

Method for Reducing Endotoxin Levels in Nucleic Acid Purification

The present invention relates to a method for reducing endotoxin levels or removing endotoxins from nucleic acids. For this a certain type of zwitterionic detergent is added during anion exchange chromatographic purification of the nucleic acids using a membrane, or monolith-based sorbent.

The demand for rapid and efficient methods for obtaining high-purity nucleic acids like plasmid DNA from biological sources is constantly increasing owing to the increasing importance of recombinant DNA for exogenous expression or therapeutic applications. In particular, the demand for purification methods which can also be carried out on a larger scale is also increasing. The use of highly pure plasmid DNA is crucial in various applications like polymerase chain reaction (PCR) amplification, DNA sequencing, in vitro mRNA synthesis, and subcloning of transgenes. Therefore, protocols for generating plasmid DNA with high yield and quality have earned serious attention.

Many known methods for the purification of, in particular, relatively large amounts of nucleic acids like plasmid DNA include a chromatographic purification step. The efficiency of this step generally also determines the efficiency and effectiveness of the manufacturing process.

A further problem in the purification of especially plasmid DNA is caused by the impurities from which the plasmid DNA is to be separated. These are firstly genomic DNA and RNA. Another impurity when purifying nucleic acids are endotoxins. Endotoxins are lipopolysaccharides (LPSs) which are located on the outer membrane of Gram-negative host cells, such as, for example, *Escherichia coli*. During lysis of the cells, LPSs and other membrane constituents are released in addition to the plasmid DNA. Endotoxins can be present in cells in a number of approximately 3.5×10^6 copies per cell (*Escherichia coli* and *Salmonella Typhimurium* Cell. and Mol. Biology, J. L. Ingraham et al. Eds., 1987, ASM) and thus exceed the number of plasmid DNA molecules by a factor of more than 104. For this reason, plasmid DNA obtained from Gram-negative host cells often contains large amounts of endotoxins. However, these substances result in a number of undesired side reactions (Morrison and Ryan, 1987, Ann. Rev. Med. 38, 417-432; Boyle et al. 1998, DNA and Cell Biology, 17, 343-348). If it is intended to

employ the plasmid DNA for gene therapy and vaccines it is of extreme importance that inflammatory or necrotic side reactions due to the impurities do not occur. There is therefore a great demand for effective methods for reducing endotoxin concentrations to the lowest possible levels.

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Known methods for reducing endotoxin levels are based on a plurality of purification steps, frequently using anion-exchange chromatography.

Firstly, the host cells are digested by known methods, such as, for example, alkaline lysis. Other lysis methods, such as, for example, the use of high pressure, boiling
10 lysis, the use of detergents or digestion by lysozyme, are also suitable.

The plasmid DNA in the medium obtained in this way, a "clarified lysate", is principally contaminated by relatively small cell constituents, chemicals from the preceding treatment steps, RNA, proteins and endotoxins. The removal of these
15 impurities usually requires a plurality of subsequent purification steps, anion-exchange chromatography being one possibility.

A disadvantage of anion-exchange chromatography is that a considerable amount of endotoxins is bound together with the plasmid DNA and cannot be sufficiently separated in this way. In order to reduce the endotoxin levels, further purification
20 steps, such as, for example, chromatographic steps (gel filtration) or precipitation with isopropanol, ammonium acetate or polyethylene glycol, are therefore necessary. Purification methods which combine chromatographic methods, such as, for example, anion-exchange chromatography, and additional endotoxin removal steps enable plasmid DNA having an endotoxin content of less than 50
25 EU/mg of plasmid DNA to be obtained. However, methods of this type are usually complex, time-consuming and of only limited suitability for the purification of relatively large amounts of DNA.

WO 95/21179 describes a method for the reduction of endotoxin levels in which a
30 clarified lysate is firstly pre-incubated with an aqueous salt solution and detergents. This is followed by purification by ion-exchange chromatography, in which the ion-exchange material is washed with a further salt solution, and the plasmid DNA is eluted and subsequently purified further, for example by isopropanol precipitation. This method likewise has the above-mentioned disadvantages.

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US6617443 discloses a method for removing endotoxins from nucleic acid preparations using a salt-free detergent solution and sorbents whose functional groups are bonded to tentacles.

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WO2009/129524 discloses a protocol suitable for purifying plasmid DNA in parallel format on small scale contacting the plasmid DNA with a zwitterionic detergent.

US6428703 describes a method for purifying biological macromolecules by contacting them with a non-ionic detergent and performing a chromatographic purification.

US2005/245733 reports a method for the reduction of endotoxins in a plasmid preparation using carbohydrate non-ionic detergents with silica chromatography or organic polymeric resins.

All of these documents show ways of purifying plasmid DNA from endotoxins. But there is nevertheless a need for a process combining enhanced performance with a high effectivity.

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Downstream processes in the biopharmaceutical and biotechnological industries usually rely on chromatographic steps with bead-based resins in a packed-bed column as the stationary phase. The resins typically have diameters between 30 and 500 μm and generally provide an efficient chromatographic technique with high binding capacity. However, the method is rather slow and represents a major cost in biomolecules production, as the transport of solute molecules to the binding sites inside resin pores is limited by intra-particle diffusion. The pressure drop over the column is high even at low flow rates and increases during processing due to bed consolidation and column blinding. Consequently, several other innovative stationary phases, including monoliths and membranes, have been developed in the last few decades as possible alternatives to classical chromatographic supports. The main advantage of using membranes or monoliths is attributed to short diffusion times, as the interactions between molecules and active sites in the membrane or monolith occur in convective through-pores rather than in stagnant fluid inside the

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resin pores. Therefore, membrane and monolith chromatography has the potential to operate at high flow rates and low pressure drops.

But as described above membrane or monolith-based chromatography, among others due to the absence of pore diffusion and the higher flow rates, might show
5 different chromatographic behavior and thus different separation properties.

It has been found that endotoxin clearance in nucleic acid purification using membrane or monolith-based chromatographic matrices can be drastically improved in combination with use of a zwitterionic detergent selected from the amine
10 oxide group without compromising nucleic acid yield and removal of host cell proteins. The selected type of detergent proved especially effective in combination with certain high-productive chromatography membrane or monolith materials. Examples provided for the proposed solution indicate for a unique potential for intensification of plasmid manufacturing at large scale over existing approaches
15 based on bead-based materials with commonly used alternative detergents such as e.g. Triton™ X100.

The present invention is therefor directed to a method for depletion or removal of endotoxins from nucleic acids comprising

- 20 a) Providing a sample comprising said nucleic acids and endotoxins
b) Subjecting the sample of step a) to a chromatographic separation on a membrane or monolith comprising anion exchange groups
whereby the sample is contacted with a zwitterionic detergent selected from the group of amine oxides.

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In a preferred embodiment step b) comprises

- i) Loading the sample comprising said nucleic acids and endotoxins onto the membrane, or monolith comprising anion exchange groups
ii) Washing the membrane or monolith with a wash buffer
30 iii) Eluting the nucleic acids bound to the membrane or monolith with an elution buffer

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In one embodiment the nucleic acids are contacted with the zwitterionic detergent by washing the membrane or monolith in step ii) with a wash buffer comprising said zwitterionic detergent.

- 5 In a preferred embodiment the zwitterionic detergent is an amine oxide.

In a very preferred embodiment, it is a N,N-Dimethyltetradecylamine N-oxide.

In a preferred embodiment the nucleic acids comprise or consist of plasmid DNA.

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In a preferred embodiment the nucleic acids are contacted with a solution comprising 0.01 to 10 % (w/v) of the zwitterionic detergent.

In a preferred embodiment the anion exchange capture material is a membrane. In

- 15 a very preferred embodiment the membrane is a hydrogel membrane.

In a preferred embodiment step ii) comprises two or more wash steps whereby one wash step is done with a wash buffer comprising ethanol.

- 20 In one embodiment the process of the invention provides for nucleic acids which are depleted from endotoxins equally effective or more effectively as with the otherwise same process but using Triton® X100 as the only detergent.

25 Definitions

Before describing the present invention in detail, it is to be understood that this invention is not limited to specific compositions or process steps, as such may vary. It must be noted that, as used in this specification and the appended claims, the singular form "a", "an" and "the" include plural referents unless the context clearly
30 dictates otherwise. Thus, for example, reference to "a ligand" includes a plurality of ligands and reference to "an antibody" includes a plurality of antibodies and the like.

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Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention is related. The following terms are defined for purposes of the invention as described herein.

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Nucleic acids that may be purified according to the method of the present invention, also called target nucleic acids, by depletion or removal of endotoxins include DNA, RNA and chimeric DNA/RNA molecules, and may be from any biological source including eukaryotic and prokaryotic cells, or may be synthetic. Nucleic acids that
10 may be purified include chromosomal DNA fragments, ribosomal RNA, mRNA, snRNAs, tRNA, plasmid DNA, viral RNA or DNA, synthetic oligonucleotides, ribozymes, and the like. Of particular interest are plasmid DNAs encoding therapeutic genes. By "therapeutic genes" is intended to include functional genes or gene fragments which can be expressed in a suitable host cell to complement a
15 defective or under-expressed gene in the host cell, as well as genes or gene fragments that, when expressed, inhibit or suppress the function of a gene in the host cell including, e.g., antisense sequences, ribozymes, transdominant inhibitors, and the like.

Thus, e.g., viral DNA or RNA may be purified from prokaryotic or eukaryotic viruses,
20 in which the viral particles are initially purified from cultures or cells permissive for viral infection in accordance with conventional techniques, e.g., from bacterial, insect, yeast, plant or mammalian cell cultures.

The term "plasmid DNA" refers to any distinct cell-derived nucleic acid entity that is
25 not part of or a fragment of the host cell's primary genome. As used herein, the term "plasmid" may refer to either circular or linear molecules composed of DNA or DNA derivatives. The term "plasmid DNA" may refer to either single stranded or double stranded molecules. Plasmid DNA includes naturally occurring plasmids as well as recombinant plasmids encoding a gene of interest including, e.g., marker genes or
30 therapeutic genes.

Plasmids are typically epigenomic circular DNA molecules having a length of between 4 and 20 kB, which corresponds to a molecular weight of between 2.6×10^6 and 13.2×10^6 Daltons often capable of autonomous replication in a producing

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cell. Even in their compact form (super coil), plasmid DNA molecules normally have a size of several hundred nm.

As used herein, and unless stated otherwise, the term "sample" refers to any composition or mixture that contains nucleic acids. Samples may be derived from biological or other sources. Biological sources include eukaryotic and prokaryotic sources, such as plant and animal cells, tissues and organs. The sample may also include diluents, buffers, detergents, and contaminating species, debris and the like that are found mixed with the target molecule. The sample may be "partially purified" (i.e., having been subjected to one or more purification steps, such as filtration steps) or may be obtained directly from a host cell or organism producing the nucleic acid (e.g., the sample may comprise harvested cell culture fluid).

The term "impurity" or "contaminant" as used herein, refers to any foreign or objectionable molecule, including one or more host cell proteins, endotoxins, lipids and one or more additives which may be present in a sample containing the nucleic acids that is being separated from one or more of the foreign or objectionable molecules using a process of the present invention. One contaminant that is depleted or removed with the process of the present invention are endotoxins.

The terms "purifying," "separating," or "isolating," as used interchangeably herein, refer to increasing the degree of purity of the target nucleic acids from a composition or sample comprising the target nucleic acids and one or more impurities. Typically, the degree of purity of the target nucleic acid is increased by removing (completely or partially) at least endotoxins from the composition.

The term "chromatography" refers to any kind of technique which separates an analyte of interest (e.g. a target nucleic acid) from other molecules present in a sample. Usually, the target nucleic acid is separated from other molecules as a result of differences in rates at which the individual molecules of the mixture bind to and/or migrate through a chromatography matrix under the influence of a moving phase.

The term "batch" refers to a certain amount of a plasmid or nucleic acid material that is intended to have a uniform character and quality within defined limits and is manufactured according to a single production order during the same manufacturing cycle with defined start and end points. In case of continuous processes a batch is typically defined based on time- and/or volume strategies.

The term "matrix" or "chromatography matrix" are used interchangeably herein and refers to a solid phase through which the sample migrates in the course of a chromatographic separation. The matrix typically comprises a base material and ligands covalently bound to the base material. The matrix of the present invention comprises or consists of a membrane, bead based resin, or monolith, preferably the base material is a membrane or monolith, most preferred a membrane.

A "ligand" is a functional group that is part of the chromatography matrix, typically it is attached to the base material of the matrix, and that determines the binding properties and interaction properties of the matrix. Examples of "ligands" include, but are not limited to, ion exchange groups, hydrophobic interaction groups, hydrophilic interaction groups, thiophilic interactions groups, metal affinity groups, affinity groups, bioaffinity groups, and mixed mode groups (combinations of the aforementioned). It is also possible that one ligand has more than one binding / interaction property. The matrix of the present invention comprises at least anion exchange groups. These might for example be strong anion exchange groups, such as trimethylammonium chloride or weak anion exchange groups, such as N,N diethylamino or DEAE. The matrix may additionally comprise further other types of ligands so that the matrix is a mixed mode matrix. Such ligands may e.g. have hydrophobic interaction groups, such as phenyl, butyl, propyl, hexyl.

The ligands can be attached to the base material of the matrix by any type of covalent attachment. Covalent attachment can for example be performed by directly bonding the functional groups to suitable residues on the base material like OH, NH₂, carboxyl, phenol, anhydride, aldehyde, epoxide or thiol etc.. It is also possible to attach the ligands via suitable linkers. It is also possible to generate the matrix by polymerizing monomers comprising the ligands and a polymerizable moiety. Examples of matrices generated by polymerization of suitable monomers are

polystyrene, polymethacrylamide or polyacrylamide based matrices generated by polymerizing suitable styrole or acryloyl monomers.

5 In another embodiment the stationary phase can be generated by grafting the ligands onto the base material or from the base material. For grafting from processes with controlled free-radical polymerisation, such as, for example, the method of atom-transfer free-radical polymerisation (ATRP), are suitable. A very preferred one-step grafting from polymerisation reaction of acrylamides, methacrylates, acrylates, methacrylates etc. which are functionalized e.g. with ionic, hydrophilic or
10 hydrophobic groups can be initiated by cerium(IV) on a hydroxyl-containing support, without the support having to be activated.

When the chromatography matrix is used in a chromatographic separation it is typically used in a separation device, also called housing, as a means for holding
15 the matrix.

In one embodiment, the device comprises a housing with an inlet and an outlet and a fluid path between the inlet and the outlet. In a preferred embodiment the device is a chromatography column. Chromatography columns are known to a person skilled in the art. They typically comprise cylindrical tubes or cartridges filled with the
20 stationary phase as well as filters and/or means for fixing the stationary phase in the tube or cartridge and optionally connections for solvent delivery to and from the tube or cartridge. The size of the chromatography column varies depending on the application, e.g. analytical or preparative. In one embodiment the column or generally the separation device is a single use device.

25 The term "anion exchange matrix" is thus used herein to refer to a chromatography matrix which carries at least anion exchange groups. That means it typically has one or more types of ligands that are positively charged under the chromatographic conditions used, such as quaternary amino groups.

30 A "buffer" is a solution that resists changes in pH by the action of its acid-base conjugate components. Various buffers which can be employed depending, for example, on the desired pH of the buffer are described in Buffers. A Guide for the Preparation and Use of Buffers in Biological Systems, Gueffroy, D., ed. Calbiochem

Corporation (1975). Non-limiting examples of buffers include MES, MOPS, MOPSO, Tris, HEPES, phosphate, acetate, citrate, succinate, and ammonium buffers, as well as combinations of these.

- 5 According to the present invention the term “buffer” or “solvent” is used for any liquid composition that is used to load, wash, elute, re-equilibrate, strip and/or sanitize the chromatography matrix.

When “loading” a chromatography column in bind and elute mode, the sample or
10 composition comprising the target molecule and one or more impurities is loaded onto a chromatography column. In preparative chromatography, the sample is preferably loaded directly without the addition of a loading buffer. If a loading buffer is used, the buffer has a composition, a conductivity and/or pH such that the target nucleic acid is bound to the stationary phase while ideally all the impurities like the
15 endotoxins are not bound and flow through the column. Typically, the loading buffer, if used, has the same or similar composition as the equilibration buffer used to prepare the column for loading.

The final composition of the sample loaded on the column is called feed. The feed may comprise the sample and the loading buffer but preferably it is only the sample.

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By “wash” or “washing” a chromatography matrix is meant passing an appropriate liquid, e.g. a buffer through or over the matrix. Typically washing is used to remove weakly bound contaminants from the matrix in bind/elute mode prior to eluting the target molecule. Additionally, wash steps can be used to reduce levels of residual
25 detergents, enhance viral clearance and/or alter the conductivity carryover during elution.

To “elute” a molecule (e.g. the target nucleic acid) from a matrix means that the molecule is removed therefrom. Elution may take place by altering the solution
30 conditions such that a buffer different from the loading and/or washing buffer competes with the molecule of interest for the ligand sites on the matrix or alters the equilibrium of the target molecule between stationary and mobile phase such that it favors that the target molecule is preferentially present in elution buffer.

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A non-limiting example is to elute a molecule from an ion exchange resin by altering the ionic strength of the buffer surrounding the ion exchange material such that the buffer competes with the molecule for the charged sites on the ion exchange material.

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A membrane as chromatographic matrix can be distinguished from particle-based chromatography by the fact that the interaction between a solute, e.g. the target nucleic acids or contaminants, and the matrix does not take place in the dead-ended pores of a particle, but mainly in the throughpores of the membrane. Exemplary
10 types of membranes are flat sheet systems, stacks of membranes, microporous polymer sheets with incorporated cellulose, polystyrene or silica-based membranes as well as radial flow cartridges, hollow fiber modules and hydrogel membranes. Preferred are hydrogel membranes. Such membranes comprise a membrane support and a hydrogel formed within the pores of said support. The membrane
15 support provides mechanical strength to the hydrogel. The hydrogel determines the properties of the final product, like pore size and binding chemistry.

The membrane support can consist of any porous membrane like polymeric membranes, ceramic based membranes and woven or non-woven fibrous material. Suitable polymeric materials for membrane supports are cellulose or cellulose
20 derivatives as well as other preferably inert polymers like polyethylene, polypropylene, polybutylenterephthalate or polyvinylidene-difluoride.

The hydrogels can be formed through in-situ reaction of one or more polymerizable monomers with one or more crosslinkers and/or one or more cross-linkable polymers to form a cross-linked gel that has preferably macropores. Suitable
25 polymerizable monomers include monomers containing vinyl or acryl groups. Preferred are monomers comprising an additional functional group that either directly forms the ligand of the matrix or is suitable for attaching the ligands. Suitable crosslinkers are compounds containing at least two vinyl or acryl groups. Further details about suitable membrane supports, monomers, crosslinkers etc. as well as
30 suitable production conditions can be found in WO04073843 and

WO2010/027955. Especially preferred are membranes made of an inert, flexible fiber web support comprising in assembly within and around the fiber web support a porous polyacrylamide hydrogel with quaternary ammonium groups (strong anion

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exchange groups), like Natrix® Q Chromatography membrane, Merck KGaA, Germany.

Depending on the membrane device used, the respective processes are conducted by different operating principles like dead-end operation, cross-flow operation and
5 radial flow operation systems. Dead-end operation is preferred.

Examples of suitable membranes to be used in the method of the present invention are

- 10 - Membranes with a polyethersulfone (PES)-based support and a cross-linked polymeric coating, functionalized with quaternary ammonium groups (strong anion exchange groups), like Mustang® Q, Pall.
- Membranes made of stabilized reinforced cellulose, functionalized with quaternary ammonium groups (strong anion exchange groups) or with DEAE groups (diethylaminoethyl, weak ion exchange groups), like Sartobind®
15 membranes, Sartorius.
- Membranes made of stabilized reinforced cellulose, comprising a hydrogel with quaternary ammonium groups (strong anion exchange groups), like Sartobind® Jumbo Membranes made of stabilized reinforced cellulose, functionalized with quaternary ammonium groups (strong anion exchange groups), like Sartobind®
20 Jumbo membranes, Sartorius.
- Membranes made of a fine fiber non-woven scaffold comprising a hydrogel with quaternary ammonium groups (strong anion exchange groups), like 3M™ Emphaze™ AEX Hybrid Purifier, 3M.
- Membranes made of an inert, flexible fiber web support comprising within and
25 around the fiber web support a porous polyacrylamide hydrogel with quaternary ammonium groups (strong anion exchange groups), like Natrix® Q Chromatography membrane, Merck KGaA, Germany.

A monolith or a monolithic sorbent, similar to a membrane, has throughpores, like
30 interconnected channels, so that liquid can flow from one side of the monolith, through the monolith, to the other side of the monolith.

Since the mobile phase is flowing through these throughpores, molecules to be separated are transported by convection rather than by diffusion. Due to their

structure monolithic sorbents show flow rate independent separation efficiency and dynamic capacity.

5 The monolith is typically formed in situ from reactant solutions and can have any shape or confined geometry, typically with frit-free construction, which guarantees convenience of operation. Preferably, monolithic materials have a binary porous structure, mesopores and macropores. The micron-sized macropores are the throughpores and ensure fast dynamic transport and low backpressure in applications; mesopores, preferably located in the walls of the throughpores,
10 contribute to sufficient surface area and thus high loading capacity.

The monoliths can be made of organic, inorganic or organic/inorganic hybrid materials. Preferred are organic polymer based monoliths.

15 The synthesis of organic polymer monoliths is typically done by a one-step polymerization providing a tunable porous structure with tailored functional groups. Generally, a pre-polymerization mixture consisting of the monomers, crosslinkers, porogenic solvents, and initiators in an appropriate ratio is polymerized in a suitable container, also called mould, determining the format of the monolith. Polymerization
20 is typically initiated by heating, use of UV radiation, microwave or γ -ray radiation in the presence of initiators. After reaction for the prescribed time at an appropriate temperature, the resulting material is typically washed with solvents to remove unreacted components and porogenic solvents.

Suitable organic polymers are polymethacrylates, polyacrylamides, polystyrenes,
25 polyurethanes, etc., like Poly(methacrylic acid–ethylene dimethacrylate), Poly(glycidyl methacrylate–ethylene dimethacrylate) or Poly(acrylamide–vinylpyridine- N,N' -methylene bisacrylamide).

Inorganic monoliths can be made of silica or other inorganic oxides. Preferably they
30 are made of silica. Silica monoliths are normally prepared via a sol–gel method with phase separation. This mainly includes hydrolysis, condensation, and polycondensation of silica precursors. Typically, tetraethoxysilane (TEOS) or tetramethylorthosilicate (TMOS) is distributed in a suitable solvent in the presence of a porogen (e.g. poly(ethylene glycol) (PEG)), followed by the addition of a

catalyst, acid or base, or a binary catalyst, acid and base in sequence. After reaction for a prescribed time, the resulting gel-like product is washed with solvents to remove unreacted precursor, porogen, and catalyst, followed by the proper post treatment, typically a heat treatment.

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Monoliths can also be made by 3D printing.

The monoliths can be modified with suitable functional groups, preferably at least ion exchange groups, to generate the targeted interaction with the sample comprising the target molecule and thus the targeted separation.

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Typically, the monoliths are contained in a housing like a column.

Particle-based resins intended for liquid chromatography are normally comprised of particles that are packed together in a tubular cylinder called column to form a bed. The packed bed shows a distinct space between the particles, so called void volume, which mainly defines the liquid fluid permeability and hydrodynamic properties of the packed bed.

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The particles typically consist of a cross-linked polymer matrix in spherical, bead-like or granular shape with relatively uniform size for improved chromatographic and hydrodynamic characteristics of the packed bed. They can have a dense structure with discrete or very small pores but usually exhibit a porous multichannel or reticular structure forming an inner pore volume and additional surface area inside the particle. The particle surface area can be modified with a variety of functional groups suitable for chromatography applications either by using functional monomers for the backbone-polymer structure, coupling of functional groups to the particle surface directly or via ligands or short polymers structures (grafts).

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Zwitterionic detergents are amphoteric surfactants with a nonpolar tail and a hydrophilic head carrying both anionic and cationic charged atomic groups. Net charge and other physicochemical properties (viscosity, solubility, critical micell formation) of the zwitterionic detergents vary as the pH of solution is adjusted. For solutions with pH at the isoelectric point, the negative charge on the surfactant molecule is exactly balanced by the positive charge on that same molecule, rendering the net charge of the detergent molecule zero.

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Amine oxides are assigned as zwitterionic detergents.

Amine oxides are compounds having the formula $R_1R_2R_3NO$, where each R_1 , R_2 , and R_3 independently of the others is an optionally substituted C1-C30 hydrocarbon chain.

Amine oxides, used especially preferably, are those in which R_1 is a C10-C18 alkyl and R_2 and R_3 are each independently C1-C4 alkyl, particularly C12-C16 alkyl dimethylamine oxides.

An especially suitable amine oxide is N,N-Dimethyltetradecylamine N-oxide (TDAO), also called Myristyldimethylamine-N-oxide, CAS number 3332-27-2.

Detailed Description

The nucleic acids to be purified according to the method of the present invention may originate from any natural, genetic-engineering or biotechnological source, such as, for example, prokaryotic cell cultures. If nucleic acids from cell preparations are to be purified, the cells are firstly digested by known methods, such as, for example, lysis. If the sample to be purified has already been pre-treated in another way, lytic digestion is unnecessary. For example, the sample can be obtained from biological material by removal of the cell debris and a precipitate of RNA, from nucleic acid samples which have already been pre-purified and, for example, are present in buffer, or alternatively from nucleic acid solutions which have been formed after amplification and still contain endotoxin impurities. Filtration, precipitation or centrifugation steps may be necessary. The person skilled in the art is able to select a suitable digestion method depending on the source of the nucleic acids to be purified. In any case, the sample to be purified should, for the method according to the invention, be present in a medium which does not form precipitates or cause other undesired side reactions on addition of a detergent solution. The sample is preferably a lysate obtained from cells, such as, for example, a clarified lysate.

For the purification of plasmid DNA from E. coli, the cells are, for example, firstly lysed by alkaline lysis with NaOH/SDS solution. Addition of an acidic potassium-containing neutralization buffer then causes the formation of a precipitate, which can be removed by centrifugation or filtration. The clear supernatant remaining, the

clarified lysate, can be employed as starting material, i.e. as sample, for the method according to the invention. It is also possible firstly to concentrate or pre-purify the clarified lysate by known methods, such as dialysis or precipitation.

5 The sample comprising the nucleic acids and the endotoxins and potentially other impurities from which the nucleic acids shall be purified and thus the endotoxins shall be removed or depleted is then subjected to a chromatographic separation on a membrane or monolith-based chromatography matrix comprising anion exchange groups. For this the sample is loaded onto the chromatography matrix. The final
10 composition of the sample loaded onto the matrix is called the feed. The feed preferably is adjusted to an electrolytic conductivity between 40 to 90 mS/cm, most preferably the feed is adjusted to a conductivity sufficiently high for preventing binding of RNA to the anion exchange material but still acceptable for capturing target nucleic acid.

15 Conductivity adjustment is done by addition of salt, salt concentrate solutions, or, respectively, dilution with a low conductivity buffer or neat water. For feed conductivity adjustment by salt supplementation preferably sodium or potassium chloride are used, but any other salt commonly used in purification applications such as e.g. salts from sulfate, acetate, carbonate/bicarbonate, phosphate or citrate might
20 be considered as well.

The feed typically shows pH values between 4.5 to 5.5 but the method might also be conducted to feeds showing pH values ranging from 4.0 up to 9.0.

Column equilibration and wash buffers are typically buffers matching the pH and conductivity of feed loaded onto the chromatography material. Typically wash
25 buffers with pH at 7.5 to 9.0 and conductivity between 5 to 90 mS/cm are selected but buffers out of that range are applicable as well.

After loading the matrix is washed with at least one wash buffer. The wash buffer might be identical to the loading buffer or different from the loading buffer.

The matrix might also be washed with 2, 3 or 4 different wash buffers. At least one
30 of the wash buffers comprises a zwitterionic detergent selected from the group of amine oxides or mixtures thereof. Typically, the concentration of the detergent in the wash is between 0.01 % and 10 % (w/v), preferably between 0.1 % and 1.5 % (w/v). Detergent wash solutions made from low conductivity wash buffers (< 40 mS/cm) with pH ranging between +/- 1 units from the isoelectric point of the zwitterionic
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detergent used are particularly suited, but buffers out of that range are applicable as well.

In another preferred embodiment, one wash buffer, preferably the last wash buffer
5 comprises ethanol in a concentration between 10 and 25% (v/v).

Preferably the pH and the ionic strength of the wash buffers is identical or similar to the pH and the ionic strength of the equilibration/loading buffer.

Elution of the target nucleic acids is then done by using an elution buffer. The elution
10 buffer has a different pH and/or different ionic strength than the equilibration/loading buffer.

In one embodiment it has a higher pH and/or a higher ionic strength than the equilibration/loading buffer. In one embodiment the pH of the elution buffer is above pH 7, preferably between pH 8.5 and 9.5. In one embodiment the elution buffer
15 comprises between 0.5 and 1.5 M NaCl.

In any case, in the method of the present invention, at least one time a wash solution comprising a zwitterionic detergent is used, preferably the zwitterionic detergent is selected from the amine oxide group or mixtures thereof. Most preferred detergent
20 is N,N-Dimethyltetradecylamine N-oxide.

The method of the present invention can be used for continuous, semi-continuous or batch chromatography using one or several chromatography columns. The various chromatography modes are known to a person skilled in the art and the
25 teaching of the present invention can be easily adapted to the respective modes by the skilled expert.

Performing nucleic acid purification according to the method of the present invention using zwitterionic detergents, target nucleic acid can be obtained with significantly
30 lower endotoxin contamination compared to purification methods omitting use of detergents.

Depending on the actual type of anion exchange capture material used for nucleic acid purification, the method results in a 30 to 600-fold enhancement of endotoxin reduction.

- 5 The types of AEX materials and the specific class of detergent, which are preferably selected for the method disclosed herein, are particularly suitable for removing or depleting endotoxins from samples comprising nucleic acids like plasmid DNA.

Starting material, i.e. sample, in volume quantities ranging from 5 to 5000 liters
10 having a nucleic acid, e.g. plasmid DNA concentration ranging from 0.02 to 1.0 mg/ml can be processed with the method of the present invention. The method is preferably applied at a scale of 50 to 500 L sample volume having plasmid titers of 0.050 to 0.200 mg/mL. Total loading of 1 to 10 mg nucleic acid, e.g. plasmid DNA, per mL volume of AEX membrane or monolith adsorber and flow rates between 1
15 to 10 volumes of the membrane or monolith device per minute are suitable with the method of the invention.

The method of the present invention is thus also suitable for large scale purification of nucleic acids and thus large scale removal or depletion of endotoxins from said nucleic acid samples. Also the amount of nucleic acids that are present in the
20 sample may vary within large ranges and may be as high as 1 mg/ml. The method also allows for very high loading of up to 20 mg nucleic acid per ml volume of the membrane or monolith.

With the method of the present invention varying amounts of target nucleic acids, especially plasmid DNA, can be processed so that for a batch 0.1 mg up to 5 kg
25 nucleic acid can be subjected to chromatographic purification.

The final endotoxin level in the target nucleic acid pool depends on the initial endotoxin level. With initial endotoxin levels around ~275,000 EU/mg target nucleic acid, with the method of the present invention final endotoxin levels as low as 10 to
30 40 EU/mg target nucleic acid can be achieved.

In one embodiment, the method of the present invention is performed by only using N,N-Dimethyltetradecylamine N-oxide as detergent. No other detergent is added to

the feed or the wash buffers or at any other time during the chromatographic purification.

The present invention is further illustrated by the following figures and examples,
5 however, without being restricted thereto.

The entire disclosure of all applications, patents, and publications cited above and below as well as the corresponding patent application US 63/318,548 filed March 10, 2022 are hereby incorporated by reference.

10

Examples

The following examples represent practical applications of the invention.

15 List of Detergents

	Chemical	Article number/ Lot number	Supplier
20	Devion™ (N,N-Dimethyltetradecylamine N-oxide)	Research sample / Lot 84184	Sigma
	Triton™X100	1.12298.1001 / K29892598	Merck

Protocols for Plasmid DNA Capture

25

Note: Small volume membrane screening devices where the system holdups are disproportionately large, very high volumes were used for wash, elution, cleaning in place (CIP) and equilibration are typical. At larger scale these values can be reduced and flow direction reversed for enhancing individual steps. This is a standard
30 practice and common knowledge for any one proficient in art.

Chromatography Materials

Material (Device)	Article number	Supplier
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5	Natrix® Q Recon Mini with 0.2 mL membrane bed volume	NXF-01	Merck Millipore
	Mustang® Q Acrodisc® with 0.18 mL membrane bed volume	MSTG25Q6	Pall Life Sciences
	CIMmultus® DEAE 1 mL packed column volume	311.5114-2	BIA Separations

Natrix® Q Protocol

- 10 • Chromatography Buffers

	Step	Buffer
	equilibrate	1M K-acetate + 160 mM NaCl, pH 5.0
	sample loading	8 kb Plasmid DNA Lysate w/o detergent
15	wash 1	100 mM Tris + 500 mM NaCl, pH 9.0
	wash 2	100 mM Tris + 500 mM NaCl, pH 9.0 with detergent at specified concentration
	wash 3	100 mM Tris + 500 mM NaCl, pH 9.0
	wash 4	20 % EtOH + 150 mM NaCl
20	elution	1 M NaCl + 100 mM Tris, pH 9.0
	CIP	2 M NaCl + 1 M NaOH
	sample pump wash	1M K-acetate + 160 mM NaCl, pH 5.0

- 25 • Chromatography Method

	Step	Volume	Flow rate
	equilibration	5 MV (membrane device volume)	2 mL/min
30	load	variable	2 mL/min
	wash 1	20 MV	2 mL/min
	wash 2	20 MV	2 mL/min

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5	wash 3	20 MV	1 mL/min
	wash 4	20 MV	1 mL/min
	elution	30 MV plasmid eluate fraction: 0 - > 25 MV	1 mL/min
	CIP	20 MV -> 15 min pause -> 20 MV	2 mL/min
	equilibration	40 MV	2 mL/min

10

- Plasmid Feed

Original 8 kb Plasmid lysate used as feed showed an initial Endotoxin level of ~275,000 EU/mg Plasmid. The lysate filtered with 0.22 µm PES media and supplemented with 175 mM NaCl required for selective binding of pDNA.

15

Mustang® Q Protocol

- Chromatography Buffers

20	Step	Buffer
	equilibrate	1M K-acetate + 375 mM NaCl, pH 5.0
	sample loading	8 kb Plasmid DNA Lysate w/o detergent
25	wash 1	100 mM Tris + 500 mM NaCl, pH 9.0
	wash 2	100 mM Tris + 500 mM NaCl, pH 9.0 with detergent at specified concentration
	wash 3	100 mM Tris + 500 mM NaCl, pH 9.0
	wash 4	20 % EtOH + 150 mM NaCl
	elution	1 M NaCl + 100 mM Tris, pH 9.0
30	CIP	2 M NaCl + 1 M NaOH
	sample pump wash	1M K-acetate + 375 mM NaCl, pH 5.0

- Chromatography Method

Step	Volume	Flow rate
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5	equilibration	5 MV	1.8 mL/min
	load	variable	1.8 mL/min
	wash 1	22 MV	1.8 mL/min
	wash 2	22 MV	1.8 mL/min
	wash 3	22 MV	1.8 mL/min
10	wash 4	22 MV	1.8 mL/min
	elution	33 MV plasmid eluate fraction: 0 - > 28 MV	0.9 mL/min
	CIP	22 MV -> 15min pause -> 22 MV	1.8 mL/min
	equilibration	40 MV	1.8 mL/min

- Plasmid Feed

15 Purification trials were conducted with original lysate filtered with 0.22 µm PES media and supplemented with 375 mM NaCl required for selective binding of pDNA.

CIMmultus® DEAE Protocol

- Chromatography Buffers

20	Step	Buffer
	equilibrate	1M K-acetate + 60 mM NaCl, pH 5.0
	sample loading	8 kb Plasmid DNA Lysate w/o detergent
	wash 1	100 mM Tris + 250 mM NaCl, pH 7.5
25	wash 2	100 mM Tris + 250 mM NaCl, pH 7.5 with detergent at specified concentration
	wash 3	100 mM Tris + 250 mM NaCl, pH 7.5
	wash 4	20 % EtOH + 150 mM NaCl
	elution	1 M NaCl + 100 mM Tris, pH 9.0
30	CIP	2 M NaCl + 1 M NaOH
	sample pump wash	100 mM Tris + 250 mM NaCl, pH 7.5

- Chromatography Method

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	Step	Volume	Flow rate
5	equilibration	3 CV (column volume)	5 mL/min
	load	variable	5 mL/min
	wash 1	5 CV	5 mL/min
	wash 2	5 CV	5 mL/min
	wash 3	5 CV	5 mL/min
10	wash 4	5 CV	5 mL/min
	elution	10 CV plasmid eluate fraction: 0 - > 5 MV	5 mL/min
	CIP	3 CV -> 10 min pause -> 2 CV	5 mL/min
	equilibration	5 CV	5 mL/min

15 • Plasmid Feed

Purification trials were conducted with original lysate filtered with 0.22 µm PES media and supplemented with 60 mM NaCl required for selective binding of pDNA.

20 **Plasmid DNA Analytics**

Purity and Quantity and Plasmid DNA in original lysate and samples collected from Matrix® Q capture trials were determined by means of analytical UV/HPLC method.

Residual amount of detergent in Plasmid eluate fractions collected from AEX capture trials was measured by means of an analytical HPLC method as described below. The method allows for direct analysis of Plasmid eluate samples without prior sample preparation for removing potentially interfering matrix components by means of e.g. solid phase extraction.

The amount of detergent in unknown eluate samples was calculated based on the analyte peak area using calibration curves obtained from standards of individual detergents in eluate buffer matrix.

The compatibility of analytical method for real plasmid samples and validity of analytical results was demonstrated by means of spike-recovery tests. For that purpose, the recovery of a defined amount of individual detergent spiked into

Plasmid eluate samples (from capture trials without use of any detergents) was verified.

5	Column type:	Oasis HLB, 25 µm 2.1x20 mm, from Waters, Cat. No.186002036		
	Column dimensions:	2.1 x20 mm (ID x L)		
	Guard column:	N/A		
10	Eluent A:	water + 0.1% v/v formic acid		
	Eluent B:	Acetonitrile + 0.1% v/v formic acid		
	Injection volume:	5- 100 µL		
15	Flow rate:	1ml/min		
	Pressure:	max. 250 bar		
	Detection:	Evaporate Light Scattering Detector (ELSD)		
20	Gradient:	time (min)	%A	%B
		0.0	95	5
		5.5	95	5
		6.0	0	100
		8.0	0	100
		8.1	95	5
		11.0	95	5

25 Results

1) Plasmid Capture with Natrix® Q

Tables R1 (Parts A and B) and R2 compare results obtained from Plasmid DNA capture trials following the Natrix® Q protocol. The membrane loading was 1.6 mg Plasmid /mL membrane volume. Original 8 kb Plasmid lysate used as feed showed an initial Endotoxin level of ~275,000 EU/mg Plasmid.

Table R1-Part A. Analytical data for Plasmid eluate pools obtained from capture trials using Natrix® Q.

5	Wash Buffer Supplement	Active Substance Concentration in Wash Buffer	Eluate Endotoxin Level in EU/mg pDNA		Residual Detergent in pDNA Pool in mg/L	
			Run1	Run2	Run 1	Run 2
	w/o	-/-	2917	6955	N/A	N/A
	Devirion™	1 % w/v	8	7	22	20

10 Table R1-Part B. Analytical data for Plasmid eluate pools obtained from capture using Natrix® Q.

15	Wash Buffer Supplement	Active Substance Concentration in Wash Buffer	Plasmid Eluate Yield (%)		Plasmid Purity in % (total A260 area)	
			Run1	Run2	Run 1	Run2
	w/o	-/-	88	95	~94	~91
	Devirion™	1 % w/v	85	92	~97	~97

20 The efficacy of endotoxin removal observed for pDNA capture using the Devirion™ wash protocol is given in Table R2.

25 Table R2. Factor of Endotoxin reduction in Plasmid eluate pools relative to the baseline experiment conducted without use of detergent. Values stated are mean values calculated from duplicate runs.

Wash Buffer Supplement	Active Substance Concentration in Wash Buffer	Endotoxin Reduction Factor
Devirion™	1 % w/v	658

30 Residual host cell protein concentration in Plasmid eluate pools obtained from Natrix Q capture are listed in Table 3.

Table R3. Removal of E.coli host cell protein (HCP) from Plasmid DNA during Natrix Q capture step. For each protocol, plasmid eluate pools collected from two consecutive runs were analyzed (referred as run1 and run 2). HCP concentration in original Plasmid lysate amounted to 3,235 µg HCP/mg pDNA.

Wash Buffer Supplement	Active Substance in Wash Buffer	Residual HCP conc. in ng/mg pDNA	
		Run 1	Run 2
w/o	-/-	439	1000
Devirion™	1 % w/v	2734	1171

2) Plasmid Capture with Mustang® Q

Tables R4 (Parts A and B) and R5 compare results obtained from Plasmid DNA capture trials with Mustang® Q. Membrane loading was 1.6 mg Plasmid /mL membrane volume. Original 8 kb Plasmid lysate used as feed showed an initial Endotoxin level of ~275,000 EU/mg Plasmid.

Table R4-Part A. Analytical data for Plasmid eluate pools obtained from capture trials using Mustang® Q.

Wash buffer Supplement	Active Substance Concentration in Wash Buffer	Eluate Endotoxin Level in EU/mg pDNA		Residual Detergent in pDNA Pool in mg/L	
		Run1	Run2	Run 1	Run 2
w/o	-/-	3,983	7,081	N/A	N/A
Devirion™	1 % w/v	18	20	10	10

Table R4-Part B. Analytical data for Plasmid eluate pools obtained from capture using Mustang® Q.

Wash buffer Supplement	Active Substance Concentration in Wash Buffer	Plasmid Eluate Yield (%)		Plasmid Purity in % (total A260 area)	
		Run 1	Run 2	Run 1	Run 2
w/o	-/-	32	33	>95	>95

Deviron TM	1 % w/v	30	29	>95	>95
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The efficacy of endotoxin removal observed for pDNA capture using the DevironTM wash protocol is given in Table R5.

Table R5. Factor of Endotoxin reduction in Plasmid eluate pools relative to the baseline experiment conducted without use of detergent. Values stated are mean values calculated from duplicate runs.

Wash Buffer Supplement	Active Substance Concentration in Wash Buffer	Endotoxin Reduction Factor
Deviron TM	1 % w/v	291

Residual host cell protein concentration in Plasmid eluate pools obtained from Mustang® Q capture runs are listed in Table 6.

Table R6. Removal of E.coli host cell protein (HCP) from Plasmid DNA during Mustang® Q capture step. Table below compares residual HCP concentrations measured in Plasmid eluate pools from Mustang® Q capture. For each protocol, plasmid eluate pools collected from two consecutive runs were analyzed (referred as run1 and run 2). HCP concentration in original Plasmid lysate amounted to 3,235 µg HCP/mg pDNA.

Wash Buffer Supplement	Active Substance in Wash Buffer	Residual HCP conc. in ng/mg pDNA	
		Run 1	Run 2
w/o	-/-	2380	6457
Deviron TM	1 % w/v	769	769

3) Plasmid Capture with CIMmultus® DEAE

Tables R7 (Parts A and B) and R8 compare results obtained from Plasmid DNA capture trials with CIMmultus® DEAE. Column loading was ~1 mg Plasmid /mL

column volume. Original 8 kb Plasmid lysate used as feed showed an initial Endotoxin level of ~275,000 EU/mg Plasmid.

5 Table R7-Part A. Analytical data for Plasmid eluates from capture trials using CIMmultus® DEAE.

Wash Buffer Supplement	Active Substance Concentration in Wash Buffer	Eluate Endotoxin Level in EU/mg pDNA		Residual Detergent in pDNA Pool in mg/L	
		Run 1	Run2	Run 1	Run 2
w/o	-/-	738	N/A	N/A	N/A
Devirion™	1 % w/v	12	35	19	20

15 Table R7-Part B. Analytical data for Plasmid eluates from capture using CIMmultus® DEAE.

Wash Buffer Supplement	Active Substance Concentration in Wash Buffer	Plasmid Eluate Yield (%)		Plasmid Purity in % (total A260 area)	
		Run 1	Run 2	Run 1	Run 2
w/o	-/-	91	N/A	92	N/A
Devirion™	1 % w/v	92	98	91	92

The efficacy of endotoxin removal observed for pDNA capture using the Devirion™ wash protocol is given in Table R8

25 Table R8. Factor of Endotoxin reduction in Plasmid eluate pools relative to the baseline experiment conducted without use of detergent. Values stated are mean values calculated from duplicate runs.

Wash Buffer Supplement	Active Substance Concentration in Wash Buffer	Endotoxin Reduction Factor
Devirion™	1 % w/v	31

Residual host cell protein concentration in Plasmid eluate pools obtained from CIMmultus® DEAE capture trials are listed in Table 9.

Table R9. Removal of E.coli host cell protein (HCP) from Plasmid DNA during CIMmultus® DEAE capture step. For each protocol, plasmid eluate pools collected from two consecutive runs were analyzed (referred as run1 and run 2). HCP concentration in original Plasmid lysate amounted to 3,235 µg HCP/mg pDNA.

Wash Buffer Supplement	Active Substance in Wash Buffer	Residual HCP conc. in ng/mg pDNA	
		Run 1	Run 2
w/o	-/-	117	N/A
Devirion™	1 % w/v	203	136

4) Endotoxin Clearance with Devirion™ Compared to Common Alternative Approach using Neutral Detergent Triton™ X100

Table R10. Comparison of Endotoxin removal in Plasmid purification following different protocols. Table below states Endotoxin reduction factors relative to baseline experiments without use of detergent.

AEX Capture Material	Endotoxin Removal Factor	
	Triton™ X100 Protocol	Devirion™ Protocol
Natrix® Q	87	658
Mustang® Q	45	291
CIMmultus® DEAE Monolith	90	31

Patent Claims

1. A method for depletion or removal of endotoxins from nucleic acids comprising
 - 5 a) Providing a sample comprising said nucleic acids and endotoxins
 - b) Subjecting the sample of step a) to a chromatographic separation on a membrane or monolith comprising anion exchange groups whereby the sample is contacted with a zwitterionic detergent selected from the group of amine oxides or mixtures thereof prior or during the
 - 10 chromatographic separation.
2. Method according to claim 1 characterized in that step b) comprises
 - i) Loading the sample comprising said nucleic acids and endotoxins onto the membrane or monolith comprising anion exchange groups
 - 15 ii) Washing the membrane or monolith with a wash buffer
 - iii) Eluting the nucleic acids bound to the membrane or monolith with an elution buffer
3. Method according to claim 1 or claim 2, characterized in that the nucleic acids are contacted with the zwitterionic detergent by washing the membrane or
- 20 monolith with a wash buffer comprising the zwitterionic detergent.
4. Method according to claim 3, characterized in that the wash buffer comprising a zwitterionic detergent comprises between 0.01% and 10%
- 25 (w/v) of the zwitterionic detergent.
5. Method according to one or more of claims 1 to 4, characterized in that the zwitterionic detergent used in the method of the invention is a C12-C16 alkyl dimethylamine oxide.
- 30 6. Method according to one or more of claims 1 to 5, characterized in that the amine oxide used in the method of the invention is N,N-Dimethyltetradecylamine N-oxide.

7. Method according to one or more of claims 1 to 6, characterized in that the nucleic acids comprise or consist of plasmid DNA.
- 5 8. Method according to one or more of claims 1 to 7, characterized in that the nucleic acids are contacted with a solution comprising 0.01 to 10 % (w/v) of the zwitterionic detergent.
9. Method according to one or more of claims 1 to 8, characterized in that in step b) a membrane is used, preferably a hydrogel membrane.
- 10 10. Method according to one or more of claims 1 to 9, characterized in that step ii) comprises two or more wash steps whereby one wash step is done with a wash buffer comprising ethanol.
- 15 11. Method according to one or more of claims 1 to 10, characterized in that in step b) the volume of the sample subjected to chromatographic separation is between 5 to 5000 liters having a plasmid DNA concentration ranging from 0.02 to 1.0 mg/ml.
- 20 12. Method according to one or more of claims 1 to 10, characterized in that in step b) the mass of nucleic acid subjected to chromatographic separation is in the range of 0.1 gm to 5 Kg for a batch.
- 25 13. Method according to one or more of claims 1 to 12, characterized in that in step b) 1 to 20 mg nucleic acids are loaded per mL volume of membrane or monolith comprising anion exchange groups.
- 30 14. Method according to one or more of claims 1 to 13, characterized in that in step b) the chromatographic separation is performed with flow rates between 1 to 10 volumes of the membrane or monolith per minute.