



(51) International Patent Classification:

C07K 1/14 (2006.01) **C12Q 1/02** (2006.01)
C07K 14/195 (2006.01) **G01N 33/53** (2006.01)

(21) International Application Number:

PCT/US2009/066223

(22) International Filing Date:

1 December 2009 (01.12.2009)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/200,655 1 December 2008 (01.12.2008) 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, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, 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, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (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, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, 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:** METHODS FOR PREPARING AEROLYSIN POLYPEPTIDES OR CONJUGATES THEREOF

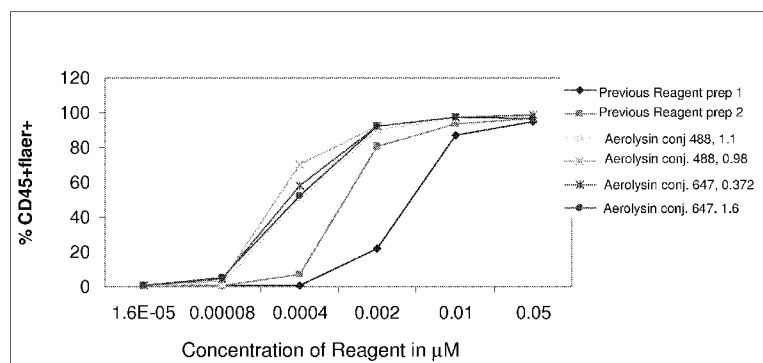


FIG. 7

(57) **Abstract:** The present disclosure relates to simplified and improved methods for preparing aerolysin polypeptides (e.g., non-lytic variant aerolysin polypeptides), or conjugates thereof, and to use of the polypeptides and conjugates. The methods for preparation include, e.g., methods for purifying aerolysin polypeptides from a sample and methods for conjugating a heterologous moiety (e.g., a detectable label) to the polypeptides.

METHODS FOR PREPARING AEROLYSIN POLYPEPTIDES OR CONJUGATES THEREOF

Sequence Listing

5 The instant application contains a Sequence Listing which has been submitted via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on November 30, 2009, is named ALXN141SeqList.txt, and is 27,870 bytes in size.

Cross-Reference to Related Application

10 This application claims the benefit of U.S. Provisional Patent Application Serial No.: 61/200,655, filed on December 1, 2008, the disclosure of which is incorporated herein by reference in its entirety.

Technical Field

15 The field of the invention is protein chemistry, molecular biology, and medical therapeutics and diagnostics.

Background

20 Aerolysin is a channel-forming cytolytic protein that is expressed by virulent *Aeromonas* species. Aerolysin is secreted from the bacterial cell as a 52 kDa precursor that is converted to the active form (activated) by proteolytic removal of a C-terminal peptide. The aerolysin precursor can be activated by host proteases as well as proteases secreted by an aerolysin-expressing bacterium. Once bound to a
25 cell, aerolysin oligomerizes to produce channels in, and ultimately lyse, the cell (Howard and Buckley (1985) *J Bacteriol* 163:336-340).

 Aerolysin selectively binds to the glycosylphosphatidylinositol (GPI) moiety that anchors many cell surface proteins to the cell membrane. There are numerous GPI-anchored proteins including, without limitation: alkaline phosphatase, 5'
30 nucleotidase acetylcholinesterase, dipeptidase, LFA-3, NCAM, PH-20, CD55, CD59, Thy-1, Qa-2, CD14, CD24, CD45, carcinoembryonic antigen (CEA), and CD52.

Purified aerolysin proteins have a number of uses including diagnostic and therapeutic applications. In one example, purified aerolysin proteins can be used to detect paroxysmal nocturnal hemoglobinuria (PNH). PNH is a rare disease caused by somatic mutation of the phosphatidylinositol glycan (PIG-A) gene, which encodes a protein product that is involved in the initial steps of GPI synthesis. The result is a reduced or absolute deficiency in GPI-anchored proteins in a clone of hematopoietic stem cells. Because of an aerolysin protein's binding properties, the presence of PIG-A deficient stem cell clone, and thus diagnosis of PNH, can be detected using, e.g., a detectably-labeled non-lytic form of the aerolysin protein.

Summary

The present disclosure relates to simplified and improved methods for preparing aerolysin polypeptides, or conjugates thereof, and to use of the polypeptides and conjugates. The methods for preparation include, e.g., methods for purifying aerolysin polypeptides from a sample and methods for conjugating a heterologous moiety (e.g., a detectable label) to the polypeptides. As is evident from the following disclosure, the aerolysin polypeptides (e.g., non-lytic variant aerolysin polypeptides) and conjugates thereof can be used in a variety of diagnostic and therapeutic methods. For example, a detectably-labeled non-lytic aerolysin protein conjugate prepared by the methods described herein can be used in diagnosing paroxysmal nocturnal hemoglobinuria (PNH). In another example, a purified aerolysin polypeptide can be used in the preparation of vaccines.

The purification and preparation methods described herein have a number of advantages over previously described methodologies. For example, the presently described methods use a single column (e.g., a DEAE column or a hydroxyapatite column) to obtain an aerolysin protein (e.g., a non-lytic variant aerolysin protein) that is as pure, or more pure, than an aerolysin protein (e.g., a non-lytic variant aerolysin protein) obtained from previous methodologies. In addition, as described in the working Examples and depicted in the Figures, the aerolysin protein (e.g., a non-lytic variant aerolysin protein) purified by the methods described herein: (i) contains fewer detectable degradation products; (ii) is more stable over time; and (iii) contains less endotoxin than aerolysin purified by the previous methods. Furthermore, the

aerolysin protein prepared by the methods described herein is more effective for detection of GPI-expressing cells.

In one aspect, the disclosure features a method for purifying aerolysin protein (e.g., a non-lytic variant aerolysin protein) from a sample. The method includes
5 providing a sample comprising aerolysin protein; separating aerolysin protein from the sample using a DEAE column or a hydroxyapatite column; and removing endotoxin from the separated aerolysin protein to thereby purify aerolysin protein from the sample.

In another aspect, the disclosure features a method for purifying an aerolysin
10 protein (e.g., a non-lytic variant aerolysin protein) from a sample, which method includes contacting a sample comprising an aerolysin protein to a DEAE column or a hydroxyapatite column under conditions in which the aerolysin protein binds to the column; eluting aerolysin protein from the column to obtain an aerolysin protein eluate; and removing endotoxin from the aerolysin protein eluate to thereby purify
15 aerolysin protein from the sample.

In another aspect, the disclosure features a method for purifying aerolysin protein (e.g., a non-lytic variant aerolysin protein) from a sample, which method includes contacting a sample comprising aerolysin protein to a DEAE column or a hydroxyapatite column to adsorb aerolysin protein on the column; washing the
20 column to remove contaminants; recovering aerolysin protein from the column; and removing endotoxin from the recovered aerolysin protein to thereby purify aerolysin protein from the sample.

In yet another aspect, the disclosure features a method for preparing an aerolysin protein (e.g., a non-lytic variant aerolysin protein) conjugate. The method
25 includes providing a sample comprising an aerolysin protein; separating aerolysin protein from the sample using a DEAE column or a hydroxyapatite column; removing endotoxin from the separated aerolysin protein to thereby purify aerolysin protein from the sample; and conjugating to the purified aerolysin protein a detectable label.

In some embodiments of any of the methods described herein, the detectable
30 label is a fluorescent label, a radioactive label, an enzymatic label, or a luminescent label. The detectable label can be, e.g., Alexa Fluor® 647, Alexa Fluor® 488, phycoerythrin, or allophycocyanin.

In some embodiments of any of the methods described herein, at least 30% or 50% of the endotoxin is removed from the sample. In some embodiments of any of the methods described herein, a filter is used to remove the endotoxin from the sample.

5 In some embodiments of any of the methods described herein, the sample can be, or contain, a bacterial cell homogenate or the supernatant from a bacterial cell culture.

In some embodiments, any of the methods described herein can further include determining the concentration of the purified aerolysin protein.

10 In some embodiments of any of the methods described herein, greater than 35 (e.g., greater than 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 55, 56, 58, 60, 65, 70, 75, 80, 85, 90, or 95 or more) % of the aerolysin protein is purified from the sample.

In some embodiments, none of the methods described herein include
15 lyophilizing and/or freeze-drying the conjugate.

In some embodiments, any of the methods described herein can include, prior to purifying the aerolysin protein from the sample, exchanging the buffer of the sample.

In yet another aspect, the disclosure features a purified aerolysin protein (e.g.,
20 a non-lytic variant aerolysin protein) prepared by any of the methods described herein.

In another aspect, the disclosure features an aerolysin protein (e.g., a non-lytic variant aerolysin protein) conjugate prepared by any of the methods for preparation described herein.

25 In some embodiments of any of the methods, proteins, or conjugates described herein, the aerolysin protein is a variant form of aerolysin protein that is non-lytic or is substantially non-lytic as compared to the wildtype form of the protein. The non-lytic or substantially non-lytic aerolysin protein can comprise, e.g., the amino acid sequence depicted in SEQ ID NO:2 or 7, wherein the threonine at position 253 is
30 substituted with a cysteine and the alanine at position 300 is substituted for a cysteine. In some embodiments, the non-lytic or substantially non-lytic aerolysin protein can comprise the amino acid sequence depicted in SEQ ID NO:10, wherein the threonine

at position 253 is substituted with a cysteine and the alanine at position 300 is substituted with a cysteine. In some embodiments, the non-lytic variant aerolysin protein comprises one or both of: (i) the amino acid sequence: GETELS (SEQ ID NO:8), wherein the threonine (T) is substituted with a cysteine; and (ii) the amino acid sequence YKADIS (SEQ ID NO:9), wherein the alanine (A) is substituted with a cysteine. In some embodiments, the non-lytic aerolysin protein comprises, or consists of, the amino acid sequence depicted in SEQ ID NO:5. The non-lytic variant aerolysin protein retains or substantially retains the ability to bind to GPI moieties on a cell.

In another aspect, the disclosure features a method for detecting the presence of a glycosylphosphatidylinositol (GPI) moiety on a cell. The method includes contacting a cell with an aerolysin protein (e.g., a non-lytic variant aerolysin protein) conjugate described herein; and detecting the binding of the aerolysin conjugate to the cell, wherein binding of the conjugate to the cell indicates that the cell expresses a GPI moiety and the absence of binding of the conjugate to the cell indicates that the cell does not express a GPI moiety.

In another aspect, the disclosure features a method for determining whether a human has paroxysmal nocturnal hemoglobinuria (PNH). The method includes contacting blood cells (e.g., white blood cells such as granulocytes) obtained from a human with an aerolysin protein (e.g., a non-lytic variant aerolysin protein) conjugate described herein; and determining whether the conjugate binds to the blood cells, wherein reduced binding of the conjugate to the blood cells, as compared to the binding of the conjugate to control blood cells, indicates that the human has PNH. In some embodiments, the control blood cells are obtained from a human that does not have PNH. In some embodiments, the blood cells include one or both of monocytes and granulocytes.

Unless otherwise defined, 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 disclosure pertains. In case of conflict, the present document, including definitions, will control. Preferred methods and materials are described below, although methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the presently disclosed methods and

compositions. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

Other features and advantages of the present disclosure, e.g., methods for preparing an aerolysin conjugate, will be apparent from the following description, the examples, and from the claims.

Brief Description of the Drawings

Fig. 1 is a chromatogram depicting the results of a size exclusion chromatography experiment to analyze the purity of a 2 mg/mL sample of a non-lytic aerolysin protein purified using a single column hydroxyapatite procedure. The X-axis represents retention time in minutes and the Y-axis represents absorbance at 280 nm (AU).

Fig. 2 is a chromatogram depicting the results of a size exclusion chromatography experiment to compare the purity of a 2 mg/mL sample of non-lytic aerolysin protein purified using a single column hydroxyapatite procedure or a single column DEAE procedure. The X-axis represents retention time in minutes and the Y-axis represents absorbance at 280 nm (AU).

Fig. 3 is a chromatogram depicting the results of a size exclusion chromatography experiment to analyze the purity of an 11 µg sample of non-lytic aerolysin protein purified using a single column hydroxyapatite procedure or a previously described method (see Buckley (1990) *Biochem Cell Biol.* 68:221-224). The X-axis represents retention time in minutes and the Y-axis represents absorbance at 280 nm (AU).

Fig. 4 is a chromatogram depicting the results of a size exclusion chromatography experiment to analyze the purity of a 2 mg/mL sample of unconjugated non-lytic aerolysin protein, non-lytic aerolysin protein conjugated to Alexa Fluor® 488, or non-lytic aerolysin protein conjugated to Alexa Fluor® 647, each of which was prepared using a single column hydroxyapatite procedure in conjunction with the conjugation methods described herein. The X-axis represents retention time in minutes and the Y-axis represents absorbance at 280 nm (AU).

Fig. 5 is a two color histogram depicting a population of monocytes that bind to both a non-lytic aerolysin protein conjugate containing Alexa Fluor® 488 and to an

antibody that binds to CD14. The conjugate binds to cells expressing GPI-anchored proteins. The Y-axis represents the log intensity of staining for the anti-CD14 antibody and the X-axis represents the log intensity of the staining for the aerolysin conjugate.

5 **Fig. 6** is a two color histogram depicting a population of monocytes that bind to both a non-lytic aerolysin protein conjugate containing Alexa Fluor® 488 and to an antibody that binds to CD24. The conjugate binds to cells expressing GPI-anchored proteins. The Y-axis represents the log intensity of staining for the anti-CD24 antibody and the X-axis represents the log intensity of the staining for the aerolysin
10 conjugate.

Fig. 7 is a line graph demonstrating that the non-lytic aerolysin protein conjugates prepared by the methods described herein detect GPI-expressing cells to a greater extent than commercially available non-lytic aerolysin protein conjugates prepared through a different method. A population of peripheral blood cells was
15 contacted to either various concentrations of commercially available non-lytic aerolysin conjugates containing Alexa Fluor® 488 (“Previous Reagents”; two different lots) or non-lytic aerolysin conjugates Alexa Fluor® 488 or Alexa Fluor® 647 (“Aerolysin Conj. 488” or “Aerolysin Conj. 647”) prepared by the methods described herein. The population was also contacted with a detectably-labeled
20 antibody that binds to CD45. The percentage of cells that bound to both the anti-CD45 antibody and the non-lytic aerolysin conjugates was determined using flow cytometry. The X-axis represents the concentration of the conjugate used and the Y-axis represents the percentage of cells in the population that were bound by both the conjugate and the anti-CD45 antibody.

25 **Fig. 8** is a line graph demonstrating that the mean fluorescence intensity (MFI) of non-lytic variant aerolysin conjugates prepared by the methods described herein is higher than the MFI of commercially available non-lytic aerolysin conjugates prepared using a previous methodology. A population of peripheral blood cells was contacted to either various concentrations of commercially available non-lytic
30 aerolysin conjugates containing Alexa Fluor® 488 (“Previous Reagents”; two different lots) or non-lytic aerolysin conjugates Alexa Fluor® 488 or Alexa Fluor® 647 (“Aerolysin Conj. 488” or “Aerolysin Conj. 647”) prepared by the methods

described herein. The mean fluorescence for each of the conjugate proteins was determined using flow cytometry. The X-axis represents the concentration of the conjugate used and the Y-axis represents the MFI.

5 Detailed Description

The disclosure features methods for preparing aerolysin polypeptides (e.g., non-lytic aerolysin polypeptides), or conjugates thereof, and use of the polypeptides and conjugates. While in no way intended to be limiting, exemplary aerolysin polypeptides and conjugates, as well as methods for their preparation and use, are
10 elaborated on below.

Aerolysin Polypeptides and Conjugates Thereof

The present disclosure provides, *inter alia*, methods for preparing aerolysin polypeptides and conjugates thereof. Aerolysin is a channel-forming cytolytic protein that is expressed by virulent *Aeromonas* species such as, but not limited to,

15 *Aeromonas hydrophila* and *Aeromonas salmonicida*. Aerolysin protein is secreted from the bacterial cell as a 52 kDa precursor that is converted to the active form (activated) by proteolytic removal of a C-terminal peptide. The aerolysin precursor can be activated by host proteases as well as proteases secreted by an aerolysin-expressing bacterium. Once bound to a cell, aerolysin oligomerizes. The heptameric
20 oligomers pierce the lipid bilayer producing channels to thereby lyse the cell. See, e.g., Howard and Buckley (1985) *J Bacteriol* 163:336-340.

Aerolysin selectively binds to the glycosylphosphatidylinositol (GPI) moiety that anchors many cell surface proteins to the cell membrane. GPI moieties generally contain a core of ethanolamine-HPO₄-6Man α 1-2Man α 1-6Man α 1-4GlcN α 1-6myo-
25 inositol-1HPO₄-diacyl-glycerol (or alkylacylglycerol or ceramide). See, e.g., Paulick and Bertozzi (2008) *Biochemistry* 47(27):6991-7000. However, a number of variations on this core structure have been reported. For example, the glycan core can be modified with side chains such as, but not limited to, phosphoethanolamine, mannose, galactose, sialic acid, or other sugars (see, e.g., Paulick and Bertozzi (2008),
30 *supra*).

GPI-anchored proteins are myriad and include, e.g., alkaline phosphatase, 5' nucleotidase acetylcholinesterase, dipeptidase, LFA-3, NCAM, PH-20, CD55,

CD59, Thy-1, Qa-2, CD14, CD33, CD16 (the Fc γ receptor III), carcinoembryonic antigen (CEA), CD24, CD66b, CD87, CD48, CD52, or any other GPI-anchored protein that is known in the art and/or set forth herein.

5 The amino acid sequences of the aerolysin polypeptide produced by each of various members of the *Aeromonas* family are highly conserved. Accordingly, an aerolysin polypeptide, as used herein, can be from any species of *Aeromonas* such as, but not limited to, *A. hydrophila*, *A. caviae*, *A. veronii* (biotype *sobria*), *A. veronii* (biotype *veronii*), *A. jandaei*, *A. salmonicida*, and *A. schubertii*.

10 In some embodiments, the aerolysin polypeptide is from *A. hydrophila* or *A. salmonicida*. In some embodiments, the aerolysin polypeptide is a proform containing a signal peptide. In some embodiments, the signal peptide can be approximately 24 (e.g., 22, 23, or 24) amino acids long. In some embodiments, the proform aerolysin polypeptide can have, or consist of, a polypeptide having the amino acid sequence depicted in SEQ ID NO:1 (the amino acid sequence of the proform aerolysin protein of *A. hydrophila*) or SEQ ID NO:6 (the amino acid sequence of the proform aerolysin protein of *A. salmonicida*).

15 In some embodiments, the aerolysin polypeptide is a form of the protein in which the signal sequence has been removed. For example, the aerolysin polypeptide can have, or consist of, a polypeptide having an amino acid sequence depicted in SEQ ID NO:2 (the amino acid sequence of the *A. hydrophila* aerolysin protein in which the signal sequence is removed) or SEQ ID NO:7 (the amino acid sequence of the *A. salmonicida* aerolysin protein in which the signal sequence is removed).

20 In some embodiments, the aerolysin polypeptide is an active form of the protein. For example, the aerolysin polypeptide can have, or consist of, a polypeptide having an amino acid sequence depicted in SEQ ID NO:3. The amino acid sequence depicted in SEQ ID NO:3 corresponds to the mature form of the aerolysin protein of *A. hydrophila* (SEQ ID NO:1) in which the signal sequence (amino acids 1-22 of SEQ ID NO:1) has been removed and a carboxy-terminal sequence (amino acids 462-492 of SEQ ID NO:1) has also been removed. In another example, the aerolysin polypeptide can have, or consist of, a polypeptide having an amino acid sequence depicted in SEQ ID NO:10, which is amino acids 1-438 of the amino acid sequence

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depicted in SEQ ID NO:7 and corresponds to the mature form of aerolysin protein of *A. salmonicida*.

As used herein, “polypeptide,” “peptide,” and “protein” are used interchangeably and mean any peptide-linked chain of amino acids, regardless of length or post-translational modification. The aerolysin polypeptides described herein can contain or be wild-type proteins or can be variants of the wild-type polypeptides that have not more than 50 (e.g., not more than one, two, three, four, five, six, seven, eight, nine, ten, 12, 15, 20, 25, 30, 35, 40, or 50) conservative amino acid substitutions. Conservative substitutions typically include substitutions within the following groups: glycine and alanine; valine, isoleucine, and leucine; aspartic acid and glutamic acid; asparagine, glutamine, serine and threonine; lysine, histidine and arginine; and phenylalanine and tyrosine.

The aerolysin polypeptides described herein also include “GPI-binding fragments” of the polypeptides, which are shorter than the full-length, proform polypeptides, but retain at least 10% (e.g., at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 50%, at least 55%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99%, at least 99.5%, or 100% or more) of the ability of the active polypeptide to bind to a GPI moiety. GPI-binding fragments of an aerolysin polypeptide include terminal as well internal deletion variants of the protein. Deletion variants can lack one, two, three, four, five, six, seven, eight, nine, ten, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid segments (of two or more amino acids) or non-contiguous single amino acids. GPI-binding fragments can be at least 40 (e.g., at least 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, or 325 or more) amino acid residues in length (e.g., at least 40 contiguous amino acid residues of SEQ ID NOs:1-7 or 10). In some embodiments, the GPI-binding fragment of an aerolysin polypeptide is less than 400 (e.g., less than 350, 325, 300, 275, 250, 225, 200, 190, 180, 170, 160, 150, 140, 130, 120, 110, 100, 95, 90, 85, 80, 75, 60, 50, 49, 48, 47, 46, 45, 44, 43, 42, 41, or 40) amino acid residues in length (e.g., less than 400 contiguous amino acid residues of SEQ ID NOs:1-7 or 10). In some embodiments, the GPI-binding fragment of an aerolysin polypeptide is at least 40, but less than 400, amino acid residues in length.

In some embodiments, the GPI-binding fragment of an aerolysin polypeptide can include, or consist of, a polypeptide having the following amino acid sequence: L
D P D S F K H G D V T Q S D R Q L V K T V V G W A V N D S D T P Q S G Y D
V T L R Y D T A T N W S K T N T Y G L S E K V T T K N K F K W P L V G E T
5 E L S I E I A A N Q S W A S Q N G G S T T T S L S Q S V R P T V P A R S K I P
V K I E L Y K A D I S Y P Y (SEQ ID NO:4). The amino acid sequence depicted in
SEQ ID NO:4 corresponds to a fragment of an aerolysin protein, the fragment
containing the GPI moiety-binding region of the protein.

In some embodiments, the GPI-binding fragment of an aerolysin polypeptide
10 can include, or consist of, a polypeptide having the following amino acid sequence: L
D P D S F K H G D V T Q S D R Q L V K T V V G W A V N D S D T P Q S G Y D
V T L R Y D T A T N W S K T N T Y G L S E K V T T K N K F K W P L V G E C
E L S I E I A A N Q S W A S Q N G G S T T T S L S Q S V R P T V P A R S K I P
15 V K I E L Y K C D I S Y P Y (SEQ ID NO:5). The amino acid sequence depicted in
SEQ ID NO:5 corresponds to a fragment of a variant aerolysin protein, the fragment
containing the GPI moiety-binding region of the protein and the variant containing
two conservative substitutions: wherein the threonine at position 253 is a cysteine and
the alanine at position 300 is a cysteine.

In some embodiments, the aerolysin polypeptide can have an amino acid
20 sequence that is, or is greater than, 70 (e.g., 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81,
82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100) %
identical to the aerolysin sequence having the amino acid sequence depicted in SEQ
ID NOs:1-3, 6, or 7 (see below).

Percent (%) amino acid sequence identity is defined as the percentage of
25 amino acids in a candidate sequence that are identical to the amino acids in a
reference sequence, after aligning the sequences and introducing gaps, if necessary, to
achieve the maximum percent sequence identity. Alignment for purposes of
determining percent sequence identity can be achieved in various ways that are within
the skill in the art, for instance, using publicly available computer software such as
30 BLAST, BLAST-2, ALIGN, ALIGN-2 or Megalign (DNASTAR) software.
Appropriate parameters for measuring alignment, including any algorithms needed to

achieve maximal alignment over the full-length of the sequences being compared can be determined by known methods.

Depending on the intended application, in some embodiments it may be preferable to use a variant aerolysin polypeptide that lacks the ability to lyse cells.

- 5 Such variant forms of the aerolysin polypeptide are known in the art and described in, e.g., Brodsky et al. (2000) *Am J Clin Pathol* 114:459-466. In some embodiments, the non-lytic, variant form of aerolysin contains, or consists of, the amino acid sequence depicted in SEQ ID NO:2 or SEQ ID NO:7 wherein the histidine at position 132 is substituted for an asparagine (His132Asn); the glycine at position 202 is a cysteine;
- 10 the threonine at position 253 is a cysteine and the alanine at position 300 is a cysteine; or the threonine at position 225 is a glycine. As described above, the variant forms will retain the ability to bind to GPI moieties. One exemplary non-lytic variant of aerolysin comprises the amino acid sequence depicted in SEQ ID NOs: 2 or 7, wherein the threonine at position 253 is a cysteine and the alanine at position 300 is a
- 15 cysteine. Another exemplary non-lytic variant of aerolysin protein comprises the amino acid sequence depicted in SEQ ID NO:10, wherein the threonine at position 253 is a cysteine and the alanine at position 300 is a cysteine. In some embodiments, the non-lytic aerolysin protein is one comprising: (i) the amino acid sequence: GETELS (SEQ ID NO:8), wherein the threonine (T) is substituted with a cysteine;
- 20 and/or (ii) the amino acid sequence YKADIS (SEQ ID NO:9), wherein the alanine (A) is substituted with a cysteine. The amino acid sequence depicted in SEQ ID NO: 8 corresponds to the amino acid sequence of the region of aerolysin protein in which T253 is present. The amino acid sequence depicted in SEQ ID NO: 9 corresponds to the amino acid sequence of the region of aerolysin protein in which A300 is present.
- 25 In some embodiments, the non-lytic form of aerolysin protein comprises the amino acid sequence depicted in SEQ ID NO:5. In some embodiments, the non-lytic form of the aerolysin protein is one described in Rossjohn et al. (1998) *Biochemistry* 37:741-746, the disclosure of which is incorporated herein by reference in its entirety. As described above, the variant forms will retain the ability to bind to GPI moieties.
- 30 In some embodiments, the variant aerolysin polypeptide has less than 10 (e.g., less than 9, 8, 7, 6, 5, 4, 3, 2, 1, or less than 1) % of the ability of the non-variant

counterpart aerolysin polypeptide to lyse target cells. In some embodiments, the variant aerolysin polypeptide has no detectable cytolytic activity.

Methods for obtaining an aerolysin polypeptide, or producing a variant of the polypeptide as described herein, are known in the art of molecular biology and exemplified in the working Examples. See, e.g., Sambrook et al. (1989) "Molecular Cloning: A Laboratory Manual, 2nd Edition," Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel et al. (1992) "Current Protocols in Molecular Biology," Greene Publishing Associates. Template DNA encoding an aerolysin polypeptide can be obtained from any of the *Aeromonas* species described herein using standard techniques (see, e.g., Sambrook et al. (1989), *supra*). For example, Howard et al. describes the isolation and characterization of a nucleic acid sequence encoding an aerolysin polypeptide from *Aeromonas hydrophila* (Howard et al. (1987) *J Bacteriol* 169(6):2869-2871). An aerolysin polypeptide isolated from *Aeromonas salmonicida* is described in Buckley (1990) *Biochem. Cell Biol.* 68:221-224 and Wong et al. (1989) *J Bacteriol.* 171:2523-2527.

Methods for determining whether a variant aerolysin polypeptide binds to a GPI moiety are known in the art and exemplified in the working examples. For example, cell-based methods for detecting the binding between a variant aerolysin polypeptide and a GPI moiety on a cell surface can be determined using flow cytometry techniques and a detectably-labeled (e.g., a fluorophore-labeled) variant aerolysin polypeptide. See, e.g., Hong et al. (2002) *EMBO J* 21(19):5047-5056.

Likewise, methods for detecting and/or quantitating the cytolytic activity of an aerolysin polypeptide of variant are also known in the art. For example, the hemolytic activity of a variant aerolysin polypeptide can be determined by contacting the variant polypeptide to normal human erythrocytes and measuring the amount of hemoglobin released from the erythrocytes. (See, e.g., Howard and Buckley (1982) *Biochemistry* 21(7):1662-1667; Avigad and Bernheimer (1976) *Infection and Immunity* 13(5):1378-1381; Garland and Buckley (1988) *Infection and Immunity* 56(5):1249-1253; and Bernheimer and Avigard (1974) *Infection and Immunity* 9:1016-1021.) A decreased amount, or the absence of, cytolytic activity by the variant, as compared to the amount of cytolytic activity possessed by the non-variant counterpart polypeptide, is an indication that the variant polypeptide has reduced or absent cytolytic activity.

In some embodiments, an aerolysin polypeptide (e.g., a non-lytic aerolysin polypeptide) can contain internal or terminal (carboxy or amino-terminal) irrelevant or heterologous amino acid sequences (e.g., sequences derived from other proteins or synthetic sequences not corresponding to any naturally occurring protein). The sequences can be, for example, an antigenic tag (e.g., FLAG, polyhistidine, hemagglutinin (HA), glutathione-S-transferase (GST), or maltose-binding protein (MBP)). Heterologous sequences can also include proteins useful as diagnostic or detectable markers, for example, luciferase, green fluorescent protein (GFP), or chloramphenicol acetyl transferase (CAT).

In some embodiments, an aerolysin polypeptide (e.g., a non-lytic aerolysin polypeptide) can be conjugated to a heterologous moiety. The heterologous moiety can be, e.g., a heterologous protein (see above), a therapeutic agent (e.g., a toxin or a drug), or a detectable label such as, but not limited to, a radioactive label, an enzymatic label, a fluorescent label, or a luminescent label. Suitable radioactive labels include, e.g., ^{32}P , ^{33}P , ^{14}C , ^{125}I , ^{131}I , ^{35}S , and ^3H . Suitable fluorescent labels include, without limitation, fluorescein, fluorescein isothiocyanate (FITC), Alexa Fluor® 488, Alexa Fluor® 647, GFP, DyLight 488, phycoerythrin (PE), propidium iodide (PI), PerCP, PE-Alexa Fluor® 700, Cy5, allophycocyanin, Cy7, and PE-Alexa Fluor® 750. Luminescent labels include, e.g., any of a variety of luminescent lanthanide (e.g., europium or terbium) chelates. For example, suitable europium chelates include the europium chelate of diethylene triamine pentaacetic acid (DTPA). Enzymatic labels include, e.g., alkaline phosphatase, CAT, luciferase, and horseradish peroxidase.

Suitable methods for conjugating detectable labels to an aerolysin polypeptide (e.g., a non-lytic aerolysin polypeptide) are known in the art of protein chemistry and are described and exemplified in the present disclosure (see below).

Methods for Preparing an Aerolysin Polypeptide or Conjugate Thereof

The disclosure also features methods for preparing any of the aerolysin polypeptides (e.g., non-lytic aerolysin polypeptides), or conjugates thereof, described herein. The methods can include, e.g., expressing the aerolysin polypeptide, purifying the polypeptide from a sample, and optionally, conjugating a heterologous moiety to the polypeptide (e.g., a detectable label).

For recombinant production of an aerolysin polypeptide (e.g., a non-lytic aerolysin polypeptide), a nucleic acid encoding the polypeptide is obtained and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the polypeptide can be readily isolated and sequenced using conventional procedures. (See, e.g., Sambrook et al. (1989), *supra* and Ausubel et al. (1992), *supra*.) The type of vector suitable for expression depends in part on the species of organism into which it will be introduced. For example, suitable expression vectors for use in *E. coli* include, without limitation, the pET vector system (Novagen®), the pGEX vector system (GE Healthcare), and the p7 and pBAD vector systems (Invitrogen™).

Suitable host cells for cloning or expressing the DNA in the vectors herein include, e.g., prokaryotic, yeast, or higher eukaryotic cells (e.g., mammalian cells). Suitable prokaryotes for use in expressing an aerolysin protein include, e.g., *E. coli* as well as various species of *Aeromonas*. In some embodiments, an aerolysin polypeptide is expressed from an expression vector in *Aeromonas salmonicida*. In some embodiments, the aerolysin polypeptide is an endogenous form of the protein produced from *Aeromonas hydrophila*. In some embodiments, the aerolysin polypeptide is an endogenous form of the protein produced from *Aeromonas salmonicida*. These examples are illustrative rather than limiting.

Methods for introducing a vector into a host cell vary depending on the species of the cell into which the vector will be introduced. For example, methods for introducing a vector into a prokaryote include, e.g., heat shock and electroporation (see, e.g., Sambrook et al. (1989), *supra*). Methods for expressing an aerolysin protein from a suitable host cell include culturing the host cell under conditions that permit the expression of the protein, which conditions are well known in the art and are described and exemplified herein (see Example 1).

Once the aerolysin polypeptide (e.g., a non-lytic aerolysin polypeptide) has been expressed, the polypeptide can be purified from cells or the medium in which the cells were cultured. The purification methods described herein include use of either a DEAE column or a hydroxyapatite column (see below; Example 2). Such columns are well known to those in the art of protein chemistry and are commercially available. For example, hydroxyapatite columns suitable for use in the methods

described herein can be obtained from BioRad (CHT® Ceramic Hydroxyapatite) and DEAE columns can be obtained from Amersham Biosciences (DEAE Sepharose CL-6B). The methods can include one or more wash steps to remove contaminating proteins or other contaminating materials (e.g., nucleic acids, carbohydrates, and/or lipids) from the column.

In some embodiments, prior to contacting the sample to the column, the method can include a “buffer exchange” step. Buffer exchange is a process wherein the solution in which the aerolysin protein is contained (e.g., Luria Broth culture media) is exchanged with a different buffer, e.g., one that is optimized for use with a DEAE column or a hydroxyapatite column. Methods for performing a buffer exchange step are well known to skilled artisans in the field of protein chemistry. For example, the buffer exchange step can be performed using dialysis or, as described in the working examples, can be performed in conjunction with a concentration step.

In some embodiments, the methods include removing endotoxin from aerolysin protein. Methods for removing endotoxin from a protein sample are known in the art and exemplified in the working examples. For example, endotoxin can be removed from a protein sample using a variety of commercially available reagents including, without limitation, the ProteoSpin™ Endotoxin Removal Kits (Norgen Biotek Corporation), Detoxi-Gel Endotoxin Removal Gel (Thermo Scientific; Pierce Protein Research Products), MiraCLEAN® Endotoxin Removal Kit (Mirus), or Acrodisc™ - Mustang® E membrane (Pall Corporation).

The endotoxin removal step can remove at least 40 (e.g., at least 42, 45, 47, 50, 52, 55, 57, 60, 62, 65, 67, 70, 72, 75, 77, 80, 82, 85, 87, 90, 92, 95, 97, or 99 or more) % of the endotoxin that was originally present in the sample. In some embodiments, the endotoxin removal step can remove at least 40 (e.g., at least 42, 45, 47, 50, 52, 55, 57, 60, 62, 65, 67, 70, 72, 75, 77, 80, 82, 85, 87, 90, 92, 95, 97, or 99 or more) % of the endotoxin that was present in the aerolysin-containing sample prior to the removal step.

Methods for detecting and/or quantitating the amount of endotoxin present in a sample (both before and after purification) are known in the art and commercial kits are available. For example, the concentration of endotoxin in a protein sample can be determined using the QCL-1000 Chromogenic kit (BioWhittaker), the limulus

amebocyte lysate (LAL)-based kits such as the Pyrotell®, Pyrotell®-T, Pyrochrome®, Chromo-LAL, and CSE kits available from the Associates of Cape Cod Incorporated.

5 It is understood that the endotoxin removal step can be performed prior to, or after, the DEAE or hydroxyapatite column step.

As described above, in some embodiments, the sample can be a cell (e.g., a bacterial cell) homogenate. In some embodiments, e.g., where the aerolysin polypeptide (e.g., a non-lytic aerolysin polypeptide) is secreted from the cells that produce it, the sample can be, or contain, the supernatant obtained from the cultured
10 cells (see the working Examples).

The term “purified” as applied to any of the aerolysin polypeptides (e.g., non-lytic variant aerolysin polypeptides) or conjugates described herein refers to a polypeptide, or conjugate thereof, that has been separated or purified from components (e.g., proteins or other naturally-occurring biological or organic
15 molecules) which naturally accompany it, e.g., other proteins, lipids, and nucleic acid in a prokaryote expressing the aerolysin polypeptide. Typically, a polypeptide is purified when it constitutes at least 60 (e.g., at least 65, 70, 75, 80, 85, 90, 92, 95, 97, or 99) %, by weight, of the total protein in a sample and contains less than 40 (e.g., less than 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20,
20 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.75, 0.5, 0.25, 0.1, 0.005, or less than 0.001) % of the endotoxin present in the sample before purification.

Methods for determining the yield or purity of an aerolysin polypeptide (e.g., a non-lytic aerolysin polypeptide) that is purified using any of the methods described herein are known in the art and include, e.g., Bradford assay, UV spectroscopy, Biuret
25 protein assay, Lowry protein assay, amido black protein assay, high pressure liquid chromatography (HPLC), mass spectrometry (MS), and gel electrophoretic methods (e.g., using a protein stain such as Coomassie Blue or colloidal silver stain). Exemplary methods for determining yield and/or purity of an aerolysin preparation are set forth in the accompanying Examples.

30 In some embodiments, the methods can also include conjugating a heterologous moiety to the purified aerolysin polypeptide. The heterologous moiety can be, e.g., any of those described herein. Methods for conjugating a heterologous

moiety to an aerolysin polypeptide are known in the art of protein chemistry. For example, two proteins can be cross-linked using any of a number of known chemical cross linkers. Examples of such cross linkers are those which link two amino acid residues via a linkage that includes a “hindered” disulfide bond. In these linkages, a disulfide bond within the cross-linking unit is protected (by hindering groups on either side of the disulfide bond) from reduction by the action, for example, of reduced glutathione or the enzyme disulfide reductase. One suitable reagent, 4-succinimidylloxycarbonyl- α -methyl- α (2-pyridyldithio) toluene (SMPT), forms such a linkage between two proteins utilizing a terminal lysine on one of the proteins and a terminal cysteine on the other. Heterobifunctional reagents that cross-link by a different coupling moiety on each protein can also be used. Other useful cross-linkers include, without limitation, reagents which link two amino groups (e.g., N-5-azido-2-nitrobenzoyloxysuccinimide), two sulfhydryl groups (e.g., 1,4-bis-maleimidobutane) an amino group and a sulfhydryl group (e.g., m-maleimidobenzoyl-N-hydroxysuccinimide ester), an amino group and a carboxyl group (e.g., 4-[p-azidosalicylamido]butylamine), and an amino group and a guanidinium group that is present in the side chain of arginine (e.g., p-azidophenyl glyoxal monohydrate).

Radioactive labels can be conjugated to the aerolysin protein by covalent or non-covalent (e.g., ionic or hydrophobic bonds). They can be bound to any part of the protein provided that the conjugation does not interfere with the ability of the aerolysin protein to bind to a GPI moiety. In some embodiments, the radioactive label can be directly conjugated to the amino acid backbone of the protein. Alternatively, the radioactive label can be included as part of a larger molecule (e.g., ^{125}I in meta- ^{125}I iodophenyl-N-hydroxysuccinimide (^{125}I mIPNHS) which binds to free amino groups to form meta-iodophenyl (mIP) derivatives of relevant proteins (see, e.g., Rogers et al. (1997) *J. Nucl. Med.* **38**:1221-1229) or chelate (e.g., to DOTA or DTPA) which is in turn bound to the protein backbone. Methods of conjugating the radioactive labels or larger molecules/chelates containing them to aerolysin proteins (e.g., non-lytic aerolysin proteins) are known in the art. Such methods involve incubating the aerolysin protein with the radioactive label under conditions (e.g., pH, salt concentration, and/or temperature) that facilitate binding of the radioactive label or chelate to the protein (see, e.g. U.S. Patent No. 6,001,329).

Methods for conjugating a fluorescent label (sometimes referred to as a “fluorophore”) to a protein (e.g., an aerolysin protein such as a non-lytic aerolysin protein) are known in the art of protein chemistry and exemplified herein in the working Examples. For example, fluorophores can be conjugated to free amino groups (e.g., of lysines) or sulfhydryl groups (e.g., cysteines) of proteins using succinimidyl (NHS) ester or TFP ester moieties attached to the fluorophores. In some embodiments, the fluorophores can be conjugated to a heterobifunctional cross-linker moiety such as sulfo-SMCC. Suitable conjugation methods involve incubating the aerolysin protein with the fluorophore under conditions that facilitate binding of the fluorophore to the protein. See, e.g., Welch and Redvanly (2003) “Handbook of Radiopharmaceuticals: Radiochemistry and Applications,” John Wiley and Sons (ISBN 0471495603). A variety of kits are commercially available for use in conjugating a fluorophore to a protein. For example, the Alexa Fluor® 488 Protein Labeling Kit and the Alexa Fluor® 647 Protein Labeling Kit (Molecular Probes, Invitrogen™). In some embodiments, the fluorophore can be conjugated to the aerolysin protein at 1-2 mol dye per mol of protein.

Methods for Using an Aerolysin Protein or Conjugate Thereof

As is clear from the following Examples, the purified aerolysin polypeptides and conjugates described herein can be used in a variety of diagnostic and therapeutic applications. For example, a non-lytic variant aerolysin protein conjugate can be used in methods for detecting the presence of GPI moieties (e.g., GPI anchored proteins) on cell surfaces.

U.S. Patent No. 5,798,218 describes the use of non-lytic aerolysin conjugates in methods for detecting Thy-1 surface protein. In another example, the non-lytic aerolysin conjugates described herein can be used in screening methods for paroxysmal nocturnal hemoglobinuria (PNH). PNH is a disease caused by somatic mutation of the phosphatidyl-inositol glycan (PIG-A) gene, which encodes a protein product that is involved in the initial steps of GPI synthesis. The result is a reduced or absolute deficiency in GPI-anchored proteins in a clone of hematopoietic stem cells. The presence of PIG-A deficient stem cell clone(s) supporting a diagnosis of PNH, can be detected using the non-lytic aerolysin conjugates described herein.

A peripheral blood cell sample is obtained from a patient suspected of having PNH. Detectably-labeled (e.g., fluorophore-labeled) non-lytic aerolysin conjugates are contacted to the blood cells for a time and under conditions in which the conjugates bind to the cells, if GPI anchored proteins are present. Optionally, the cells can be incubated with a lytic agent to remove erythrocytes from the cell sample. The cell sample is then subjected to flow cytometry analysis to determine the percentage of certain leukocyte populations that are bound to the conjugate. An elevated percentage of granulocytes or monocytes that do not bind to the conjugate, as compared to the percentage of control cells that are not bound to the conjugate, is an indication that patient has clones of blood cells with a GPI-antigen deficiency, which is consistent with a diagnosis of PNH. Such diagnostic methods are described in detail in, e.g., Peghini and Fehr (2005) *Cytometry Part B (Clinical Cytometry)* 67B:13-18 and Sutherland et al. (2007) *Cytometry Part B (Clinical Cytometry)* 72B:167-177.

Additional methods for using the non-lytic aerolysin polypeptides and conjugates described herein in diagnostic methods are exemplified in the accompanying Examples.

Therapeutic Uses

In addition to the above-described diagnostic methods, the purified aerolysin polypeptides and conjugates thereof can be used in a variety of therapeutic applications. In one example, purified aerolysin polypeptides can be used in the preparation of vaccines. Purified aerolysin polypeptide can be contacted to preparations of enveloped virus comprising a glycosylphosphatidylinositol-anchored protein in their membrane to thereby attenuate the virus in the preparation. See, e.g., U.S. Patent Application Publication No. 20020012671 and U.S. Patent No. 6,495,315, the disclosures of each of which are incorporated by reference in their entirety.

In another example, acetylated forms of the purified aerolysin polypeptides can be used for treating certain cancers such as prostate cancer. See, e.g., U.S. Patent Application Publication No. 20060264364, the disclosure of which is incorporated by reference in its entirety.

Kits

Also featured herein are kits for use in: (i) the purification of an aerolysin protein (e.g., a non-lytic aerolysin protein); (ii) the preparation of an aerolysin conjugate (e.g., a non-lytic aerolysin conjugate); (iii) detecting the presence of a GPI moiety on a cell surface; or (iv) diagnosing a human as having paroxysmal nocturnal hemoglobinuria (PNH).

Kits for use in the purification of aerolysin protein can include one or more of: a DEAE or hydroxyapatite column; one or more equilibration or wash buffers; one or more buffers for use in buffer exchange; one or more reagents for use in concentrating a protein sample; one or more reagents for use in determining the concentration of a protein in a sample; one or more reagents for removing endotoxin from a protein sample; and optionally, instructions for how to purify an aerolysin protein.

Kits for use in the preparation of an aerolysin conjugate (e.g., a non-lytic aerolysin protein conjugate) can include, e.g., a source of purified aerolysin protein purified by the methods described herein; one or more detectable labels (e.g., one or more fluorophores) such as any of the ones described herein; one or more reagents for conjugating a detectable label to a protein; and optionally, instructions for conjugating a detectable label to a protein. The kits can also include, e.g., any of the reagents in the purification kits described above.

Kits for use in detecting the presence of a GPI expressing cell can include, e.g., a detectably-labeled non-lytic aerolysin conjugate, a control sample containing a GPI expressing cell or GPI bound particle; and optionally, instructions for detecting the presence of a GPI expressing cell. The kits can also include one or more means for obtaining a biological sample (e.g., a blood sample) from a human and/or any of the kit components described above.

Kits for diagnosing a human as having PNH can include, without limitation, one or more of a detectably-labeled non-lytic aerolysin conjugate; a control cell sample (e.g., one containing monocytes and/or granulocytes expressing GPI moieties on their surface); optionally, instructions for diagnosing a human as having PNH; and optionally, any of the kit components described above.

In some embodiments, any of the kits described herein can contain one or more reagents for expressing an aerolysin protein (e.g., a non-lytic aerolysin protein)

in a cell. For example, the kits can contain a culture medium (e.g., LB), a vector containing a nucleic acid encoding an aerolysin protein (e.g., a non-lytic variant aerolysin protein), a cell suitable for expressing aerolysin, an antibiotic, and/or an agent to induce expression (e.g., where the vector contains an inducible promoter) such as IPTG or an analog thereof.

The following examples are intended to illustrate, not limit, the invention.

10

Examples

Example 1. Expression of Aerolysin in *Aeromonas salmonicida*

Aeromonas salmonicida bacteria were transformed with a plasmid encoding an *A. salmonicida*-derived non-lytic aerolysin polypeptide and containing an ampicillin resistance gene. The aerolysin polypeptide is rendered non-lytic by way of two substitutions: the threonine at position 253 is substituted with a cysteine and the alanine at position 300 is substituted for a cysteine. The *Aeromonas* strain secretes the expressed non-lytic aerolysin polypeptide into the culture medium and was used to allow for more facile collection and purification of the expressed polypeptide.

The *Aeromonas* was inoculated into 30 mL of Luria Broth (LB) containing MgSO₄, glucose, and ampicillin. The culture was incubated at 30°C with shaking overnight. The next morning, the optical density of the culture at 600 nm was determined to be between 3 and 6. Approximately 5 to 6 mL of the starter culture was added to 356 mL of LB (including 40 mL of 10 x Davis buffer with MgSO₄; 4 mL of 20% w/v glucose; 400 µL of 100 mg/mL ampicillin; and 320 µL of 50 mg/mL kanamycin). The 10x Davis buffer contains 20.9g K₂HPO₄, 28.45g Na₂HPO₄·7H₂O, 8.98g KH₂PO₄, 9.11g NaH₂PO₄, and 3.97g (NH₄)₂SO₄, and is at a pH of 7.5.

The larger culture was incubated at 30°C with shaking until the optical density at 600 nm of the culture reached between about 0.4 to 0.9. Next, 400 µl of 1M IPTG was added to the culture followed by another incubation below 30°C with shaking overnight.

Once the optical density at 600 nm of the culture reached greater than 5, the culture material including cells and media was centrifuged once for 15 minutes at 10,000 rpm and then the supernatant was filtered using a 0.2 μ m filter to remove any excess bacteria. Next, with constant stirring 24 mL of 100 mM 1, 10-phenanthroline (in ethanol) was added to the clarified supernatant to inhibit zinc metalloproteases as well as polypropylene glycol to reduce foaming in subsequent steps.

Next, protein present in the clarified supernatant was concentrated by passing the supernatant through a filter with a 10 kDa cut-off using an osmotic pump (set to no more than 10 psi). Concentration on the protein was carried out until the void volume remaining in the filter reservoir was approximately 130 mL. Following the concentration step, a buffer exchange step was performed by adding 1 L of 20 mM Na₂HPO₄, 300 mM NaCl pH 6.0 (prepared as 17.54 mL 1M NaH₂PO₄, 2.46 mL 1M Na₂HPO₄, 60 mL 5M NaCl, and H₂O to adjust volume to 1 L) and concentrating the volume back down to the void volume. The buffer exchange step was repeated one additional time.

The concentrated, buffer-exchanged protein fraction was subjected to ultracentrifugation two times, each for 2 hours at 4°C.

The protein yield determined after the fermentation step is shown in Table 1.

Table 1

<u>step</u>	<u>abbreviation</u>	<u>conc.</u>	<u>Starting</u> <u>volume</u>	<u>End</u> <u>volume</u>	<u>milligram</u>	<u>%</u> <u>recovery</u> <u>(of total)</u>
fermentation		Estimate 0.5 mg/mL	1L	70 mL	35	100
Ceramic Hydroxyapatite	CHP	1.13 mg/mL	70 mL	15	17	48.5%
Mustang Q filter	MQF	1 mg/mL	15	15	15	42.8%
Concentrate prior to	MQF conc	2.67 mg/mL	5	1.5	4.12	35.3%

<u>step</u>	<u>abbreviation</u>	<u>conc.</u>	<u>Starting</u> <u>volume</u>	<u>End</u> <u>volume</u>	<u>milligram</u>	<u>%</u> <u>recovery</u> <u>(of total)</u>
conjugation**						
Conjugated to 488	MQF-488	0.85 mg/mL	0.5	1	0.85	22.5%
**Not all MQF material was used in conjugation steps; had all of the purified protein obtained from this fermentation been used in conjugation, about 7.8 mg of Alexafluor 488-aerolysin would have been obtained instead of 0.85 mg						

Example 2. Purification of Aerolysin Polypeptide

To purify the non-lytic aerolysin protein from the buffer-exchanged protein sample prepared as above, was applied at 0.5 ml /min to a ceramic hydroxyl-apatite (CHP) column was previously equilibrated with at least 5 column volumes of 20 mM NaPO₄ buffer pH 6.0 containing 300 mM NaCl, hereafter referred to as buffer A. The column was then eluted with a linear gradient of buffer A to buffer B (300 mM NaCl, 150 mM NaPO₄, pH 6) over 250 mL, while collecting 5 mL fractions throughout at a flow rate of 0.5 mL/minute.

The “peak” fractions containing aerolysin protein were determined using absorbance at 280 nm, then pooled, and the purity determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Blue staining. The aerolysin protein was found to migrate on the gel as a 50 kDa dimer. The total protein collected using this procedure was about 35 mg from 70 mL of culture or a yield of about 48.5%.

Next, endotoxin was removed from the pooled aerolysin fractions. The pooled sample was filter through an equilibrated Mustang E filter (Pall Corporation, catalogue no. MSTG25E3). Protein yields following MQ filtration were determined to be approximately 88.5% of the concentration of the input material and cumulative recovery of 42.8% from the starting material (Table 1).

In an alternative single column method, aerolysin protein was also purified using a DEAE sepharose column. The column was packed with 50 mL resin (DEAE sepharose Cl6B; Amersham Biosciences/GE Healthcare) according to manufacturer's instructions. The column was equilibrated using at least 5 column volumes of buffer C (20 mM HEPES and 1 mM EDTA at pH 7.4). The prepared aerolysin protein sample (which underwent a buffer exchange step to replace the buffer with buffer C) was loaded onto the column at 0.3-0.5 mL per minute, while collecting 5 mL fractions. The column was eluted with a gradient of buffer C to buffer D (20 mM HEPES, 1 mM EDTA, and 400 mM NaCl at pH 7.4) over 250 mL, while collecting 5 mL fractions at a flow rate of 0.5 mL/minute. The "peak" fractions containing aerolysin protein were determined using absorbance at 280 nm, then pooled, and the purity of the pooled fractions determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Blue staining as well as absorbance at 280 nm using an extinction coefficient of $A_{280}=0.4$ mg/mL aerolysin. The aerolysin protein was found to migrate on the gel as a 50 kDa dimer.

The protein yield determined after the purification over ceramic hydroxyapatite and subsequent filtration step is shown in Table 1.

Example 3. Conjugation of Purified Aerolysin to a Fluorophore

To conjugate a fluorophore to the non-lytic aerolysin, the purified aerolysin sample was concentrated to approximately 2 mg of protein per mL and then subjected to a desalting column. The concentrated, purified protein was buffer-exchanged into phosphate buffered saline (PBS).

One vial of either Alexa Fluor® 488 or Alexa Fluor® 647 coupling dye was incubated with 500 μ L of the aerolysin sample for 60 minutes essentially as described by the manufacturers instructions (see, e.g., instructions for Alexa Fluor® 647 Protein Labeling Kit A20173, or Alexa Fluor® 488 Protein Labeling Kit A10235, from Molecular Probes, Invitrogen™). The reactions were quenched by running the samples over the gel filtration column provided, according to the kit instructions.

The total yield of protein as well as the dye/protein ratio was assessed using the molar extinction coefficients for the fluorophores. The extinction coefficient for aerolysin is $1 A_{280}=0.4$ mg/mL.

Optimal dye-protein conjugation was determined to be between 1.5-2 moles dye incorporated per mole protein. The protein conjugates were analyzed using SEC-HPLC, MALDI-TOF and Coomassie Simply Blue Safestain (Invitrogen). Aliquots of the conjugated proteins were prepared and stored away from light, at 4°C, in PBS containing 2 mM azide.

The protein yields determined after conjugation with Alexa Fluor® 488 are shown in Table 1.

10 Example 4. Analysis of the Purified Aerolysin and Conjugates

A series of size exclusion chromatography (SEC) experiments were performed to determine the purity of the non-lytic aerolysin protein purified using the single hydroxyapatite or DEAE column. The chromatogram of a sample containing 2 mg/mL aerolysin protein purified using the hydroxyapatite column analyzed by SEC is shown in Fig. 1. The aerolysin in the sample was determined to be greater than 90% pure. The chromatogram depicted in Fig. 2 shows a chromatogram of a sample containing 2 mg/mL aerolysin purified using a DEAE column and a chromatogram of a sample containing 2 mg/mL aerolysin purified using a hydroxyapatite column, both of which were greater than 90% pure.

Another SEC experiment was performed to compare the non-lytic aerolysin protein purified using the single column method described herein and non-lytic aerolysin protein purified using a previously described purification method (see, e.g., Buckley (1990) *Biochem Cell Biol.* 68:221-224). The chromatogram depicted in Fig. 3 shows the chromatogram of a sample containing 11.5 µg of aerolysin protein prepared using the single column hydroxyapatite method described herein and the chromatogram of a sample containing 11.5 µg of aerolysin protein prepared using a previously described method. As shown in Fig. 3, the aerolysin protein prepared by the methods described herein contains fewer lower molecular weight species.

In yet another experiment, samples containing 2 mg/mL unconjugated aerolysin, 2 mg/mL aerolysin conjugated to Alexa Fluor® 488, or 2 mg/mL aerolysin conjugated to Alexa Fluor® 647 (purified and prepared using the methods described herein) were subjected to SEC analysis to determine the purity of the product. As

shown in Fig. 4, each of the samples eluted with relative retention times consistent with their expected rank ordering of size.

Example 5. Detection of GPI-expressing Cells Using the Conjugates

5 Non-lytic aerolysin conjugates containing Alexa Fluor® 488 (prepared using the above methods) were used to detect the presence of GPI-linked proteins on the surface of cells. A population of peripheral blood cells was contacted with the non-lytic aerolysin conjugate and then subjected to analysis using flow cytometry.

10 A population of blood cells was contacted with the conjugate (0.98 mg/mL) as well as detectably-labeled antibodies that bind to two monocyte markers CD45 and CD64 and a detectably labeled antibody that binds to CD14. The population was then subjected to multiparametric analysis using flow cytometry. First, cells that bound to the anti-CD45 and anti-CD64 antibodies (monocytes) were selected for analysis, and from this population of cells, cells that bound to CD14 and the conjugate were
15 analyzed. As shown in Fig. 5, the conjugate bound very well to CD14 positive cells. As mentioned above, CD14 is a GPI-anchored protein.

A population of blood cells was contacted with the non-lytic conjugate (0.98 mg/mL) as well as detectably-labeled antibodies that bind to two granulocyte markers CD45 and CD15 and a detectably labeled antibody that binds to CD24. The
20 population was then subjected to multiparametric analysis using flow cytometry. First, cells that bound to the anti-CD45 and anti-CD15 antibodies (granulocytes) were selected for analysis, and from this population of cells, cells that bound to CD24 and the conjugate were analyzed. As shown in Fig. 6, the conjugate bound very well to CD24 positive cells. As mentioned above, CD24 is a GPI-anchored protein.

25 These results indicate that the Alexa Fluor® 488-aerolysin conjugate derived by the disclosed method can be used to detect cells that express GPI-anchored proteins.

Next, to determine whether non-lytic aerolysin conjugates prepared by the methods described herein were able to detect GPI-expressing cells as well as, or better
30 than, a commercially available aerolysin conjugate made by the above-described previous methodology, the following experiment was performed. A population of peripheral blood cells was contacted to either various concentrations of commercially

available aerolysin conjugates containing Alexa Fluor® 488 (“Previous Reagents”; two different lots) or aerolysin conjugates Alexa Fluor® 488 or Alexa Fluor® 647 (“Aerolysin Conj. 488” or “Aerolysin Conj. 647”) prepared by the methods described herein. The population was also contacted with a detectably-labeled antibody that
5 binds to CD45. The percentage of cells in the population that bound to both the non-lytic conjugate and the anti-CD45 antibody was determined using flow cytometry. As mentioned above, CD45 is a GPI-anchored protein. As shown in Fig. 7, the conjugates prepared by the methods described herein were more effective at detecting GPI-expressing cells than the commercially available product. In addition, the mean
10 fluorescence was determined for the commercially available aerolysin conjugates and compared to the conjugates prepared by the above methods. While conjugates containing Alexa Fluor® 647 exhibited the highest mean fluorescence intensity (MFI), the MFI for the instantly prepared conjugates containing Alexa Fluor® 488 was also consistently higher than the commercially available aerolysin- Alexa Fluor®
15 488 conjugates (Fig. 8).

While the present disclosure has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the disclosure. In addition, many modifications may be made
20 to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present disclosure. All such modifications are intended to be within the scope of the disclosure.

What is claimed is:

1. A method for purifying aerolysin protein from a sample, the method comprising:
providing a sample comprising aerolysin protein;
5 separating aerolysin protein from the sample using a DEAE column or a hydroxyapatite column; and
removing endotoxin from the separated aerolysin protein to thereby purify aerolysin protein from the sample.
- 10 2. A method for purifying an aerolysin protein from a sample, the method comprising:
contacting a sample comprising an aerolysin protein to a DEAE column or a hydroxyapatite column under conditions in which the aerolysin protein binds to the column;
15 eluting aerolysin protein from the column to obtain an aerolysin protein eluate; and
removing endotoxin from the aerolysin protein eluate to thereby purify aerolysin protein from the sample.
- 20 3. A method for purifying aerolysin protein from a sample, the method comprising:
contacting a sample comprising aerolysin protein to a DEAE column or a hydroxyapatite column to adsorb aerolysin protein on the column;
washing the column to remove contaminants;
recovering aerolysin protein from the column; and
25 removing endotoxin from the recovered aerolysin protein to thereby purify aerolysin protein from the sample.
4. A method for preparing an aerolysin protein conjugate, the method comprising:
providing a sample comprising an aerolysin protein;
30 separating aerolysin protein from the sample using a DEAE column or a hydroxyapatite column;

removing endotoxin from the separated aerolysin protein to thereby purify aerolysin protein from the sample; and

conjugating to the purified aerolysin protein a detectable label to prepare an aerolysin protein conjugate.

5

5. The method of any one of claims 1-4, wherein the aerolysin protein is a non-lytic variant aerolysin protein.

6. The method claim 5, wherein the non-lytic variant aerolysin protein comprises the amino acid sequence depicted in SEQ ID NO:5.

7. The method of claim 5, wherein the non-lytic variant aerolysin protein comprises one or both of: (i) the amino acid sequence: GETELS (SEQ ID NO:8), wherein the threonine (T) is substituted with a cysteine; and (ii) the amino acid sequence YKADIS (SEQ ID NO:9), wherein the alanine (A) is substituted with a cysteine.

8. The method of claim 5, wherein the non-lytic aerolysin protein comprises the amino acid sequence depicted in SEQ ID NOs: 2, 7, or 10, wherein the threonine at position 253 is substituted with a cysteine and the alanine at position 300 is substituted with a cysteine.

9. The method of any one of claims 4-8, wherein the detectable label is a fluorescent label.

10. The method of any one of claims 4-8, wherein the detectable label is a radioactive label, an enzymatic label, or a luminescent label.

11. The method of claim 9, wherein the detectable label is Alexa Fluor® 647 or Alexa Fluor® 488.

30

12. The method of claim 9, wherein the detectable label is phycoerythrin or allophycocyanin.

13. The method of any one of claims 1-12, wherein at least 30% of the endotoxin is removed from the sample.

5 14. The method of any one of claims 1-12, wherein at least 50% of the endotoxin is removed from the sample.

15. The method of any one of claims 1-14, wherein the endotoxin is removed using a filter.

10

16. The method of any one of claims 1-15, wherein the sample is a bacterial cell homogenate.

15 17. The method of any one of claims 1-15, wherein the sample comprises the supernatant from a bacterial cell culture.

18. The method of any one of claims 1-17, further comprising determining the concentration of the purified aerolysin protein.

20 19. The method of any one of claims 1-18, wherein greater than 35% of the aerolysin protein is purified from the sample.

20. The method of any one of claims 1-18, wherein greater than 40% of the aerolysin protein is purified from the sample.

25

21. The method of any one of claims 4-20, wherein the method does not comprise lyophilizing the conjugate.

30 22. The method of any one of claims 1-21, further comprising, prior to purifying the aerolysin protein from the sample, exchanging the buffer of the sample.

23. A purified aerolysin protein prepared by the method of any one of claims 1-3.

24. An aerolysin conjugate prepared by the method of any one of claims 4-22.

25. A non-lytic aerolysin conjugate prepared by the method of any one of claims 5-
5 22.

26. A method for detecting the presence of a glycosylphosphatidylinositol (GPI) moiety on a cell, the method comprising:

10 contacting a cell with the non-lytic aerolysin conjugate of claim 25; and
 detecting the binding of the non-lytic aerolysin conjugate to the cell, wherein
binding of the conjugate to the cell indicates that the cell expresses a GPI moiety and
the absence of binding of the conjugate to the cell indicates that the cell does not
express a GPI moiety.

15 27. A method for determining whether a human has paroxysmal nocturnal hemoglobinuria (PNH), the method comprising:

 contacting blood cells obtained from a human with the non-lytic aerolysin
conjugate of claim 25; and
 determining whether the non-lytic conjugate binds to the blood cells, wherein
20 reduced binding of the conjugate to the blood cells, as compared to the binding of the
conjugate to control blood cells, indicates that the human has PNH.

28. The method of claim 27, wherein the control blood cells are obtained from a
human that does not have PNH.

25

29. The method of claim 27 or 28, wherein the blood cells comprise one or both of monocytes and granulocytes.

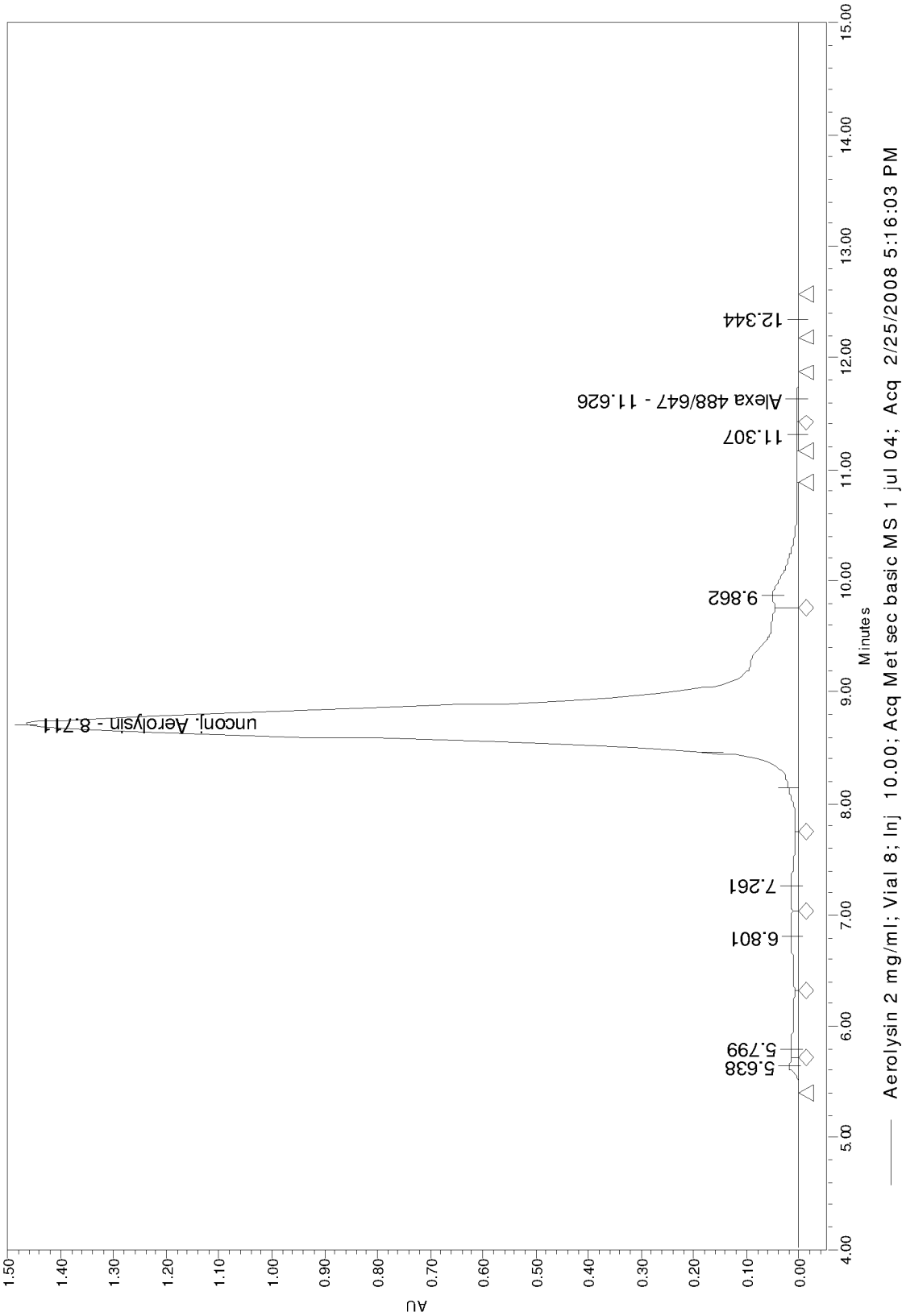


FIG. 1

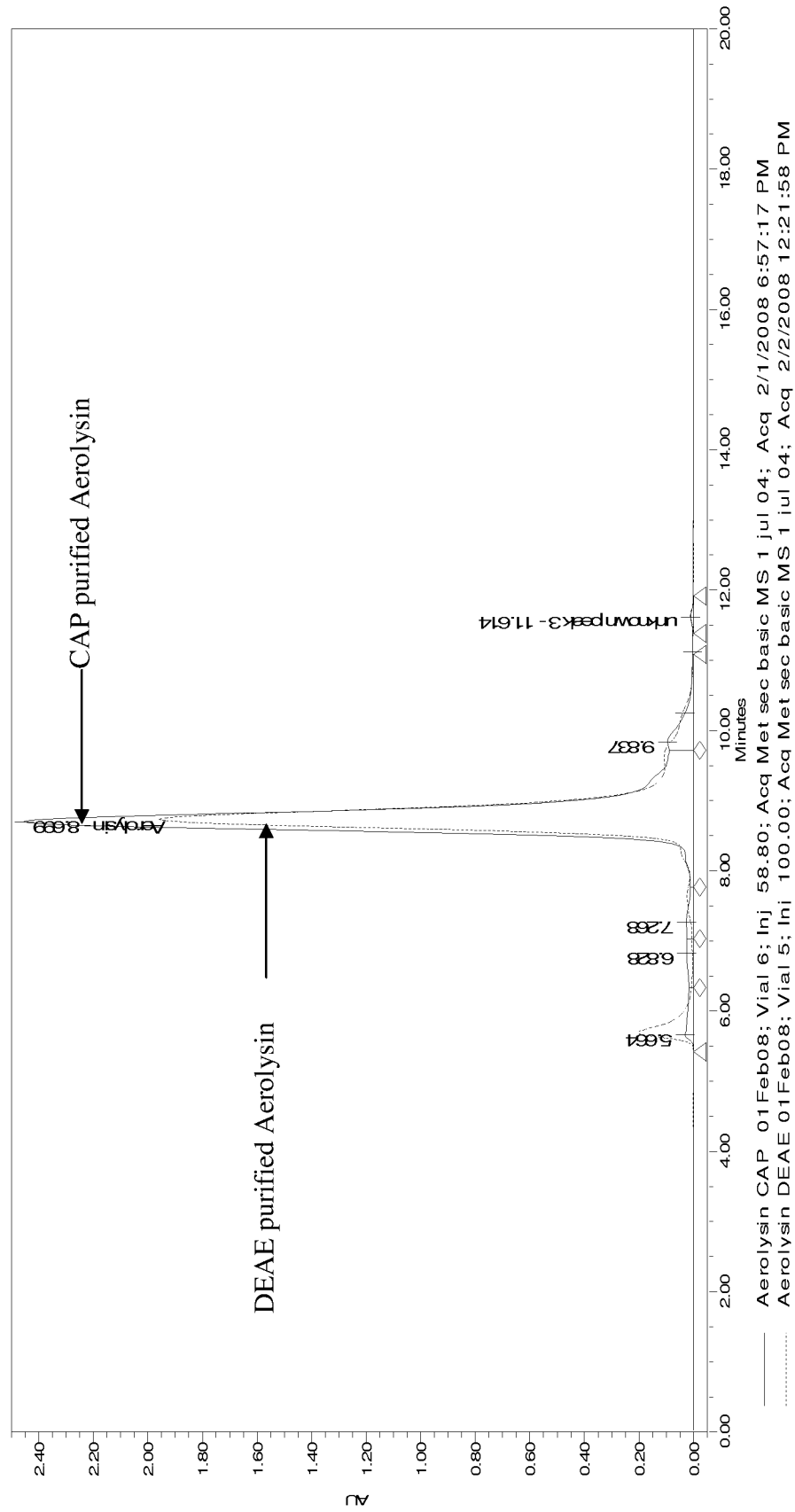


FIG. 2

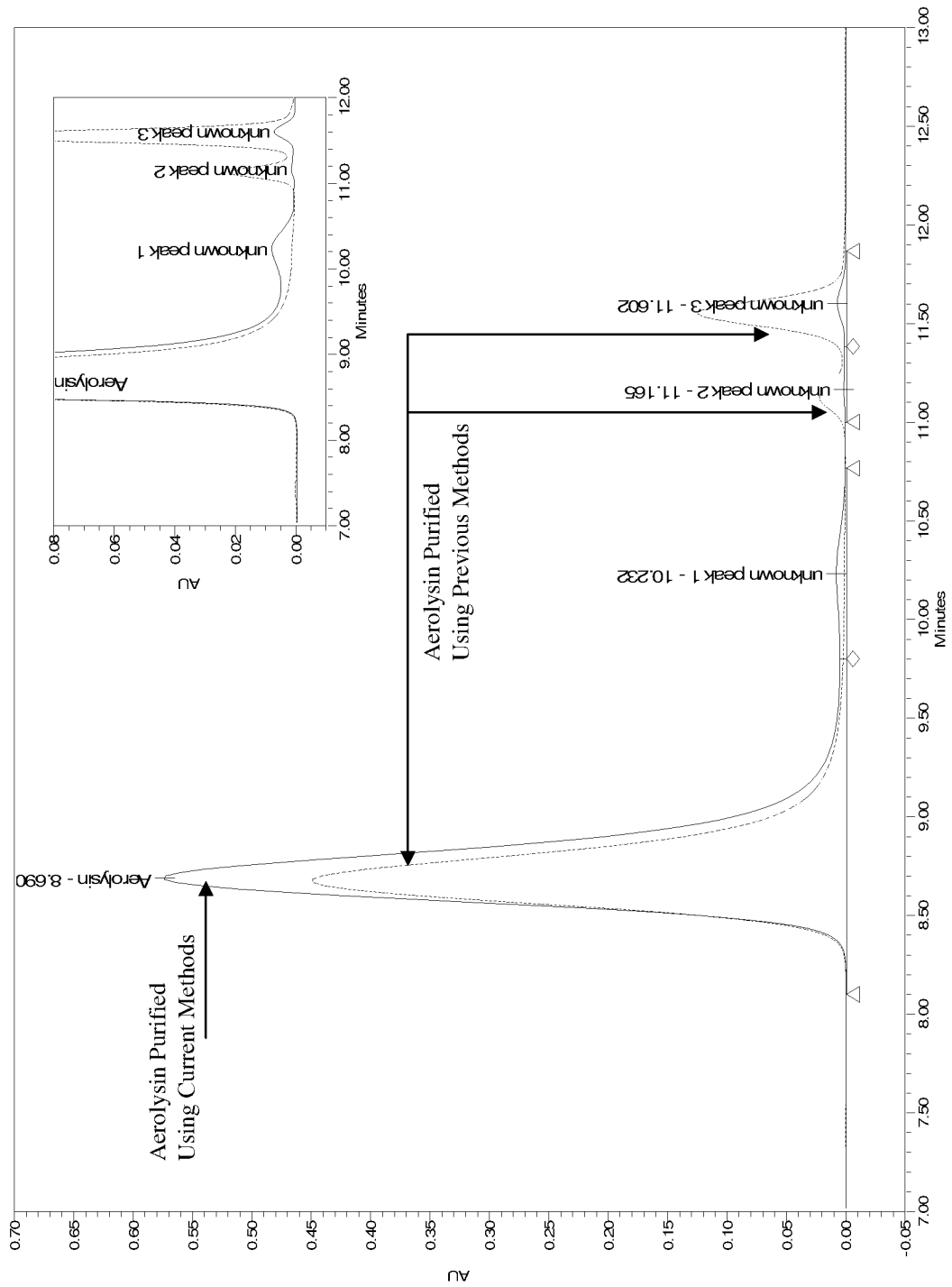


FIG. 3

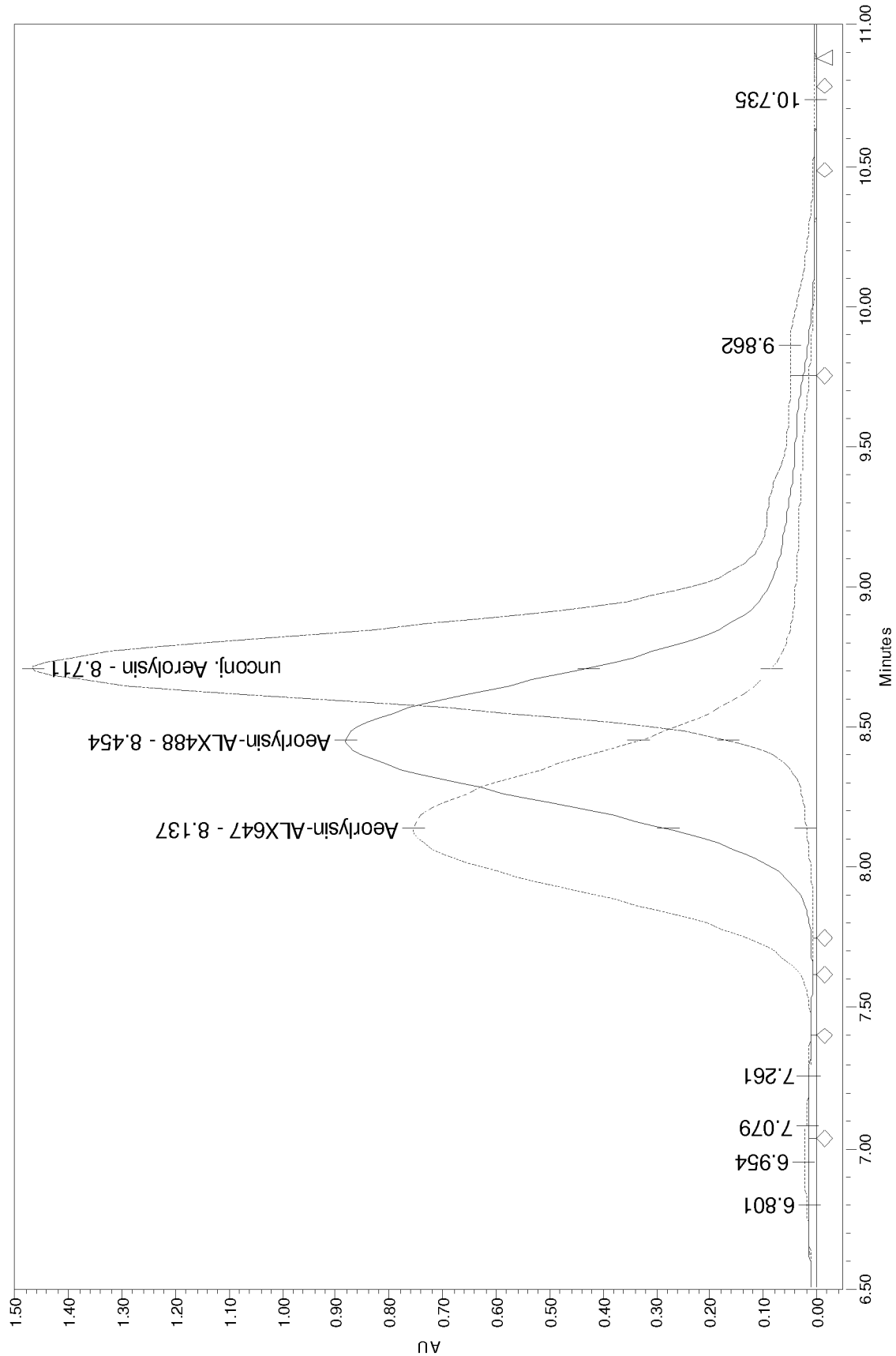


FIG. 4

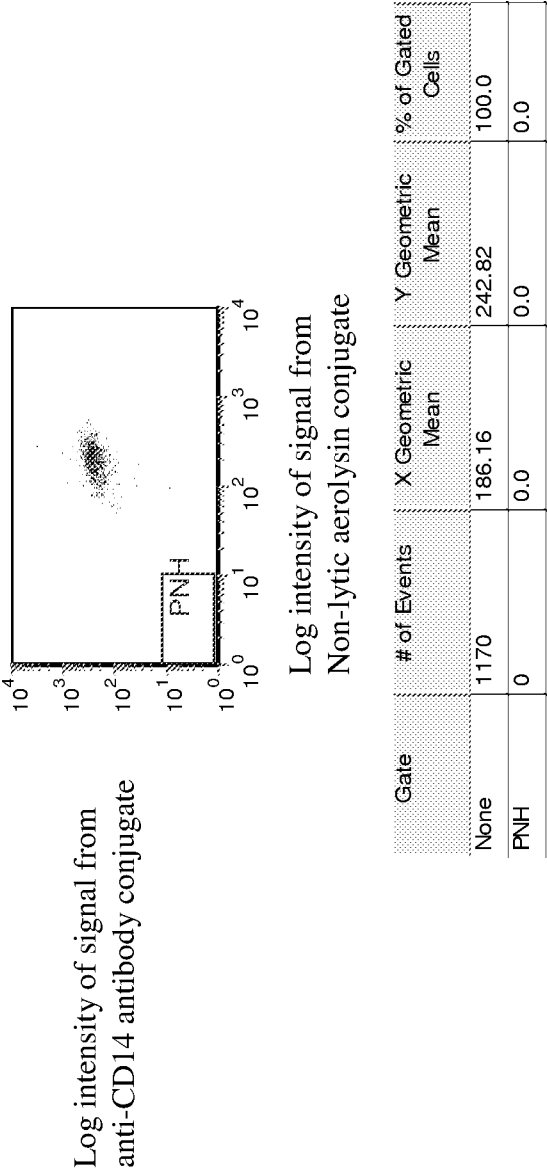
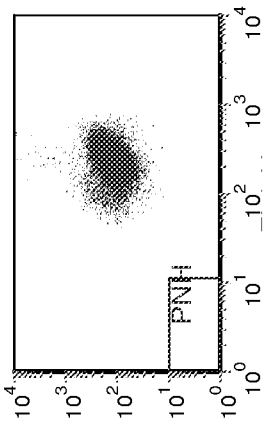


FIG. 5



Log intensity of signal from
anti-CD24 antibody conjugate

Log intensity of signal from
Non-lytic aerolysin conjugate

Gate	# of Events	X Geometric Mean	Y Geometric Mean	% of Gated Cells
None	29481	225.95	120.22	100.0
PNH	0	0.0	0.0	0.0

FIG. 6

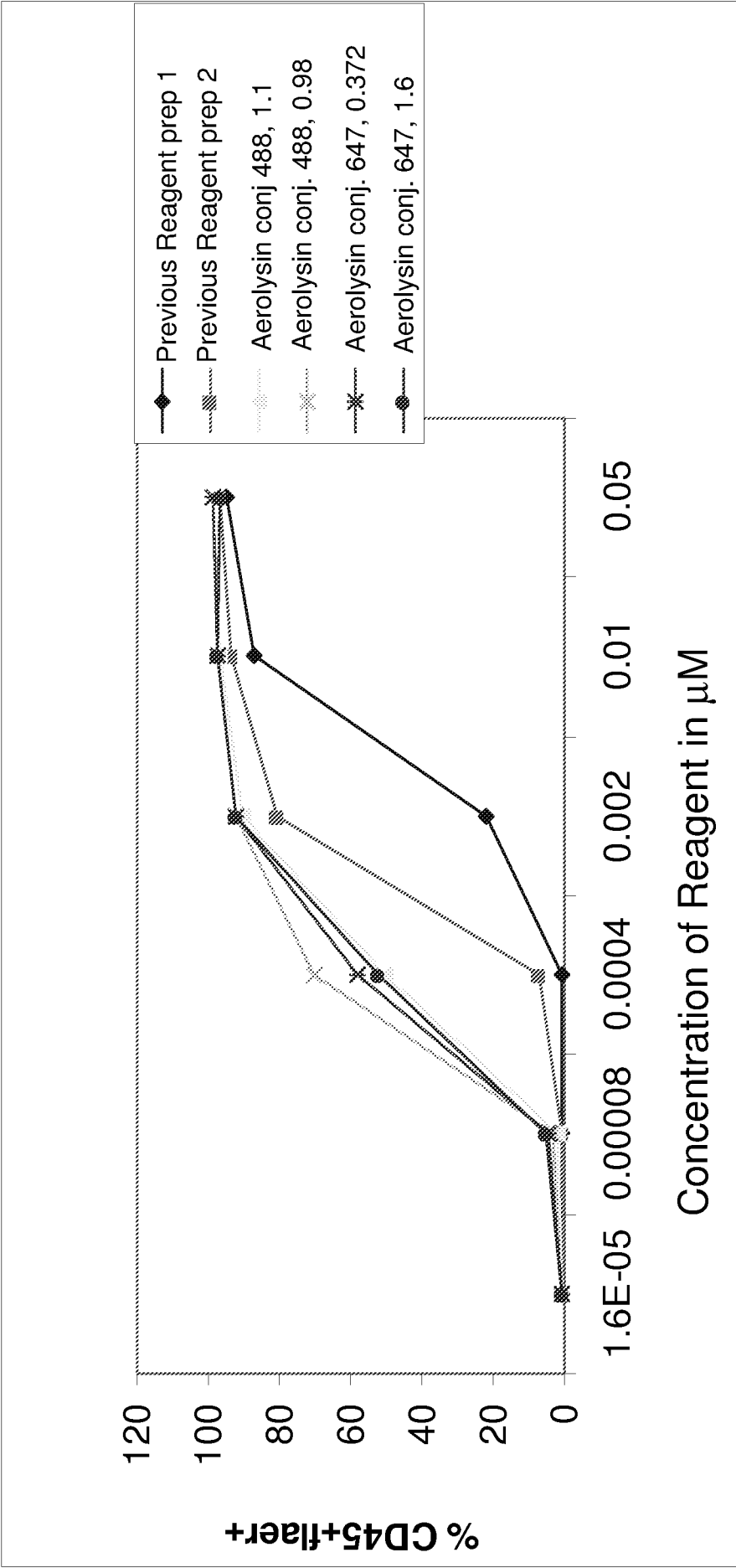


FIG. 7

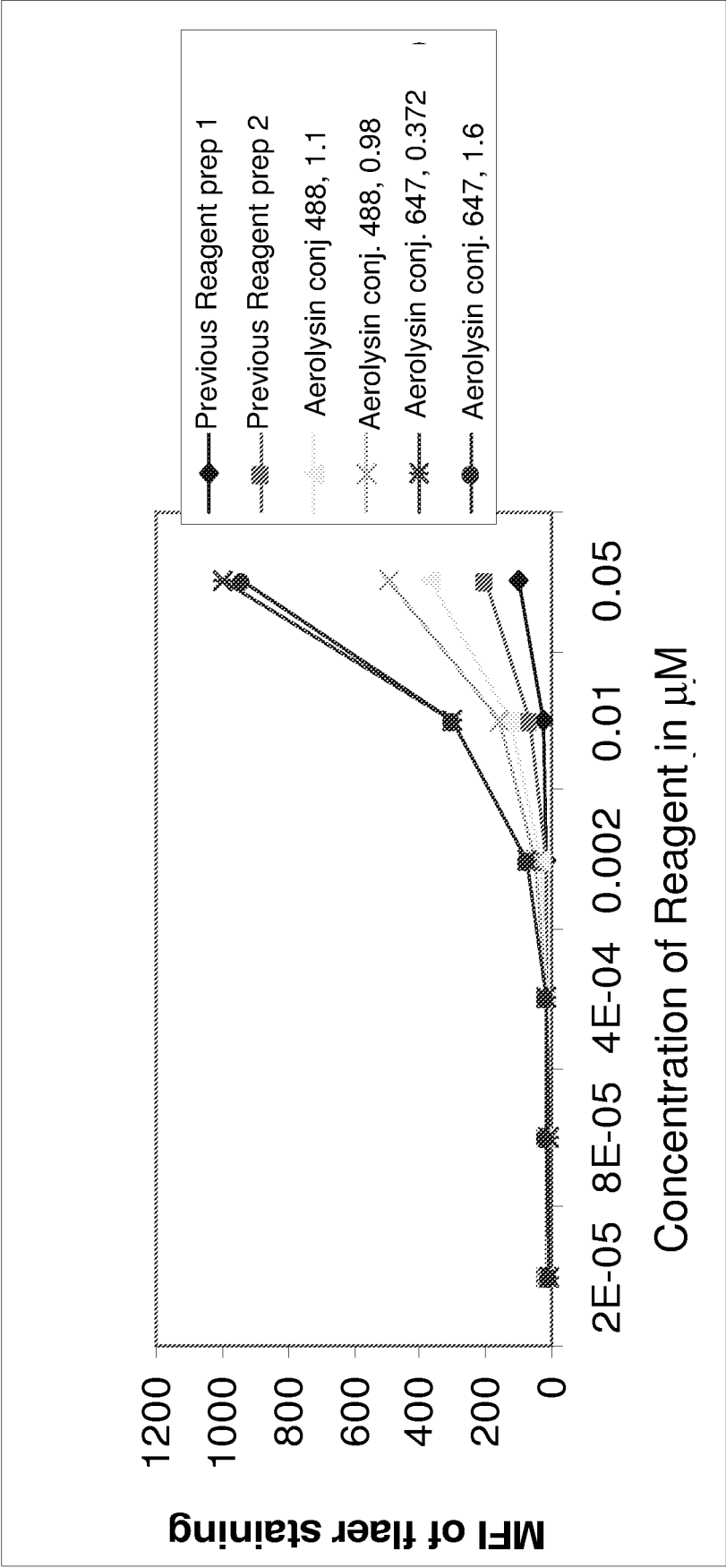


FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2009/066223

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C07K 1/14 (2010.01)

C12Q 1/02 (2010.01)

C07K 14/195 (2010.01)

G01N 33/53 (2010.01)

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)

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

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

MEDLINE, CAPLUS, BIOSIS, EPODOC, WPI (Aerolysin, purify, remove, isolate, separate, extract; produce, endotoxin, non-lytic, non-lysing, non-cytolytic, DEAE, hydroxyapatite, diethylaminoethyl, hydroxylapatite, durapatite, HA-chromatography, GPI, Glycosylphosphatidylinositol, PNH, Paroxysmal nocturnal hemoglobinuria, Marchiafava Micheli); GenomeQuest (SEQ ID NOs: 2, 5, 7, 8, 9, 10 with claimed substitutions)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 1999/036778 A1 (UNIVERSITY OF VICTORIA INNOVATION & DEVELOPMENT CORPORATION & JOHNS HOPKINS UNIVERSITY) 22 July 1999 See page 4, line 33 – page 5, line 5; page 9, lines 16-24; claims 1, 2, 6, 7	23-29
X	US 2007/0274919 A1 (DERTINGER, S.D.) 29 November 2007 See ([0009]; claims 1, 2	23-29
X	US 5798218 A (BUCKLEY, J.T.) 25 August 1998 See column 3, lines 21-35; column 9, lines 49-51; column 10, lines 39 – column 11, lines 37; example 1; table 2	1-5, 9-25



Further documents are listed in the continuation of Box C



See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"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
"E"	earlier application or patent but published on or after the international filing date	"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
"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)	"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
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
21 January 2010

Date of mailing of the international search report

27 JAN 2010

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2009/066223

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2002/038799 A2 (UNIVERSITY OF VICTORIA INNOVATION AND DEVELOPMENT CORPORATION) 16 May 2002 See page 20, lines 4-12; example 1	1-4, 9-24
X	LJUNGH, A. et al., "Separation and characterization of enterotoxin and two haemolysins from <i>Aeromonas hydrophila</i> " Acta Path. Microbiol. Scand. Sect. B, 1981, vol. 89, pages 387-397 See page 388, column 1, paragraph 5-column 2, paragraph 1	1-4, 9-24
X	HOWARD, S.P. & BUCKLEY, J.T., "Activation of the hole-forming toxin aerolysin by extracellular processing" J. Bacteriol., 1985, vol. 163, no. 1, pages 336-340 See page 336, column 2, paragraph 4-page 337, column 1, paragraph 1.	1-4, 9-24
X	BUCKLEY, J.T. & HOWARD, S.P., "Aerolysin from <i>Aeromonas hydrophila</i> " Meth. Enzymol., 1988, vol. 165, pages 193-199 See page 195, paragraph 4-page 196, paragraph 1	1-4, 9-24
X	ROSE, J.M. et al., "Purification and chemical characterization of a cholera toxin-cross-reactive cytolytic enterotoxin produced by a human isolate of <i>Aeromonas hydrophila</i> " Infect. Immun., 1989, vol. 57, no. 4, pages 1165-1169 See page 1166, column 2, paragraph 2	1-4, 9-24
X	BUCKLEY, J.T., "Purification of cloned proaerolysin released by a low protease mutant of <i>Aeromonas salmonicida</i> " Biochem. Cell Biol., 1990, vol. 68, 221-224 See page 222, column 2, paragraph 1	1-4, 9-24
X	WARE, R.E. et al., "Circulating PIG-A mutant T lymphocytes in healthy adults and patients with bone marrow failure syndromes including PNH" Blood, 2000, vol. 96, no. 11, page 711 See whole document	23, 24
X	WARE, R.E. et al., "Circulating PIG-A mutant T lymphocytes in healthy adults and patients with bone marrow failure syndromes" Exp. Hematol., 2001, vol. 29, no. 12, pages 1403-1409 See page 1404, column 1, paragraph 4-5	23, 24

Information on patent family members

PCT/US2009/066223

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member	
WO 1999/036778	AU 23261/99	CA 2318127	US 6593095
US 20070274919	NONE		
US 5798218	NONE		
WO 2002/038799	AU 23316/02	US 20050266512	

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX