



(51) International Patent Classification:

B01D 15/36 (2006.01) C07K 1/18 (2006.01)
B01D 15/38 (2006.01) C07K 16/00 (2006.01)
B01J 39/26 (2006.01)

(21) International Application Number:

PCT/US2019/022687

(22) International Filing Date:

18 March 2019 (18.03.2019)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/644,234 16 March 2018 (16.03.2018) US

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(81) Designated States (*unless otherwise indicated, for every*

kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ,
CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO,
DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,
HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP,
KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,
OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,
SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every*

kind of regional protection available): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ,
UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,
TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,
EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,
MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: PURIFICATION OF TARGET MOLECULES

(57) Abstract: The present invention provides a method of purifying a target molecule in a sample containing the target molecule, for example, an acidic target molecule, that utilizes a multimodal ligand.



WO 2019/178587 A1

PURIFICATION OF TARGET MOLECULES

The Sequence Listing for this application is labeled "Seq-List.txt" which was created on March 14, 2019 and is 11 KB. The entire content of the sequence listing is incorporated
5 herein by reference in its entirety.

BACKGROUND OF THE INVENTION

Downstream purification of recombinant proteins pose multiple challenges which affect the process productivity. The manufacture of recombinant proteins, especially the ones
10 lacking affinity handles, often requires multiple chromatography steps and excessive feed conditioning, resulting in high production costs and relatively low yields. Thus, there remains a need in the art to develop robust purification methods that permit for the purification of target molecules, such as very low expression, non-affinity tagged acidic recombinant proteins from *Escherichia coli* (*E. coli*). The subject application provides methods addressing
15 such needs.

BRIEF DESCRIPTION OF THE INVENTION

In certain embodiments of the present invention, a sample comprising the target molecule, such as an acidic target molecule, is subjected to chromatography on
20 chromatography medium that utilizes a multimodal ligand or is by nature multimodal. In such an embodiment, the sample containing the target molecule is applied to a chromatography medium that utilizes a multimodal chromatography medium and which is equilibrated with a buffer having a pH that is equal to or higher than the pI of the target molecule (for example, up to 3.00 units higher than the pI of the target molecule). In some
25 embodiments, the pH of the equilibration buffer has a pH that is equal to or up to 3.0 units higher than the pI of the target molecule or is as high as about 9.50. Other embodiments utilize buffers that are higher than the pI of the target molecule and are within about 0.01 and about 3.00 unit of the pI of the target molecule, about 0.01 and about 2.50 unit of the pI of the target molecule, about 0.01 and about 2.00 unit of the pI of the target molecule, or about 0.01
30 and about 1.50 unit of the pI of the target molecule. In certain embodiments, the pH will be selected to be about 0.1 unit to about 1.5 units higher than the pI of the target molecule. In certain embodiments, it is in the range of about 0.1 unit to about 1 unit higher, about 0.2 unit

to about 1.5 units higher or about 0.5 to about 1.0 unit higher than the pI of the target molecule. The target molecule can be eluted from the multimodal chromatography media using a buffer containing an alkaline earth metal chloride and subjected to further chromatography steps for additional purification as the intent of the final material requires.

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BRIEF DESCRIPTION OF THE FIGURES

FIG. 1: Capture chromatography with Nuvia aPrime 4A media – Protocol and results.

FIG. 2: DoE and final capture results. A) Contour plots for purity and recovery with the various salt/additive concentrations and pH levels. B) Optimized Capture chromatography with Nuvia aPrime 4A media – Protocol and results.

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FIG. 3: SDS-PAGE analysis from the two-step purification workflow involving Nuvia aPrime 4A media.

DEFINITIONS

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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.

20

As used herein, the singular forms “a”, “an” and “the” are intended to include the plural forms as well, unless the context clearly indicates otherwise. Further, to the extent that the terms “including”, “includes”, “having”, “has”, “with”, or grammatical variations thereof are used in either the detailed description and/or the claims, such terms are intended to be inclusive in a manner similar to the term “comprising”. The transitional terms/phrases (and any grammatical variations thereof) “comprising”, “comprises”, “comprise”, include the phrases “consisting essentially of”, “consists essentially of”, “consisting”, and “consists” and any grammatical variations thereof.

25

The phrases “consisting essentially of” or “consists essentially of” indicate that the claim encompasses embodiments containing the specified materials or steps and those that do not materially affect the basic and novel characteristic(s) of the claim.

30

Except as otherwise defined herein, the term “about” or “approximately” means a variation of 0 to 10%, 0 to 5%, or up to 1% of a given value. Thus, the terms “about” or “approximately” can be expressed as a variation (error range) of 0-10% around the value ($X \pm 10\%$).

In the present disclosure, ranges are stated in shorthand, so as to avoid having to set out at length and describe each and every value within the range. Any appropriate value within the range can be selected, where appropriate, as the upper value, lower value, or the terminus of the range. For example, a range of 0.1-1.0 represents the terminal values of 0.1 and 1.0, as well as the intermediate values of 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and all intermediate ranges encompassed within 0.1-1.0, such as 0.2-0.5, 0.2-0.8, 0.7-1.0, etc.

As used herein, the term “target molecule” refers to any molecule, substance or compound that is to be isolated, separated or purified from one or more other components, e.g. impurities or contaminants, in a sample. For the purposes of this disclosure, a target molecule can be a protein or polypeptide that has an acidic pI (such a target molecule that may be referred to as an “acidic target molecule”), for example a pI <7.0, less than 6.5, less than 6.0, less than 5.5, or less than 5.0. Other embodiments provide an “acidic target molecule” that has a pI of between 3.5000 and 6.9999, between 4.00 and 6.75, between 4.5 and 6.5 or between 5.0 and 6.0. Non-limiting examples of suitable acidic target molecules include Exotoxin A or CRM197. A “target molecule” can also include a protein or polypeptide that has a basic pI, a “basic target molecule”, for example a pI greater than 7.0, a pI greater than 7.1, greater than 7.5, greater than 8.0, greater than 8.5, or greater than 9.0. Other embodiments provide a “basic target molecule” that has a pI of between 7.0001 and 9.5000, between 7.2 and 9.0, between 7.5 and 8.8 or between 7.5 and 8.5. Suitable examples of “basic target molecule(s)” include antibodies, antigen binding fragments (Fab), constant region fragments (Fc), proteins, peptides, recombinant proteins, other natural compounds that have an basic pI. In the context of the pI of a protein, the term “about” is meant to provide that the pI is within ± 0.1 or ± 0.2 unit of the protein pI.

In certain embodiments of the present invention, a sample comprising the target molecule, such as an acidic target molecule, is subjected to chromatography on chromatography medium that utilizes a multimodal ligand or is by nature multimodal. In such an embodiment, the sample containing the target molecule is applied to a chromatography medium that utilizes a multimodal chromatography medium and which is equilibrated with a buffer having a pH that is equal to or higher than the pI of the target molecule (for example, up to 3.00 units higher than the pI of the target molecule). In some embodiments, the pH of the equilibration buffer has a pH that is equal to or up to 3.0 units higher than the pI of the target molecule or is as high as about 9.5. Other embodiments

utilize equilibration buffers that are higher than the pI of the target molecule and are within about 0.01 and about 3.00 unit of the pI of the target molecule, about 0.01 and about 2.50 unit of the pI of the target molecule, about 0.01 and about 2.00 unit of the pI of the target molecule, or about 0.01 and about 1.50 unit of the pI of the target molecule. In certain
5 embodiments, the pH will be selected to be about 0.1 unit to about 1.5 units higher than the pI of the target molecule. In certain embodiments, it is in the range of about 0.1 unit to about 1 unit higher, about 0.2 unit to about 1.5 units higher or about 0.5 to about 1.0 unit higher than the pI of the target molecule. The target molecule can be eluted from the multimodal chromatography media using a buffer containing an alkaline earth metal chloride and
10 subjected to further chromatography steps for additional purification as the intent of the final material.

Exemplary polypeptides that can be “target molecules” include, e.g., antibodies or antigen binding fragments, Fc fusion proteins, renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid
15 hormone; thyroid stimulating hormone; lipoproteins; α -1-antitrypsin; insulin α -chain; insulin β -chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrand factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or tissue-type plasminogen activator (t-PA); bombesin; thrombin;
20 hemopoietic growth factor; tumor necrosis factor- α and - β ; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1- α); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin α -chain; relaxin β -chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as β -lactamase; DNase; IgE; a
25 cytotoxic T-lymphocyte associated antigen (CTLA) (e.g., CTLA-4); inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF- β ; platelet-derived growth factor (PDGF); fibroblast growth factor such as α FGF and
30 β FGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF- α and TGF- β , including TGF- β I, TGF- β 2, TGF- β 3, TGF- β 4, or TGF- β 5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(I-3)-IGF-I (brain IGF-I), insulin-like growth factor

binding proteins (IGFBPs); CD proteins such as CD3, CD4, CD8, CD 19 CD20, CD34, and CD40; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon- α , - β , and - γ ; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigens, such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CDI Ia, CDI Ib, CDI Ic, CD 18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments and/or variants of any of the above-listed polypeptides.

As used herein, and unless stated otherwise, the term “sample” refers to any composition or mixture that contains a target molecule. Samples may be derived from biological or other sources. Biological sources include eukaryotic sources like animals or humans or prokaryotic sources, such as bacterial cell lines (e.g., E. coli based systems for the production of target molecules). In certain preferred embodiments, the sample comprises fermentation media from a eukaryotic or prokaryotic cell culture that produces the target molecule. Other embodiments the sample may be a bacterial cell lysates obtained from a culture that expresses the target molecule. The sample may be diluted with a volume of a buffer, such as an equilibrating buffer for a given chromatographic media, in various ratios. For example, the sample can be diluted at a ratio of 1:X (Sample Volume:Buffer Volume), where X is any integer from 1 to 1000. In some embodiments, X is an integer from 1 to 10 or 1 to 100.

The terms “purifying,” “separating,” or “isolating,” may be used interchangeably herein and refer to increasing the degree of purity of a target molecule by separating it from a sample comprising the target molecule and one or more other components, e.g. impurities such as other proteins and/or polypeptides. Typically, the degree of purity of the target molecule is increased by removing (completely or partially) at least one impurity from the composition.

The term “impurity” or “contaminant” (or grammatical variants thereof) as used herein, refers to any molecule other than the target molecule. Non-limiting examples include a biological macromolecule such as DNA, RNA, one or more host cell proteins, nucleic acids, endotoxins, lipids, impurities of synthetic origin and one or more additives which may

be present in a sample containing the target molecule that is being separated from within the sample.

The term “chromatography” refers to any kind of technique which separates a target molecule from other molecules (components) present in a mixture. Usually, the target molecule is separated from other molecules as a result of differences in rates at which the individual molecules of the mixture migrate through a stationary medium or media under the influence of a moving phase, or in bind and elute processes. Examples for chromatographic separation processes are reversed phase chromatography, ion exchange chromatography, size exclusion chromatography, affinity chromatography, hydrophobic interaction chromatography and multimodal chromatography. The medium can be a combination of a media, ligand, or any other absorptive surface used to perform chromatography in the format of absorption between a stationary and liquid phase.

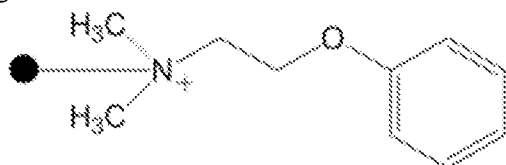
A “buffer” is a solution that resists changes in pH by the action of its acid-base conjugate components. Various buffers which can be employed depending, for example, on the desired pH of the buffer are described in Buffers. A Guide for the Preparation and Use of Buffers in Biological Systems, Gueffroy, D., ed. Calbiochem Corporation (1975). Non-limiting examples of buffers include MES (2-(N-morpholino)ethanesulfonic acid), MOPS (3-(N-morpholino)propanesulfonic acid), MOPSO (3-Morpholino-2-hydroxypropanesulfonic acid), Tris (2-Amino-2-(hydroxymethyl)-1,3-propanediol), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), phosphate, acetate, borate, citrate, succinate, and ammonium buffers, as well as combinations thereof.

“Bind-elute mode” as it relates to the invention herein, refers to an operational approach to chromatography in which the buffer conditions are established so that both a target molecule and undesired contaminants bind to the multimodal chromatography media when the sample is applied. Contaminants can be washed from the media and the target molecule eluted from the media using buffers comprising additives as disclosed herein. Bind and elute chromatography and batch mode chromatography, where medium is added to a tank of feed rather than the feed being passed through a stationary device (e.g. column or membrane), shall be considered the same as the mechanism of purification is identical.

In certain embodiments, the buffers include one or more “additive” that are salts composed of alkaline earth metal chlorides. Non-limiting examples include calcium chloride, magnesium chloride and the like.

The term “medium”, “media” or “chromatography media” are used interchangeably herein and refer to any kind of membrane, particulate media, sorbent, solid or gel phase media which, in a separation process, separates a target molecule from other molecules present in a mixture. Usually, the target molecule is separated from other molecules as a result of differences in rates at which the individual molecules of the mixture migrate through the media under the influence of a moving phase, or in bind and elute processes. The media can be put in columns or cartridges or used in bulk.

A “ligand” is a functional group that is attached to the chromatography media and that determines the binding properties of the media. 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 (also referred to as multimodal) groups (combinations of the aforementioned ligands). In some embodiments, the stationary phase (chromatography medium) does not have the ligand, and acts as the ligand itself (for example, hydroxyapatite or fluorapatite). With respect to the disclosed methods a preferred ligand for purifying a target molecule is:



, where the ●— represents a solid support to which the ligand is attached.

The term “ion-exchange” and “ion-exchange chromatography” refers to the chromatographic process in which a target molecule in a mixture interacts with a charged compound (a ligand) that is attached to a chromatography support such that the target molecule interacts non-specifically with the charged compound more than solute impurities or contaminants in the mixture. The impurities in the mixture elute from a column of the ion exchange material faster than the target molecule, which is bound to the chromatography media. “Ion-exchange chromatography” specifically includes cation exchange, anion exchange, and mixed mode ion exchange chromatography. Anion exchange chromatography can bind the target molecule via ligands on the chromatography media which is then followed by elution of the target molecule while impurities or contaminants in the sample flow through the chromatography media.

The term “anion exchange media” is used herein to refer to a chromatography media which is positively charged, e.g. having one or more positively charged ligands, such as quaternary amino groups, attached thereto. There are a number of commercially available chromatography media for anion exchange chromatography and the present invention is not limited to any specific media and/or ligand. Ligands suitable for use in the disclosed invention include DEAE and QAE. Various types of anion exchange media can be used, including DEAE-Sephadex, QAE-Sephadex, DEAE-Sephacel, DEAE-cellulose and DEAE-Sephacel-FF. According to one embodiment, the anion exchange ligand is DEAE.

“Multimodal, or mixed-mode chromatography media” refers to a chromatographic solid phase that substantially involves a combination of two or more chemical mechanisms. The terms “multimodal” and “mixed-mode” can be used interchangeably in this description. Examples of chemical mechanisms that can be combined in multimodal medias include but are not limited to any combination of two or more of the following: cation exchange, anion exchange, hydrophobic interaction, hydrophilic interaction, hydrogen bonding, pi-pi bonding, and metal affinity. The solid phase can be a porous particle, nonporous particle, membrane, or monolith.

“Hydroxyapatite” refers to a multimodal media comprising an insoluble hydroxylated mineral of calcium phosphate with the structural formula $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. Its dominant modes of interaction are phosphoryl cation exchange and calcium metal affinity. Hydroxyapatite is commercially available in various forms, including but not limited to ceramic, crystalline and composite forms. Composite forms contain hydroxyapatite microcrystals entrapped within the pores of agarose or other beads.

“Fluorapatite” refers to a multimodal media comprising an insoluble fluoridated mineral of calcium phosphate with the structural formula $\text{Ca}_{10}(\text{PO}_4)_6\text{F}_2$. Its dominant modes of interaction are phosphoryl cation exchange and calcium metal affinity. Fluorapatite is commercially available in various forms, including but not limited to ceramic and crystalline composite forms.

“Ceramic” hydroxyapatite (CHT) or “ceramic” fluorapatite (CFT) refer to forms of the respective minerals in which nanocrystals are agglomerated into particles and fused at high temperature to create stable ceramic microspheres suitable for chromatography applications. Commercial examples of ceramic hydroxyapatite include, but are not limited to CHT Type I and CHT Type II. Commercial examples of fluorapatite include, but are not

limited to CFT Type I and CFT Type II. Unless specified, CHT and CFT refer to roughly spherical particles of any average diameter, including but not limited to about 10, 20, 40, and 80 microns. The choice of hydroxyapatite or fluorapatite, the type, and average particle diameter can be determined by the skilled artisan.

5 There are a number of commercially available chromatography media for HIC, and the present invention is not limited to any specific media and/or ligand. Thus, in general terms, the HIC media typically comprises one or more hydrophobic ligands. Non-limiting examples of such ligands include hydrophobic groups such as phenyl, propyl, isopropyl, butyl, n-butyl, t-butyl, octyl, n-octyl, and the like that are bound to a solid support.

10 When “loading” a separation column in bind and elute mode, a buffer is used to load the sample or composition comprising the target molecule and one or more impurities onto a chromatography column (e.g., an ion exchange column). The buffer has a conductivity and/or pH such that the target molecule is bound to the chromatography media while ideally all the impurities are not bound and flow through the column. The pH of the buffer may be the
15 same as, or, preferably, higher than, the pI of the target molecule. Typically the buffer in which the sample is loaded on the chromatography media is called loading buffer or sample buffer. Loading or sample buffers can have a pH that is that is equal to or higher than the pI of the target molecule (for example, up to 3.00 units higher than the pI of the target molecule or a pH as high as 9.5). Thus, in some embodiments, the pH of the loading or sample buffer
20 has a pH that is equal to or up to 3.00 units higher than the pI of the target molecule. Other embodiments utilize a loading or sample buffer that is higher than the pI of the target molecule and is within about 0.01 and about 3.00 unit of the pI of the target molecule, about 0.01 and about 2.50 unit of the pI of the target molecule, about 0.01 and about 2.00 unit of the pI of the target molecule, or about 0.01 and about 1.50 unit of the pI of the target molecule.
25 In certain embodiments, the pH will be selected to be about 0.1 unit to about 1.5 units higher than the pI of the target molecule. In certain embodiments, it is in the range of about 0.1 unit to about 1 unit higher, about 0.2 unit to about 1.5 units higher or about 0.5 to about 1.0 unit higher than the pI of the target molecule.

30 The term “equilibrating” refers to the use of a buffer to equilibrate the chromatography media prior to loading the target molecule. Typically, the loading buffer is used for equilibrating the chromatography media and may also be referred to as an “equilibrating buffer”. The equilibrating buffer may or may not contain an alkaline earth

metal chloride. Equilibrating buffers can have a pH that is equal to or higher than the pI of the target molecule (for example, up to 3.00 units higher than the pI of the target molecule or a pH as high as 9.5). In some embodiments, the pH of the equilibration buffer has a pH that is equal to or up to 3.0 units higher than the pI of the target molecule. Other embodiments
5 utilize an equilibration buffer that is higher than the pI of the target molecule and is within about 0.01 and about 3.00 unit of the pI of the target molecule, about 0.01 and about 2.50 unit of the pI of the target molecule, about 0.01 and about 2.00 unit of the pI of the target molecule, or about 0.01 and about 1.50 unit of the pI of the target molecule. In certain embodiments, the pH will be selected to be about 0.1 unit to about 1.5 units higher than the pI
10 of the target molecule. In certain embodiments, it is in the range of about 0.1 unit to about 1 unit higher, about 0.2 unit to about 1.5 units higher or about 0.5 to about 1.0 unit higher than the pI of the target molecule.

By “wash” or “washing” a chromatography media is meant passing an appropriate liquid, e.g. a buffer through or over the media. Typically washing is used to remove weakly
15 bound contaminants from the media prior to eluting the target molecule and/or to remove non-bound or weakly bound target molecule after loading. Typically, the wash buffer and the loading buffer are the same. However, in some embodiments, a wash buffer can contain additives not found in the loading buffer. In this case, typically, the washing buffer differs from loading buffer and may contain a detergent or detergents (such as polysorbate 80) or
20 have different properties, such as low or high pH or high conductivity (e.g., buffers containing salts, such as NaCl in a concentration of 10-2000 mM or KCl in a concentration of 10-2000 mM, etc.). Wash buffers can have a pH that is equal to or higher than the pI of the target molecule (for example, up to 3.00 units higher than the pI of the target molecule or a pH as high as 9.5). In some embodiments, the pH of the wash buffer has a pH that is equal to
25 or up to 3.0 units higher than the pI of the target molecule. In other embodiments, the wash buffer has a pH that is higher than the pI of the target molecule and is within about 0.01 and about 3.00 unit of the pI of the target molecule, about 0.01 and about 2.50 unit of the pI of the target molecule, about 0.01 and about 2.00 unit of the pI of the target molecule, or about 0.01 and about 1.50 unit of the pI of the target molecule. In certain embodiments, the pH will be
30 selected to be about 0.1 unit to about 1.5 units higher than the pI of the target molecule. In certain embodiments, it is in the range of about 0.1 unit to about 1 unit higher, about 0.2 unit

to about 1.5 units higher or about 0.5 to about 1.0 unit higher than the pI of the target molecule.

To “elute” a target molecule from a chromatography medium means that the target molecule is removed from the chromatography medium. In the case of this disclosure, the target molecule can be eluted by the addition of an “additive” to the a buffer (which may be referred to as an “elution or wash buffer”). The additive can be provided in the form of a concentration gradient (elution gradient) that is applied to the chromatography medium (the elution buffer is formulated such that the concentration/amount of the additive in the elution buffer increases over time) and the target molecule is eluted or isocratic elution can be used to elute the target molecule from the chromatography media (the elution buffer contains a constant concentration/amount of the additive). As discussed above, alkaline earth metal chlorides are additives can be added to a wash buffer in order to form an “elution buffer”. Elution buffers can have a pH that is equal to or higher than the pI of the target molecule (for example, up to 3.00 units higher than the pI of the target molecule or a pH as high as 9.5). In some embodiments, the pH of the wash buffer has a pH that is equal to or up to 3.0 units higher than the pI of the target molecule. In other embodiments, the wash buffer has a pH that is higher than the pI of the target molecule and is within about 0.01 and about 3.00 unit of the pI of the target molecule, about 0.01 and about 2.50 unit of the pI of the target molecule, about 0.01 and about 2.00 unit of the pI of the target molecule, or about 0.01 and about 1.50 unit of the pI of the target molecule. In certain embodiments, the pH will be selected to be about 0.1 unit to about 1.5 units higher than the pI of the target molecule. In certain embodiments, it is in the range of about 0.1 unit to about 1 unit higher, about 0.2 unit to about 1.5 units higher or about 0.5 to about 1.0 unit higher than the pI of the target molecule.

Also provided are methods of purifying a target molecule. In an embodiment, the method comprises contacting a sample comprising the target molecule with multimodal medium under conditions that allow the target molecule to bind to the multimodal medium, thereby separating the target molecule from a contaminant that does not bind to the multimodal medium. The resulting purified target molecule is subsequently collected by elution. An intermediate step of washing the multimodal medium may be performed if desired.

In some bind-elute mode embodiments, loading (e.g., binding the target molecules, such as antibodies or non-antibody proteins to the chromatography media), and optionally washing, is performed at a pH above 7, e.g., between 7-8 or 7-9, where the pH is equal to or higher than the pI of the target molecule and is within about 0.01 and about 3.0 unit of the pI of the target molecule when the pH is higher than the pI of the target molecule. Some exemplary bind and elute conditions are:

binding condition: 0-2000 mM NaCl or 100-300 mM NaCl or 0-10mM MgCl₂ or 0-10mM CaCl₂, pH 3.5-9.5 in an appropriate buffer (e.g., MES, MOPS, MOPSO, Tris, HEPES, phosphate, acetate, citrate, succinate, borate, ammonium buffers, or combinations thereof), said buffer having a pH equal to or higher than the pI of the target protein and being within about 0.01 and about 3.0 unit of the pI of the target molecule where the buffer pH is higher than the pI of the target molecule;

elution condition: 0-1000 mM MgCl₂ or CaCl₂, pH 7.0-9.0 or 6.0-9.0, using an appropriate buffer, such as MES, MOPS, MOPSO, Tris, HEPES, phosphate, borate, acetate, citrate, succinate, ammonium buffers, or combinations thereof wherein the elution buffer has a pH that is equal to or higher than the pI of the target molecule and is within about 0.01 and about 3.0 unit of the pI of the target molecule where the pH of the elution buffer is higher than the pI of the target molecule.

Optionally, the chromatography media can be washed under conditions such that some components of the sample are removed from the chromatography media but the target molecules remain immobilized on (bound to) the media. In some embodiments, the target molecule is subsequently eluted by applying a concentration gradient of a buffer comprising an alkaline earth metal chloride to the chromatography media or utilizing isocratic elution of the target molecule by applying a buffer containing a constant concentration of an alkaline earth metal chloride to the chromatography media in order to elute the target molecule from the chromatography. As discussed above, the pH of the wash buffer and the elution buffer are higher than the pI of the target molecule and are within about 0.01 and about 3.0 unit of the pI of the target molecule.

The method may further comprise a "polishing step". Thus, certain embodiments of the method further comprises performance of one or more additional chromatographic steps ("polishing" step(s)). In particular embodiments, the methods of the invention include a polishing step of performing chromatographic separation using a Hydrophobic

Interaction Chromatography (HIC) media, anion exchange chromatography using a media such as DEAE, hydroxyapatite, ceramic hydroxyapatite (CHT), fluorapatite, ceramic fluorapatite (CFT), or combinations thereof.

5 Exotoxin A (amino acid sequence), SEQ ID NO: 1

MHLIPHWIPLVASLGLLAGGSSASAAEEAFDLWNECAKACVLDLKDGVRRSSRMSVD
 PAIADTNGQGVHLHYSMVLEGGNDALKLAIDNALSITSDGLTIRLEGGVEPNKPVRY
 YTRQARGSWSLNWLVPIGHEKPSNIKVFIHELNAGNQLSHMSPIYTIEMGDELLAKL
 ARDATFFVRAHESNEMQPTLAISHAGVSVVMAQAQPRREKRWSEWASGKVLCLLD
 10 PLDGVYNYLAQQRCNLDDTWEGKIYRVLAGNPAKHDLDIKPTVISHRLHFPEGGLA
 ALTAHQACHLPLETFTRHRQPRGWEQLEQCGYPVQRLVALYLAARLSWNQVDQVI
 RNALASPGSGGDLGEAIREQPEQARLALTLAAESERFVRQGTGNDEAGAASADVV
 SLTCPVAAGECAGPADSGDALLERNYPTGAEFLGDGGDVSFSTRGTQNWTVRLLQ
 AHRQLEERGYVFGYHGTFLAAQSVFVGVRARSQDLDAIWRGFYIAGDPALAYG
 15 YAQDQEPDARGIRNGALLRVYVPRSSLPGFYRTGLTLAAPEAADEVERLIGHPLPL
 RLDAITGPEEEGGRLETILGWPLAERTVVIPSAIPTDPRNVGGDLDPSSIPDQEQAISAL
 PDYASQPGKPPREDLK

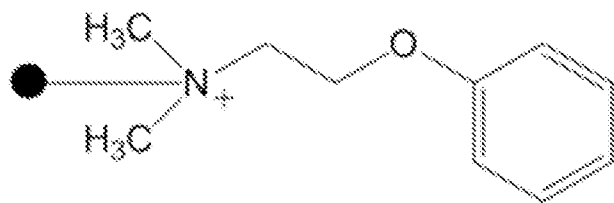
CRM197 (amino acid sequence), SEQ ID NO: 2

20 GADDVVDSSKSFVMENFSSYHGTPGYVDSIQKGIQKPKSGTQGNYYDDDWKEFYST
 DNKYDAAGYSVDNENPLSGKAGGVVKVTYPGLTKVLALKVDNAETIKKELGLSLTE
 PLMEQVGTEEFIKRFGDGASRVVLSLPAEGSSSVEYINNWEQAKALSVELEINFETR
 GKRQDAMYEYMAQACAGNRVRRSVGSSLSCINLDWDVIRDKTKTKIESLKEHGPI
 25 KNKMSSEPNKTVSEEKAKQYLEEFHQTALEHPELSELKTVTGTNPVFAGANYAAWA
 VNVAQVIDSETADNLEKTTAALSILPGIGSVMGIADGAVHHNTEEIVAQSIALSSLMV
 AQAIPLVGELVDIGFAAYNFVESIINLFQVVHNSYNRPAYSPGHKTQPFLHDGYAVS
 WNTVEDSIIRTGFQGESGHDIKITAENTPLPIAGVLLPTIPGKLDVNKSKTHISVNGRKI
 RMRCRAIDGDVTFCRPKSPVYVGNGVHANLHVAFHRSSSEKHSNEISSDSIGVLGYQ
 30 KTVDHTEKVNKLSLFFFEIKS

The following non-limiting embodiments are also provided:

1. A method of purifying an target molecule from a sample comprising:

a) contacting an sample comprising said target molecule to a multimodal chromatography (MMC) medium comprising a ligand under conditions that allow the target molecule to bind to the multimodal chromatography media, said ligand being



, wherein  represents a solid support to which said ligand is bound; and

b) eluting the target molecule from the multimodal chromatography media, thereby forming a multimodal eluate,

wherein the MMC media has been equilibrated with an equilibration buffer having a pH that is equal to or higher than the pI of the target molecule and, if the equilibration buffer is higher than the pI or the target molecule, the pH of the equilibration buffer is within about 0.01 and about 3.0 units of the pI of the target molecule.

2. The method of embodiment 1, said method further comprising:

a) loading the multimodal eluate to an polishing media under conditions that allow the target molecule to bind to the polishing media; and

b) eluting the target molecule from the polishing media.

3. The method of embodiment 2, wherein the polishing media is selected from hydroxyapatite, fluorapatite, ceramic hydroxyapatite, ceramic fluorapatite, anion exchange chromatography media, hydrophobic interaction chromatography media and combinations thereof.

4. The method of embodiments 1-3, wherein the sample comprising the target molecule contains one or more contaminants selected from proteins, nucleic acids, lipopolysaccharides and combinations thereof.

5. The method of embodiment 4, wherein the sample comprises:

a) fermentation media containing said target molecule obtained from a prokaryotic or eukaryotic cell culture; or

b) a prokaryotic cell lysate containing said target molecule.

6. The method of embodiment 1, wherein in step a), the sample that is contacted to the multimodal chromatography media is diluted in a buffer.

7. The method of embodiments 1-3, wherein the multimodal chromatography media has been equilibrated with a buffer (equilibration buffer) that has a pH that is higher than the pI of the target molecule and is within about 0.01 and about 2.5 unit of the pI of the target molecule.

8. The method of embodiments 1-3, further comprising, after step a), washing the multimodal chromatography media with a wash buffer that has a pH that is higher than the pI of the target molecule and is within about 0.01 and about 3.0 unit of the pI of the target molecule.

9. The method of embodiment 8, wherein the wash buffer comprises 0-2.0 M NaCl.

10. The method of embodiments 1-3, wherein in step b) the target molecule is eluted from the multimodal chromatography media with an elution buffer that has a pH that is higher than the pI of the target molecule and is within about 0.01 and about 3.0 units of the pI of the target molecule and comprises an alkaline earth metal chloride in a concentration of 0.01-1.0M.

11. The method of embodiment 10, wherein in step (b) the target molecule is eluted from the multimodal chromatography media with an elution buffer comprising an alkaline earth metal chloride in a concentration of 0-500 mM, 0-750 mM, 100mM-500mM, 100mM-750 mM, 250 mM to 500 mM or 250 mM to 750 mM and the alkaline earth metal chloride is applied as a gradient or isocratically to the multimodal chromatography media.

12. The method of embodiment 11, wherein the alkaline earth metal chloride is MgCl_2 or CaCl_2 or a mixture thereof.

13. The method of any preceding embodiment, wherein the buffer is a MES (2-(N-morpholino)ethanesulfonic acid) buffer, MOPS (3-(N-morpholino)propanesulfonic acid) buffer, MOPSO (3-Morpholino-2-hydroxypropanesulfonic acid) buffer, Tris (2-Amino-2-(hydroxymethyl)-1,3-propanediol) buffer, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, phosphate buffer, acetate buffer, borate buffer, citrate buffer, succinate buffer, ammonium buffer or is a combination thereof.

14. The method of embodiment 6, wherein the sample is diluted with equilibrating buffer.

15. The method of embodiment 14, wherein the sample is diluted at a 1:1 ratio with equilibrating buffer prior to loading onto the multimodal chromatography media.

16. The method of embodiments 1-3, wherein the target molecule is exotoxin A or CRM197.

17. The method of embodiment 10, wherein the buffer containing the alkaline earth metal chloride is applied in a concentration gradient to the multimodal chromatography media to elute said target molecule.

18. The method of embodiment 10, wherein a fixed concentration of alkaline earth metal chloride in a buffer is applied to the multimodal chromatography media to elute said target molecule.

19. The method of embodiment 11 or 12, wherein the buffer containing the alkaline earth metal chloride is applied in a concentration gradient to the multimodal chromatography media to elute said target molecule.

20. The method of embodiment 11 or 12, wherein a fixed concentration of alkaline earth metal chloride in a buffer is applied to the multimodal chromatography media to elute said target molecule.

21. The method of any preceding embodiment, wherein the elution buffer has a pH of about 6.0 to about 9.5, about 6.0 to about 8.5, about 6.5 to about 8.0 or about 6.5 to about 7.5.

22. The method of any preceding embodiment, wherein the equilibration buffer has a pH of about 6.0 to about 9.5, about 6.0 to about 8.5, about 6.5 to about 8.0 or about 6.5 to about 7.5.

23. The method of any preceding embodiment, wherein the wash buffer has a pH of about 6.0 to about 9.5, about 6.0 to about 8.5, about 6.5 to about 8.0 or about 6.5 to about 7.5.

24. The method of any preceding embodiment, wherein the wash buffer has a pH of about 6.0 to about 9.5, about 6.0 to about 8.5, about 6.5 to about 8.0 or about 6.5 to about 7.5.

25. The method of any preceding embodiment, wherein the elution buffer has a pH of about 6.0 to about 9.5, about 6.0 to about 8.5, about 6.5 to about 8.0 or about 6.5 to about 7.5.

26. The method of any preceding embodiment, wherein the equilibration buffer has a pH of about 6.0 to about 9.5, about 6.0 to about 8.5, about 6.5 to about 8.0 or about 6.5 to about 7.5.

27. The method of any preceding embodiment, wherein the target molecule is an acidic target molecule.

28. The method of embodiments 1-3, wherein the target molecule is an acidic target molecule.

29. The method of embodiment 28, wherein the target molecule is an acidic target molecule and has a pI of between 5.0 and 6.5.

30. The method of embodiment 10, wherein the target molecule is an acidic target molecule.

31. The method of embodiment 30, wherein the target molecule is an acidic target molecule and has a pI of between 5.0 and 6.5.

32. The method of any preceding embodiment, wherein the equilibration buffer has a pH equal to the pI of the target molecule.

33. The method of any preceding embodiment, wherein the wash buffer has a pH equal to the pI of the target molecule.

34. The method of any preceding embodiment, wherein the elution buffer has a pH equal to the pI of the target molecule.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

5 Following are examples which illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

EXAMPLE 1 –

This example demonstrates an exemplary purification scheme for a target molecule.

10 This scheme identified optimal capture conditions leading to the single step purification of the target molecule to >80% purity and >90% yield. A further polishing step with another chromatography media increased the purity levels to >95% and the final recovery to >75%.

Results

15 Capture Purification Media Screening:

Table 1: Summary of all the media tested and their results				
Chromatography Media	Binding	Recovery	Purity	Elution Conditions
Bio-Rad Laboratories Nuvia™ aPrime 4A	✓	✓	✓	✓
Pall Biotech Hypercel™ STAR AX	✗	*	*	*
Tosoh Bioscience Toyopearl® Butyl 650M	✗	*	*	*
Tosoh Bioscience Toyopearl® Phenyl 600M	✗	*	*	*
Pall Biotech CMM Hypercel™	✗	*	*	*
Pall Biotech HEA Hypercel™	✗	*	*	*
Pall Biotech PPA Hypercel™	✓	✗	*	✗

* Indicates criteria were not evaluated due to another critical criteria not being satisfied by chromatographic media.

Only Nuvia aPrime 4A, a multimodal medium, met the capture screening requirements of efficient binding and recovery of the target molecule under mild binding conditions (Table 1).

5

Initial Capture Purification study with Nuvia aPrime 4A:

Capture chromatography was therefore performed with Nuvia aPrime 4A. At modest salt concentrations and neutral pH, the majority of impurities such as host cell proteins, lipids, and nucleic acids, were eliminated in the column flow-through fractions (Figure 1).

10 Interestingly, the target molecule was not eluted from this column by high concentrations of sodium chloride (data not shown). Instead, a divalent metal ion containing buffer was found effective for recovering the target. Table 2 illustrates various conditions examined for elution of the target molecule and based on the purity and recovery results (Figure 2A), the final elution was performed (Figure 2B).

15 DoE for elution with varying pH, NaCl and Additive 1 (MgCl₂)conc.

Table 2: Summary of all the elution conditions tested	
Step	Buffer
Equilibrate	25 mM Tris, pH 7.0
Load	Flocculated SCRB-981 fermentate diluted 1:1 with 25 mM Tris pH 7.0 (8 mS/cm, pH 7.0, A280 = 11.958)

Wash	25 mM Tris + 200 mM NaCl, pH 7.0
Elution	NaCl (100-400mM), Additive 1 (Concentrations 1-3), pH (7.0-9.0)
Strip	0.5 M NaOH

Polish Purification with another mixed-mode resin:

The eluate from the capture step was loaded directly onto the second chromatographic media, for polish purification, resulting in over 95% purity and close to quantitative recovery of the target molecule (Figure 3).

Conclusions:

This is an effective purification workflow option to purify proteins which are expressed at low levels and lack affinity handles. Both the chromatography media used in this method are macroporous particles, which offer efficient mass transfer in a wide range of operation flow rates. Moreover, the gentle chromatography conditions and the easy step transition allow maximum protection of the target molecule integrity. The two-step workflow is robust and readily scalable for process production.

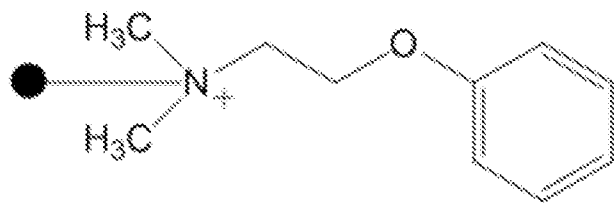
It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims. In addition, any elements or limitations of any invention or embodiment thereof disclosed herein can be combined with any and/or all other elements or limitations (individually or in any combination) or any other invention or embodiment thereof disclosed herein, and all such combinations are contemplated within the scope of the invention without limitation thereto.

CLAIMS

We claim:

1. A method of purifying an target molecule from a sample comprising:

a) contacting an sample comprising said target molecule to a multimodal chromatography (MMC) medium comprising a ligand under conditions that allow the target molecule to bind to the multimodal chromatography media, said ligand being



, wherein ●— represents a solid support to which said ligand is bound; and

b) eluting the target molecule from the multimodal chromatography media, thereby forming a multimodal eluate,

wherein the MMC media has been equilibrated with an equilibration buffer having a pH that is equal to or higher than the pI of the target molecule and, if the equilibration buffer is higher than the pI or the target molecule, the pH of the equilibration buffer is within about 0.01 and about 3.0 units of the pI of the target molecule.

2. The method of claim 1, said method further comprising:

a) loading the multimodal eluate to an polishing media under conditions that allow the target molecule to bind to the polishing media; and

b) eluting the target molecule from the polishing media.

3. The method of claim 2, wherein the polishing media is selected from hydroxyapatite, fluorapatite, ceramic hydroxyapatite, ceramic fluorapatite, anion exchange chromatography media, hydrophobic interaction chromatography media and combinations thereof.

4. The method of claims 1-3, wherein the sample comprising the target molecule contains one or more contaminants selected from proteins, nucleic acids, lipopolysaccharides and combinations thereof.

5. The method of claim 4, wherein the sample comprises:

- a) fermentation media containing said target molecule obtained from a prokaryotic or eukaryotic cell culture; or
- b) a prokaryotic cell lysate containing said target molecule.

6. The method of claim 1, wherein in step a), the sample that is contacted to the multimodal chromatography media is diluted in a buffer.

7. The method of claims 1-3, wherein the multimodal chromatography media has been equilibrated with a buffer (equilibration buffer) that has a pH that is higher than the pI of the target molecule and is within about 0.01 and about 2.5 unit of the pI of the target molecule.

8. The method of claims 1-3, further comprising, after step a), washing the multimodal chromatography media with a wash buffer that has a pH that is higher than the pI of the target molecule and is within about 0.01 and about 3.0 unit of the pI of the target molecule.

9. The method of claim 8, wherein the wash buffer comprises 0-2.0 M NaCl.

10. The method of claims 1-3, wherein in step b) the target molecule is eluted from the multimodal chromatography media with an elution buffer that has a pH that is higher than the pI of the target molecule and is within about 0.01 and about 3.0 units of the pI of the target molecule and comprises an alkaline earth metal chloride in a concentration of 0.01-1.0M.

11. The method of claim 10, wherein in step (b) the target molecule is eluted from the multimodal chromatography media with an elution buffer comprising an alkaline earth

metal chloride in a concentration of 0-500 mM, 0-750 mM, 100mM-500mM, 100mM-750 mM, 250 mM to 500 mM or 250 mM to 750 mM and the alkaline earth metal chloride is applied as a gradient or isocratically to the multimodal chromatography media.

12. The method of claim 11, wherein the alkaline earth metal chloride is MgCl_2 or CaCl_2 or a mixture thereof.

13. The method of any preceding claim, wherein the buffer is a MES (2-(N-morpholino)ethanesulfonic acid) buffer, MOPS (3-(N-morpholino)propanesulfonic acid) buffer, MOPSO (3-Morpholino-2-hydroxypropanesulfonic acid) buffer, Tris (2-Amino-2-(hydroxymethyl)-1,3-propanediol) buffer, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, phosphate buffer, acetate buffer, borate buffer, citrate buffer, succinate buffer, ammonium buffer or is a combination thereof.

14. The method of claim 6, wherein the sample is diluted with equilibrating buffer.

15. The method of claim 14, wherein the sample is diluted at a 1:1 ratio with equilibrating buffer prior to loading onto the multimodal chromatography media.

16. The method of claims 1-3, wherein the target molecule is exotoxin A or CRM197.

17. The method of claim 10, wherein the buffer containing the alkaline earth metal chloride is applied in a concentration gradient to the multimodal chromatography media to elute said target molecule.

18. The method of claim 10, wherein a fixed concentration of alkaline earth metal chloride in a buffer is applied to the multimodal chromatography media to elute said target molecule.

19. The method of claim 11 or 12, wherein the buffer containing the alkaline earth metal chloride is applied in a concentration gradient to the multimodal chromatography media to elute said target molecule.

20. The method of claim 11 or 12, wherein a fixed concentration of alkaline earth metal chloride in a buffer is applied to the multimodal chromatography media to elute said target molecule.

21. The method of any preceding claim, wherein the elution buffer has a pH of about 6.0 to about 9.5, about 6.0 to about 8.5, about 6.5 to about 8.0 or about 6.5 to about 7.5.

22. The method of any preceding claim wherein the equilibration buffer has a pH of about 6.0 to about 9.5, about 6.0 to about 8.5, about 6.5 to about 8.0 or about 6.5 to about 7.5.

23. The method of any preceding claim, wherein the wash buffer has a pH of about 6.0 to about 9.5, about 6.0 to about 8.5, about 6.5 to about 8.0 or about 6.5 to about 7.5.

24. The method of any preceding claim, wherein the wash buffer has a pH of about 6.0 to about 8.5, about 6.5 to about 8.0 or about 6.5 to about 7.5.

25. The method of any preceding claim, wherein the elution buffer has a pH of about 6.0 to about 8.5, about 6.5 to about 8.0 or about 6.5 to about 7.5.

26. The method of any preceding claim, wherein the equilibration buffer has a pH of about 6.0 to about 8.5, about 6.5 to about 8.0 or about 6.5 to about 7.5.

27. The method of any preceding claim, wherein the target molecule is an acidic target molecule.

28. The method of claims 1-3, wherein the target molecule is an acidic target molecule.

29. The method of claim 28, wherein the target molecule is an acidic target molecule and has a pI of between 5.0 and 6.5.

30. The method of claim 10, wherein the target molecule is an acidic target molecule.

31. The method of claim 30, wherein the target molecule is an acidic target molecule and has a pI of between 5.0 and 6.5.

32. The method of any preceding claim, wherein the equilibration buffer has a pH equal to the pI of the target molecule.

33. The method of any preceding claim, wherein the wash buffer has a pH equal to the pI of the target molecule.

34. The method of any preceding claim, wherein the elution buffer has a pH equal to the pI of the target molecule.

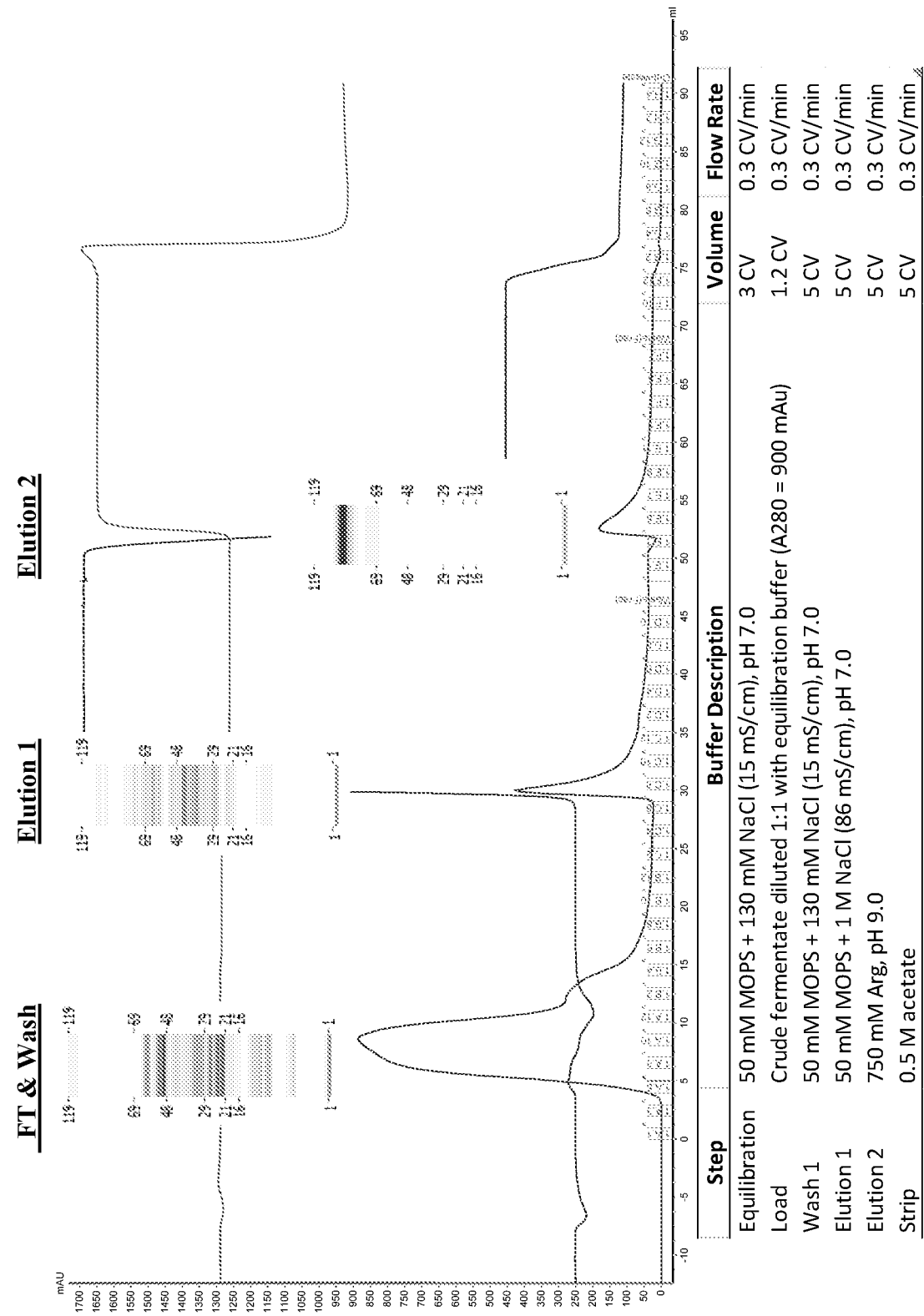
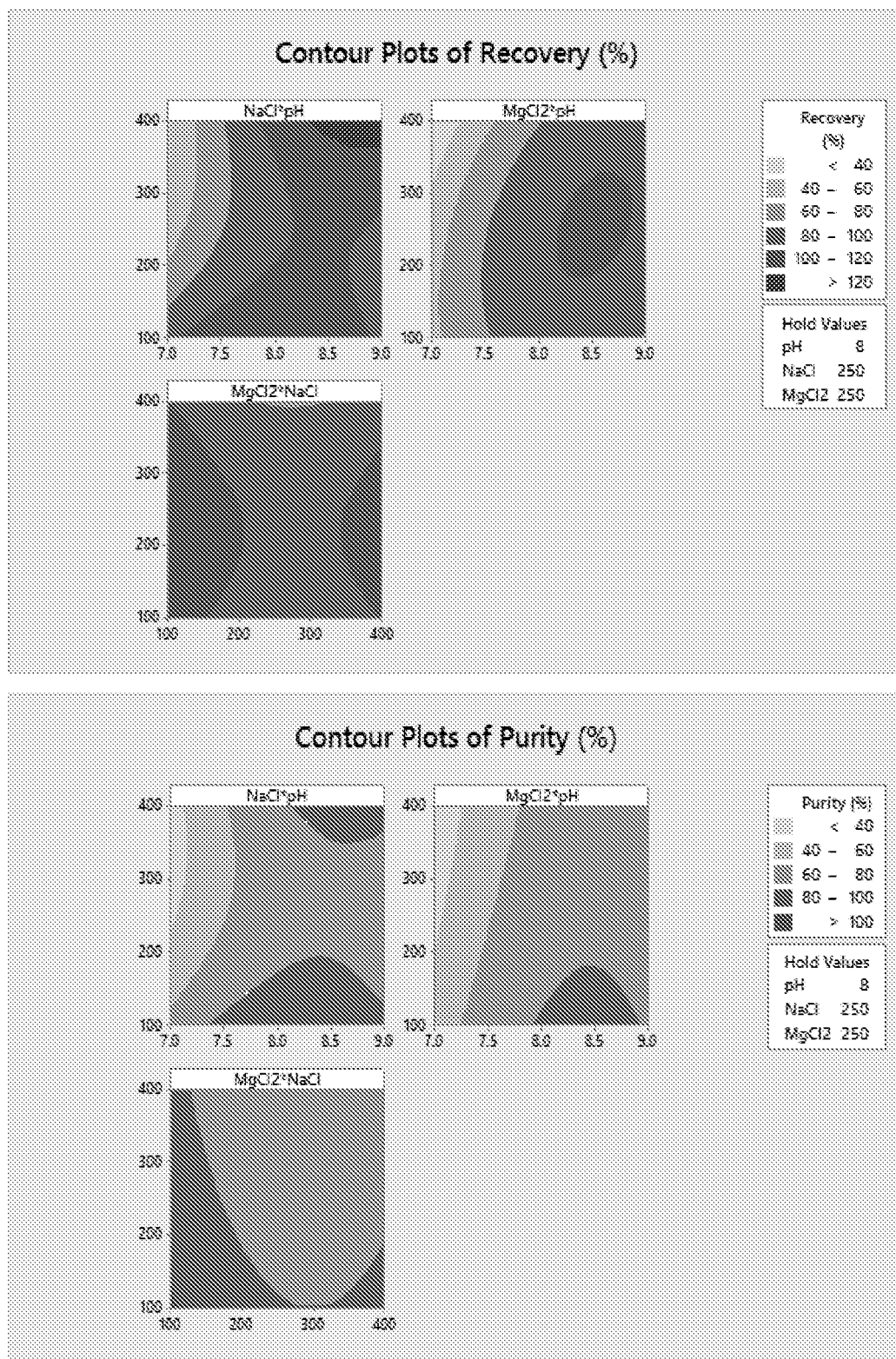


FIGURE 1

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**FIGURE 2A**

3/4

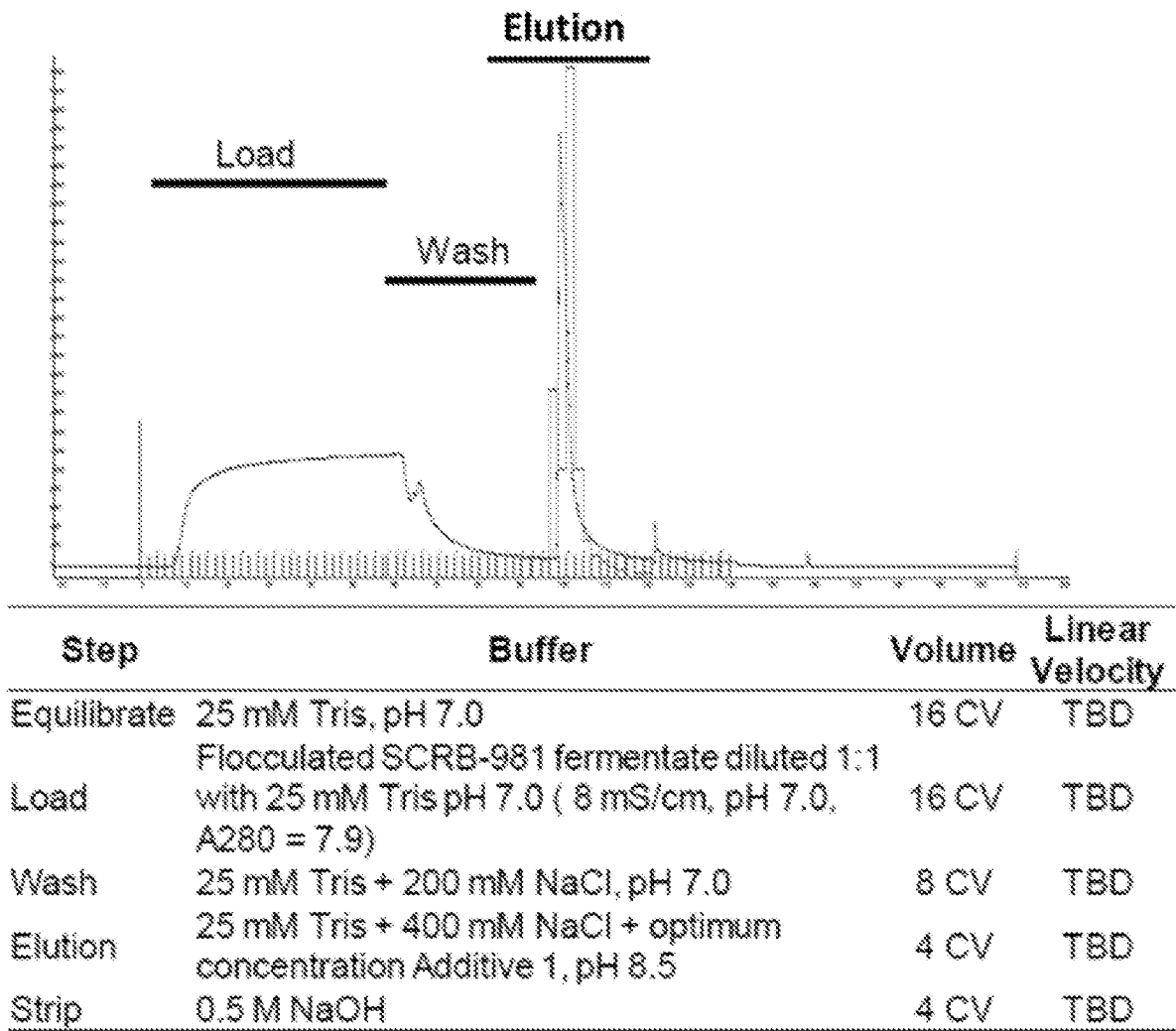


FIGURE 2B

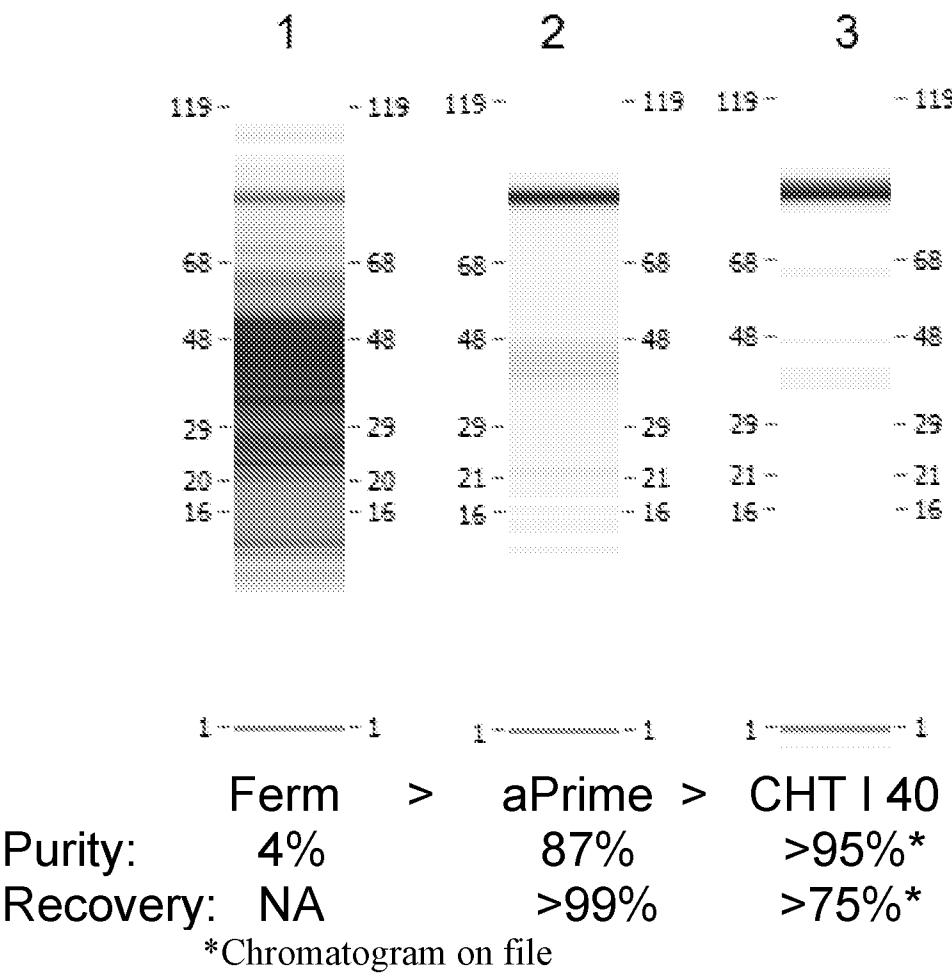


FIGURE 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2019/022687

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - B01D 15/36; B01D 15/38; B01J 39/26; C07K 1/18; C07K 16/00 (2019.01)

CPC - B01J 39/26; B01D 15/36; B01D 15/38; C07K 1/18; C07K 16/00 (2019.05)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2015/0258539 A1 (BIO-RAD LABORATORIES INC et al) 17 September 2015 (17.09.2015) entire document	1-12, 14-18, 28-31
A	US 2017/0022479 A1 (BIO-RAD LABORATORIES INC) 26 January 2017 (26.01.2017) entire document	1-12, 14-18, 28-31
A	US 2017/0073379 A1 (SCARAB GENOMICS LLC) 16 March 2017 (16.03.2017) entire document	1-12, 14-18, 28-31
A	US 5,308,735 A (KANDA et al) 03 May 1994 (03.05.1994) entire document	1-12, 14-18, 28-31
A	US 2014/0193876 A1 (GOERKE et al) 10 July 2014 (10.07.2014) entire document	1-12, 14-18, 28-31

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

15 May 2019

Date of mailing of the international search report

29 MAY 2019

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PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/022687

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 13, 19-27, 32-34
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.