds which the inventors demonstrate to prevent RNA degradation, significantly improving upon the limitations associated with cryopreservation. The benefits of this invention are not limited to the

preservation of integrity alone but encompass logistical, economic, and environmental aspects, thereby representing a transformative approach to RNA sample management.

The described innovation has multiple potential usage areas, e.g., in biological and medical research, as well as in clinical applications, in which biological samples are to be collected and stored until the RNA of the sample is analyzed. Such analyses include, but are not limited to, characterizing the transcriptional state of the biological sample or the detection of specific RNA sequences within the sample, such as a molecular marker molecule, bacterial, or viral RNA.

The described innovation could be incorporated in a larger device or kit containing a filter step used to purify the sample before it touches the absorbent material of the RNA storage device. A filter may capture incompletely lysed cell debris (e.g., mesh filter in spin-down column or compression syringe), as alternative to the cell debris removal by centrifugation.

Definitions

By "polymer" we include the meaning of any of a class of natural or synthetic substances that are multiples of simpler chemical units called monomers. In an embodiment, the polymer is a non-protein polymer. In an embodiment, the polymer is a non-biological polymer. By "non-biological" we include the meaning of a molecule or agent not normally found in a biological system.

In a particular embodiment, such polymers (i.e. sulfonated and/or carboxylated polymers, as described herein) are vinyl polymers. In a further embodiment, the relevant monomers (i.e. sulfonated and/or carboxylated monomers, as described herein) are vinyl polymers.

By "vinyl polymer" we include the meaning of products from the polymerization of vinyl monomers. By "vinyl monomers" we include the meaning of monomers containing vinyl groups, i.e. small molecules containing carbon-carbon double bonds.

Salt forms of any of the monomers, polymers and polysaccharides described herein may also be used in the methods of the present invention. Any salt form used should not comprise a cation which inhibits any part of the method of the invention, such as the PCR reaction. Salts that may be used include acid addition salts and base addition salts. Examples of addition salts include those derived from mineral acids, such as hydrochloric, hydrobromic, phosphoric, metaphosphoric, nitric and sulphuric acids; from organic acids, such as tartaric, acetic, citric, malic, lactic, fumaric, benzoic, glycolic, gluconic, succinic, arylsulphonic acids; and from metals such as sodium, magnesium, potassium or calcium. In an embodiment, the salt form is a sodium salt.

It will be appreciated that for salt forms of the RNase-inhibiting polymer or monomer, the counter ion may be exchanged to another counter ion. Indeed, it is common knowledge in chemistry that a functional charged molecule can be paired with various counter ions.

As a specific example, sodium alginate (NaAlg) having sodium (Na^+) as counter ion could be replaced by potassium alginate (KAlg) having potassium (K^+) as counter ion.

Unsalted forms of the polymers and polysaccharides described herein may also be used in the methods of the present invention.

By "sulfonated polymer" we include the meaning of a repeated chain of molecules wherein a sulfonate residue appears at least once per unit in

the chain. In an embodiment, the sulfonated polymer comprises one sulfonate group per unit.

By "carboxylated polymer" we include the meaning of a repeated chain of molecules wherein a carboxylate residue appears at least once per unit in the chain. In an embodiment, the carboxylated polymer comprises one carboxyl group per unit.

By "sulfonated monomer" we include the meaning of a compound that is non-repeating and that contains at least one sulfonate residue.

By "carboxylated monomer" we include the meaning of a compound that is non-repeating and that contains at least one carboxylated residue.

By "polysaccharide" we include the meaning of polymeric carbohydrates composed of repeating units, e.g. monosaccharides or disaccharides, linked together by glycosidic bonds. Polysaccharide compounds such as glycosaminoglycans are also included. Polysaccharides are known in the art and include but are not limited to, cellulose, amylose, dextran, and heparin. Native heparin has a molecular weight ranging from 3 to 30 kDa, although the average molecular weight of most commercial heparin preparations is in the range of 12 to 15 kDa. Dextrans are available in multiple molecular weights ranging from 3 kDa to 2 MDa. The molecular weight of amylose varies between several thousand and one-half million daltons with a degree of polymerization of 1000–10,000 glucose units.

By "functionalised" we include the meaning that the polysaccharide comprises one or more acidic group. In an embodiment, the functionalised polysaccharide is a sulfated and/or carboxylated polysaccharide.

By "sulfated polysaccharide" we include the meaning of a chain of repeating units linked together by glycosidic bonds wherein a sulfate residue appears at least once per unit in the chain. The repeating unit may be a monosaccharide or a disaccharide. In an embodiment, the sulfated polysaccharide comprises one sulfate group per monosaccharide.

By "carboxylated polysaccharide" we include the meaning of a chain of repeating units linked together by glycosidic bonds wherein a carboxylate residue appears at least once per unit in the chain. The repeating unit may be a monosaccharide or a disaccharide. In an embodiment, the carboxylated polysaccharide comprises one carboxyl group per monosaccharide.

By non-ionic detergent, surfactants containing no charged group, we include but are not limited to Triton X-100, nonyl phenoxypolyethoxylethanol (NP)-40, Tween-20, Tween-80, digitonin.

By ionic detergent, detergents have a hydrophilic head group that is charged and can be either negatively (anionic) or positively (cationic) charged, we include but are not limited to sodium dodecyl sulfate (SDS), sarkosyl, sodium deoxycholate.

By zwitter-ionic detergent we include 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS).

Chaotropic agents, that have the ability to disrupt hydrogen bonding and other non-covalent interactions between molecules, such as guanidinium thiocyanate, sodium iodide, and guanidinium hydrochloride, may also act as lysis agent and may replace detergent.

By "Triton X-100" we include Triton X-100 ($C_{14}H_{22}O(C_2H_4O)_n$) is a nonionic surfactant that has a hydrophilic polyethylene oxide chain (on average it has 9.5 ethylene oxide units) and an aromatic hydrocarbon

lipophilic or hydrophobic group. The hydrocarbon group is a 4-(1,1,3,3-tetramethylbutyl)-phenyl group.

In an embodiment, the agent is selected from the group consisting of: sodium alginate, dextran sulfate, polyvinyl sulfonic acid, vinyl sulfonic acid, heparin, fucoidan, HEPES.

In an embodiment, the agent inhibits RNase. By "inhibit" in the context of the activity of Rnase, we include the meaning that the activity of at least one RNase is reduced in a sample to which an agent of the invention is added, compared to the activity in an analogous sample to which the agent is not added. Inhibition is not limited to complete inhibition or inactivation of a given RNase. In a given application, it may be that some low level of RNase activity can be tolerated that will not have a detrimental effect on the outcome of the reaction, purification and/or assay being performed (in this case, preparation of a cDNA sequencing library). In an embodiment, the agent inhibits the activity of an RNase by at least 10%, such as at least 20%, 30%, 40% or 50% compared to the activity in an analogous sample to which the agent is not added. In an embodiment, the agent inhibits the activity of an RNase by at least 50%, such as at least 60%, 70%, 80% or 90%, such as by 95% compared to the activity in an analogous sample to which the agent is not added. It will be appreciated that the extent to which the polymer inhibits the activity of RNase depends on the concentration of the polymer, the RNase concentration and the conditions of the reaction.

"Substantial inhibition" is achieved when the RNase activity in a sample is below the level that is tolerable in a given application (i.e., the preparation of a cDNA sequencing library, or other applications where inhibition of RNase activity is desired). The level of inhibition that is substantial will then depend upon the application in which the inhibitory

agents are employed. In contrast, the term inactivation is used when there is no detectable level of activity of a given RNase. An RNase that is inactivated need not be rendered irreversibly inoperative. Agents of this invention may exhibit inhibition of certain RNases and inactivation of other RNases.

Examples of RNases that can be inactivated, inhibited and/or removed using the agents and/or devices described herein include eukaryotic RNases (e.g., mammalian RNases or fungal RNases) and prokaryotic RNases. Specifically exemplary RNases include RNase A, RNase B, RNase C, RNase 1, RNase T1, and bacterial RNase (e.g., those of *E. coli*).

In an embodiment, the agent reduces and/or prevents RNA degradation. By "reduces and/or prevents RNA degradation" we include the meaning that the degradation of RNA is reduced in a sample to which an agent of the invention is added, compared to the degradation of RNA in an analogous sample to which the agent is not added. In an embodiment, the agent reduces the degradation of RNA by at least 10%, such as at least 20%, 30%, 40% or 50% compared to the degradation of RNA in an analogous sample to which the agent is not added. In an embodiment, the agent reduces the degradation of RNA by at least 50%, such as at least 60%, 70%, 80% or 90%, such as by 95% compared to the degradation of RNA in an analogous sample to which the agent is not added.

The agents of the invention can be used alone or in combination with other agents of the invention in the methods described herein, such as in a device comprising an absorbent material impregnated with one or more of such agents.

In an embodiment, the RNase is selected from the group comprising RNase A and/or B; and/or C; and/or *E. coli* RNase. Preferably the agent

(i.e. RNase inhibitor) does not inhibit or affect fidelity or processivity of modifying enzymes like reverse transcriptases, DNA polymerases, and/or transposases under the given reaction conditions.

The amount and integrity of RNA in a stored sample can be inspected by generating a full-length cDNA library using reverse transcription and PCR amplification of the full-length cDNA (e.g., using Smart-seq2 (Picelli 2023, Picelli 2014)). Yield of cDNA and cDNA size distribution of the samples can be inspected using gel electrophoresis, e.g., quantitatively using Agilent Bioanalyzer 2100 High sensitivity DNA chips. The shape of full-length cDNA traces is known in the field to reflect the quality and integrity of the underlying mRNA sample (Trombetta 2014), and an intact library (satisfactory library) is expected to have a peak around ~2kb, reflecting the median length of full-length mRNA transcripts in human cells. Exact patterns of spikes in the cDNA traces can further be experiment- or cell-type specific, as abundant cell-type-specific or condition-specific RNA transcripts, for which one or a few transcripts account for a large proportion of total cellular transcripts lead to additional peaks on the Bioanalyzer trace.

Inactivation and/or inhibition is carried out by contacting a biological medium which may contain RNase with one or more agents or devices as described herein. By "biological medium" we include the meaning of any liquid in which a biological reaction or assay can be carried out or performed during the preparation of a cDNA library, which might be detrimentally affected by the presence of one or more active RNases. Biological medium includes any buffers (e.g. storage and lysis buffers) and reagents employed in the preparation of a cDNA sequencing library. The inhibitory agents described herein can, for example, be added along with reagents (e.g., prior to, or simultaneous with reagents) to inactivate or inhibit RNases that might be present in a reaction mixtures. The inhibitory agents can be bound to the internal and/or

external surfaces (e.g., glass, plastic, or fiber material) of absorbent material. Thus, the inhibitory agents can be bound in a material such as a membrane, for example a cotton or paper sheet pre-soaked in the RNase inhibitory agent, onto which a biological sample is added for storage and subsequent elusion and processing into an RNA-sequencing library.

Preparation of agent-coated surfaces can be achieved using an *in situ* polymerization method or by incubating material or surfaces with the inhibitory agent, such as in an aqueous solution comprising the agent or agents. Those of ordinary skill in the art will appreciate that other means for directly or indirectly (through a linker) coupling of agents of this invention to surfaces are available in the art and can be employed in the practice of this invention.

By "the method includes the use of an agent", we include the meaning that the agent is incorporated into a method of generating a cDNA library in such a way that RNase is inhibited. For example, the agent may already be included in a buffer that is used in the method or the agent may be added to one of the buffers used in the method before the method is carried out. The agent may be present in a reaction vessel (such as a multi-well plate) prior to be method being carried out. In an embodiment, the method includes the addition of the agent. In an embodiment, the method includes the use and/or addition of an effective amount of the agent. By "effective amount" we include the meaning of the amount of an agent or the combined amount of a mixture of agents which is used in or added to a biological medium containing one or more RNases to observe inhibition (as defined above) of at least one of the one or more RNases, whilst not substantially interfering with the biological reactions necessary for the generation of a cDNA sequencing library (e.g. first strand synthesis reaction and/or subsequent PCR reactions).

By "not substantially interfering" we include that addition of the agent does not negatively affect the biochemical reactions necessary for the generation of a cDNA sequencing library (e.g. first strand synthesis reaction and/or subsequent PCR reactions) such that the final yield of cDNA is not substantially decreased and that original RNA molecules are accurately recorded in the resulting sequencing library. This can be measured as the number of genes detected in a samples and the proportion of reads mapping to specific regions of the genome (exonic, intronic, intergenic). "Not substantially interfering" also includes polymerase processivity along the length of RNA transcripts and accuracy of nucleotide-sequence replication during RT and PCR during generation of the sequencing library, so that markedly increased frequency of incorrectly inserted bases does not occur. It will be appreciated that some level of decrease can be tolerated. It will be appreciated that methods and use of devices according to the present may include a step of RNA purification. Such purification may include e.g. RNA precipitation or purification that removes some or all of the one or more RNA inhibitors.

The amounts or combined amounts of agents of this invention that are inhibitory toward a given RNase or mixture of RNases or which render one or more RNases inactive can be readily determined by one of ordinary skill in the art without undue experimentation in view of the teachings herein and in view of what is generally known in the art.

For example, the purity and/or yield of RNA and cDNA retrieved in the presence of the agent can be measured using a spectrophotometer, fluorometer or Bioanalyzer/Fragment Analyzer and compared to the purity and/or yield of RNA retrieved in the absence of the agent. The quality of a total RNA prep can be assessed for signs of degradation by running a portion on an agarose or acrylamide gel or by using an instrument such as the Agilent Bioanalyzer. Examples of methods for

assessing the quantity of RNA include using: UV absorbance, fluorescence, and an Agilent Bioanalyzer. RNA and resulting cDNA can be analysed by fluorometry for quantification and the Bioanalyzer (or equivalent device) for quantification and RNA integrity evaluation. A fluorometer (such as Life Technologies' Qubit) measures the concentration of RNA or DNA bound to a fluorescent dye. The concentration of RNA and cDNA can also be estimated from a Bioanalyzer or Fragment Analyzer trace. Another way to quantitate RNA is by measuring the absorbance at 260 nm. In case of full-length cDNA libraries, the size distribution of yielded cDNA after reverse transcription and PCR amplification provides an accurate readout of the underlying RNA integrity. Mammalian mRNA and full-length cDNA samples should display a characteristic peak at approximately 2000 bp, reflecting the length distribution of mRNAs in mammalian cells. Degradation, due to failed RNase inhibition, display an mRNA and cDNA size distribution skewed towards shorter fragments.

The agents of this invention can be used in combination or can be combined with any art-known RNase inhibitor (that are or are not agents of this invention) to achieve a desired inhibitory effect on or inactivation of one or more RNases.

It will be appreciated that the preparation of a cDNA library is the first step in a method of RNA sequencing (RNA-seq). By "RNA sequencing" or "RNA-seq" we include the meaning of a genomic approach for the detection and quantitative analysis of RNA molecules in a biological sample by the readout of nucleotide sequences. RNA-seq is a multi-purpose methodology that is increasingly used in biological, biomedical and clinical settings. RNA-seq can for example be useful for studying cellular states and responses *in vivo* and *in vitro* by studying protein-encoding mRNA molecules as well as non-protein-coding RNAs (collectively termed the 'transcriptome'). RNA-seq is also a useful

methodology to detect foreign biological material or infection in a sample, such as that of an RNA virus or bacteria transcribing their nucleic acid. RNA-seq is furthermore a useful readout in various in vitro applications and synthetic biology utilizing RNA.

The method and devices of the invention may be used for bulk RNA sequencing or single-cell RNA sequencing methods. By "bulk RNA sequencing" we include the meaning of the sequencing of RNA isolated from pools of cells, including tissues, blood, secretions, tissue sections etc. By "bulk RNA sequencing" we also include the meaning of the sequencing of RNA from pools of cells, including tissues, blood, secretions, tissue setions etc. By "single cell RNA sequencing" or "scRNAseq" we include the meaning of the sequencing of RNA isolated from an individual cell which allows comparison of the transcriptomes of individual cells. Single-cell RNA-seq methods can also be used to detect RNA of parts or sub-compartments of a cell. The performance of scRNAseq methods can be characterized using single cells (generally containing 10-30 pg of total RNA in case of mammalian cells) or low amounts of input RNA, such as 10-100 pg of total RNA from an pool of RNA extracted from multiple cells.

It will also be appreciated that the methods of the present invention can be used as part of any "multiomics" method, i.e., a method that combines preparing a cDNA sequencing library from one part or fraction of the sample material and measurement of another biological modality from another part or fraction of the same sample, such as for example a DNA or protein library.

Where reference is made herein to a method comprising two or more defined steps, the defined steps can be carried out in any order or simultaneously (except where the context excludes that possibility), and the method can include one or more other steps which are carried out

before any of the defined steps, between two of the defined steps, or after all the defined steps (except where the context excludes that possibility). Moreover, the method can be paused following one or more steps and resumed at a later stage, if technically appropriate to do so.

By "cDNA sequencing library" (may also be termed "next generation sequencing (NGS) library") we include the meaning of a collection of complementary DNA (cDNA) fragments, which together constitute some portion of the transcriptome of a single cell or a plurality of cells. The collection of cDNA fragments in the library include a partial or complete sequencing platform adapter sequence at their termini useful for sequencing using a sequencing platform of interest.

Once prepared, the cDNA sequencing library can be subject to a full-length transcript, or 3'/5'-end sequencing protocol. By a "full-length transcript sequencing protocol" we include the meaning of methods that generates sequencing-read coverage across most of the length of the RNA transcripts, such as for example in Smart-seq (Ramsköld, D. *et al*. Nat. Biotechnol. 30, 777–782 (2012)); Smart-seq2 (Picelli, S. *et al*. Nat. Methods 10, 1096–1098 (2013)); Smart-seq3 (Hagemann-Jensen, M. et al., 2020 and WO2020136438A1); and Smart-seq3xpress (Hagemann-Jensen, M. et al., Nat Biotechnol 40, 1452–1457 (2022)).

By "3'/5'-end sequencing protocol" we include the meaning of methods that generates sequencing-read coverage in either 3' or 5' end of the RNA transcripts coverage across most of the length of the RNA transcripts, such as for example in STRT-seq-2i (Hochgerner, H. *et al*. Sci. Rep. 7, 16327 (2017)); SCRB-seq (Soumillon, M., Cacchiarelli, D., Semrau, S., van Oudenaarden, A. & Mikkelsen, T. S. Preprint at bioRxiv https://doi.org/10.1101/003236 (2014)).

By "sequencing platform adapter sequence" or "sequencing platform adapter construct" we include the meaning of a nucleic acid construct that includes at least a portion of a nucleic acid domain (e.g., a sequencing platform adapter nucleic acid sequence) utilized by a sequencing platform of interest.

Most of the current sequencing platforms use clonal amplification to create clusters of identical molecules that are tethered next to each other on a solid support. For the Illumina platform the clusters are attached to the surface of a flow-cell, while for the 454, IonTorrent, and SOLiD platforms the clusters are generated on beads using emulsion PCR. Regardless of the platform, two types of adapter sequence are generally required: (1) platform-dependent domains that are required for clonal amplification and attachment to the sequencing support; and (2) a sequencing primer binding domain for priming the sequencing reaction. In addition, several optional elements may be present, including sequence tags to allow for multiplexing (known as barcodes or indices), unique molecular identifiers (UMIs), and/or a second sequence-priming site to allow for sequencing of the insert from the other side (known as paired-end sequencing).

In certain aspects, a sequencing platform adapter sequence includes one or more nucleic acid domains selected from: a platform-dependent domain that specifically binds to a surface-attached sequencing platform oligonucleotide (e.g., the P5 or P7 oligonucleotides attached to the surface of a flow cell in an Illumina® sequencing system); a sequencing primer binding domain (e.g., a domain to which the Read 1 or Read 2 primers of the Illumina® platform may bind); a barcode domain (e.g., a domain that uniquely identifies the sample source of the nucleic acid being sequenced to enable sample multiplexing by marking every molecule from a given sample with a specific barcode or "tag"); a barcode sequencing primer binding domain (a domain to

which a primer used for sequencing a barcode binds); a unique molecular identification domain (e.g., a molecular index tag, such as a randomized tag of 4, 6, or other number of nucleotides) for uniquely marking molecules of interest to determine expression levels based on the number of instances a unique tag is sequenced; or any combination of such domains.

In certain aspects, a barcode domain (e.g., sample index tag combination including a unique index or unique dual indexes (UDIs)) and a unique molecular identifier (UMI) domain (i.e., molecule index tag) may be included in the same nucleic acid domain. A sequencing platform adapter construct, when present, may include one or more nucleic acid domains of any length and sequence suitable for the sequencing platform of interest. In certain aspects, the nucleic acid domains are from 4 to 200 nts in length. For example, the nucleic acid domains may be from 4 to 100 nts in length, such as from 6 to 75, from 8 to 50, or from 10 to 40 nts in length. The sequencing platform adapter construct may include a nucleic acid domain that is from 2 to 8 nucleotides in length, such as from 9 to 15, from 16 to 22, from 23 to 29, or from 30 to 36 nts in length.

Such sequencing platform adapter constructs can be added to each end of the insert during the first- and/or second-strand synthesis steps. In this case the reverse transcriptase primer can contain an overhanging or nested sequence that does not anneal to the RNA template but contains at least a portion of the adapter sequences. In a similar manner the forward PCR primer can contain over-hanging sequences and therefore introduce such adapters. Alternatively, adapters can be introduced via ligation. This approach is used in the Illumina TruSeq Small RNA kit, the NEBNext Small RNA prep kit, and in the SOLiD RNA kits from Life Technologies. These kits use ligation procedures that allow two different

adapters to be ligated onto each end of the target RNA. These adapters are then used to prime the first- and second-strand synthesis reactions resulting in cDNAs terminated by the appropriate adapter sequences.

It will be appreciated that the nucleotide sequences of nucleic acid domains useful for sequencing on a sequencing platform of interest may vary and/or change over time. Adapter sequences are typically provided by the manufacturer of the sequencing platform (e.g., in technical documents provided with the sequencing system and/or available on the manufacturer's website). Based on such information, the sequence of any sequencing platform adapter domains, such as the template switch oligonucleotide, first strand cDNA primer, amplification primers, and/or the like, may be designed to include all or a portion of one or more nucleic acid domains in a configuration that enables sequencing the nucleic acid insert (corresponding to the template RNA) on the platform of interest.

By "sequencing" we include the meaning of high throughput sequencing. By "high throughput sequencing" we include the meaning of the simultaneous or near simultaneous sequencing of thousands of nucleic acid molecules. High throughput sequencing is sometimes referred to as "next generation sequencing (NGS)" or "massively parallel sequencing".

Sequencing platforms of interest include, but are not limited to, sequencing platforms provided by Illumina® (e.g., the NextSeq™, HiSeq™, MiSeq™, NovaSeq™ and/or Genome Analyzer™ sequencing systems); Ion Torrent™ (e.g., the Ion PGM™ and/or Ion Proton™ sequencing systems); Pacific Biosciences (e.g., the PACBIO RS II sequencing system); Life Technologies™ (e.g., a SOLiD sequencing system); Roche (e.g., the 454 GS FLX+ and/or GS Junior sequencing

systems); MGI Tech Co., Ltd. "MGI" (e.g., the DNBSEQ-T7™, DNBSEQ-G400™, DNBSEQ

In a particular embodiment, the method comprises:

i. preparing a lysate and thereby releasing a plurality of RNA molecules from one or more cells, tissue, tissue extract or cell extract concomitant with or followed by application of the RNA molecules and/or lysate to a carrier impregnated with an agent wherein the agent is according to any embodiment disclosed herein;
ii. eluting the RNA from the carrier
iii. synthesizing a plurality of cDNA strands from the RNA molecules by reverse transcription; and
iv. processing the cDNA strands to generate a cDNA sequencing library.

It is appreciated that the time between in particular step i. and step ii. may be up to 30 days, such as up to 25 days, such as up to 20 days, such as up to 14 days such as up to 10 days, such as up to 7 days, such as up to 6 days, such as up to 5 days, such as up to 4 days, such as up to 3 days, such as up to 2 days, such as up to a day.

Releasing a plurality of RNA molecules from one or more cells or cell extract can be achieved by, for example, heating or freeze-thaw of cells, or by the use of detergents, chaotropic agents, mechanical methods, or other chemical methods, or by a combination of these, in the presence of an aqueous solution comprising the agent. Mechanical methods for homogenizing tissues include using cryo-grinding with a mortar/pestle, shearing using a rotor-stator homogenizer or a Dounce homogenizer, sonication, or bead-beating. After homogenization two methods are commonly used to recover RNA from the cell lysate: (1) extraction with organic solvents; or (2) solid-phase extraction on silica.

In an embodiment, releasing a plurality of RNA molecules from one or more cells or cell extract, tissue or other sample, in the presence of an aqueous solution comprising the agent comprises contacting one or more cells or cell extract with an aqueous solution to release RNA molecules. The RNA molecules are preferably poly(A) containing RNA molecules, such as mRNA molecules, and are typically present in and released from the cytoplasm of the lysed cell.

By "aqueous solution" we include the meaning of any liquid solution that can be used in a method of liberating RNA from cells. Such aqueous solutions include buffers such as sample collection buffers and lysis buffers. Examples of suitable buffers include, PBS, Tris, sodium-acetate, HEPES, MOPS. In an embodiment, the aqueous solution may be a sample collection buffer. A sample collection buffer may not comprise a detergent and/or chaotropic agent. For example, a sample collection buffer could contain the bulk sample of intact cells without detergent, and the RNA can be extracted through another means, such as using Trizol, phenol, and/or a commercially available RNA extraction kit. In an embodiment, the aqueous solution may be a lysis buffer.

By "lysis buffer" we include the meaning of a buffer used for the purpose of breaking open cells. Examples of suitable lysis buffer to which the agent could be added are described herein and are described in known protocols for preparing a cDNA library. For example, the lysis buffer may comprise enzymes (e.g. Proteinase K), detergents (e.g. Triton X-100, SDS, NP-40/Igepal, Tween-20, sodium deoxycholate, and CHAPS) and/or chaotropic agent (i.e. (compounds that disrupt both hydrophobic and hydrogen-bond interactions, such as guanidine salts) together with the agent. For instance, Triton X-100 could be used as a

detergent when lysing cells. Guanidinium is a strong protein denaturant capable of denaturing recalcitrant proteins such as RNases. In an embodiment, the buffer is a lysis buffer comprising 0.1-1% Triton X-100 and the agent. In an embodiment the buffer is a lysis buffer comprising 0.1% Triton X-100. A mild lysis procedure can advantageously be used to prevent the release of nuclear chromatin, thereby avoiding genomic contamination of the cDNA library, and to minimize degradation of mRNA. For example, heating the cells at 72°C for 2-10 minutes in the presence of mild detergent (together with the agent) is generally sufficient to lyse cells.

The term "one or more cells" refers to any number of (e.g. unlysed) cells desired to be analysed. One or more cells may include at least 1 cell, at least 10 cells, or alternatively at least 25 cells, or alternatively at least 50 cells, or alternatively at least 100 cells, or alternatively at least 200 cells, or alternatively at least 500 cells, or alternatively at least 1000 cells, or alternatively 5,000 cells or alternatively 10,000 cells. One or more cells may include from 10 to 100 cells, or alternatively from 50 to 200 cells, alternatively from 100 to 500 cells, or alternatively from 100 to 1000, or alternatively from 1,000 to 5,000 cells. One or more cells may include 10,000 cells, 20,000 cells, 30,000 cells, 40,000 cells, 50,000 cells, 60,000 cells, 70,000 cells, 80,000 cells, 90,000 cells or alternatively 100,000 cells.

The "one or more cells or cell extract" comprises template RNA and may be derived from any sample of interest, including but not limited to, a single cell, a plurality of cells (e.g., cultured cells), a tissue, an organ, or an organism (e.g., bacteria, yeast, or higher eukaryotic organisms, such as a plant, or a mouse, or a worm, or the like). In certain embodiments, the one or more cells or cell extract are derived from a tissue, organ, and/or the like of a mammal (e.g., a human, a rodent

(e.g., a mouse), or any other mammal of interest). The one or more cells or cell extract can be derived from live samples, non-conserved samples, preserved samples, embalmed samples and/or fixed samples. In certain aspects, the RNA molecules are liberated into an aqueous solution comprising the agent from one or more cells in a fixed biological sample, e.g., formalin-fixed, formaldehyde/paraformaldehyde-fixed, paraffin-embedded (FFPE) tissue. RNA from one or more cells in FFPE tissue may be released using commercially available kits - such as the NucleoSpin® FFPE RNA kits by Clontech Laboratories, Inc. (Mountain View, CA).

Further non-limiting examples for samples from which one or more cells or cell extract can be derived from includes a cell culture sample, blood, serum, plasma, reticulocytes, lymphocytes, any product prepared from blood or lymph, bone marrow tissue, cerebrospinal fluid, sweat, tear, saliva, sputum, amniotic fluid, seminal fluid, vaginal excretion, serous fluid, synovial fluid, pericardial fluid, peritoneal fluid, pleural fluid, transudates, exudates, cystic fluid, bile, urine, gastric fluid, intestinal fluid, or faecal samples), any type of tissue biopsy (e.g. a tumour biopsy, a muscle biopsy, a liver biopsy, a kidney biopsy, a bladder biopsy, a bone biopsy, a cartilage biopsy, a skin biopsy, a pancreas biopsy, a biopsy of the intestinal tract, a thymus biopsy, a uterus biopsy, a testicular biopsy, an eye biopsy or a brain biopsy), or any other biological material that may harbor RNA molecules. Suitable samples containing cells further comprise clinical samples (which are samples provided by a patient), biological swabs and biological washes. Suitable samples containing cells may be fresh or may have been stored (e.g. cryopreserved), such as at -80°C. Furthermore, in general, cells from any population can be used in the methods, such as a population of prokaryotic or eukaryotic single-celled organisms including bacteria or yeast.

Further, the biological sample may comprise one or more viruses.

After obtaining an RNA preparation that is suitable for RNA-seq (step 1) the RNA is typically converted to double-stranded complementary DNA (cDNA). Currently available sequencing technologies require a DNA template with platform-specific "adaptor" sequences at either end of each molecule. Generating the cDNA, adding the adaptors, and (if necessary) amplifying the DNA for sequencing encompasses steps (ii) and/or (iii) of the method described herein.

In order to convert RNA to DNA the RNA must be used as a template for a DNA polymerase. Most DNA polymerases cannot use RNA as a template. However, retroviruses encode a unique type of polymerase known as reverse transcriptases, which are able to synthesize DNA using an RNA template.

By "RNA molecules" we include the meaning of the template ribonucleic acid (RNA) liberated from the one or more cells or contained within the cell extract. It may be a polymer of any length composed of ribonucleotides, e.g., 10 nts or longer, 20 nts or longer, 50 nts or longer, 100 nts or longer, 500 nts or longer, 1000 nts or longer, 2000 nts or longer, 3000 nts or longer, 4000 nts or longer, 5000 nts or longer or more nts. In certain aspects, the template ribonucleic acid (RNA) is a polymer composed of ribonucleotides, e.g., 10 nts or less, 20 nts or less, 50 nts or less, 100 nts or less, 500 nts or less, 1000 nts or less, 2000 nts or less, 3000 nts or less, 4000 nts or less, or 5000 nts or less, 10,000 nts or less, 25,000 nts or less, 50,000 nts or less, 75,000 nts or less, 100,000 nts or less. The template RNA may be any type of RNA (or sub-type thereof) including, but not limited to, a messenger RNA (mRNA), a microRNA (miRNA), a small interfering RNA (siRNA), a transacting small interfering RNA (ta-siRNA), a natural small interfering

RNA (nat-siRNA), a ribosomal RNA (rRNA), a transfer RNA (tRNA), a small nucleolar RNA (snoRNA), a small nuclear RNA (snRNA), a long non-coding RNA (lncRNA), a non-coding RNA (ncRNA), a transfer-messenger RNA (tmRNA), a precursor messenger RNA (pre-mRNA), or any combination of RNA types thereof or subtypes thereof.

As discussed herein, the inhibitory agents can be bound to an absorbent material (e.g., fiber material, sponge, paper, etc) and so forms or is comprised in a device.

In an embodiment, the absorbent material is a fibrous material, such as paper. Fibrous materials may comprise cotton fibre, glass fibre, polymer, cellulose, or a combination thereof. It will be appreciated that the fibrous material is suitable for binding RNA. Chemical groups on paper surface (e.g., hydroxyl and carboxyl groups) can help immobilize chemical reagents on paper. For example, cellulose contains hydroxyl groups on its surface and has the properties of hydrophilicity, easy usability, high porosity, high mechanical strength. Cotton fiber, a natural material, also contains hydroxyl groups on its surface. Glass fiber is a kind of synthetic fiber and is formed of silica-based thin strands. Such fibrous material can be 100 µm – 500 µm thick. Examples of fibrous material include filter paper such as Flinders Technology Associates (FTA) cards®, Nobuto filter paper, Whatman® paper, and iBlot Filter Paper.

Absorbent material may also be in the form of e.g. polyethylene foam.

Absorbent material may also be in the form of polyurethane foam.

Accordingly, the invention also provides a solid support comprising the agent as defined in any of the embodiments herein.

It will be appreciated that the solid support is capable of binding to the agent defined herein, or otherwise containing the agent defined herein.

By "binding to the agent" we include the meaning that the agent is immobilised onto a solid support such as an absorbent material. Immobilisation may be via a covalent or non-covalent interaction.

Preparation of agent-coated supports can be achieved using an *in situ* polymerization method or by incubating material or surfaces with the inhibitory agent. Those of ordinary skill in the art will appreciate that other means for directly or indirectly (through a linker) coupling of agents of this invention to solid supports are available in the art and can be employed in the practice of this invention.

Where immobilisation is via a non-covalent interaction, the support may be coated with a moiety that binds non-covalently to the agent. Additionally or alternatively, the agent can be adsorbed to the support either through the porous nature of the support or absorbent material, or through weak hydrophobic and/or polar interactions between the support and the agent.

Where immobilisation is via a covalent interaction, the support may be coated with a pre-activated functional group to covalently immobilize the agent to the surface. Any suitable system for covalent interactions may be used, including ELISA principles, and pre-activated surfaces to facilitate covalent bonds. Also, any suitable commercially available support that allows for covalent interactions can be used, such as those available from Corning®.

By "containing" we include the meaning that the solid support has one or more pores within which the agents as defined herein can be retained. In most cases, biological samples (e.g., whole blood, saliva, urine, tissue and cells and lysates thereof) need be stored and transported at low temperature before analysis. Tubes, bottles and refrigerators are normally utilized to collect and store samples. Compared to these methods, absorbent material has the advantages of

low cost, porous structure, portability and ease of use. Thus, improved paper-based (and other absorbent material) sample storage and collection methods are required.

The inventors have shown that absorbent material pre-incubated (also referred to as impregnated) with the agents defined herein allow storage and collection of RNA containing samples at room temperature and the eluted RNA is compatible with RNA sequencing. The absorbent material can be stored at room temperature. After storage, the RNA can be eluted and analysed for RNA quality, cDNA yield by methods known in the art and as described herein.

It will be appreciated that the absorbent material is suitable for receiving a liquid solution comprising the agent. In an embodiment, the absorbent material is pre-incubated with a liquid solution comprising an effective amount of the agent, and may be dried to remove all or most of the liquid phase. Effective amounts include 1µg agent /mL water to 1000mg agent /mL water.

In an embodiment the absorbent material is made of cotton fiber or wood pulp, or other cellulose-based materials.

In an embodiment the absorbent material is polyethylene foam and the agent is sodium alginate.

In an embodiment the absorbent material is polyethylene foam and the agent is PVSA.

In an embodiment the absorbent material is polyethylene foam and the agent is heparin.

In an embodiment the absorbent material is polyethylene foam and the agent is dextran sulfate.

In an embodiment the absorbent material is polyurethane foam and the agent is sodium alginate.

In an embodiment the absorbent material is polyurethane foam and the agent is PVSA.

In an embodiment the absorbent material is polyurethane foam and the agent is heparin.

In an embodiment the absorbent material is polyurethane foam and the agent is dextran sulfate.

In an embodiment the device is in the form of a column.

In an embodiment such a column is in a support, such as in a tube.

In an embodiment, the elusion procedure of such a column can include centrifugation.

Aspects of the present disclosure also include kits. As used herein, the term "kit" refers to one or more suitably aliquoted compositions or reagents for use in the methods of the present disclosure, preferably together with a device as disclosed. Some components of the kits may be packaged either in aqueous or lyophilized form, while other may be dry or substantially dry. The container means of the kits may include at least one vial, test tube, flask, bottle, syringe, or other container means, into which a component may be placed, and preferably, suitably aliquoted. Where there is more than one component in the kit, the kit also will generally contain a second, third, or other additional container into which the additional components may be separately placed. However, various combinations of components may be contained in a vial. The kits of the present disclosure also will typically include a means for containing the reagent containers in close confinement for commercial sale. Such containers may include injection or blow

moulded plastic containers into which the desired vials are retained, for example.

The term "absorbent material" includes but is not limited to cellulose paper, such as filter paper, alpha cellulose cotto paper (such as e.g. Whatman filter paper) wood pulp paper such as coffee filter (tree pulp) and foams such as e.g. polyethylene foam or polyurethane foam. By absorbent is intended that the material is able to absorb and retain a liquid such as an aqueous solution of an RNase inhibitor and/or other compounds, or a biological sample such as e.g. a lysate obtained from a tissue or cell culture or other sources. Absorbent materials according to the present invention may absorb a liquid, undergo drying and be able to absorb a liquid or sample multiple times such as at least once. During drying it is contemplated that only the aqueous phase is removed from the absorbent material, whereas solutes and/or other components may retain absorbed in the material.

By soaking or wetting an absorbent material with a solution of e.g. one or more RNase inhibitors according to the invention, the material may, optionally subsequent to drying, be said to be impregnated with said one or more RNase inhibitors.

Solutes or components absorbed in the absorbent material may be subject to elution from the material. This includes elution of RNA, such as for use in reverse transcription. It is contemplated that such elution may also release RNase inhibitor from the absorbent material, rendering the eluted RNA under continued protection from RNase activity.

To prevent microbial growth, antimicrobial agents can be incorporated into absorbent materials, sample buffers, and lysis buffers. These agents may include compounds such as sodium azide, triclosan, sorbic acid, and benzoic acid, as well as metal-based compounds like silver

nitrate, silver sulfadiazine, silver nanoparticles, and copper-based compounds such as copper sulfate and copper oxide. Additional antimicrobial agents and combinations may be used depending on specific needs and circumstances, including the biological sample source, sampling environment, and storage conditions.

Vinylsulfonic acid (VSA) is a organosulfur compound with the chemical formula CH2=CHSO3H, and is a sulfonated monomer. Polymerization of VSA gives polyvinylsulfonic acid.

Sodium alginate $(NaC6H_7O_6)_n$, NaAlg, is the sodium salt of alginic acid, and is a carboxylated polysaccharine derived from algae with a repeating disaccharide block. The structure of the repeating blocks are (1→4)-linked β-D-mannuronate (M) and α-L-guluronate (G) residues.

Heparin is a member of the glycosaminoglycan family of carbohydrates and consists of a variably sulfated repeating disaccharide unit. The repeating unit consists of is glucosamine and uronic acid.

Dextran sulfate is a sulfated polymer consisting of (1→6)-α-linked anhydroglucose molecules. It has a molecular weight of greater than 500,000 Daltons.

Fucoidan is a sulfated polysaccharine found in algae consisting predominantly fucose sugar molecules. It has a molecular weight ranging from approximately 50-1000 kiloDaltons.

General

It should be understood that any feature and/or aspect discussed above in connections with the compounds according to the invention apply by analogy to the methods described herein.

The terms “a” and “an” are used as meaning one or more, such at least one.

The following figures and examples are provided below to illustrate the present invention. They are intended to be illustrative and are not to be construed as limiting in any way.

The examples demonstrate that effective dry-storage devices for biological RNA samples can be constructed by impregnating an absorbent material with sodium alginate (NaAlg), dextran sulfate (DexSulf), fucoidan, heparin, polyvinyl sulfonic acid (PVSA), vinyl sulfonic acid (VSA), and/or 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), onto which lysed samples can be placed and absorbed.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Various chemical compounds preventing RNA degradation in RNA dry-storage devices after 1 day of storage. Bioanalyzer traces from amplified cDNA libraries generated from (a) 25 lysed cells, or the equivalent of 25 cells-worth of RNA eluted from lysed cells stored for 1 day on cellulose paper material pre-treated with (b) NaAlg, (c) fucoidan, (d) heparin, (e) VSA , (f) PVSA, (g) HEPES, (h) DexSulf, (i) MES, (j) VPA, (k) PVPA, (l) chondroitin, (m) MOPS, or (n) TAPS at various concentrations stated in each panel, or (o) water. For all panels, the x-axis represents fragment size in base pairs and the y axis represents fluorescence units (FU) relating to detected cDNA abundance. Tick marks on the y-axis correspond to increments of 500 FUs. Concentrations from left to right for each row denote decreases in the inhibitor by 10-fold.

FIG. 2. Various chemical compounds preventing RNA degradation in RNA dry-storage devices after 3 days of storage. Bioanalyzer traces from amplified cDNA libraries generated from the

equivalent of 25 cells-worth of RNA eluted from lysed cells stored for 3 days on cellulose paper material pre-treated with (a) NaAlg, (b) fucoidan, (c) heparin, (d) VSA , (e) PVSA, (f) HEPES, (g) DexSulf, (h) MES, (i) VPA, (j) PVPA, (k) chondroitin, (l) MOPS, or (m) TAPS at various concentrations stated in each panel, or (n) water. For all panels, the x-axis represents fragment size in base pairs and the y axis represents fluorescence units (FU) relating to detected cDNA abundance. Tick marks on the y-axis correspond to increments of 500 FUs. Concentrations from left to right for each row denote decreases in the inhibitor by 10-fold.

FIG. 3. Effect of elution time on RNA library yield. Bioanalyzer traces from amplified cDNA libraries generated from the equivalent of 25 cells-worth of RNA eluted from lysed cells stored for 1 day on cellulose paper material and eluted. (a) The eluted samples were incubated at room temperature for 2 minutes prior to Smart-seq2 cDNA preparation. (b) The eluted samples were incubated at room temperature for 5 minutes prior to Smart-seq2 cDNA preparation. (c) The eluted samples were incubated at room temperature for 30 minutes prior to Smart-seq2 cDNA preparation. For all panels, the x-axis represents fragment size in base pairs and the y axis represents fluorescence units (FU) relating to detected cDNA abundance.

FIG. 4. Various chemical compounds preventing RNA degradation in RNA dry-storage devices after 1 day of dry storage and 6 days of elution. Bioanalyzer traces from amplified cDNA libraries generated from the equivalent of 25 cells-worth of RNA eluted from lysed cells stored dry for 1 day in cellulose paper material and then kept incubated in elution solution for an additional 6 days

("1+6"). Cellulose paper material was pre-treated with (a) NaAlg, (b) fucoidan, (c) heparin, (d) VSA, (e) PVSA, (f) HEPES, (g) DexSulf, (h) MES, (i) VPA, (j) PVPA, (k) chondroitin, (l) MOPS, or (m) TAPS at various concentrations stated in each panel, or (n) water. For all panels, the x-axis represents fragment size in base pairs and the y axis represents fluorescence units (FU) relating to detected cDNA abundance. Tick marks on the y-axis correspond to increments of 500 FUs. Concentrations from left to right for each row denote decreases in the inhibitor by 10-fold.

FIG. 5. Comparison of different materials used as sample carrier in RNA dry-storage. Bioanalyzer traces from amplified cDNA libraries generated from the equivalent of 25 cells-worth of RNA eluted from lysed cells stored in various materials treated with various amounts of PVSA (3000 µg/mL, 300 µg/mL, 30 µg/mL) or water (left to right). The stated PVSA concentrations apply to all materials displayed column-wise. Samples were stored on (a) polyethylene foam, (b) α-cellulose sheet, (c) wood pulp filter, (d) cellulose sheet, or (e) PES filter for samples eluted after 1 day. (f-j) Same as panel *(a-f)* but for samples eluted after 6 days. (k-o) Same as panel *(a-f)* but for sample carriers placed in elution solution after 1 day of dry storage and then kept incubated in elution solution for an additional 5 days ("1+5"). For all panels, the x-axis represents fragment size in base pairs and the y axis represents fluorescence units (FU) relating to detected cDNA abundance. Tick marks on the y-axis correspond to increments of 500 FUs.

FIG. 6. Polyurethane foam and polyethylene foam are suitable sample carrier materials for RNA dry-storage. Bioanalyzer traces

from amplified cDNA libraries generated from the equivalent of 25 cells-worth of RNA eluted from lysed cells stored in various materials treated with two concentrations of PVSA (300 µg/mL and 30 µg/mL, left to right). The stated PVSA concentrations apply to all materials displayed column-wise. Samples were stored on (a) polyethylene foam, (b) polyurethane foam, (c) wood pulp filter (d) cellulose sheet, or (e) PES filter for samples eluted after 3 days. For all panels, the x-axis represents fragment size in base pairs and the y axis represents fluorescence units (FU) relating to detected cDNA abundance. Tick marks on the y-axis correspond to increments of 500 FUs.

FIG. 7. Combinatorial analysis of materials and chemical inhibitors for RNA preservation. Bioanalyzer traces from amplified cDNA libraries generated from the equivalent of 25 cells-worth of RNA eluted from lysed cells stored in various materials pre-treated with the following inhibitors and concentrations: (a) 400 µg/ml NaAlg, (b) 10k µg/ml fucoidan, (c) 20 µg/ml heparin, (d) 200k µg/ml VSA, (e) 20k µg/ml HEPES, (f)) 250 µg/ml DexSulf, and (g) water (no inhibitor). Samples were stored on polyethylene foam, polyurethane foam, wood pulp filter, cellulose sheet, or PES filter (left to right) for samples eluted after 3 days. The stated dry-storage matrix applies to all materials displayed column-wise. For all panels, the x-axis represents fragment size in base pairs and the y axis represents fluorescence units (FU) relating to detected cDNA abundance. Tick marks on the y-axis correspond to increments of 500 FUs.

FIG. 8. Long-term RNA preservation in polyurethane foam.

Bioanalyzer traces from amplified cDNA libraries generated from the equivalent of 25 cells-worth of RNA eluted from lysed cells stored in

various materials treated with various amounts of PVSA (3000 µg/mL, 300 µg/mL, 30 µg/mL) or water (left to right). The stated PVSA concentrations apply to all materials displayed column-wise. Samples were stored on (a) polyurethane foam, (b) α-cellulose sheet, (c) wood pulp filter (d) cellulose sheet, or (e) PES filter for samples eluted after 11 months. For all panels, the x-axis represents fragment size in base pairs and the y axis represents fluorescence units (FU) relating to detected cDNA abundance. Tick marks on the y-axis correspond to increments of 500 FUs.

EXAMPLES

EXAMPLE 1 - Development of Dry-storage Devices for Effortless Collection and Preservation of RNA Samples

Methods

Preparation of matrix material for RNA storage devices

As matrix for sample storage, we prepared 3 x 4 mm^2 rectangles of cotton fibre cellulose filter paper. These absorbent materials, were placed into 200 µL microcentrifuge tubes containing various chemical compounds, diluted in water to different concentrations (described below). We impregnated the absorbent materials in the following agents, at five different concentrations per chemical compound: sodium alginate (NaAlg), fucoidan, heparin, vinyl sulfonic acid (VSA), polyvinyl sulfonic acid (PVSA), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), dextran sulfate (DexSulf), 2-(N-morpholino)ethanesulfonic acid (MES), vinyl phosphonic acid (VPA), polyvinylphosphonic acid (PVPA), chondroitin, 3-(N-morpholino)propanesulfonic acid (MOPS), tris(hydroxymethyl)methylamino]propanesulfonic acid (TAPS). The concentrations tested for these agents in our study were: 40000, 4000,

400, 40, 4 µg/mL for NaAlg; 10000, 1000, 100, 10, 1 µg/mL for fucoidan; 2000, 200, 20, 2, 0.2 µg/mL for heparin; 200000, 20000, 2000, 200, 20 µg/mL VSA; 30000, 3000, 300, 30, 3 µg/mL for PVSA; 20000, 2000, 200, 20, 2 µg/mL for HEPES; 2500, 250, 25, 2.5, 0.25 µg/mL for DexSulf; 40000, 4000, 400, 40, 4 µg/mL for MES; 200000, 20000, 2000, 200, 20 µg/mL for VPA; 100000, 10000, 1000, 100, 10 µg/mL for PVPA; 20000, 2000, 200, 20, 2 µg/mL for chondroitin; 100000, 10000, 1000, 100, 10 µg/mL for MOPS; and 20000, 2000, 200, 20, 2 µg/mL for TAPS. As a negative control, we impregnated the absorbent material in water. Thus, we tested 66 different impregnations (i.e., storage device conditions). For all mentioned dilutions, as well as in all following experiments and elusions, we used nuclease-free water (Ambion, Ref AM9932). Specifically, impregnation was done by soaking the absorbent material to saturation (15 minutes incubation at room temperature) in the different chemical compounds and concentrations. The impregnated absorbent material was then lifted out from the solution using stainless steel tweezers, moved into sterile 24-well polystyrene plates with ventilating lids (loose-fitting lids protecting samples from dust but allowing evaporation), and let dry at room temperature for 24 hours.

Cell culture, collection, and lysis

We cultured human cell samples to be tested on the storage devices. Human embryonic kidney (HEK293FT) cells were cultured and expanded in standard medium (DMEM/10% FBS) in 5% CO_2 and 37°C. Aliquots of 2 million cells were collected in a 1.5 mL microcentrifuge tube, washed with 1x PBS, pelleted by brief centrifugation upon which the PBS supernatant was discarded, and frozen in a -80°C deep freezer. For the following storage experiments, cell pellets were diluted in a cell lysis solution, consisting of 1000 µL 1% Triton-X100, and pipetted up and down to achieve a lysed cell suspension. The lysed cell suspension was vortexed for 30 seconds then centrifuged at 300 g for 3 minutes to pellet

potential cell debris, such as incompletely lysed nuclei and other potentially remaining cell compartments. Consequently, the lysed cell suspension contained the input equivalent of 2000 lysed HEK293FT cells per µL.

RNA sample storage in absorbent material

The dried, pre-treated absorbent materials (sample devices) were spotted with 1.25 µL of lysed cell suspension corresponding to 2500 lysed cells (lysed supernatant after centrifugation, as described in the previous section), by pipetting the suspension onto the middle of the sample devices and letting the sample absorb and dry into the material. The lysed-cell-spotted storage devices were then incubated/dried for 1 or 3 days at room temperature in 24-well polystyrene plates with ventilating lids (loose-fitting lids protecting samples from dust but allowing evaporation).

Sample elusion and full-length cDNA library preparation

Upon 1, or 3 days of storage, the cell-spotted absorbent materials were individually placed in the wells of a sterile 96-well polystyrene plate, and sample material was eluted by adding 100 µL of nuclease-free water followed by incubation at room temperature for 3 minutes. The 96-well plate was sealed using plastic PCR sealing film, vortexed for 20 seconds, and the plates were finally centrifuged at 300 g for 1 minute. The resulting eluates were immediately used as RNA input to generate full-length cDNA libraries using the Smart-seq2 protocol (Picelli 2013, Picelli 2014), using 1 µL of eluate as input to the Smart-seq2 reaction. This volume (1 µL) corresponded to an original input of 25 lysed cells, not taking potential losses into count, e.g., due to potential trapping of material inside the absorbent material. We reasoned that RNA sample material absorbed and dried into the storage device material might require prolonged elusion time for maximum release into the elusion

solution. We tested effects of elusion time on yield by varying sample elusion time, incubating the absorbent material containing sample for either 2, 5, or 30 min in water (300 µg/mL PVSA in the absorbent material in this experiment), upon which 1 µL of eluate was used to generate cDNA using Smart-seq2. In addition, we generated "1+6 day" samples for each condition, where after elution on day 1, the absorbent material was not removed from the elusion solution but kept bathing in the liquid at room temperature for an additional 6 days, upon which 1 µL of eluate was used to generate cDNA using Smart-seq2. We reasoned that 6-day elusion should fully hydrate the absorbent material likely resulting in nearly complete release of RNA sample material absorbed and bound within the storage device material. Smart-seq2 cDNA library generation was performed as follows: Smart-seq2 lysis buffer composition was 2.5mM dNTP/each and 2.5mM Smart seq2 oligo-dT (5′-AAGCAGTGGTATCAACGCAGAGTACT30VN-3′) with 1 µL of eluant in a total buffer volume 4.5 µL. Samples in lysis buffer were incubated at 72°C for 3 min in a Bioer Life ECO thermocycler to hybridize the oligo-dT and placed on ice prior to first-strand synthesis. The following reverse transcriptase reaction contained 1x Superscript II buffer, 5mM DTT, 1M betaine, 10 mM $MgCl_2$, 1 µM Smart-seq2 TSO (5′-AAGCAGTGGTATCAACGCAGAGTACATrGrG+G-3′), and 10 U Superscript II for a total reaction volume of 10 µL. RT thermocycles were 42°C for 90 min, followed by 10 cycles of 42°C for 2 min and 50°C for 2 min. The following cDNA amplification reaction contained 1x KAPA HiFi HotStart Ready Mix and 80 nM ISPCR primers (5′-AAGCAGTGGTATCAACGCAGAGT-3′); total reaction volume 25 µL. Thermocycles for Smart-seq2 cDNA amplification were 98°C for 3 min, followed by 18 cycles of 98°C for 20 sec, 67°C for 15 sec, and 72°C for 6 min, followed by a final incubation at 72°C for 5 min. Amplified cDNA samples were bead purified (AMPure XP, Beckman) at a ratio of 0.8:1 beads:cDNA (20 µL beads:25 µL cDNA for Smart-seq2) and inspected on

a Bioanalyzer 2100 (High Sensitivity DNA kit, Agilent). Input control libraries (samples going into the storage devices) were generated directly from the original lysed-cell supernatant (i.e., samples not kept in storage devices) diluted in nuclease-free water so that their input to the Smart-seq2 reactions corresponded to 25 lysed cells.

cDNA yield and quality

To inspect cDNA yield and cDNA size distribution of the samples in each condition, the resulting purified Smart-seq2 cDNA libraries were analyzed using Agilent Bioanalyzer 2100 High sensitivity DNA chips. The shape of full-length cDNA traces is known in the field to reflect the quality and integrity of the underlying mRNA sample (Trombetta 2014), and an intact library (satisfactory library) is expected to have a peak around ~2kb, reflecting the median length of full-length mRNA transcripts in human cells. Exact patterns of spikes in the cDNA traces can further be experiment- or cell-type specific, as abundant cell-type-specific or condition-specific RNA transcripts, for which one or a few transcripts account for a large proportion of total cellular transcripts lead to additional peaks on the Bioanalyzer trace.

Results

Inspecting the cDNA yield and size distribution resulting from each condition, we first expectedly observed intact full-length cDNA libraries from the positive control samples (original lysed-cell supernatant diluted in water) (**Fig. 1a**), demonstrating that the lysed-cell input material spotted onto the storage devices contained intact mRNA. Thus, potential differences in cDNA traces after storage in the different pre-treated absorbent materials would reflect the devices' capability to preserve the RNA sample over time and its release during sample elusion.

Storage day 1: Inspecting the cDNA traces of samples from the different dry-storage devices after 1 day of storage, we observed pronounced differences both in yield and cDNA size distribution (**Fig. 1b-o**), demonstrating distinct variation in their capacity to preserve the RNA sample. Notably, storage devices impregnated with the following agents resulted in satisfactory cDNA libraries in terms of yield and fragment-size distribution: NaAlg (4000, 400 µg/mL); fucoidan (10000 µg/mL); heparin (20, 2 µg/mL); VSA (200000, 20000 µg/mL); PVSA (3000, 300, 30 µg/mL); HEPES (20000, 2000 µg/mL); DexSulf (250 µg/mL) (**Fig. 1b-h**). Lower-concentration treatments using these mentioned agents produced degraded traces, i.e., size-distribution shifted towards shorter fragments or the lack of sufficient cDNA yield. Out of the working agents and concentrations, the following produced superior yield and/or size-distribution profiles compared to other working conditions: NaAlg (4000 µg/mL), VSA (200000 µg/mL), PVSA (3000, 300 µg/mL), and HEPES (20000 µg/mL). Of note, very high concentrations of the chemicals used to impregnate the storage device may negatively affect reverse transcription and/or PCR in cDNA library preparation (Smart-seq2). That is, cDNA library amplification would fail due to RT/PCR inhibition of the detection assay rather than the lack of intact RNA in the high-concentration condition. RT/PCR-inhibiting amounts of (RNA-protecting) impregnation agents in the eluate could be removed by subjecting the eluate to a conventional RNA purification step, such as RNA precipitation or bead purification. Therefore, rationally, the results should be interpreted as RNA preservation being successfully achieved if satisfactory cDNA traces are achieved *above* a given concentration threshold, i.e., NaAlg (≥400 µg/mL); fucoidan (≥10000 µg/mL); heparin (≥ 2 µg/mL); VSA (≥20000 µg/mL); PVSA (≥30 µg/mL); HEPES (≥2000 µg/mL); DexSulf (≥250 µg/mL). Conversely, storage devices impregnated with the following agents failed to produce acceptable cDNA libraries in any concentration range tested: MES, VPA, PVPA, chondroitin,

MOPS, TAPS, and water only (negative control) (**Fig. 1i-o**), demonstrating that these compounds were unsuitable for the construction of an RNA dry-storage device.

Storage day 3: cDNA traces of samples from the variously impregnated storage conditions after 3 days of storage revealed a very similar pattern as observed at storage day 1, reenforcing our conclusions regarding preferred chemical treatments to achieve RNA dry-storage devices (**Fig. 2**). Indeed, we observed that impregnation with the following agents resulted in satisfactory cDNA libraries: NaAlg (4000, 400 µg/mL); fucoidan (10000 µg/mL); heparin (20, 2 µg/mL); VSA (200000, 20000 µg/mL); PVSA (3000, 300 µg/mL); HEPES (20000, 2000 µg/mL); DexSulf (250 µg/mL) (**Fig. 2a-g**). Notably, one low-concentration conditions that produced satisfactory libraries at day 1, namely PVSA 30 µg/mL, resulted in a more degraded profile at day 3 (**Fig. 2e**), highlighting concentration dependency in the devices' ability to prevent RNA degradation over time. Conversely, storage devices impregnated with the following agents failed to produce acceptable cDNA libraries in any concentration range tested also on day 3: MES, VPA, PVPA, chondroitin, MOPS, TAPS, and water only (negative control), again demonstrating that these compounds were unsuitable for the construction of an RNA dry-storage device (**Fig. 2h-n**).

Effects of elusion time: We compared the cDNA traces resulting from varying elusion time, having the absorbent material containing sample elute for 2, 5, or 30 min. We expectedly observed that elusion time did not notably affect size distribution (i.e., RNA integrity) (**Fig. 3**). However, elusion time did indeed affect cDNA yield obtained from the storage devices, producing higher yield when incubation time was increased (**Fig. 3**). This can be understood, to reach maximum recovery of RNA from storage device, which is bound and dried, potentially

crystallized, inside the absorbent material, sufficient time for hydrolysis or release is needed.

Storage for 1+6 days: We inspected cDNA libraries generated from "1+6 day" samples for each storage devices impregnated with the various chemical compounds, i.e., samples where after elution on day 1, the absorbent material was not removed from the elusion solution but kept bathing in the liquid at room temperature for an additional 6 days to assure maximum hydrolysis. We once again observed, similarly to our results from day 1 and 3 treatments, that conditions with NaAlg, fucoidan, heparin, VSA, PVSA, HEPES, and DexSulf produced satisfactory cDNA traces (**Fig. 4**). However, for most conditions, we observed that cDNA yields tended to be considerably higher upon 6-day elusion compared to that produced from brief (3 min) elusion of day 1 samples (**Fig. 1**). These results reinforce our conclusion from the previously mentioned experiments in which elusion time was varied 1, 5, 30 min (**Fig. 3**), i.e., that elusion time is an important consideration for maximizing RNA recovery from dry-storage devices, and importantly extending this conclusion to devises of all tested chemical impregnation agents tested within the present study.

Conclusion

Here, we have provided efficient dry-storage devices for the preservation of biological samples, by experimentally demonstrating preservation of RNA from lysed human cell samples within/on an absorbent material, when the material is impregnated with one of the following chemical agents: NaAlg (≥400 µg/mL); fucoidan (≥10000 µg/mL); heparin (≥2 µg/mL); VSA (≥20000 µg/mL); PVSA (≥30 µg/mL); HEPES (≥2000 µg/mL); DexSulf (≥250 µg/mL). Combining chemical agents may allow for use of lower concentrations of the individual chemical agents and/or

allow for improved RNA preservation. At the same time, we identified several chemical agents not suitable for this task: MES, VPA, PVPA, chondroitin, MOPS, TAPS. In our experiments, RNA stability was demonstrated up to day 3 of dry storage at room temperature and atmospheric conditions; it is expected that longer term stability also feasible. Furthermore, we have demonstrated that elusion time affects RNA recovery from the dry-storage device, and that eluted RNA samples from the dry-storage devices produced intact full-length cDNA profiles even upon a 6-day elusion from the dry-storage device (incubation at room temperature). Given that RNA kept in dry state is considered to be more stable and less exposed to RNases compared to when eluted in water phase, RNA can be kept stably in dry storage devices for considerable amounts of time. In our example, we used human embryonic kidney (HEK) cells as biological input sample to the RNA storage device. However, usefulness is contemplated with various other biological input materials, such as for example lysed tissues, blood, biopsies, solid or liquid tumors and extracts, saliva, culture cells. Human, mammalian and other animal or plant RNA as well as microbial RNA could be sampled, stored, and detected from the device. A product kit may be developed to include one of the identified chemical RNase inhibitors (e.g., NaAlg, fucoidan, heparin, VSA, PVSA, HEPES, DexSulf) also in the sample lysis buffer. In such a product kit, a filter capturing incompletely lysed cell debris may be included (e.g., mesh filter in spin-down column or compression syringe), as alternative to the cell debris removal by centrifugation. We furthermore envision that separation or selection of sampled RNA based on physical or chemical RNA features (e.g., RNA size, three-dimensional structure, or chemical modification) could be achieved within the device's absorbent and chemical-RNase-inhibitor-impregnated material by modifying the device. For example, size selection could be achieved by manufacturing the device into an elongated shape and separating RNA by diffusion speed through the material. In our

experiments, we generated full-length cDNA libraries (Smart-seq2), demonstrating the devices' suitability for transcriptome-wide RNA-seq workflow.

RNA separation within the storage device, e.g. by integrating a gradient of absorbency or pore size within the matrix. This could be tailored to facilitate the selective passage of RNA molecules of specific sizes or configurations. By engineering a gradated material—beginning with a looser, larger-pored section that transitions to a denser, smaller-pored one—larger RNA fragments could be retained earlier in the matrix, while smaller fragments travel further, enabling size-based stratification directly within the deviceMoreover, by functionalizing different sections of the matrix with chemically distinct agents, it may be possible to target and isolate RNA molecules with specific chemical modifications. For instance, a section of the matrix could be functionalized to have an affinity for methylated RNA, thus capturing and segregating these molecules from the rest of the RNA population during the diffusion process. Additionally, the application of a controlled electric field across the device could induce directional movement of RNA molecules, enhancing separation based on their charge-to-mass ratio—a technique reminiscent of gel electrophoresis. This method could be finely tuned to improve the precision of RNA separation by exploiting the minute differences in the electrical properties of RNA molecules. For the isolation of RNA based on its three-dimensional structure, the device could incorporate binding domains or molecules with shape-specific affinity. This could be particularly useful for capturing uniquely structured RNA, such as tRNA or rRNA, which could be bound and thus separated from mRNA. In terms of device design, incorporating microfluidic channels could lead to more advanced control over the flow and distribution of the sample, allowing for a more precise separation of RNA molecules based on their physical and chemical characteristics. These channels could be

lined with various absorbent materials, each section providing a different selection pressure or chemical environment. Finally, integrating sensor technology within the device could offer real-time monitoring and characterization of the RNA as it is separated, providing immediate analytical data. Such a device would not only serve as a storage medium but also as a tool for RNA analysis, significantly streamlining the process from sample storage to RNA sequencing and identification.

These envisioned advancements could increase the functionality of RNA storage devices, transforming them from mere preservation tools to comprehensive RNA processing and analysis platforms.

Multiple other downstream RNA detection and analysis methods could be utilized in the RNA-detection steps, such as for example, but not limited to, RT-PCR, RT-LAMP (loop-mediated isothermal amplification), direct RNA-sequencing, RNA-FISH. In summary, there is provided useful tools for RNA sampling and storage from various sources and for various purposes.

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Example 2 Comparative Analysis of Absorbent Materials for Enhanced RNA Dry-Storage

Methods

Assessing Substitute Materials for Improved RNA Sample Storage Devices

We prepared and tested five distinct materials as matrices within dry-storage devices for lysed biological samples: cellulose filter paper (3 x 4 mm^2), alpha cellulose Whatman filter paper (4 x 4 mm^2), wood pulp coffee filter (tree pulp) (4 x 7 mm^2), polyethersulfone (PES) membrane filter (3 x 5 mm^2), polyethylene foam (4 x 3 x 3 mm^2). These materials were immersed (impregnated) in varying concentrations of PVSA (3000, 300, 30 µg/mL) or water (as a negative control) for 15 minutes. Subsequently, the saturated materials were extracted using stainless steel tweezers and transferred to sterile 24-well polystyrene plates capped with ventilated lids—these loose-fitting covers shield the samples from contaminants while permitting evaporation. The assemblies were then left to dry at ambient room temperature for a duration of 24 hours.

Cell culture, collection, and lysis

We cultured human cell samples to be tested on the storage devices. Human embryonic kidney (HEK293FT) cells were cultured and expanded in standard medium (DMEM/10% FBS) in 5% CO_2 and 37°C. Aliquots of 2 million cells were collected in a 1.5 mL microcentrifuge tube, washed with 1x PBS, pelleted by brief centrifugation upon which the PBS supernatant was discarded, and frozen in a -80°C deep freezer. For the following storage experiments, cell pellets were diluted in a cell lysis solution, consisting of 1000 µL 1% Triton-X100, and pipetted up and down to achieve a lysed cell suspension. The lysed cell suspension was vortexed for 30 seconds then centrifuged at 300 g for 3 minutes to pellet potential cell debris, such as incompletely lysed nuclei and other potentially remaining cell compartments. Consequently, the lysed cell

suspension contained the input equivalent of 2000 lysed HEK293FT cells per µL.

Sample storage on paper material

The dried, pre-treated materials (sample devices) were spotted with 1.25 µL of lysed cell suspension corresponding to 2500 lysed cells by pipetting the suspension onto the middle of the sample devices and letting the sample absorb and dry into the material. The lysed-cell-spotted storage devices were then incubated/dried for 1 or 6 days at room temperature in 24-well polystyrene plates with ventilating lids.

Full-length cDNA library preparation

Upon 1, or 6 days of storage, the cell-spotted storage devices were individually placed in the wells of a sterile 96-well polystyrene plate, and sample material was eluted by adding 100 µL of nuclease-free water followed by incubation at room temperature for 3 minutes. The 96-well plate was sealed using plastic PCR sealing film, vortexed for 20 seconds, and the plates were finally centrifuged at 300 g for 1 minute. The resulting eluates were immediately used as RNA input to generate full-length cDNA libraries using the Smart-seq2 protocol (Picelli 2013, Picelli 2014), using 1 µL of eluate as input to the Smart-seq2 reaction ("input" of 25 cells). In addition, we generated "1+5 day" samples for each condition, where after elution on day 1, the storage material was not removed from the elusion solution but kept bathing in the liquid at room temperature for an additional 5 days, upon which 1 µL of eluate was used to generate cDNA using Smart-seq2. Smart-seq2 cDNA library generation was performed as follows: Smart-seq2 lysis buffer composition was 2.5mM dNTP/each and 2.5mM Smart seq2 oligo-dT (5'-AAGCAGTGGTATCAACGCAGAGTACT30VN-3') with 1 µL of eluant in

a total buffer volume 4.5 µL. Samples in lysis buffer were incubated at 72°C for 3 min in a Bioer Life ECO thermocycler to hybridize the oligo-dT and placed on ice prior to first-strand synthesis. The following reverse transcriptase reaction contained 1x Superscript II buffer, 5mM DTT, 1M betaine, 10 mM $MgCl_2$, 1 µM Smart-seq2 TSO (5'-AAGCAGTGGTATCAACGCAGAGTACATrGrG+G-3'), and 10 U Superscript II for a total reaction volume of 10 µL. RT thermocycles were 42°C for 90 min, followed by 10 cycles of 42°C for 2 min and 50°C for 2 min. The following cDNA amplification reaction contained 1x KAPA HiFi HotStart Ready Mix and 80 nM ISPCR primers (5'-AAGCAGTGGTATCAACGCAGAGT-3'); total reaction volume 25 µL. Thermocycles for Smart-seq2 cDNA amplification were 98°C for 3 min, followed by 18 cycles of 98°C for 20 sec, 67°C for 15 sec, and 72°C for 6 min, followed by a final incubation at 72°C for 5 min. Amplified cDNA samples were bead purified (AMPure XP, Beckman) at a ratio of 0.8:1 beads:cDNA (20 µL beads:25 µL cDNA for Smart-seq2) and inspected on a Bioanalyzer 2100 (High Sensitivity DNA kit, Agilent).

cDNA yield and quality

To inspect cDNA yield and cDNA size distribution of the samples in each condition, the resulting purified Smart-seq2 cDNA libraries were analyzed using Agilent Bioanalyzer 2100 High sensitivity DNA chips. The shape of full-length cDNA traces is known in the field to reflect the quality and integrity of the underlying mRNA sample (Trombetta 2014), and an intact library (satisfactory library) is expected to have a peak around ~2kb, reflecting the median length of full-length mRNA transcripts in human cells. Exact patterns of spikes in the cDNA traces can further be experiment- or cell-type specific, as abundant cell-type-specific or condition-specific RNA transcripts, for which one or a few transcripts account for a large proportion of total cellular transcripts lead to additional peaks on the Bioanalyzer trace.

Results

Sample absorption into materials: Upon pipetting the lysed cell suspension onto the different materials, we noticed that the cellulose filter paper, alpha cellulose Whatman filter paper, wood pulp filter, and polyethylene foam quickly absorbed the small volume (1.25 µL) of lysed-cell sample dispensed onto the different materials, i.e., the released sample rapidly entered into the matrix of these materials. In contrast, the polyethersulfone (PES) membrane filter demonstrated poor absorption; a noticeable quantity of the lysed-cell sample remained on its surface, which can be attributed to the material's hydrophobic characteristics.

Storage day 1: We inspected the Bioanalyzer traces resulting from cDNA libraries generated from the different materials eluted after 1 day in dry storage and observed pronounced differences in cDNA library yield for the different materials (**Fig. 5a-e**). Out of the five materials tested, polyethersulfone stood out in that samples stored in this material failed to produce any satisfactory full-length cDNA traces (**Fig. 5e**). The samples stored in the other materials: cellulose filter paper, alpha cellulose Whatman filter paper, coffee filter, and polyethylene foam; all produced successful full-length cDNA traces at least in some PVSA pre-treatments. For each of these working materials, the 300 µg/mL PVSA treatment repeatedly produced the highest cDNA yield. Intriguingly, cDNA yield and fragment size distribution varied among the different working materials. Notably, we found that using polyethylene as sample carrier resulted in considerably higher yield than gained from the others (**Fig. 5a-d**). The other functional materials —alpha cellulose, wood pulp filter, and cellulose— exhibited less clear

variance in their performance. The outcomes of these experiments highlight the impact of material selection on both the yield and integrity of RNA in storage devices, with polyethylene foam emerging as the most effective in our experiments.

Storage day 6: Inspecting the Bioanalyzer traces resulting from cDNA libraries generated from the different materials and eluted after 6 days of storage, we observed a similar pattern as observed for day 1 (**Fig. 5f-j**), reenforcing our conclusions regarding preferred materials. The following materials were found to be functioning: cellulose filter, alpha cellulose, wood pulp filter, and polyethylene foam. Polyethersulfone did not. Notably, the superiority of polyethylene as storage matrix, in terms of increased yield and retainment of longer cDNA fragments among functioning materials, became even more distinct at day 6 than previously observed for day 1.

Storage for 1+5 days: We evaluated cDNA libraries derived from "1+5 day" samples for material impregnated with 3000, 300, 30, or 0 µg/mL PVSA — i.e., samples where after elution on day 1, the absorbent material was not removed from the elusion solution but kept soaking in the liquid at room temperature for an additional 5 days to assure complete hydrolysis (**Fig. 5k-o**). Once more, cellulose filter, alpha cellulose, wood pulp filter, and polyethylene foam proved effective, while polyethersulfone did not. This further reinforced the superiority of polyethylene foam, which provided the highest yield as depicted in **Figure 5k**.

Conclusion

In this study, we assessed various materials employed as carriers in dry-storage devices for preserving lysed biological samples. Our findings reveal that the material selection critically influences the stored sample's RNA quality and integrity over time. We successfully utilized several absorbent materials, including cellulose filter paper, Whatman alpha cellulose filter paper, wood pulp coffee filters, and polyethylene foam. Conversely, using polyethersulfone as sample carrier failed to produce satisfactory cDNA profiles, likely due to its hydrophobic nature, which is expected to impede efficient absorption of aqueous samples. Notably, polyethylene foam consistently emerged as the superior material for use in the sample devices. Our results suggest that optimizing the both the choice of chemical RNase inhibitor *and* the material of the carrier — for instance, replacing cotton fiber cellulose used throughout EXAMPLE 1 with polyethylene foam — could enhance the efficacy of RNA dry-storage devices further.

In summary, we have developed valuable tools for the sampling and storage of RNA from diverse sources, catering to a range of applications.

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Trombetta, J.J., et al. Preparation of Single-Cell RNA-Seq Libraries for Next Generation Sequencing. Curr Protoc Mol Biol 107, 4 22 21-24 22 17 (2014).

EXAMPLE 3

Synergistic Chemical Impregnation of RNA Dry-Storage Devices

Rationale

Multiple agents of the invention can be combined to achieve a mix of RNase inhibiting agents that could be used to impregnate RNA dry-storage devices. Thus combining two or more of the chemical agents each demonstrated to be functional as RNase-inhibiting agent in the RNA dry-storage devices presented in EXAMPLE 1 were useful in RNA storage devices. An enhanced capability to preserve RNA was gained from combinatorial effects of the different chemical agents in cocktail, relative to a single RNase inhibitor alone. This is likely due to the different individual chemical, structural and molecular properties of sodium alginate (NaAlg), fucoidan, heparin, vinyl sulfonic acid (VSA), polyvinyl sulfonic acid (PVSA), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), and dextran sulfate (DexSulf). For example, these agents may be differently effective in inhibiting different classes of RNases, such as for example RNAse A, RNase, B, RNase C, which are structurally non-identical, due to differences in conformational and steric properties both of RNases and RNase-inhibiting agents. Thus, experiments where we combined two or more chemical RNase inhibitors in various amounts and tested their combined efficacy in inhibiting RNase activity in biological samples were carried out.

Methods

Building on the framework established in EXAMPLE 1 and EXAMPLE 2, we explored the synergistic effects of combined chemical treatments on the efficacy of RNA storage devices. To this end, we prepared matrices of absorbent materials, e.g., polyethylene foam or cotton fiber, known

for their absorption qualities, and subject them to a cocktail of chemical impregnations. The chemicals selected for combinatorial testing were that showed promise individually in EXAMPLE 1: sodium alginate (NaAlg), fucoidan, heparin, vinyl sulfonic acid (VSA), polyvinyl sulfonic acid (PVSA), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), and dextran sulfate (DexSulf). We hypothesized that combinations of these could utilize the individual strengths of each chemical as an RNase inhibiting agent. The specific concentrations for the combined chemical treatments were informed from the optimal single-agent concentrations identified in EXAMPLE 1. We argued that it is likely that the concentration of individual chemical RNase inhibitors could be decreased when multiple agents are combined in a mix, as each would contribute RNase inhibition. The effects of combined chemical treatment on the efficacy of RNA storage devices were tested similarly as previously performed in EXAMPLE 1 and 2. That is: treating absorbent materials with cocktail of chemical impregnations; spotting these devices with lysed cell material (e.g., HEK293FT); incubating the devices carrying the biological samples at room temperature for several days or even weeks; then eluting the RNA samples and subjecting the eluates to cDNA library generation (e.g., using Smart-seq2); and evaluating the resulting cDNA on a Bioanalyzer 2100 (High Sensitivity DNA kit, Agilent) and/or by DNA sequencing.

EXAMPLE 4

Combinatorial analysis of absorbent materials and chemical RNase inhibitors achieving devices for long-term RNA dry storage

Methods

Preparation of RNA dry-storage devices

Assessing polyurethane foam as matrix for RNA dry-storage: We prepared and tested five distinct materials as dry-storage devices for lysed cell samples: cellulose filter paper (3 x 4 mm^2), wood pulp coffee filter (tree pulp) (4 x 7 mm^2), polyethersulfone (PES) membrane filter (3 x 5 mm^2), polyethylene foam (4 x 3 x 3 mm^3), and polyurethane foam (4 x 4 x 4 mm^3). We impregnated the materials in 300 or 30 µg/ml polyvinyl sulfonic acid (PVSA). Subsequently, the saturated materials were extracted using stainless steel tweezers and transferred to sterile 48-well polystyrene plates capped with ventilated lids—these loose-fitting covers shield the samples from contaminants while permitting evaporation. The assemblies were then left to dry at ambient room temperature for a duration of 24 hours.

Assessing different materials in combination with various chemical RNase inhibitors for RNA dry-storage: We prepared and tested five distinct materials as dry-storage devices for lysed cell samples: cellulose filter paper (3 x 4 mm^2), wood pulp coffee filter (tree pulp) (4 x 7 mm^2), polyethersulfone (PES) membrane filter (3 x 5 mm^2), polyethylene foam (4 x 3 x 3 mm^3), and polyurethane foam (4 x 4 x 4 mm^3). We impregnated the absorbent materials in the following compounds and concentrations: 400 µg/ml NaAlg, 10000 µg/ml fucoidan, 20 µg/ml heparin, 200000 µg/ml VSA, 20000 µg/ml HEPES, and 250 µg/ml DexSulf. As control, we also impregnated the absorbent material in water. Subsequently, the saturated materials were extracted using stainless steel tweezers and transferred to sterile 48-well polystyrene plates capped with ventilated lids—these loose-fitting covers shield the samples from contaminants while permitting evaporation. The assemblies were then left to dry at ambient room temperature for a duration of 24 hours.

Assessing various materials for long-term RNA dry-storage (11 months):

We prepared and tested five distinct materials as matrices for long-term dry-storage devices for lysed cell samples: cellulose filter paper (3 x 4 mm^2), alpha-cellulose Whatman filter paper (4 x 4 mm^2), wood pulp coffee filter (tree pulp) (4 x 7 mm^2), polyethersulfone (PES) membrane filter (3 x 5 mm^2), polyurethane foam (4 x 4 x 4 mm^3). These materials were immersed (impregnated) in varying concentrations of PVSA (3000, 300, 30 µg/mL) or water for 15 minutes. Subsequently, the materials were extracted using stainless steel tweezers and transferred to sterile 24-well polystyrene plates capped with ventilated lids—these loose-fitting covers shield the samples from contaminants while permitting evaporation. The assemblies were then left to dry at ambient room temperature for a duration of 24 hours. Subsequently, the impregnated materials were used for long-term (11 months) storage tests of lysed cells as described in the following sections.

Cell culture, collection, and lysis

We cultured human cell samples to be tested on the RNA dry-storage devices. Human embryonic kidney (HEK293FT) cells were cultured and expanded in standard medium (DMEM/10% FBS) in 5% CO2 and 37°C. Aliquots of 2 million cells were collected in a 1.5 mL microcentrifuge tube, washed with 1x PBS, pelleted by brief centrifugation upon which the PBS supernatant was discarded, and frozen in a -80°C deep freezer. For the following storage experiments, cell pellets were diluted in a cell lysis solution, consisting of 1000 µL 1% Triton-X100, and pipetted up and down to achieve a lysed cell suspension. The lysed cell suspension was vortexed for 30 seconds then centrifuged at 300 g for 3 minutes to pellet

potential cell debris, such as incompletely lysed nuclei and other potentially remaining cell compartments. Consequently, the lysed cell suspension contained the input equivalent of 2000 lysed HEK293FT cells per µL.

RNA sample storage in absorbent material

The dried, pre-treated absorbent materials (sample devices) were spotted with 1.25 µL of lysed cell suspension corresponding to 2500 lysed cells (lysed supernatant after centrifugation, as described in the previous section), by pipetting the suspension onto the middle of the sample devices and letting the sample absorb and dry into the material. The lysed-cell-spotted storage devices were then incubated/dried for 3 days (combinatorial) or 11 months (long term storage) at room temperature in 24-well polystyrene plates with ventilating lids (loose-fitting lids protecting samples from dust but allowing evaporation).

Sample elusion and full-length cDNA library preparation

Upon 3 days (combinatorial), or 11 months (long-term), of storage, the cell-spotted absorbent materials were individually placed in the wells of a sterile 96-well polystyrene plate, and sample material was eluted by adding 100 µL of nuclease-free water followed by incubation at room temperature for 3 minutes. The 96-well plate was sealed using plastic PCR sealing film, vortexed for 20 seconds, and the plates were finally centrifuged at 300 g for 1 minute. The resulting eluates were immediately

used as RNA input to generate full-length cDNA libraries using the Smart-seq2 protocol (Picelli 2013, Picelli 2014), using 1 µL of eluate as input to the Smart-seq2 reaction. This volume (1 µL) corresponded to an original input of 25 lysed cells, not taking potential losses into count, e.g., due to potential trapping of material inside the absorbent material. Smart-seq2 cDNA library generation was performed as follows: Smart-seq2 lysis buffer composition was 2.5mM dNTP/each and 2.5mM Smart seq2 oligo-dT (5′-AAGCAGTGGTATCAACGCAGAGTACT30VN-3′) with 1 µL of eluant in a total buffer volume 4.5 µL. Samples in lysis buffer were incubated at 72°C for 3 min in a Bioer Life ECO thermocycler to hybridize the oligo-dT and placed on ice prior to first-strand synthesis. The following reverse transcriptase reaction contained 1x Superscript II buffer, 5mM DTT, 1M betaine, 10 mM MgCl2, 1 µM Smart-seq2 TSO (5′-AAGCAGTGGTATCAACGCAGAGTACATrGrG+G-3′), and 10 U Superscript II for a total reaction volume of 10 µL. RT thermocycles were 42°C for 90 min, followed by 10 cycles of 42°C for 2 min and 50°C for 2 min. The following cDNA amplification reaction contained 1x KAPA HiFi HotStart Ready Mix and 80 nM ISPCR primers (5′-AAGCAGTGGTATCAACGCAGAGT-3′); total reaction volume 25 µL. Thermocycles for Smart-seq2 cDNA amplification were 98°C for 3 min, followed by 18 cycles of 98°C for 20 sec, 67°C for 15 sec, and 72°C for 6 min, followed by a final incubation at 72°C for 5 min. Amplified cDNA samples were bead purified (AMPure XP, Beckman) at a ratio of 0.8:1 beads:cDNA (20 µL beads:25 µL cDNA for Smart-seq2) and inspected on a Bioanalyzer 2100 (High Sensitivity DNA kit, Agilent).

cDNA yield and quality

To inspect cDNA yield and cDNA size distribution of the samples in each condition, the resulting purified Smart-seq2 cDNA libraries were analyzed

using Agilent Bioanalyzer 2100 High sensitivity DNA chips. The shape of full-length cDNA traces is known in the field to reflect the quality and integrity of the underlying mRNA sample (Trombetta 2014), and an intact library (satisfactory library) is expected to have a peak around ~2kb, reflecting the median length of full-length mRNA transcripts in human cells. Exact patterns of spikes in the cDNA traces can further be experiment- or cell-type specific, as abundant cell-type-specific or condition-specific RNA transcripts, for which one or a few transcripts account for a large proportion of total cellular transcripts lead to additional peaks on the Bioanalyzer trace.

Results

Polyurethane foam is a suitable sample carrier material for RNA dry-storage

We impregnated polyethylene foam, polyurethane foam, wood pulp, cellulose, and polyethersulfone with 300 or 30 µg/mL PVSA, and added lysed HEK cell suspension to each of the materials and let to dry, as described in the Methods. Upon storage for 3 days at room temperature, we eluted and subjected the samples to full-length Smart-seq2 cDNA library generation using 1 µL input volume to the reactions, corresponding to an original input of 25 lysed cells if not taking potential losses e.g. due to potential trapping of material inside the absorbent material. To inspect RNA integrity of the samples, we used microfluidic gel electrophoresis (Agilent Bioanalyzer high-sensitivity DNA chips) and inspected the full-length Smart-seq2 cDNA libraries generated from eluates. In agreement with our earlier results (**Example 1-2**), polyethylene foam, wood pulp, and cellulose pre-treated with 300 µg/mL PVSA yielded high-quality cDNA libraries with the characteristic high-molecular-weight peak around 2 kbp, indicating successful RNA

preservation throughout dry storage in these conditions (**Fig. 6, left panels**). In further agreement with our previous data, samples stored in polyethersulfone did not yield cDNA (**Fig. 6e**). Additionally, we here observed high-quality cDNA reflecting intact mRNA in the polyurethane foam condition (300 µg/mL PVSA) (**Fig. 6b**). Polyethylene and polyurethane foam pre-treated with 30 µg/mL PVSA yielded cDNA libraries while producing partially degraded profiles, signified by cDNA fragment-size distributions shifted towards shorter lengths (**Fig. 6, right panels**). Conversely, we observed a lack of cDNA library formation from samples stored in wood pulp, cellulose and polyethersulfone pre-treated with 30 µg/mL PVSA, indicating severe RNA degradation in these conditions.

Together, these results replicated our previous experimental data regarding materials (**Example 1-2**) and additionally revealed polyurethane foam to be a suitable matrix for RNA-dry-storage when treated with the chemical RNase inhibitor PVSA, notably demonstrating a performance on par with polyethylene foam. cDNA library yield observed at lower PVSA concentration (30 µg/mL) for samples stored in polyethylene foam and polyurethane foam, but the lack thereof in the other materials at this RNase-inhibitor concentration, suggest that polyethylene and polyurethane are preferred matrixes in this context compared to the other materials tested.

Combinatorial analysis of sample carrier materials and chemical inhibitors for RNA dry-storage

We combinatorically characterized how different absorbent materials performed together with various chemical compounds serving as RNase inhibitors. For this testing, we selected six of the top-performing chemical RNase inhibitors based on our previous experimental data (**Example 1-2**), specifically: sodium alginate (NaAlg) (400 µg/mL), fucoidan (10,000

µg/mL), heparin (20 µg/mL), vinyl sulfonic acid (VSA) (200,000 µg/mL), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) (20,000 µg/mL), and dextran sulfate (250 µg/mL), or water. Utilizing all these RNase-inhibitor conditions, we impregnated and dried five different materials: polyethylene foam, polyurethane foam, wood pulp, cellulose and polyethersulfone (in total 35 conditions evaluated considering all combinations). Using the same procedures and sample amounts as in our previous experiments, lysed HEK cell samples were spotted onto the impregnated materials and the samples were left to dry for 3 days at room temperature. Upon 3 days of storage, we eluted the samples and processed RNA into cDNA library using Smart-seq2 (Methods). Resulting cDNA yield and fragment-size distributions were inspected by microfluidic gel electrophoresis (Agilent Bioanalyzer high-sensitivity DNA chips) (**Fig. 7**). This analysis showed that all inhibitor and material combination produced cDNA libraries, with exception of polyethersulfone. However, across the RNase-inhibitor compounds tested, we observed a prominent trend of increased cDNA yield and quality (importantly reflecting RNA integrity) from samples stored in polyethylene and polyurethane foam compared to the other materials.

In summary, while the tested chemical RNase inhibitors worked across matrix materials (polyethersulfone representing the exception), polyethylene and polyurethane foam performed better as storage matrix – importantly demonstrating that the combination of material and chemical RNase inhibitor is a critical consideration for constructing successful RNA dry-storage devices.

Achieving dry-storage devices for long-term preservation of RNA samples

We evaluated different materials in extreme long-term storage (11 months) of dried cell lysate kept at room temperature, focusing on

evaluating RNA integrity. For this experiment, we included five different materials: polyurethane foam, alpha-cellulose, wood pulp, cellulose, and polyethersulfone; with each material being impregnated with PVSA at the concentration of 3000 µg/mL, 300 µg/mL, or 30 µg/mL, or water. As in previously described experiments, HEK cell lysate was added onto the pre-impregnated materials and left to dry in plastic dishes with lid, allowing evaporation but avoiding dust. After 11 months in storage at room temperature, we eluted the samples and processed RNA into cDNA library generation using Smart-seq2 (Methods). As in previously described experiments, we used 1 µL eluate input in the Smart-seq2 reactions, corresponding to the original input of 25 lysed cells if not taking potential losses e.g. due to potential trapping of material inside the absorbent material. Resulting cDNA yield and fragment-size distributions were inspected by microfluidic gel electrophoresis (Agilent Bioanalyzer high-sensitivity DNA chips) (**Fig. 8**). Remarkably, in the polyurethane foam 3000 µg/mL and polyurethane foam 300 µg/mL conditions, we observed high cDNA yield with the fragment-size distribution peaking ~2 kbp (**Fig. 8a**), signifying the presence of mostly intact RNA in the eluates. Conversely, all other materials failed to yield intact cDNA libraries. Notably, neither the polyurethane foam 30 µg/mL PVSA condition nor the polyurethane foam treated with water yielded cDNA libraries, demonstrating that the polyurethane material alone was insufficient for preserving RNA integrity.

Our long-term experiment demonstrates severe differences in performance between materials, and that RNA is lysed cell material can be effectively preserved in dry form for unexpectedly long durations of time if the right combination of chemical RNase inhibitor condition and matrix material is used.

Conclusion

In this Example, we have evaluated various materials as sample carriers for dry storage of lysed cell samples, focusing on RNA integrity. We evaluated various combinations of chemical RNase inhibitors and materials. Our findings highlight that not only the choice of RNase inhibitor, but also the choice of material, is critical for preserving RNA quality in lysed cell samples kept in dry storage. Our experiments demonstrated that polyurethane and polyethylene foams are superior support materials for the preservation of RNA integrity across various RNase inhibitors tested compared to other materials (cellulose, wood pulp, alpha-cellulose, and polyethersulfone). Our long-term storage experiments demonstrate that RNA from lysed cell samples can be preserved in dry form for remarkably long periods with the appropriate choice of storage material and chemical RNase inhibitor. We demonstrated that polyurethane foam treated with 3000 or 300 µg/mL PVSA effectively maintained RNA quality and integrity in lysed human cell samples after 11 months kept at room temperature. While our shorter-term experiments demonstrated that the polyethylene and polyurethane foam yielded higher RNA recovery compared to other materials tested, our 11-month test revealed a drastic advantage of the foam material – indicating that the choice of material becomes more critical upon longer-term storage. Higher concentrations of PVSA (3000 or 300 µg/mL) enhanced RNA recovery, in line with the reasoning that effective RNase inhibition requires a minimum concentration threshold of chemical RNase inhibitor. Thus, together with our previous data (Examples 1-2) which demonstrated the importance of the choice of chemical RNase inhibitor to achieve an effective RNA-dry-storage device, we here further emphasize important differences between materials.

In summary, selecting the right combination of chemical RNase inhibitor and carrier material is essential for creating effective RNA dry-

storage devices, which we here successfully achieved. These findings pave the way for robust solutions in applications and devices designed for RNA sample collection and preservation in biological and clinical samples in dry form. Such devices could be valuable for field sampling, space-efficient long-term storage, sample shipment, home-based sampling, and many other settings. Our approach similarly enable the dry preservation of synthetically produced RNA molecules. Concludingly, we have developed valuable tools for sample storage of RNA from cell samples that cater to a range of applications and devices for sampling and storage of biological and clinical samples containing RNA.

References

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Items I

1. A storage device for a biological sample comprising an absorbent material impregnated with a solution of at least one chemical RNase inhibitor wherein the at least one RNase inhibitor or RNase inhibitors is/are selected from the group comprising sodium alginate (NaAlg), heparin, fucoidan, vinyl sulfonic acid (VSA), polyvinyl sulfonic acid (PVSA), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), and dextran sulfate (DexSulf).

2. The storage device for a biological sample according to item 1 wherein the absorbent material is impregnated with only one RNase inhibitor and the RNase inhibitor is sodium alginate (NaAlg).

3. The storage device for a biological sample according to item 2 wherein the absorbent material is impregnated with NaAlg in the concentration of at least 200µg/mL, such as at least 300µg/mL, such as at least 350µg/mL, such as at least 400µg/mL, such as at least 500µg/mL, such as at least 600µg/mL, such as at least 700µg/mL, such as at least 1000µg/mL, such as at least 2000µg/mL, such as at least 3000µg/mL, such as at least 4000µg/mL, such as at least 5000µg/mL, such as at least 8000µg/mL, such as at least 10,000µg/mL, such as at least 20,000µg/mL, such as at least 40,000µg/mL, such as at least 80,000µg/mL.

4. The storage device for biological samples according to item 1 wherein the absorbent material is impregnated with at least two chemical RNase inhibitor selected from the group comprising sodium alginate (NaAlg), heparin, fucoidan, vinyl sulfonic acid (VSA), polyvinyl sulfonic acid (PVSA), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), and dextran sulfate (DexSulf).

5. The storage device for biological samples according to any one of the above items wherein the impregnated absorbent material is dry.

6. The storage device for biological samples according to any one of the above items wherein the absorbent material is selected from the group comprising cellulose paper, alpha cotton cellulose

paper, wood pulp paper, polyurethane foam, and polyethylene foam.

7. The storage device for biological samples according to any one of the above items for use as an RNA storage device.

8. The storage device for biological samples according to item 7 for use as RNA storage for a pre-lysed biological sample.

9. A kit comprising a storage device according to any one of the preceding items and a container comprising a lysis buffer.

10. A method of preserving and eluting RNA from a biological sample comprising the steps of

   a. Providing a storage device or a kit according to any one of the preceding items

   b. Providing to said storage device a biological sample comprising RNA

   c. Optionally allowing the storage device comprising said biological sample to dry

   d. Eluting RNA from said storage device comprising said biological sample

Items II

1. A storage device for a biological sample comprising an absorbent material impregnated with a solution of at least one chemical RNase inhibitor wherein the at least one RNase inhibitor or RNase inhibitors is/are selected from the group consisting of sodium alginate (NaAlg), fucoidan, vinyl sulfonic acid (VSA), and 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES).

2. The storage device for a biological sample according to item 1 wherein the absorbent material is impregnated with only one RNase inhibitor and the RNase inhibitor is sodium alginate (NaAlg).

3. The storage device for a biological sample according to item 2 wherein the absorbent material is impregnated with NaAlg in the concentration of at least 200µg/mL, such as at least 300µg/mL, such as at least 350µg/mL, such as at least 400µg/mL, such as at least 500µg/mL, such as at least 600µg/mL, such as at least 700µg/mL, such as at least 1000µg/mL, such as at least 2000µg/mL, such as at least 3000µg/mL, such as at least 4000µg/mL, such as at least 5000µg/mL, such as at least 8000µg/mL, such as at least 10,000µg/mL, such as at least 20,000µg/mL, such as at least 40,000µg/mL, such as at least 80,000µg/mL.

4. The storage device for biological samples according to item 1 wherein the absorbent material is impregnated with at least two chemical RNase inhibitor(s) selected from the group consisting of sodium alginate (NaAlg), fucoidan, vinyl sulfonic acid (VSA), polyvinyl sulfonic acid (PVSA), and 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), .

5. The storage device for biological samples according to any one of the above items wherein the impregnated absorbent material is dry.

6. The storage device for biological samples according to any one of the above items wherein the absorbent material is selected from the group comprising cellulose paper, alpha cotton cellulose paper, wood pulp paper, polyurethane foam, and polyethylene foam.

7. The storage device for biological samples according to any one of the above items for use as an RNA storage device.

8. The storage device for biological samples according to item 7 for use as RNA storage for a pre-lysed biological sample.

9. A kit comprising a storage device according to any one of the preceding items and a container comprising a lysis buffer.

10. A method of preserving and eluting RNA from a biological sample comprising the steps of

    a. Providing a storage device or a kit according to any one of the preceding items

    b. Providing to said storage device a biological sample comprising RNA

    c. Optionally allowing the storage device comprising said biological sample to dry

    d. Eluting RNA from said storage device comprising said biological sample

CLAIMS

1. A storage device for a biological sample comprising an absorbent material impregnated with a solution of at least one chemical RNase inhibitor wherein the at least one RNase inhibitor or RNase inhibitors is/are selected from the group comprising sodium alginate (NaAlg), heparin, fucoidan, vinyl sulfonic acid (VSA), polyvinyl sulfonic acid (PVSA), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), and dextran sulfate (DexSulf).

2. The storage device for a biological sample according to claim 1 wherein the absorbent material is impregnated with only one RNase inhibitor and the RNase inhibitor is sodium alginate (NaAlg).

3. The storage device for a biological sample according to claim 1 or 2 wherein the absorbent material is impregnated with NaAlg in the concentration of at least 200µg/mL, such as at least 300µg/mL, such as at least 350µg/mL, such as at least 400µg/mL, such as at least 500µg/mL, such as at least 600µg/mL, such as at least 700µg/mL, such as at least 1000µg/mL, such as at least 2000µg/mL, such as at least 3000µg/mL, such as at least 4000µg/mL, such as at least 5000µg/mL, such as at least 8000µg/mL, such as at least 10,000µg/mL, such as at least 20,000µg/mL, such as at least 40,000µg/mL, such as at least 80,000µg/mL.

4. The storage device for a biological sample according to claim 1 wherein the absorbent material is impregnated with only one RNase inhibitor and the RNase inhibitor is heparin.

5. The storage device for biological samples according to claim 1 or 4 wherein the absorbent material is impregnated with heparin in the concentration of at least 1 µg/mL, such as at least 1.5 µg/mL,

such as at least 2 µg/mL, such as at least 3µg/mL, such as at least 5µg/mL, such as at least 10µg/mL, such as at least 15µg/mL, such as at least 20µg/mL, such as at least 100µg/mL, such as at least 200µg/mL, such as at least 300µg/mL, such as at least 500µg/mL, such as at least 1,000µg/mL, such as at least 2000µg/mL, such as at least 4000µg/mL.

6. The storage device for biological samples according to claim 1 wherein the absorbent material is impregnated with only one RNase inhibitor and the RNase inhibitor is fucoidan.

7. The storage device for biological samples according to claim 1 or 6 wherein the absorbent material is impregnated with fucoidan in the concentration of at least 5000µg/mL, such as at least 10,000µg/mL, such as at least 15,000µg/mL, such as at least 20,000µg/mL, such as at least 30,000µg/mL, such as at least 40,000µg/mL, such as at least 50,000µg/mL, such as at least 60,000µg/mL, such as at least 70,000µg/mL, such as at least 80,000µg/mL, such as at least 90,000µg/mL, such as at least 100,000µg/mL.

8. The storage device for biological samples according to claim 1 wherein the absorbent material is impregnated with only one RNase inhibitor and the RNase inhibitor is vinyl sulfonic acid (VSA).

9. The storage device for biological samples according to claim 1 or 8 wherein the absorbent material is impregnated with vinyl sulfonic acid (VSA) in the concentration of at least at least 10,000 µg/mL, such as at least 15,000 µg/mL, such as at least 20,000 µg/mL, such as at least 30,000 µg/mL, such as at least 50,000 µg/mL, such as at least 100,00µg/mL, such as at least

150,000µg/mL, such as at least 200,000µg/mL, such as at least 300,000µg/mL, such as at least 400,000µg/mL.

10. The storage device for biological samples according to claim 1 wherein the absorbent material is impregnated with only one RNase inhibitor and the RNase inhibitor is polyvinyl sulfonic acid (PVSA).

11. The storage device for biological samples according to claim 1 or 10 wherein the absorbent material is impregnated with polyvinyl sulfonic acid (PVSA) in the concentration of at least 15µg/mL, such as at least 20µg/mL, such as at least 30µg/mL, such as at least 50µg/mL, such as at least 100µg/mL, such as at least 300µg/mL, such as at least 500µg/mL, such as at least 1000µg/mL, such as at least 3000µg/mL, such as at least 5000µg/mL, such as at least 10,000µg/mL, such as at least 30,000µg/mL, such as at least 60,000µg/mL.

12. The storage device for biological samples according to claim 1 wherein the absorbent material is impregnated with only one RNase inhibitor and the RNase inhibitor is 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES).

13. The storage device for biological samples according to claim 1 or 12 wherein the absorbent material is impregnated with 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) in the concentration of at least 1000µg/mL, such as at least 2000µg/mL, such as at least 3000µg/mL, such as at least 5000µg/mL, such as at least 10,000µg/mL, such as at least 20,000µg/mL, such as at least 30,000µg/mL, such as at least 40,000µg/mL, such as at least 80,000µg/mL.

14. The storage device for biological samples according to claim 1 wherein the absorbent material is impregnated with only one RNase inhibitor and the RNase inhibitor is dextran sulfate (DexSulf).

15. The storage device for biological samples according to claim 1 or 14 wherein the absorbent material is impregnated with dextran sulfate (DexSulf) in the concentration of as at least 100µg/mL, such as at least 200µg/mL, such as at least 250µg/mL, such as at least 300µg/mL, such as at least 400µg/mL. such as at least 500µg/mL, such as at least 600µg/mL, such as at least 1,000µg/mL, such as at least 1,500µg/mL, such as at least 2,000µg/mL, such as at least 2,500µg/mL, such as at least 3,000µg/mL, such as at least 5,000µg/mL, such as at least 10,000µg/mL

16. The storage device for biological samples according to any one of claims 1, 3, 5, 7, 9, 11, 13, or 15, wherein the wherein the absorbent material is impregnated with at least two chemical RNase inhibitors selected from the group comprising sodium alginate (NaAlg), heparin, fucoidan, vinyl sulfonic acid (VSA), polyvinyl sulfonic acid (PVSA), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), and dextran sulfate (DexSulf).

17. The storage device for biological samples according to any one of the above claims wherein the impregnated absorbent material is dry.

18. The storage device for biological samples according to any one of the above claims wherein the absorbent material is selected from the group comprising cellulose paper, alpha cotton cellulose paper, wood pulp paper, polyurethane foam, and polyethylene foam.

19. The storage device for biological samples according to any one of the above wherein the absorbent material is cellulose paper.

20. The storage device for biological samples according to any one of the above wherein the absorbent material is alpha cotton cellulose paper.

21. The storage device for biological samples according to any one of the above wherein the absorbent material is wood pulp paper.

22. The storage device for biological samples according to any one of the above wherein the absorbent material is polyethylene foam.

23. The storage device for biological samples according to any one of the above wherein the absorbent material is polyurethane foam.

24. The storage device for biological samples according to any one of the above claims for use as an RNA storage device.

25. The storage device for biological samples according to claim 24 for use as RNA storage for a cell suspension.

26. The storage device for biological samples according to claim 24 for use as RNA storage for a pre-lysed biological sample.

27. The storage device for biological samples according to claim 26 wherein the pre-lysed biological sample is selected from the group comprising tissue, blood, biopsies, liquid biopsies, tumor extraction, saliva, and cell culture.

28. The storage device for biological samples according to any one of the above for use in generation of an RNA-sequencing library.

29. The storage device according to claims 28 wherein the RNA-sequencing library is a single-cell RNA-sequencing library.

30. The storage device for biological samples according to any one of the preceding claims wherein the absorbent material is in the form of a filter.

31. A kit comprising a storage device according to any one of the preceding claims and a container comprising a lysis buffer.

32. The kit according to claim 31 wherein the lysis buffer comprises at least one detergent.

33. The kit according to claim 32 wherein the detergent is selected from the group comprising Triton X-100, nonyl phenoxypolyethoxylethanol (NP)-40, Tween-20, Tween-80, and digitonin.

34. The kit according to any one of claims 31 to 33 wherein the lysis buffer is an aqueous solution of said at least one detergent.

35. The kit according to any one of claims 34 to 34 wherein the lysis buffer further comprises a chemical RNase inhibitor selected from the group comprising sodium alginate (NaAlg), heparin, fucoidan, vinyl sulfonic acid (VSA), polyvinyl sulfonic acid (PVSA), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), and dextran sulfate (DexSulf).

36. Use of the storage device or the kit according to any one of the preceding claims for use in storing a biological sample and optionally for subsequent elution of RNA.

37. Use according to claim 35 wherein the biological sample is a pre-lysed sample selected from the group comprising tissue, blood, biopsies, liquid biopsies, solid or liquid tumors and extracts, saliva, cultured cells, or microbial sample.

38. A method for preserving RNA from a biological sample comprising the steps of

a. Providing a storage device or a kit according to any one of the preceding claims

b. Providing to said storage device a biological sample comprising RNA

c. Optionally allowing the storage device comprising said biological sample to dry

39. A method of preserving and eluting RNA from a biological sample comprising the steps of

a. Providing a storage device or a kit according to any one of the preceding claims

b. Providing to said storage device a biological sample comprising RNA

c. Optionally allowing the storage device comprising said biological sample to dry

d. Eluting RNA from said storage device comprising said biological sample

40. The method according to claim 39 wherein step d is carried out for at least 1 minute, such as at least 5 minutes, such as at least 6, minutes such as at least 7 minutes, such as at least 8 minutes, such as at least 9 minutes, such as at least 10 minutes, such as at least 15 minutes, such as at least 20 minutes, such as at least 25 minutes, such as at least 30 minutes, such as at least 45 minutes, such as at least 60 minutes, such as at least 2 hours, such as at least 3 hours, such as at least 6 hours, such as at least 12 hours, such as at least 24 hours, such as at least 2 days, such

as at least 3 days, such as at least 4 days, such as at least 5 days, such as at least 6 days, such as at least 1 week.

41. The method according to claim 39 or 40 wherein step c. is included and followed by a step of storing the storage device with the biological sample for 1, 2, 3, 4, 5, 6, 7 days, or more, such as ten days, two weeks, three weeks, one month, two months, three months, four months, five months, six months, seven months, eight months, nine months, ten months, eleven months, a year, or two years before step d. is carried out.

42. The method according to any one of claims claim 39 to 41 wherein step d. is followed by step e., an RNA purification step.

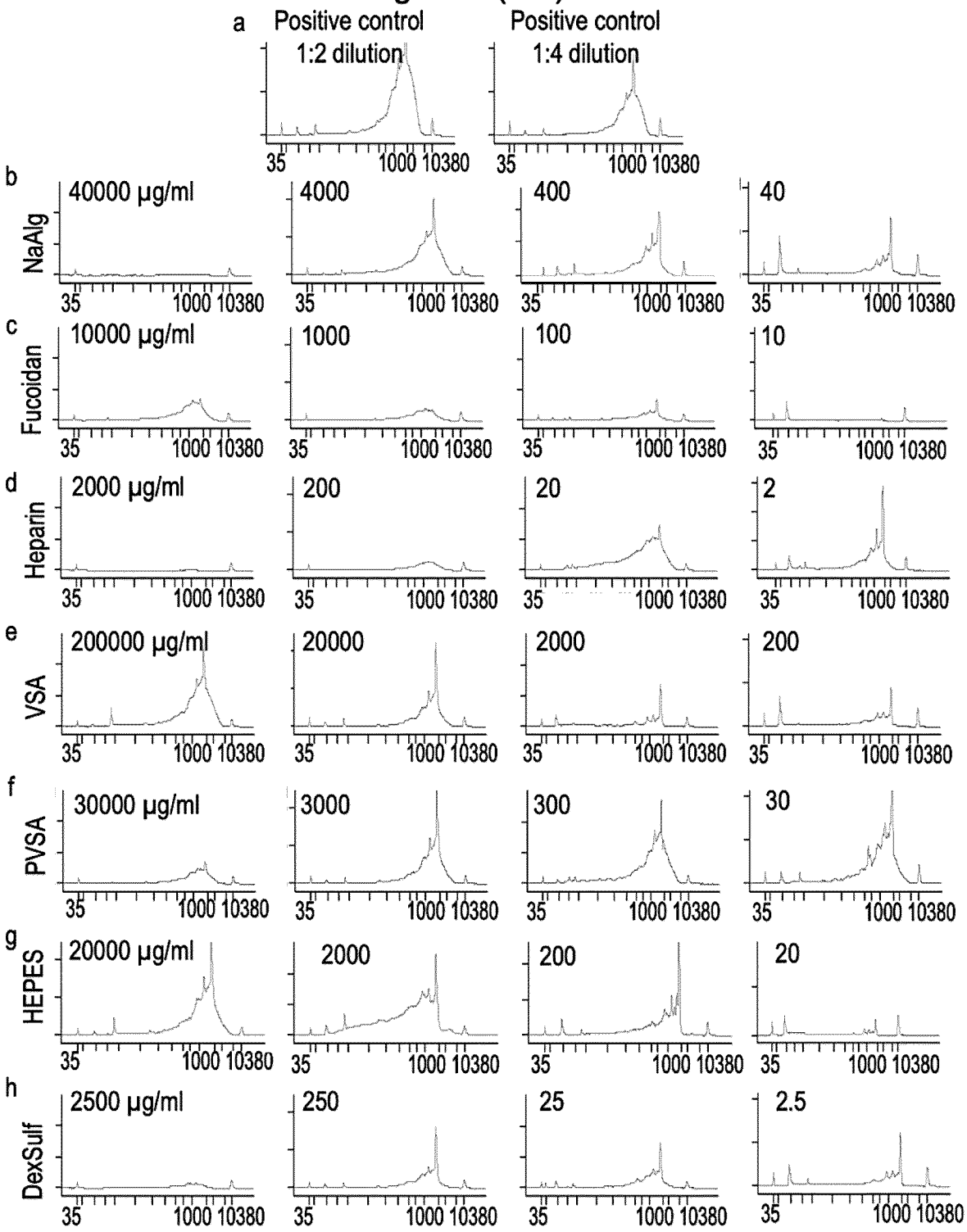
43. The method according to claim 42 wherein step e. reduces the amount of chemical RNase inhibitor in the eluate.

44. The method according to any one of claims 42 to 43 wherein step e. is followed by reverse transcription of the RNA.

45. The method according to any one of claims 42 to 43 wherein step e. is followed by the generation of an RNA-sequencing library.

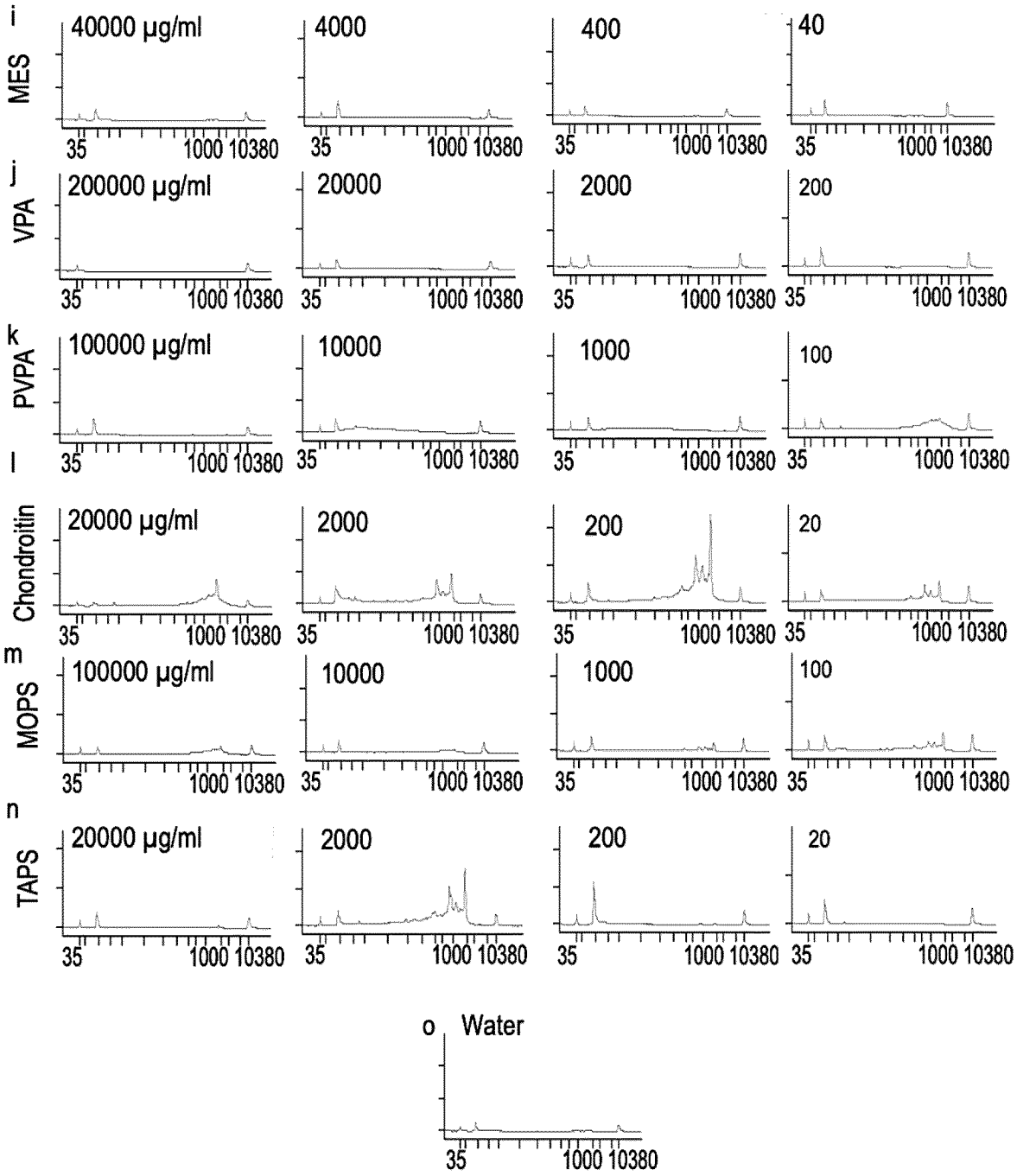
Day 1

Figure 1 (a-h)



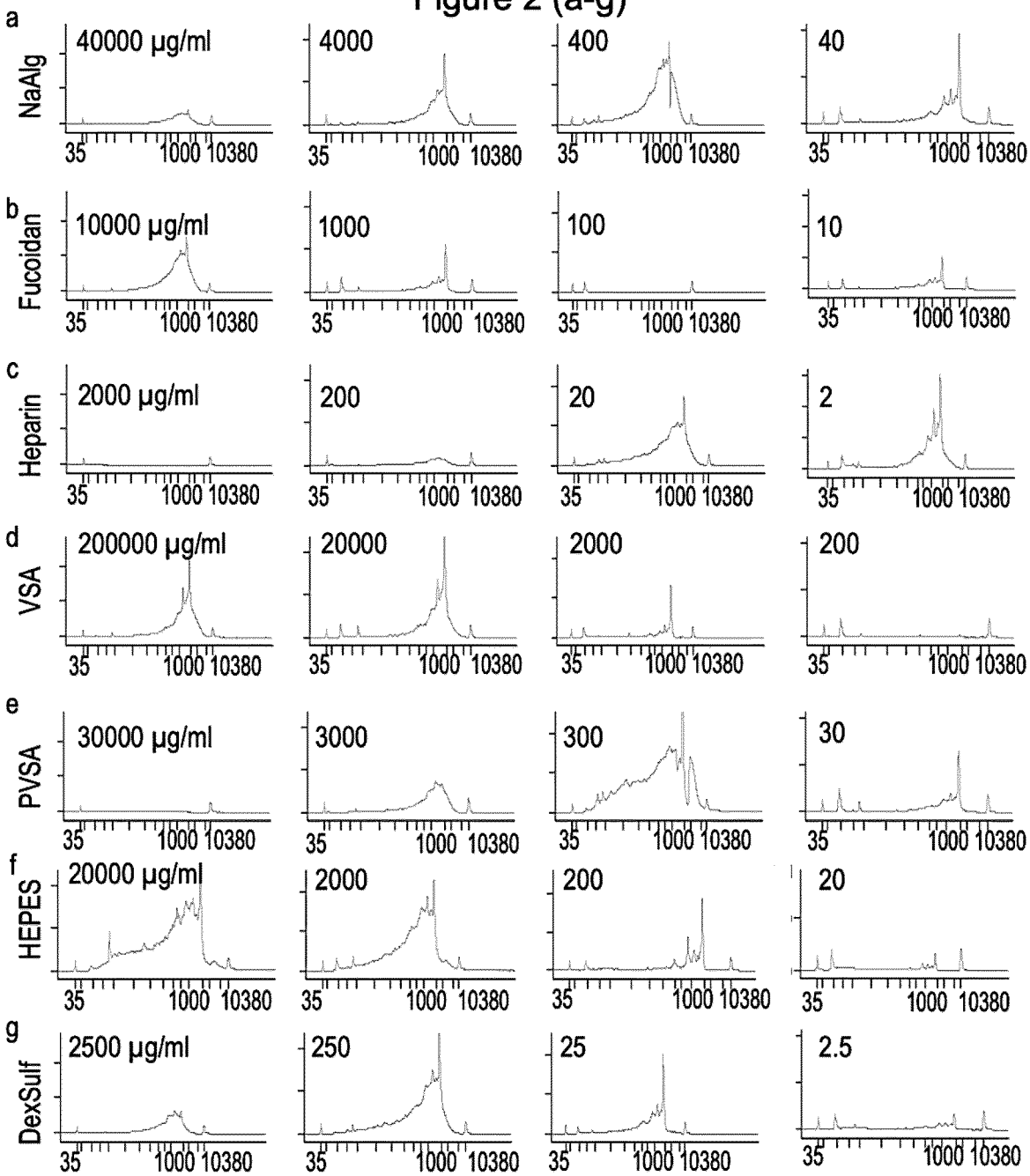
Day 1

Figure 1 (i-o)



Day 3

Figure 2 (a-g)



Day 3

Figure 2 (h-n)

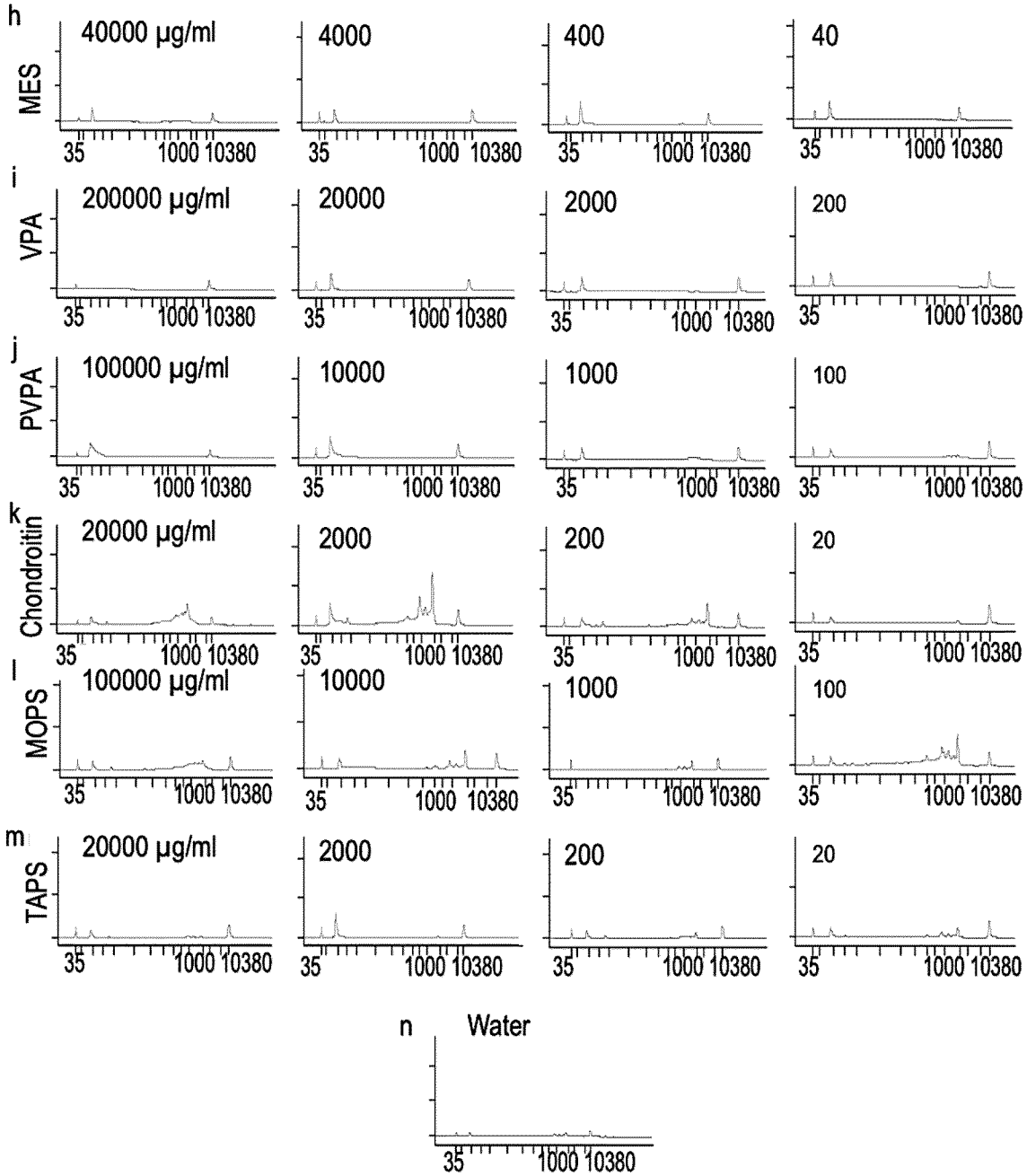
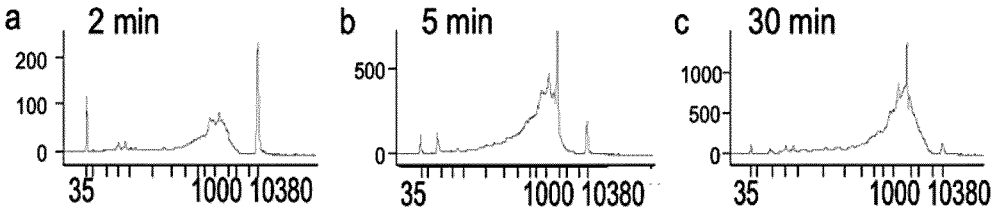
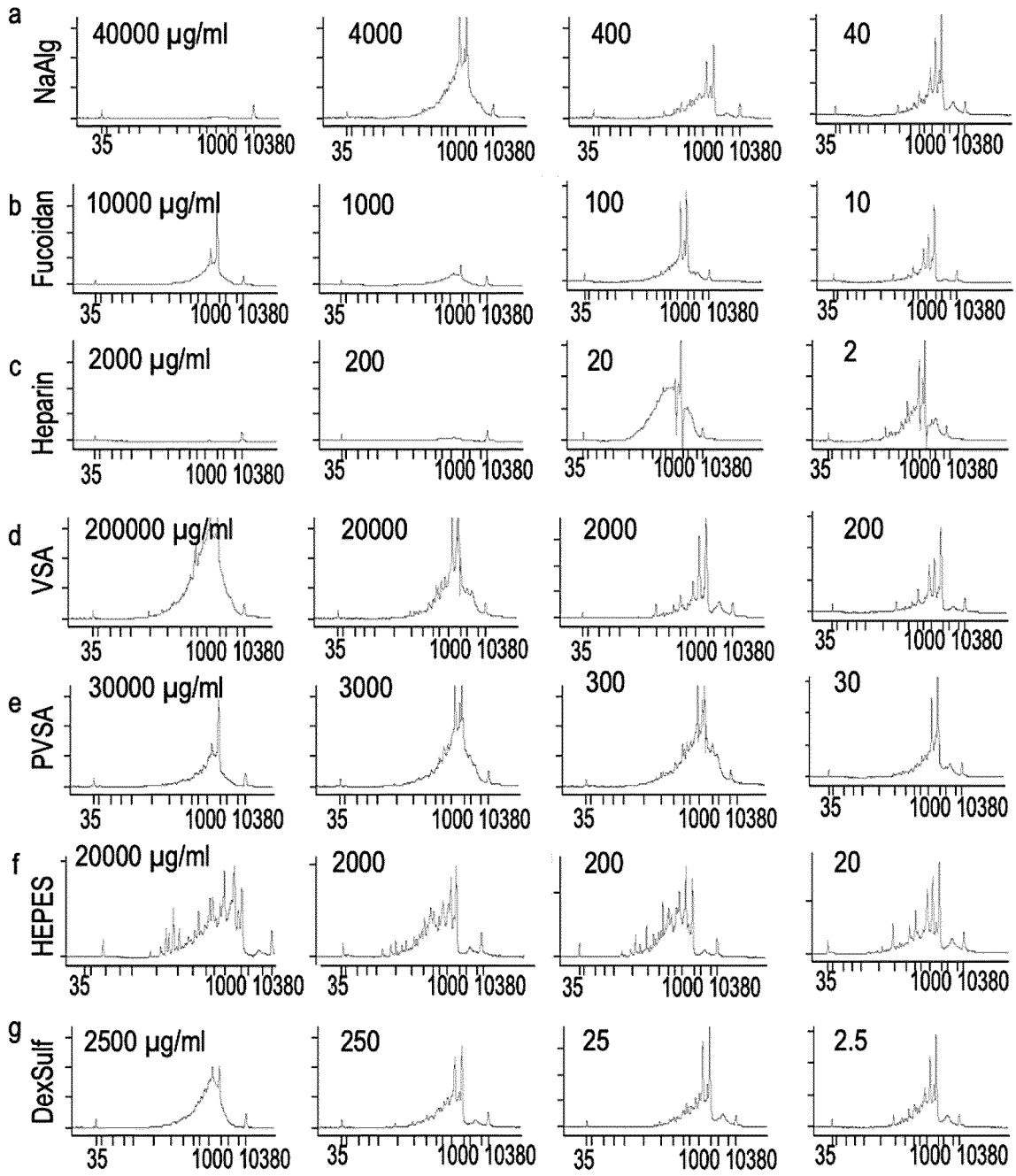


Figure 3


a
2 min
200
100
0
35
1000 10380
b
5 min
500
0
35
1000 10380
c
30 min
1000
500
0
35
1000 10380

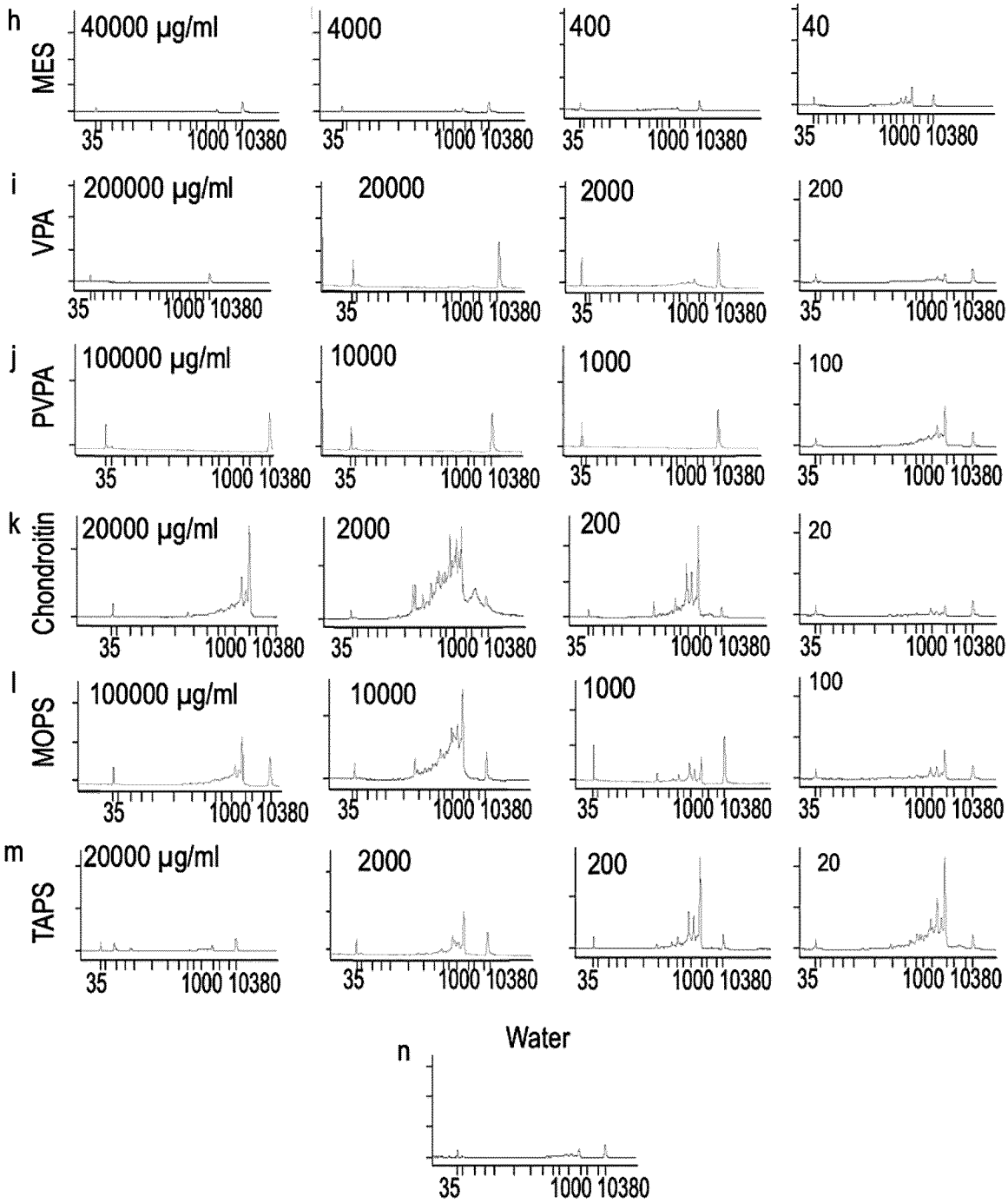
Day 1+6

Figure 4 (a-g)



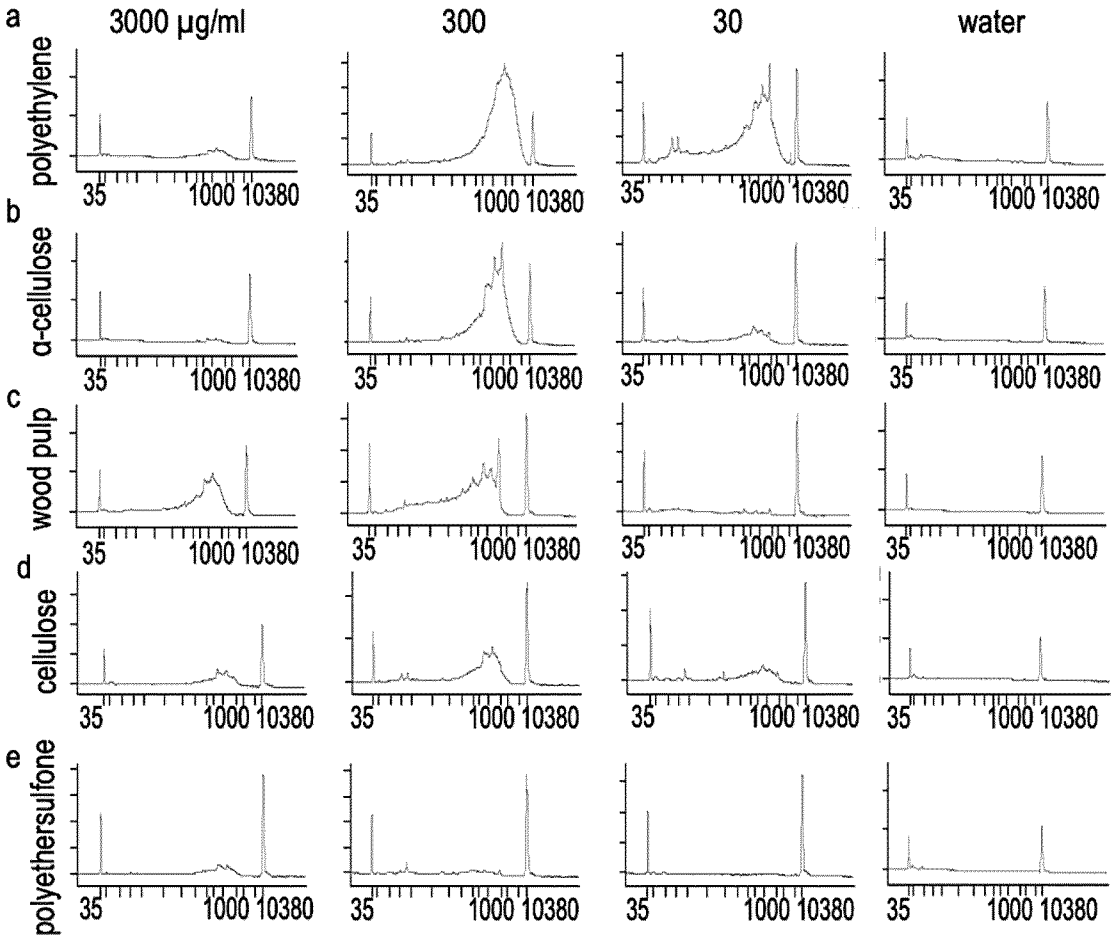
Day 1+6

Figure 4 (h-n)



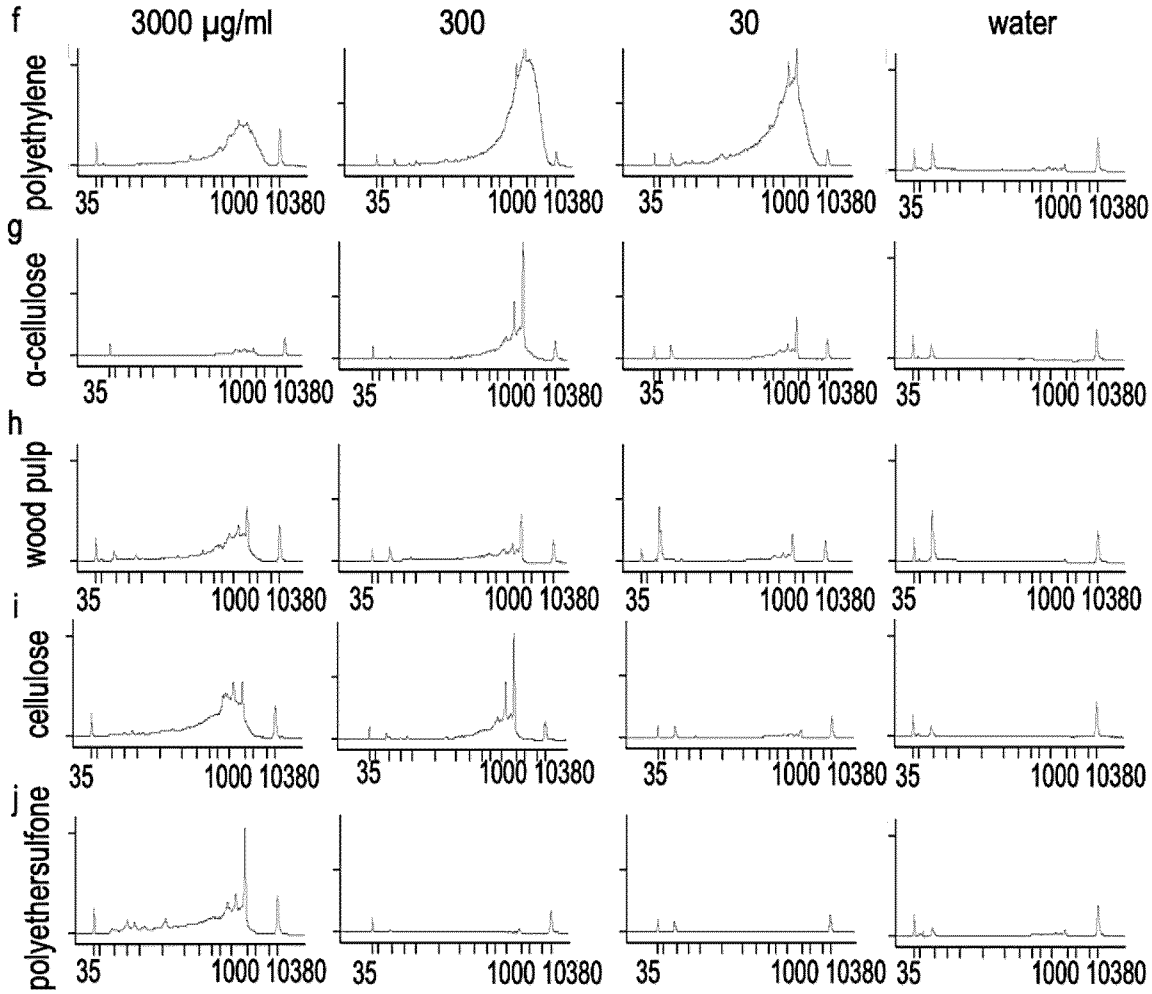
Day 1

Figure 5 (a-e)



Day 6

Figure 5 (f-j)



Day 1+5

Figure 5 (k-o)

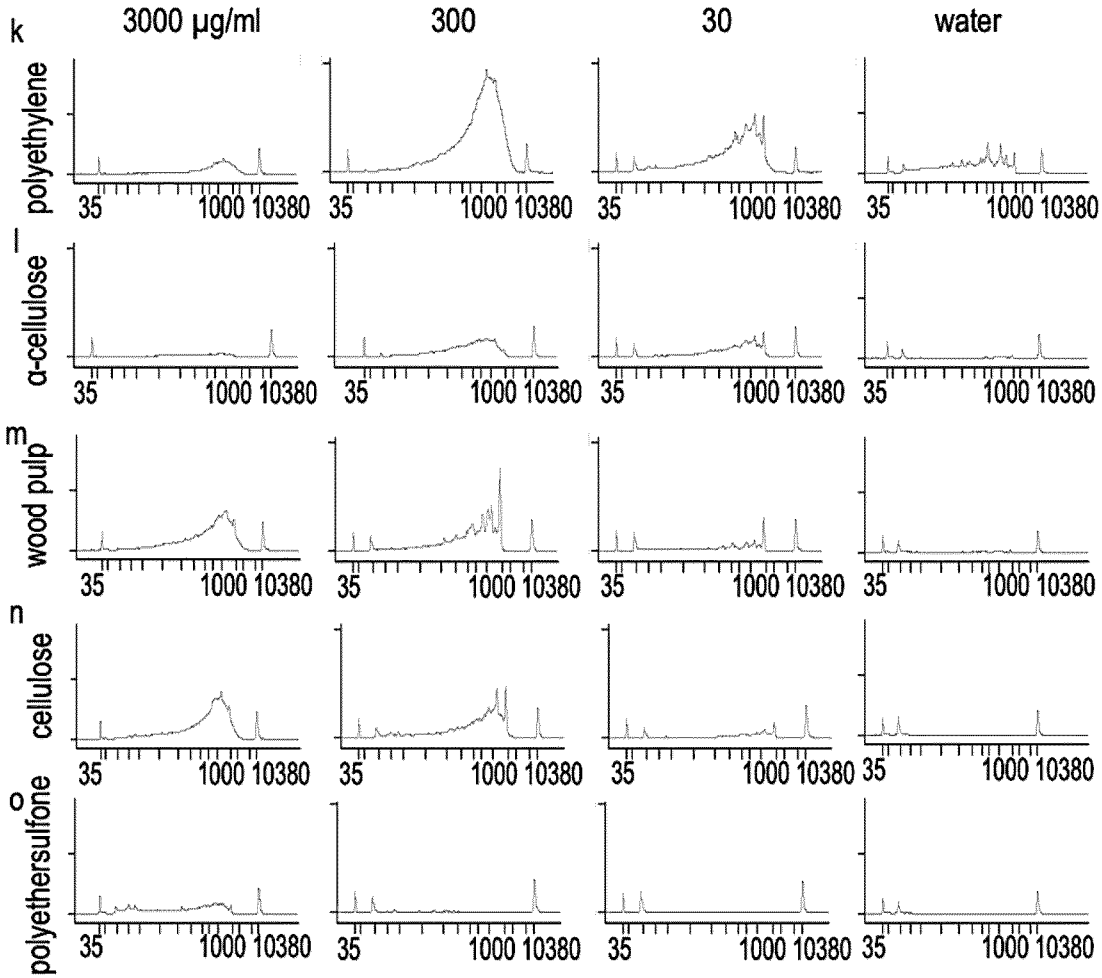


Figure 6 (a-e)

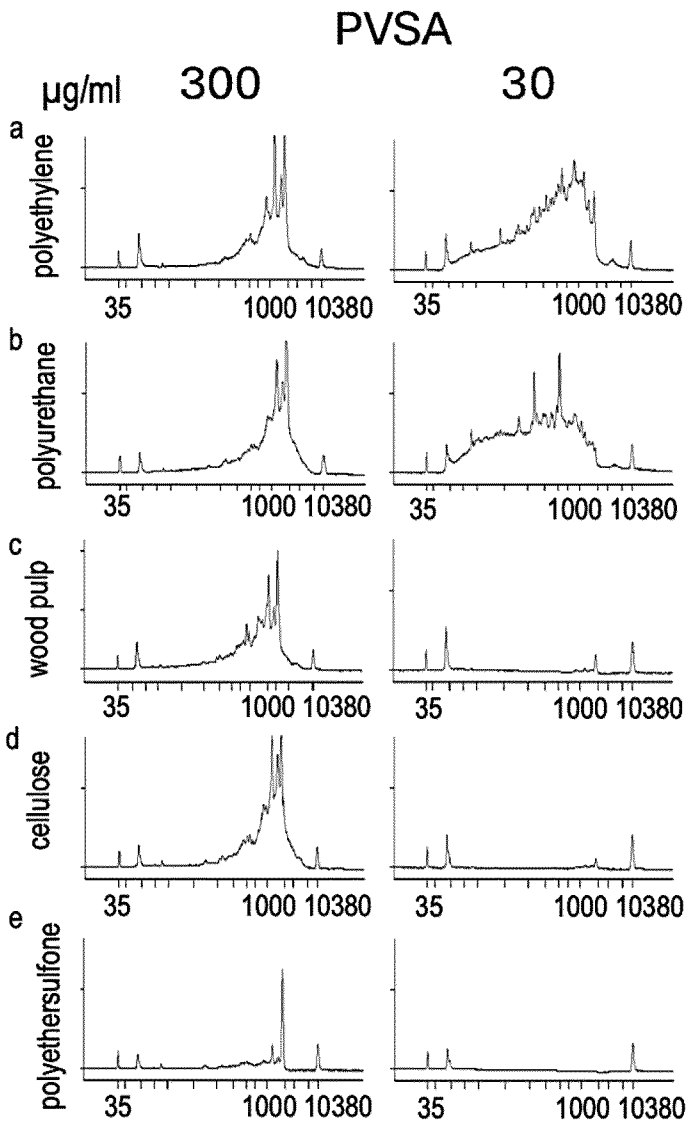
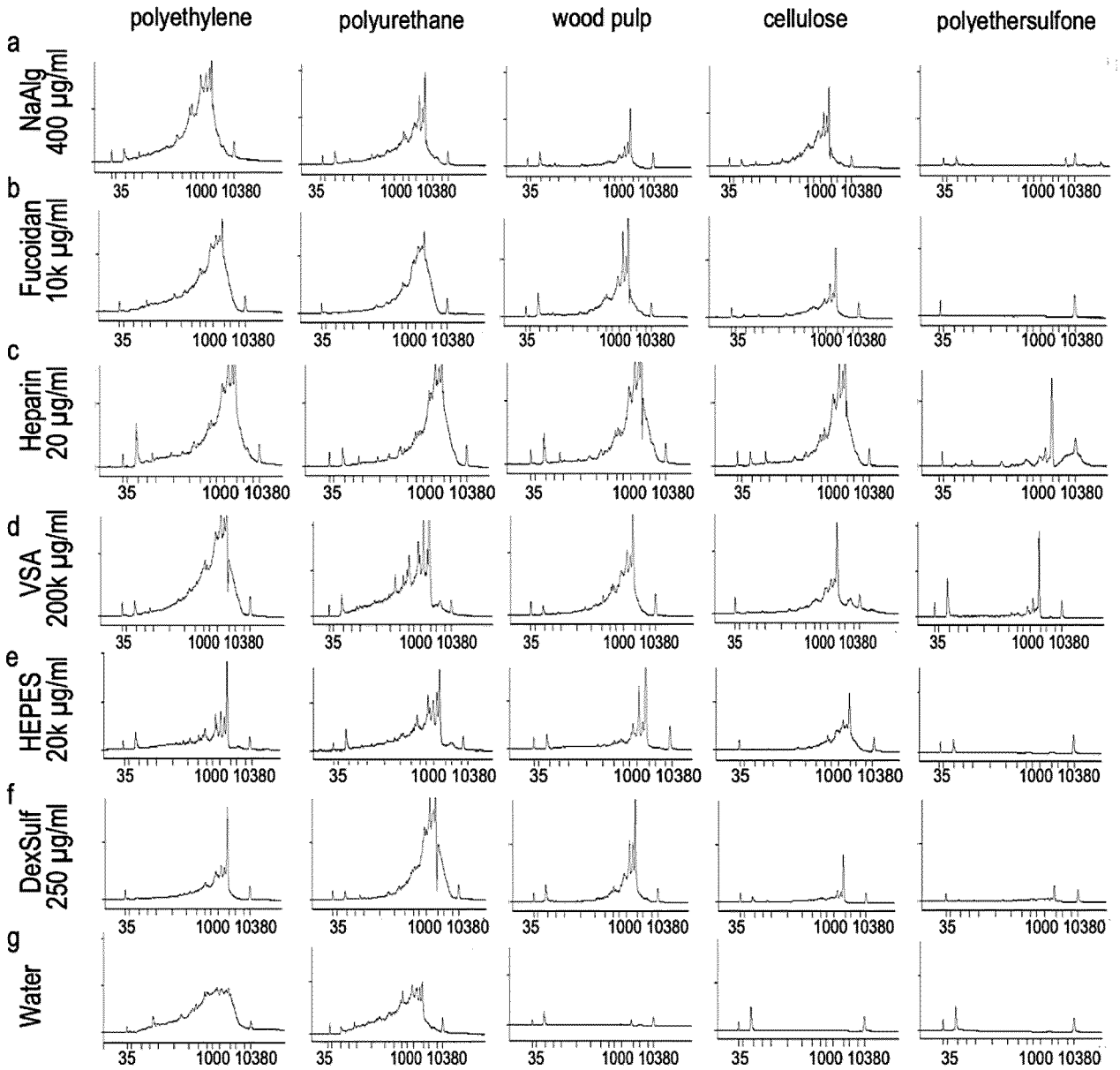

PVSA
µg/ml
300
30
a
polyethylene
b
polyurethane
c
wood pulp
d
cellulose
e
polyethersulfone
35
1000
10380

Figure 7 (a-g)


polyethylene
polyurethane
wood pulp
cellulose
polyethersulfone
a
NaAlg 400 µg/ml
b
Fucoidan 10k µg/ml
c
Heparin 20 µg/ml
d
VSA 200k µg/ml
e
HEPES 20k µg/ml
f
DexSulf 250 µg/ml
g
Water
35
1000
10380

Month 11

Figure 8 (a-e)