



(43) International Publication Date
13 September 2012 (13.09.2012)

- (51) **International Patent Classification:**
C07H 21/00 (2006.01) *G01N 33/53* (2006.01)
- (21) **International Application Number:**
PCT/US2012/028632
- (22) **International Filing Date:**
9 March 2012 (09.03.2012)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**
61/451,227 10 March 2011 (10.03.2011) US
- (71) **Applicant (for all designated States except US):** **SOMA-LOGIC, INC.** [US/US]; 2945 Wilderness Place, Boulder, Colorado 80301 (US).
- (72) **Inventors; and**
- (75) **Inventors/Applicants (for US only):** **OCHSNER, Urs** [US/US]; 1953 Ivy Street, Denver, Colorado 80220 (US). **KATILIUS, Evaldas** [LT/US]; 2184 Jarosa Lane, Superior, Colorado 80027 (US). **JANJIC, Nebojsa** [US/US]; 6973 Carter Trail, Boulder, Colorado 80301 (US).
- (74) **Agents:** **SWANSON & BRATSCHUN, L.L.C.** et al.; 8210 SouthPark Terrace, Littleton, Colorado 80120 (US).
- (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, QA, RO, RS, RU, RW, 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, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, 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, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))
- with sequence listing part of description (Rule 5.2(a))

(54) **Title:** APTAMERS FOR *CLOSTRIDIUM DIFFICILE* DIAGNOSTICS

(57) **Abstract:** The present disclosure relates generally to the field of nucleic acids and, more particularly, to aptamers capable of binding to toxins produced by *Clostridium difficile*; diagnostic kits and methods comprising such aptamers; and methods of making and using such aptamers.



WO 2012/122540 A1

APTAMERS FOR *CLOSTRIDIUM DIFFICILE* DIAGNOSTICS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Application Serial No. 61/451,227, which was filed on March 10, 2011, the disclosure of which is incorporated herein in its entirety.

FIELD OF THE INVENTION

[0001] The present disclosure relates generally to the field of nucleic acids and more particularly to aptamers capable of binding to toxins produced by *C. difficile* and useful for diagnostic tests for *C. difficile*. The disclosure further relates to materials and methods for diagnosing *C. difficile* contamination or infection.

[0002] Incorporated by reference herein in its entirety is the Sequence Listing entitled "20120221SequenceListing005741_ST25.txt", created March 9, 2012, size of 9 kilobytes.

BACKGROUND

[0003] The following description provides a summary of information relevant to the present disclosure and is not an admission that any of the information provided or publications referenced herein is prior art to the present disclosure.

[0004] *C. difficile* infection (CDI) has been on the rise worldwide over the last several years. The clinical and economic consequences are substantial, with more than half a million cases and estimated costs of 3.2 billion dollars per year for CDI management in the U.S. alone (O'Brien, J.A., *et al.*, Infect. Control Hosp. Epidemiol., 2007. 28(11): p. 1219-27).

[0005] CDI is an inflammatory condition of the large bowel characterized by diarrhea and can range in severity from mild to fulminant. More severe CDI syndromes are pseudomembranous colitis and toxic megacolon. Most CDI cases occur in elderly patients in a hospital setting or in nursing homes. Hospitalization, however, increases the risk of colonization also for healthy adults. In the U.S., CDI hospitalizations and CDI-related case-fatality rates doubled between 2000 and 2005. A number of recent outbreaks have been reported in which CDI cases were primarily clonal in nature. A strain type classified as BI/NAP1/027 was responsible for more than half of the cases, and hallmarks of this epidemic

“outbreak” strain are high morbidity and mortality, higher resistance to antibiotics (*e.g.* fluoroquinolones), the presence of a *tcdC* variant gene, and toxin hyper-production (Freeman, J., *et al.*, Clin. Microbiol. Rev., 2010. 23(3): p. 529-49; Rupnik, M., M.H. Wilcox, and D.N. Gerding, Nat. Rev. Microbiol., 2009. 7(7): p. 526-36).

[0006] Use of antibiotics is a strong predisposing factor for CDI due to the disruption of the normal gut flora that otherwise suppresses *C. difficile*. Ingestion of spores is the main route of colonization of the human gut by *C. difficile*. Spores are extremely resistant to disinfectants and can persist in the environment for more than 12 months with little loss of viability or pathogenicity. Spores are also implicated in the 20–25% of CDI cases which relapse after treatment. Current treatment regimens for CDI are vancomycin or metronidazole. Several new, more selective agents that hold promise to reduce CDI recurrence rates are in clinical development.

[0007] The inflammation of the intestinal lining is caused by two toxins (toxin A and toxin B) that are expressed by some *C. difficile* strains. Toxin A and toxin B are glucosyltransferases that target small host GTPases in the Ras superfamily. They are encoded on the 19.6 kb pathogenicity locus and strains lacking these toxin genes are non-pathogenic. Toxinogenic strains can be further classified into toxinotypes according to sequence variability within the pathogenicity locus. Both toxins contribute to CDI, as shown by using isogenic mutants that produced either toxin A or toxin B alone and were cytotoxic *in vitro* and virulent *in vivo* (Kuehne, S.A., *et al.*, Nature, 2010. 467(7316): p. 711-3). A vaccine prototype based on inactivated toxins A and B (toxoids) and anti-toxin monoclonal antibodies are being studied for their effectiveness in preventing recurrent CDI.

[0008] Toxin A and toxin B are structurally related, large toxins of MW~300 kDa, and consist of an amino-terminal catalytic domain (glucosyltransferase), a central peptidase C80 domain, a translocation domain, and multiple carboxy-terminal β -hairpin repeats. The mechanism of action of the clostridial toxins has been shown to involve binding of these β -hairpin repeats to carbohydrates present on the surface of gastrointestinal cells, endopeptidase-mediated cleavage, and internalization of the catalytic domain (Pfeifer, G., *et al.*, J. Biol. Chem., 2003. 278(45): p. 44535-41).

[0009] Some *C. difficile* strains produce a binary toxin which possesses ADP-ribosyltransferase activity. Although its role in pathogenesis is unclear, the presence of binary toxin is a good marker for the epidemic outbreak strain BI/NAP1/027. The binary toxin consists of two subunits, which are the actin ADP-ribosyltransferase binary toxin A chain and the pore-forming binary toxin B chain. They are secreted from the bacterial cells

as separate polypeptides and have the potential to combine to form a potent cytotoxin which has been shown to kill Vero cells (Sundriyal, A., *et al.*, Protein Expr. Purif., 2010. 74(1): p. 42-8).

[0010] Rapid and accurate CDI diagnosis is important for patient care, infection control and surveillance. The *C. difficile* toxins A and B are of high clinical diagnostic relevance since they are sufficiently pathogen-specific targets and the demonstration of their presence is important for CDI diagnosis. All currently used CDI diagnostic tests are qualitative and belong to one of three types, (i) cytotoxin assay (tissue culture), (ii) non-molecular toxin tests (EIA), and (iii) molecular tests (PCR).

[0011] The tissue culture-based cytotoxin assay is considered the gold standard, but is cumbersome and not routinely performed by most clinical laboratories. In essence, this assay detects *C. difficile* toxin via the toxin's cytopathic effect in cell culture that can be neutralized with specific anti-sera. The cytotoxicity assay detects as little as 10 pg of toxin B and is the recommended confirmatory test for 510(k) submissions in the "Draft Guidance for Industry and Food and Drug Administration Staff Establishing the Performance Characteristics of *In Vitro* Diagnostic Devices for the Detection of *Clostridium difficile*" that was released in November 2010 FDA,

<http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm234868.htm>. 2010.

[0012] Molecular tests for CDI are available from several diagnostic companies. The Cepheid GeneXpert™ test is based on multiplex PCR (*tcdB*, *cdt*, *tcdC*), with advertised sensitivity and specificity of >95% and time-to-result of 30 min. The Meridian illumigene™ *C. difficile* test detects the presence of the toxin producing region by isothermal loop amplification and advertised to provide results in under an hour. The BD GeneOhm™ *Cdiff* assay is a real-time PCR method for the detection of toxin B gene (*tcdB*) direct from stool samples, with an assay protocol time of less than two hours, sensitivity of 93.8% and specificity of 95.5%. Gen-Probe offers the Prodesse ProGastro Cd test which also detects the toxin B gene (*tcdB*) by PCR and is advertised to provide results in three hours with a sensitivity of 91.7% and specificity of 94.7%.

[0013] Non-molecular tests for *C. difficile* toxin detection in stool samples from patients with suspected CDI are also available. Enzyme immunoassays (EIAs) are the most widely used rapid detection methods for *C. difficile* common antigen and toxin A/B antigens, but traditional EIAs have modest sensitivity and specificity. Among the well-type EIAs, the Meridian Premier™ Toxins A/B test and the Techlab TOX A/B II™ test are considered the

best-performing ELISAs and detect both toxins in stool specimens in less than 1 hour. These assays had about 80% sensitivity and 98% specificity when tested independently. The toxin B antibodies for the PremierTM Toxins A/B (Meridian) and for the *C. difficile* TOX A/B IITM (TechLab) were able to detect 125 pg and 250 pg of toxin B, respectively, when tested side by side (Novak-Weekley, S.M. and M.H. Hollingsworth. Clin Vaccine Immunol, 2008. 15(3): p. 575-8). Many other well-type EIAs assays have been brought to market (GA's *C. difficile* antigen, R-Biopharm's RidascreenTM Toxin A/B; Remel's ProSpectTM Toxin A/B) but are used less often in the U.S. Membrane EIA assays performed with lateral flow devices are the Meridian ImmunoCardTM Toxins A&B, the Techlab Tox A/B Quik ChekTM, and the Remel XpectTM assays.

[0014] There is one automated test on the market, bioMérieux's VIDASTM *C. difficile* Toxin A&B, which combines toxin testing and culture based identification with the API[®] 20A strip and automated bacterial genotyping with the DiversiLab[®] system.

[0015] Aptamer-based *C. difficile* toxin tests, like EIAs, have the advantage over molecular tests that they do not require big investments in equipment or expensive reagents. Aptamers have several distinct advantages over antibodies that are currently used in non-molecular assays, such as EIAs: aptamers generally have lower molecular weight, provide higher multiplexing capabilities (low cross-reactivity, universally-applicable assay conditions), chemical stability (to heat, drying, and solvents, reversible renaturation), provide ease of reagent manufacturing, consistent lot-to-lot performance and can be produced at lower cost.

[0016] Aptamers can be generated against virtually any protein target, not only toxins A/B, but also binary toxin for which there is no antibody-based test of which Applicants are aware. Detection and read-out methods can be the same as for existing tests, thus minimizing equipment needs and training requirements.

SUMMARY

[0017] The present disclosure provides various aptamers that bind to toxins produced by *C. difficile*. Included are diagnostic kits and diagnostic methods comprising such aptamers; and methods of making and using such aptamers.

[0018] The provided aptamers bind to *C. difficile* toxin A, toxin B, binary toxin A chain, or binary toxin B chain. Diagnostic methods are provided for detecting a *C. difficile*

toxin which comprise an aptamer to a toxin produced by *C. difficile* including but are not limited to pull-down assays, dot blot assays, PCR assays and sandwich assays.

[0019] The provided aptamers optionally comprise at least one pyrimidine modified at a C-5 position and may comprise at least one additional chemical modification. Also provided are aptamers and methods for identifying or producing such aptamers which bind to a *C. difficile* toxin which have a slow off-rate from the toxin. Further provided are aptamers and methods for identifying or producing such aptamers which bind to a *C. difficile* toxin which have nuclease resistance.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] Figure 1A illustrates the crystal structure of *C. difficile* toxin A (rTcdA) including five carboxy-terminal receptor-binding repeats (Ho, J.G., *et al.* Proc. Natl. Acad. Sci. USA, 2005, 102(51): p. 18373-8); shows the purification of toxin A as a recombinant tagged protein purified via affinity chromatography with Ni-NTA agarose and Strep-Tactin resin using the His-tag and Strep tag of toxin A; and PCR amplification of corresponding portion of the toxin gene encoding toxin A (*tcdA*). Figure 1B illustrates the crystal structure of *C. difficile* toxin B (rTcdB) amino-terminal catalytic domain (Reinert, D.J. *et al.* (2005), J. Mol. Biol. 351: 973-981); shows the purification of toxin B as a recombinant tagged protein purified via affinity chromatography with Ni-NTA agarose and Strep-Tactin resin using the His-tag and Strep tag of toxin B; and PCR amplification of corresponding portion of the toxin gene encoding toxin B (*tcdB*). Figure 1C illustrates the crystal structure of the full-length *C. difficile* binary toxin A chain (rCdtA) (Sundrival, A., *et al.*, J Biol. Chem. 2009, 284(42): p. 28713-9); shows the purification of binary toxin A chain as a recombinant tagged protein purified via affinity chromatography with Ni-NTA agarose and Strep-Tactin resin using the His-tag and Strep tag of the binary toxin A chain; and PCR amplification of corresponding portion of the toxin gene encoding the binary toxin A chain (*cdtA*). Figure 1D illustrates the modeled structure of the full-length *C. difficile* binary toxin B chain (rCdtB); shows the purification of binary toxin B chain as a recombinant tagged protein purified via affinity chromatography with Ni-NTA agarose and Strep-Tactin resin using the His-tag and Strep tag of the binary toxin B chain; and PCR amplification of corresponding portion of the toxin gene encoding the binary toxin B chain (*cdtB*).

[0021] Figure 2A illustrates the results of a pull-down assay of recombinant and native toxin A using toxin A aptamers showing high specificity over the control proteins

toxin B or BSA. Figure 2B illustrates the results of a pull-down assay of recombinant and native toxin B using toxin B aptamers showing high specificity over the control proteins recombinant and native toxin A. Figure 2C illustrates the results of a pull-down assay of binary toxin with aptamers to binary toxin A chain showing specificity for binary toxin A chain over binary toxin B chain and control protein BSA. Figure 2D illustrates the results of a pull-down assay of recombinant and native toxin A as well as recombinant and native toxin B with random aptamers showing no proteins present in the pull-down fraction.

[0022] Figure 3A illustrates detection of *C. difficile* toxin A on dot blots using biotinylated aptamers to toxin A and streptavidin-alkaline phosphatase conjugate. Figure 3B illustrates detection of *C. difficile* toxin B on dot blots using biotinylated aptamers to toxin B and streptavidin-alkaline phosphatase conjugate.

[0023] Figure 4A illustrates quantitative detection of toxin A by qPCR of aptamers eluted from a sample containing aptamers in complex with toxin A wherein unbound aptamers have been removed before quantitative measurement of aptamers as proxy measurement for toxin A. Figure 4B illustrates quantitative detection of toxin B by qPCR of aptamers eluted from a sample containing aptamers in complex with toxin B wherein unbound aptamers have been removed before quantitative measurement of aptamers as proxy measurement for toxin B.

[0024] Figure 5A illustrates results of detection of *C. difficile* toxin A via streptavidin plate sandwich (aptamer-target-antibody) assay, using biotinylated aptamers to toxin A and mouse monoclonal antibodies to toxin A detected with goat-anti-mouse antibodies. Figure 5B illustrates results of detection of *C. difficile* toxin B via streptavidin plate sandwich (aptamer-target-antibody) assay, using biotinylated aptamers to toxin B and mouse monoclonal antibodies to toxin B detected with goat-anti-mouse antibodies.

[0025] Figure 6 illustrates results of detection of *C. difficile* toxin A and B via sandwich (antibody-target-aptamer) assays on nitrocellulose, wherein monoclonal antibodies are spotted onto nitrocellulose and air dried, blocked, samples containing toxin A or B added, washed, biotinylated aptamers added, washed, and developed with streptavidin-alkaline phosphatase conjugate.

[0026] Figure 7 illustrates results of detection of *C. difficile* binary toxin A chain in a sandwich (aptamer-target-aptamer) assay with a first biotinylated aptamer to binary toxin A chain attached a streptavidin bead, addition of target, and addition of a second, radiolabeled aptamer to binary toxin A chain.

[0027] Figure 8 illustrates relevant steps of a Catch 1 – Catch 2 assay.

[0028] Figure 9 illustrates examples of C-5 modified pyrimidines which may be used in the methods of making aptamers described herein.

DETAILED DESCRIPTION

[0029] Reference will now be made in detail to representative embodiments of the invention. While the invention will be described in conjunction with the enumerated embodiments, it will be understood that the invention is not intended to be limited to those embodiments. On the contrary, the invention is intended to cover all alternatives, modifications, and equivalents that may be included within the scope of the present invention as defined by the claims.

[0030] One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in and are within the scope of the practice of the present invention. The present invention is in no way limited to the methods and materials described.

[0031] Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art(s) to which this invention belongs. Although any methods, devices, and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods, devices and materials are now described.

[0032] All publications, published patent documents, and patent applications cited in this disclosure are indicative of the level of skill in the art(s) to which the disclosure pertains. All publications, published patent documents, and patent applications cited herein are hereby incorporated by reference to the same extent as though each individual publication, published patent document, or patent application was specifically and individually indicated as being incorporated by reference.

[0033] As used in this disclosure, including the appended claims, the singular forms “a,” “an,” and “the” include plural references, unless the content clearly dictates otherwise, and are used interchangeably with “at least one” and “one or more.” Thus, reference to “an aptamer” includes mixtures of aptamers, and the like.

[0034] As used herein, the term “about” represents an insignificant modification or variation of the numerical value such that the basic function of the item to which the numerical value relates is unchanged.

[0035] As used herein, the term “aptamer clone” refers to an aptamer of a particular nucleotide sequence. Aptamer clones are identified herein by “Aptamer ID No.” as well as by “SEQ ID NO:.”

[0036] As used herein, "competitor molecule" and "competitor" are used interchangeably to refer to any molecule that can form a non-specific complex with a non-target molecule. A "competitor molecule" or "competitor" is a set of copies of one type or species of molecule. "Competitor molecules" or "competitors" refer to more than one such set of molecules. Competitor molecules include oligonucleotides, polyanions (e.g., heparin, single-stranded salmon sperm DNA, and polydextrans (e.g., dextran sulphate)), abasic phosphodiester polymers, dNTPs, and pyrophosphate. In the case of a kinetic challenge that uses a competitor, the competitor can also be any molecule that can form a non-specific complex with an aptamer. Such competitor molecules include polycations (e.g., spermine, spermidine, polylysine, and polyarginine) and amino acids (e.g., arginine and lysine).

[0037] As used in tables 4, 6, 8 and 10, the term “count” refers to the number of occurrences of a particular aptamer sequence among all aptamers that were cloned and sequenced from a pool that resulted from SELEX.

[0038] As used herein, the term “dot blot” refers to an assay wherein a mixture containing the target molecule to be detected is applied directly onto a substrate as a dot followed by detection of the presence of the target molecule by an affinity molecule, wherein the affinity molecule may be, but is not limited to, an aptamer or antibody.

[0039] The term “each” when used herein to refer to a plurality of items is intended to refer to at least two of the items. It need not require that all of the items forming the plurality satisfy an associated additional limitation.

[0040] As used herein, the terms “comprises,” “comprising,” “includes,” “including,” “contains,” “containing,” and any variations thereof, are intended to cover a non-exclusive inclusion, such that a process, method, product-by-process, or composition of matter that comprises, includes, or contains an element or list of elements does not include only those elements but may include other elements not expressly listed or inherent to such process, method, product-by-process, or composition of matter.

[0041] As used herein, “consensus sequence,” when used in reference to a series of related nucleic acids, refers to a nucleotide sequence that reflects the most common choice of base at each position in the sequence where the series of related nucleic acids has been subjected to mathematical and/or sequence analysis.

[0042] As used herein, the term “nucleotide” refers to a ribonucleotide or a deoxyribonucleotide, or a modified form thereof, as well as an analog thereof. Nucleotides include species that include purines (*e.g.*, adenine, hypoxanthine, guanine, and their derivatives and analogs) as well as pyrimidines (*e.g.*, cytosine, uracil, thymine, and their derivatives and analogs).

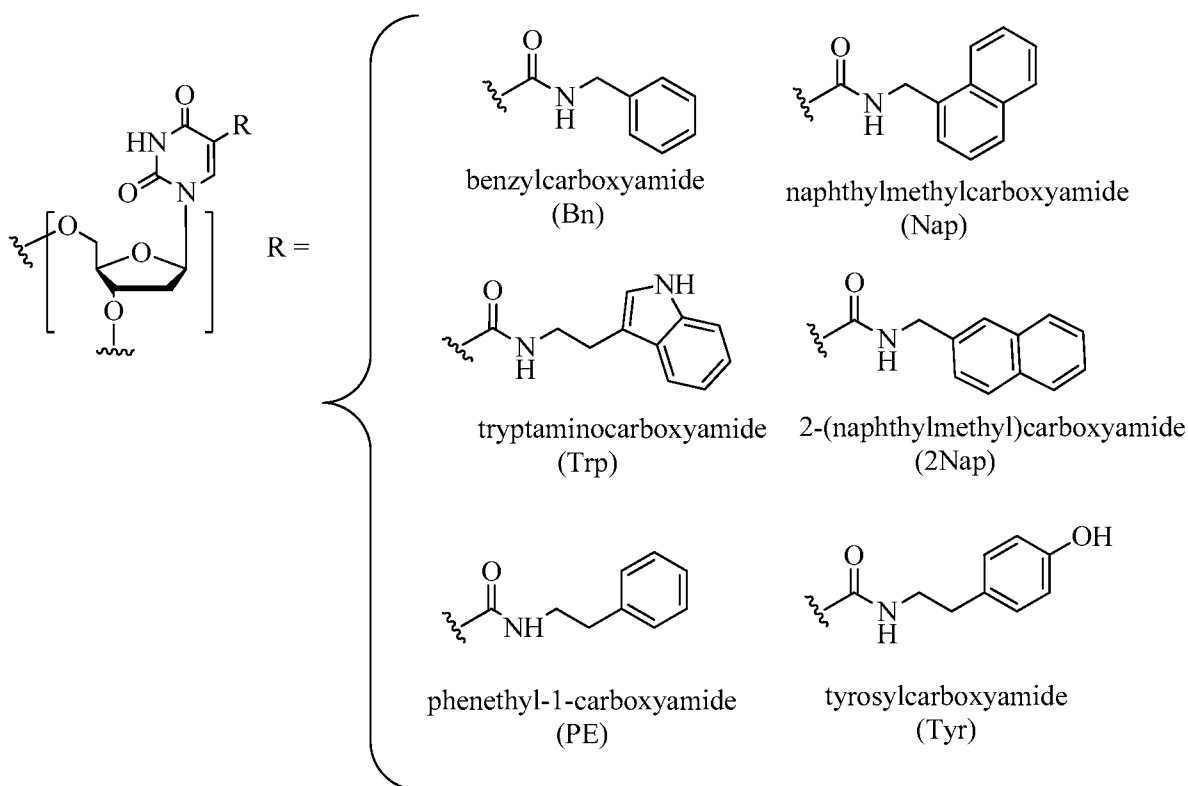
[0043] As used herein, “nucleic acid,” “oligonucleotide,” and “polynucleotide” are used interchangeably to refer to a polymer of nucleotides and include DNA, RNA, DNA/RNA hybrids and modifications of these kinds of nucleic acids, oligonucleotides and polynucleotides, wherein the attachment of various entities or moieties to the nucleotide units at any position are included. The terms “polynucleotide,” “oligonucleotide,” and “nucleic acid” include double- or single-stranded molecules as well as triple-helical molecules. Nucleic acid, oligonucleotide, and polynucleotide are broader terms than the term aptamer and, thus, the terms nucleic acid, oligonucleotide, and polynucleotide include polymers of nucleotides that are aptamers but the terms nucleic acid, oligonucleotide, and polynucleotide are not limited to aptamers.

[0044] As used herein, the terms “modify”, “modified”, “modification”, and any variations thereof, when used in reference to an oligonucleotide, means that at least one of the four constituent nucleotide bases (*i.e.*, A, G, T/U, and C) of the oligonucleotide is an analog or ester of a naturally occurring nucleotide. In some embodiments, the modified nucleotide confers nuclease resistance to the oligonucleotide. A pyrimidine with a substitution at the C-5 position is an example of a modified nucleotide. Modifications can include backbone modifications, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine, and the like. Modifications can also include 3' and 5' modifications, such as capping. Other modifications can include substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (*e.g.*, methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, *etc.*) and those with charged linkages (*e.g.*, phosphorothioates, phosphorodithioates, *etc.*), those with intercalators (*e.g.*, acridine, psoralen, *etc.*), those containing chelators (*e.g.*, metals, radioactive metals, boron, oxidative metals, *etc.*), those containing alkylators, and those with modified linkages (*e.g.*, alpha anomeric nucleic acids, *etc.*). Further, any of the hydroxyl groups ordinarily present on the sugar of a nucleotide may be replaced by a phosphonate group or a phosphate group; protected by standard protecting groups; or activated to prepare additional linkages to additional nucleotides or to a solid support. The 5' and 3' terminal OH groups can be phosphorylated or substituted with

amines, organic capping group moieties of from about 1 to about 20 carbon atoms, polyethylene glycol (PEG) polymers in one embodiment ranging from about 10 to about 80 kDa, PEG polymers in another embodiment ranging from about 20 to about 60 kDa, or other hydrophilic or hydrophobic biological or synthetic polymers. In one embodiment, modifications are of the C-5 position of pyrimidines. These modifications can be produced through an amide linkage directly at the C-5 position or by other types of linkages.

[0045] Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including 2'-O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, α -anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside. As noted above, one or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include embodiments wherein phosphate is replaced by P(O)S ("thioate"), P(S)S ("dithioate"), (O)NR₂ ("amidate"), P(O)R, P(O)OR', CO or CH₂ ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (-O-) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. Substitution of analogous forms of sugars, purines, and pyrimidines can be advantageous in designing a final product, as can alternative backbone structures like a polyamide backbone, for example.

[0046] As used herein, the term "C-5 modified pyrimidine" refers to a pyrimidine with a modification at the C-5 position including, but not limited to, those moieties illustrated in Figure 9. Examples of a C-5 modified pyrimidine include those described in U.S. Pat. Nos. 5,719,273 and 5,945,527. Examples of a C-5 modification include substitution of deoxyuridine at the C-5 position with a substituent independently selected from: benzylcarboxamide (alternatively benzylaminocarbonyl) (Bn), naphthylmethylcarboxamide (alternatively naphthylmethylaminocarbonyl) (Nap), tryptaminocarboxamide (alternatively tryptaminocarbonyl) (Trp), tyrosylcarboxamide (alternatively tyrosylaminocarbonyl) (Tyr), 2-naphthylmethylcarboxamide (alternatively 2-naphthylmethylaminocarbonyl) (2Nap) and phenethyl-1-carboxamide (alternatively phenethyl-1-aminocarbonyl) (PE), as illustrated immediately below.



[0047] Chemical modifications of a C-5 modified pyrimidine can also be combined with, singly or in any combination, 2'-position sugar modifications, modifications at exocyclic amines, and substitution of 4-thiouridine and the like.

[0048] Representative C-5 modified pyrimidines include: 5-(N-benzylcarboxamide)-2'-deoxyuridine (BndU), 5-(N-benzylcarboxamide)-2'-O-methyluridine, 5-(N-benzylcarboxamide)-2'-fluorouridine, 5-(N-tryptaminocarboxamide)-2'-deoxyuridine (TrpdU), 5-(N-tryptaminocarboxamide)-2'-O-methyluridine, 5-(N-tryptaminocarboxamide)-2'-fluorouridine, 5-(N-naphthylmethylcarboxamide)-2'-deoxyuridine (NapdU), 5-(N-naphthylmethylcarboxamide)-2'-O-methyluridine, 5-(N-naphthylmethylcarboxamide)-2'-fluorouridine, 5-(N-tyrosylcarboxamide)-2'-deoxyuridine (TyrdU), 5-(N-tyrosylcarboxamide)-2'-O-methyluridine, 5-(N-tyrosylcarboxamide)-2'-fluorouridine, 5-(N-(2-naphthylmethyl)carboxamide)-2'-deoxyuridine (2NapdU), 5-(N-(2-naphthylmethyl)carboxamide)-2'-O-methyluridine, 5-(N-(2-naphthylmethyl)carboxamide)-2'-fluorouridine, 5-(N-phenethyl-1-carboxamide)-2'-deoxyuridine (PEdU), 5-(N-phenethyl-1-carboxamide)-2'-O-methyluridine, or 5-(N-phenethyl-1-carboxamide)-2'-fluorouridine.

[0049] If present, a modification to the nucleotide structure can be imparted before or after assembly of a polymer. A sequence of nucleotides can be interrupted by non-nucleotide

components. A polynucleotide can be further modified after polymerization, such as by conjugation with a labeling component.

[0050] As used herein, the term “at least one pyrimidine,” when referring to modifications of a nucleic acid, refers to one or more, two or more, three or more, four or more, five or more, or all pyrimidines in the nucleic acid, indicating that any or all occurrences of any or all of C, T, or U in a nucleic acid may be modified or not.

[0051] As used herein, the terms "kinetically challenge" and "kinetic challenge" refer to a process of enrichment for an aptamer affinity complex from a set of complexes that includes an aptamer affinity complex and non-specific complexes, by applying kinetic pressure and making use of the different affinity characteristics of the constituents of such classes of complexes, including dissociation rates. A kinetic challenge generally results in an increase in specificity, since aptamer-non-target complexes are typically reduced compared to aptamer-target complexes. As used herein, the term "kinetic pressure" refers to a means for providing an opportunity for the natural dissociation of complexes and/or inhibiting the rebinding of molecules that dissociate from a complex naturally. Kinetic pressure can be applied by the addition of a competitor molecule, or by sample dilution, or by extensive washes when complexes are bound to a solid support, or by any other means known to one skilled in the art. As one of ordinary skill in the art will appreciate, because a kinetic challenge generally depends upon the differing dissociation rates of aptamer affinity complexes and aptamer-non-target complexes, the duration of the kinetic challenge is chosen so as to retain a high proportion of aptamer affinity complexes while substantially reducing the number of aptamer-non-target complexes. For a kinetic challenge to be effective, the dissociation rate for the aptamer affinity complex is preferably significantly lower than those for aptamer-non-target complexes. Since an aptamer can be selected to include particular properties, the constituents of an aptamer affinity complex can be designed to have a comparatively low dissociation rate, *i.e.* slow off rate.

[0052] As used herein, “nucleic acid ligand,” “aptamer,” and “clone” are used interchangeably to refer to a non-naturally occurring nucleic acid that has a desirable action on a target molecule. A desirable action includes, but is not limited to, binding of the target, catalytically changing the target, reacting with the target in a way that modifies or alters the target or the functional activity of the target, covalently attaching to the target (as in a suicide inhibitor), and facilitating the reaction between the target and another molecule. In one embodiment, the action is specific binding affinity for a target molecule, such target molecule being a three dimensional chemical structure other than a polynucleotide that binds to the

nucleic acid ligand through a mechanism which is independent of Watson/Crick base pairing or triple helix formation, wherein the aptamer is not a nucleic acid having the known physiological function of being bound by the target molecule. Aptamers to a given target include nucleic acids that are identified from a candidate mixture of nucleic acids, where the aptamer is a ligand of the target, by a method comprising: (a) contacting the candidate mixture with the target, wherein nucleic acids having an increased affinity to the target relative to other nucleic acids in the candidate mixture can be partitioned from the remainder of the candidate mixture; (b) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and (c) amplifying the increased affinity nucleic acids to yield a ligand-enriched mixture of nucleic acids, whereby aptamers of the target molecule are identified. It is recognized that affinity interactions are a matter of degree; however, in this context, the “specific binding affinity” of an aptamer for its target means that the aptamer binds to its target generally with a much higher degree of affinity than it binds to other, non-target, components in a mixture or sample. An “aptamer” or “nucleic acid ligand” is a set of copies of one type or species of nucleic acid molecule that has a particular nucleotide sequence. An aptamer can include any suitable number of nucleotides. “Aptamers” refer to more than one such set of molecules. Different aptamers can have either the same or different numbers of nucleotides. Aptamers may be DNA or RNA and may be single stranded, double stranded, or contain double stranded or triple stranded regions.

[0053] As used herein, IUPAC nucleotide ambiguity codes are: M = A or C; R = A or G; W = A or N; S = C or G; Y = C or N; K = G or N (N represents the pool-specific modified dU).

[0054] As used herein, “plateau” refers to a region of a binding curve (in which the fraction of aptamers bound increases up the y-axis and the concentration of target increases to the right on the x-axis) where a plateau is reached as increasing target concentration causes relatively little change in the fraction of aptamers bound to target. The plateau percentage provided herein is relative to 100% of aptamers being bound to target.

[0055] As used herein, “protein” is used synonymously with “peptide”, “polypeptide”, or “peptide fragment.” A “purified” polypeptide, protein, peptide, or peptide fragment is substantially free of cellular material or other contaminating proteins from the cell, tissue, or cell-free source from which the amino acid sequence is obtained, or substantially free from chemical precursors or other chemicals when chemically synthesized.

[0056] As used herein, “pull-down assay” refers to an assay which comprises removal of a target from solution wherein the removal is accomplished by a selective affinity

interaction between the target and a second molecule. In one embodiment, the molecule having selective affinity for the target molecule is an aptamer. In another embodiment, the molecule having selective affinity for the target is an antibody.

[0057] As used herein, “PCR” refers to a polymerase chain reaction used to amplify the copy number of a DNA molecule. As used herein, “qPCR” or “quantitative PCR” refers to a polymerase chain reaction that is used to amplify and simultaneously quantify a targeted DNA molecule.

[0058] As used herein, “sandwich assay” refers to an assay capable of detecting the presence of or quantitating the amount of a target of interest. The assay requires the use of two different affinity molecules capable of binding two different, non-overlapping (noncompetitive) regions on a target of interest. Affinity molecules include but are not limited to aptamers and antibodies.

[0059] As used herein, “substrate” refers to a surface, including but not limited to the surface of a plate, bead or membrane to which an organic molecule can attach. A substrate may or may not comprise a first molecule that mediates attachment of a second molecule, such as a substrate comprising streptavidin which can mediate attachment of biotin or a molecule comprising a biotin moiety. In one embodiment, the substrate is nitrocellulose.

[0060] As used herein, “test sample” refers to a sample in which the presence or amount of one or more analytes of interest (*e.g.* *C. difficile* toxin A, toxin B, binary toxin A chain, or binary toxin B chain) are unknown and to be determined in an assay, preferably a diagnostic test comprising an aptamer. In one embodiment, the test sample can be a “biological sample” such as cellular and non-cellular biological material, including, but not limited to, tissue samples, blood, serum, other bodily fluids, and excrement. In another embodiment, the test sample can be an “environmental sample” which can be obtained from water, soil or air. Normally no prior culturing is necessary for detection of *C. difficile* in environmental samples.

The SELEX Method

[0061] The terms “SELEX” and “SELEX process” are used interchangeably herein to refer generally to a combination of (1) the selection of nucleic acids that interact with a target molecule in a desirable manner, for example binding with high affinity to a protein, with (2) the amplification of those selected nucleic acids. The SELEX process can be used to identify aptamers with high affinity to a specific target molecule or biomarker.

[0062] SELEX generally includes preparing a candidate mixture of nucleic acids, binding of the candidate mixture to the desired target molecule to form an affinity complex, separating the affinity complexes from the unbound candidate nucleic acids, separating and isolating the nucleic acid from the affinity complex, purifying the nucleic acid, and identifying a specific aptamer sequence. The process may include multiple rounds to further refine the affinity of the selected aptamer. The process can include amplification steps at one or more points in the process. See, *e.g.*, U.S. Patent No. 5,475,096, entitled “Nucleic Acid Ligands.” The SELEX process can be used to generate an aptamer that covalently binds its target as well as an aptamer that non-covalently binds its target. See, *e.g.*, U.S. Patent No. 5,705,337 entitled “Systematic Evolution of Nucleic Acid Ligands by Exponential Enrichment: Chemi-SELEX.”

[0063] The SELEX process can be used to identify high-affinity aptamers containing modified nucleotides that confer improved characteristics on the aptamer, such as, for example, improved *in vivo* stability or improved delivery characteristics. Examples of such modifications include chemical substitutions at the ribose and/or phosphate and/or base positions. SELEX process-identified aptamers containing modified nucleotides are described in U.S. Patent No. 5,660,985, entitled “High Affinity Nucleic Acid Ligands Containing Modified Nucleotides,” which describes oligonucleotides containing nucleotide derivatives chemically modified at the 5'- and 2'-positions of pyrimidines. U.S. Patent No. 5,580,737, see *supra*, describes highly specific aptamers containing one or more nucleotides modified with 2'-amino (2'-NH₂), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe). See also, U.S. Patent Application Publication No. 2009/0098549, entitled “SELEX and PHOTOSELEX,” which describes nucleic acid libraries having expanded physical and chemical properties and their use in SELEX and photoSELEX.

[0064] The nuclease resistant oligonucleotides include at least one pyrimidine modified at the C-5 position with a group selected from those set forth in Figure 9. In various embodiments, the modifications include substitution of deoxyuridine at the C-5 position with a substituent independently selected from: benzylcarboxamide (Bn), naphthylmethylcarboxamide (Nap), tryptaminocarboxamide (Trp), tyrosylcarboxamide (Tyr), (2-naphthylmethyl)carboxamide (2Nap), and phenethyl-1-carboxamide (PE) as illustrated above.

[0065] SELEX can also be used to identify aptamers that have desirable off-rate characteristics. See U.S. Patent Publication No. 2009/0004667, entitled “Method for Generating Aptamers with Improved Off-Rates,” which describes improved SELEX methods

for generating aptamers that can bind to target molecules. Methods for producing aptamers and photoaptamers having slower rates of dissociation from their respective target molecules are described. The methods involve contacting the candidate mixture with the target molecule, allowing the formation of nucleic acid-target complexes to occur, and performing a slow off-rate enrichment process wherein nucleic acid-target complexes with fast dissociation rates dissociate and do not reform, while complexes with slow dissociation rates remain intact. Additionally, the methods include the use of modified nucleotides in the production of candidate nucleic acid mixtures to generate aptamers with improved off-rate performance (see U.S. Patent Publication No. 2009/0098549, entitled "SELEX and PhotoSELEX").

[0066] "Target" or "target molecule" or "target" refers herein to any compound upon which a nucleic acid can act in a desirable manner. A target molecule can be a protein, peptide, nucleic acid, carbohydrate, lipid, polysaccharide, glycoprotein, hormone, receptor, antigen, antibody, virus, pathogen, toxic substance, substrate, metabolite, transition state analog, cofactor, inhibitor, drug, dye, nutrient, growth factor, cell, tissue, any portion or fragment of any of the foregoing, *etc.*, without limitation. Virtually any chemical or biological effector may be a suitable target. Molecules of any size can serve as targets. A target can also be modified in certain ways to enhance the likelihood or strength of an interaction between the target and the nucleic acid. A target can also include any minor variation of a particular compound or molecule, such as, in the case of a protein, for example, minor variations in amino acid sequence, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component, which does not substantially alter the identity of the molecule. A "target molecule" or "target" is a set of copies of one type or species of molecule or multimolecular structure that is capable of binding to an aptamer. "Target molecules" or "targets" refer to more than one such set of molecules. Embodiments of the SELEX process in which the target is a peptide are described in U.S. Patent No. 6,376,190, entitled "Modified SELEX Processes Without Purified Protein." In the instant case, the targets include *C. difficile* toxin A, toxin B, binary toxin, binary toxin A chain, or binary toxin B chain.

Methods of Identifying or Producing an Aptamer to a *C. difficile* toxin

[0067] The present disclosure provides methods of identifying or producing a slow off-rate aptamer that binds to a toxin produced by *C. difficile* wherein said toxin is selected from toxin A, toxin B, binary toxin A chain, and binary toxin B chain, the method comprising:

(a) preparing a candidate mixture of nucleic acids, wherein the candidate mixture comprises modified nucleic acids in which one, several or all pyrimidines in at least one, or each, nucleic acid of the candidate mixture comprises a chemical modification at a C-5 position; (b) contacting the candidate mixture with a target which is said toxin produced by *C. difficile* and exposing the candidate mixture to a slow off-rate enrichment process, wherein nucleic acids having a slow rate of dissociation from the target relative to other nucleic acids in the candidate mixture bind the target, forming nucleic acid-target molecule complexes; (c) partitioning slow off-rate nucleic acids from the candidate mixture; and (d) amplifying the slow off-rate nucleic acids to yield a mixture of nucleic acids enriched in nucleic acid sequences that are capable of binding to the target molecule with a slow off-rate, whereby a slow off-rate aptamer to the target molecule may be identified. The methods of identifying or producing a slow off rate aptamer to a *C. difficile* toxin may comprise at least one pyrimidine wherein a chemical modification at a C-5 position is independently chosen from at least one of the modifications shown in Figure 9. The methods of identifying or producing a slow off rate aptamer to a *C. difficile* toxin may comprise at least one pyrimidine wherein a chemical modification at a C-5 position is independently chosen from benzylcarboxamide, naphthylmethylcarboxamide, tryptaminocarboxamide, tyrosylcarboxamide, 2-naphthylmethylcarboxamide and phenethyl-1-carboxamide. The methods of identifying or producing a slow off rate aptamer to a *C. difficile* toxin may comprise at least one additional chemical modification, wherein said at least one additional chemical modification is a chemical substitution at one or more positions independently selected from the group consisting of a ribose position, a deoxyribose position, a phosphate position, and a base position. Further, the methods of identifying or producing a slow off rate aptamer to a *C. difficile* toxin may comprise at least one additional chemical modification, wherein said at least one additional chemical modification is independently selected from the group consisting of a 2'-position sugar modification, a 2'-amino (2'-NH₂), a 2'-fluoro (2'-F), a 2'-O-methyl (2'-OMe) a modification at a cytosine exocyclic amine, a substitution of 5-bromouracil, a substitution of 5-bromodeoxyuridine, a substitution of 5-bromodeoxycytidine, a backbone modification, methylation, a 3' cap, and a 5' cap. The methods of identifying or producing a slow off rate aptamer to a *C. difficile* toxin may comprise a slow off-rate enrichment process selected from incubation of a candidate mixture with a competitor molecule, dilution of a candidate mixture, or dilution of a candidate mixture in the presence of a competitor molecule.

[0068] The present disclosure further provides methods of producing an aptamer having a slow rate of dissociation from a toxin produced by *C. difficile* wherein said toxin is selected from toxin A, toxin B, binary toxin A chain, and binary toxin B chain, said method comprising the step of preparing or synthesizing an aptamer based on a nucleic acid sequence identified by a process comprising the steps of: (a) preparing a candidate mixture of nucleic acids, wherein the candidate mixture comprises modified nucleic acids in which one, several or all pyrimidines in at least one, or each, nucleic acid of the candidate mixture comprises a chemical modification at a C-5 position; (b) contacting the candidate mixture with a target which is said toxin produced by *C. difficile* and exposing the candidate mixture to a slow off-rate enrichment process, wherein nucleic acids having a slow rate of dissociation from the target molecule relative to other nucleic acids in the candidate mixture bind the target molecule, forming nucleic acid-target molecule complexes; (c) partitioning slow off-rate nucleic acids from the candidate mixture; and (d) amplifying the slow off-rate nucleic acids to yield a mixture of nucleic acids enriched in nucleic acid sequences that are capable of binding to the target molecule with a slow off-rate, whereby a slow off-rate aptamer to the target molecule is identified. Such methods of producing an aptamer having a slow rate of dissociation from a *C. difficile* toxin may comprise at least one pyrimidine wherein a chemical modification at a C-5 position is independently chosen from at least one of the modifications shown in Figure 9. The methods of producing an aptamer having a slow rate of dissociation from a *C. difficile* toxin may comprise at least one pyrimidine wherein a chemical modification at a C-5 position is independently chosen from benzylcarboxamide, naphthylmethylcarboxamide, tryptaminocarboxamide, tyrosylcarboxamide, 2-naphthylmethylcarboxamide and phenethyl-1-carboxamide. The methods of producing an aptamer having a slow rate of dissociation from a *C. difficile* toxin may comprise at least one additional chemical modification, wherein said at least one additional chemical modification is a chemical substitution at one or more positions independently selected from the group consisting of a ribose position, a deoxyribose position, a phosphate position, and a base position. Further, the methods of producing an aptamer having a slow rate of dissociation from a *C. difficile* toxin may comprise at least one additional chemical modification, wherein said at least one additional chemical modification is independently selected from the group consisting of a 2'-position sugar modification, a 2'-amino (2'-NH₂), a 2'-fluoro (2'-F), a 2'-O-methyl (2'-OMe) a modification at a cytosine exocyclic amine, a substitution of 5-bromouracil, a substitution of 5-bromodeoxyuridine, a substitution of 5-bromodeoxycytidine, a backbone modification, methylation, a 3' cap, and a 5' cap. The methods of producing an

aptamer having a slow rate of dissociation from a *C. difficile* toxin may comprise a slow off-rate enrichment process selected from incubation of a candidate mixture with a competitor molecule, dilution of a candidate mixture, or dilution of a candidate mixture in the presence of a competitor molecule.

[0069] The present disclosure further provides methods for producing a nuclease resistant aptamer that binds to a toxin produced by *C. difficile* wherein said toxin is selected from toxin A, toxin B, binary toxin A chain, and binary toxin B chain, the method comprising preparing or synthesizing said nuclease resistant aptamer based on a nucleic acid sequence identified by a process comprising: (a) preparing a candidate mixture of modified nucleic acids, wherein the candidate mixture comprises modified nucleic acids in which at least one pyrimidine in at least one, or in each, nucleic acid of the candidate mixture comprises a chemical modification at a C-5 position; (b) contacting the candidate mixture with a target which is said toxin produced by *C. difficile*, wherein nucleic acids having an increased affinity to the target molecule relative to other nucleic acids in the candidate mixture bind the target molecule, forming nucleic acid-target molecule complexes; (c) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and (d) amplifying the increased affinity nucleic acids to yield a mixture of nucleic acids enriched in nucleic acid sequences that are capable of binding to the target molecule with increased affinity and that are nuclease resistant, whereby a nuclease resistant aptamer to the target molecule is identified. The methods of producing a nuclease resistant aptamer to a *C. difficile* toxin may comprise at least one pyrimidine wherein a chemical modification at a C-5 position is independently chosen from at least one of the modifications shown in Figure 9. The methods of producing a nuclease resistant aptamer to a *C. difficile* toxin may comprise at least one pyrimidine wherein a chemical modification at a C-5 position is independently chosen from benzylcarboxamide, naphthylmethylcarboxamide, tryptaminocarboxamide, tyrosylcarboxamide, 2-naphthylmethylcarboxamide and phenethyl-1-carboxamide. The methods of producing a nuclease resistant aptamer to a *C. difficile* toxin may comprise at least one additional chemical modification, wherein said at least one additional chemical modification is a chemical substitution at one or more positions independently selected from the group consisting of a ribose position, a deoxyribose position, a phosphate position, and a base position. Further, the methods of producing a nuclease resistant aptamer to a *C. difficile* toxin may comprise at least one additional chemical modification, wherein said at least one additional chemical modification is independently selected from the group consisting of a 2'-position sugar modification, a 2'-amino (2'-NH₂), a 2'-fluoro (2'-F), a 2'-O-methyl (2'-OMe) a

modification at a cytosine exocyclic amine, a substitution of 5-bromouracil, a substitution of 5-bromodeoxyuridine, a substitution of 5-bromodeoxycytidine, a backbone modification, methylation, a 3' cap, and a 5' cap.

Aptamers

[0070] The aptamers to *C. difficile* toxins of the instant disclosure were identified using the improved SELEX method for identifying aptamers having slow off-rates, as described above. The form of *C. difficile* toxins used in the selection process were recombinant toxins prepared by PCR amplification of desired gene fragments from *C. difficile* genomic DNA, as described in Example 1.

[0071] SELEX was performed using purified His-tag proteins obtained from over-expression of cloned *C. difficile* toxin gene fragments. Libraries of 40mer random sequences were used that contained one of six modified nucleotides, 5-tyrosylcarboxyamide-dU (TyrdU), 5-benzylcarboxyamide-dU (BndU), 5-naphthylmethylcarboxyamide-dU (NapdU), 5-tryptaminocarboxyamide-dU (TrpdU), 5-(2-naphthylmethyl)carboxyamide (2NapdU), or 5-phenethyl-1-carboxyamide (PEdU) instead of dU. Seven or eight rounds of selection were carried out, and a kinetic challenge with dextran sulfate was applied in rounds 2-8. The aptamer pools obtained after the last round of SELEX were tested for affinity to their targets in filter binding assays, and the K_d 's and plateaus were determined (Table 2). All pools with sufficient affinity (K_d of ~10 nM or below) were cloned and the sequences of at least 48 clones per pool were determined.

Aptamers to Toxin A

[0072] For toxin A, the aptamer pool 4943 (TrpdU) had excellent affinity with a K_d =2.42 nM. Pools 4936 (TyrdU) and 4939 (NapdU) were active, with K_d 's of 11.5 and 10.8 nM, respectively. Pools 5564 (2NapdU) and 5577 (2NapdU) had good affinity, with K_d 's of 4.63 and 6.40 nM. For toxin A, the aptamer pool 5570 (PEdU) was the best, with a K_d =1.61 nM. Aptamer clones with good affinities for toxin A were isolated from all of the pools with TrpdU, TyrdU, NapdU, 2NapdU, and PEdU modified nucleotides (Table 3); the sequences of *C. difficile* toxin A aptamers are listed on Table 4. In addition to identifying aptamer clones with good binding affinity to toxin A, consensus sequences between such aptamer clones were identified.

[0073] The leading aptamer clone from pool 5570 (PEdU) was 5570-54, with K_d =0.12 nM for the recombinant toxin A and K_d =6.91 nM for native toxin A.

[0074] Some aptamer clones demonstrated excellent affinity for both recombinant toxin A and native toxin A, *e.g.* aptamer clone 4943-51 (TrpdU) had $K_d=1.23$ nM for recombinant toxin A and $K_d=1.78$ nM for native toxin A; aptamer clone 5564-49 (2NapdU) had $K_d=1.13$ nM for recombinant toxin A and $K_d=1.78$ nM for native toxin A.

[0075] Some aptamer clones demonstrated relatively little drop off of affinity between recombinant toxin A and native toxin A, *e.g.* aptamer clone 5577-1 (2NapdU) had $K_d=1.59$ nM for recombinant toxin A and $K_d=4.97$ nM for native toxin A; aptamer clone 5577-3 (2NapdU) had $K_d=1.73$ nM for recombinant toxin A and $K_d=5.52$ nM for native toxin A; aptamer clone 4943-60 (TrpdU) had $K_d=2.65$ nM for recombinant toxin A and $K_d=4.57$ nM for native toxin A.

[0076] In addition to identifying aptamer clones with good binding affinity to toxin A, consensus sequences between such aptamer clones were identified.

Aptamers to Toxin B

[0077] The affinities of aptamers for toxin B were generally very good and correlated well between the 68.8 kDa amino-terminal catalytic domain of *C. difficile* toxin B fragment that had been used in SELEX and the 270 kDa native, full-length toxin B. Aptamer clones with sub-nanomolar K_d 's for toxin B were isolated from all of the pools with TyrDU, BndU, NapdU, TrpdU, 2NapdU and PEdU modified nucleotides (Table 5); the sequences of *C. difficile* toxin B aptamers are listed in Table 6, with the best clones shown in bold.

[0078] The highest-affinity aptamers were clones with NapdU or TrpdU modified nucleotides. Five aptamers demonstrated very low K_d 's of <0.1 nM: aptamer clone 4940-1 (NapdU) had $K_d=0.04$ nM for recombinant toxin B and $K_d=0.06$ for native toxin B; aptamer clone 4940-23 (NapdU) had $K_d=0.07$ nM for recombinant toxin B and $K_d=0.09$ nM for native toxin B; aptamer clone 4940-27 (NapdU) had $K_d=0.10$ nM for recombinant toxin B and $K_d=0.09$ nM for native toxin B; aptamer clone 4944-5 (TrpdU) had $K_d=0.08$ nM for recombinant toxin B and $K_d=0.09$ nM for native toxin B; and aptamer clone 4944-30 (TrpdU) had $K_d=0.06$ nM for recombinant toxin B and $K_d=0.08$ nM for native toxin B. Aptamer clones with good affinities for toxin B were isolated from all of the pools with TrpdU, TyrDU, NapdU, 2NapdU, BndU and PEdU modified nucleotides (Table 5); the sequences of *C. difficile* toxin B aptamers are listed on Table 6. In addition to identifying aptamer clones with good binding affinity to toxin B, consensus sequences between such aptamer clones were identified.

Aptamers to Binary Toxin (A chain)

[0079] SELEX with the recombinant binary toxin A chain (CdtA) yielded active aptamers with TrpdU, 2NapdU and PEDU modified nucleotides (Table 7). The sequences and common sequence patterns of CdtA aptamers are shown in Table 8. Cloning of pool 4758 (TrpdU) revealed clone 4758-6 which comprised 18% of the sequences in that pool and showed good affinity ($K_d=0.86$ nM) to CdtA binary toxin. Twenty sequences from 2NapdU pools were obtained, most of them with subnanomolar affinity, and several sequence patterns shared between these 2NapdU clones were identified. PEDU pools contained five active aptamers.

Aptamers to Binary Toxin (B chain)

[0080] SELEX with recombinant binary toxin B chain (CdtB) yielded active aptamers with 2NapdU modified nucleotides (Table 9). The sequences and common sequence patterns of CdtB aptamers are shown in Table 10. The most active clone was 5556-51 $K_d=1.68$ nM.

[0081] The present disclosure provides aptamers to toxins produced by *C. difficile* identified using the SELEX method and listed in Tables 4, 6, 8 and 10. Aptamers to toxins produced by *C. difficile* that are substantially homologous to any of the listed aptamers and that have a substantially similar ability to bind the respective toxin produced by *C. difficile* as that of an aptamer selected from the group of aptamers set forth in Tables 4, 6, 8 and 10 are also encompassed by the present disclosure. Further, aptamers to the respective toxin produced by *C. difficile* that have substantially the same structural form as the aptamers identified herein and that have a substantially similar ability to bind the respective toxin produced by *C. difficile* as that of an aptamer selected from the group of aptamers set forth in Tables 4, 6, 8 and 10 are also encompassed by the present disclosure.

[0082] In one aspect, the present disclosure provides an aptamer that specifically binds to a toxin produced by *C. difficile* and includes a primary nucleic acid sequence. In one embodiment, the primary nucleic acid sequence is selected from a sequence disclosed in Table 4, 6, 8, or 10. In other embodiments, the primary nucleic acid sequence is selected such that it is at least about 75% identical, at least about 80% identical, at least about 85% identical, at least about 90% identical, or at least about 95% identical to a primary nucleic acid sequence disclosed in Table 4, 6, 8 or 10.

[0083] The terms “sequence identity”, “percent sequence identity”, “percent identity”, “% identical”, “% identity”, and variations thereof, when used in the context of two or more nucleic acid sequences, are used interchangeably to refer to two or more sequences or

subsequences that are the same or have a specified percentage of nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using a sequence comparison algorithm or by visual inspection. For sequence comparisons, typically one sequence acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith and Waterman, *Adv. Appl. Math.*, 1981. 2:482, by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.*, 1970. 48:443, by the search for similarity method of Pearson and Lipman, *Proc. Nat'l. Acad. Sci. USA*, 1988. 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by visual inspection (see generally, Ausubel, F. M. *et al.*, *Current Protocols in Molecular Biology*, pub. by Greene Publishing Assoc. and Wiley-Interscience (1987)).

[0084] One example of an algorithm that is suitable for determining percent sequence identity is the algorithm used in the basic local alignment search tool (hereinafter "BLAST"), see, *e.g.* Altschul *et al.*, *J. Mol. Biol.*, 1990. 215:403-410 and Altschul *et al.*, *Nucleic Acids Res.*, 1997. 15:3389-3402. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (hereinafter "NCBI"). The default parameters used in determining sequence identity using the software available from NCBI, *e.g.*, BLASTN (for nucleotide sequences) are described in McGinnis *et al.*, *Nucleic Acids Res.*, 2004. 32:W20-W25.

[0085] As used herein, when describing the percent identity of a nucleic acid, such as an aptamer to a toxin produced by *C. difficile*, the sequence of which is at least, for example, about 95% identical to a reference nucleotide sequence, it is intended that the nucleic acid sequence is identical to the reference sequence except that the nucleic acid sequence may include up to five point mutations per each 100 nucleotides of the reference nucleic acid sequence. In other words, to obtain a desired nucleic acid sequence, the sequence of which is at least about 95% identical to a reference nucleic acid sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or some number of nucleotides up to 5% of the total number of nucleotides in the reference sequence

may be inserted into the reference sequence (referred to herein as an insertion). These mutations of the reference sequence to generate the desired sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence. The reference (query) sequence may be any one of the entire nucleotide sequences shown in Table 4, 6, 8 or 10, or any fragment of any of these sequences.

[0086] In one aspect, a consensus sequence selected from the group consisting of SEQ ID NOs: 5, 11, 15, 23, 28, 32, 47, 66, 75, 83, 90, 95, 98, 110, 124, 125, 134, 139, 145, 151, 152 or 157 can be modified to comprise at least one insertion, one deletion and/or one transposition. In one embodiment, the consensus sequence selected from the group consisting of SEQ ID NOs: 5, 11, 15, 23, 28, 32, 47, 66, 75, 83, 90, 95, 98, 110, 124, 125, 134, 139, 145, 151, 152 or 157 is modified such that at least one nucleotide is inserted into the consensus sequence. In another embodiment, a consensus sequence selected from the group consisting of SEQ ID NOs: 5, 11, 15, 23, 28, 32, 47, 66, 75, 83, 90, 95, 98, 110, 124, 125, 134, 139, 145, 151, 152 or 157 is modified such that at least one nucleotide is deleted from the consensus sequence. In another embodiment, a consensus sequence selected from the group consisting of SEQ ID NOs: 5, 11, 15, 23, 28, 32, 47, 66, 75, 83, 90, 95, 98, 110, 124, 125, 134, 139, 145, 151, 152 or 157 is modified such that at least one nucleotide is transposed from one location in the consensus sequence to another location in the consensus sequence. It is also recognized that a consensus sequence selected from the group consisting of SEQ ID NOs: 5, 11, 15, 23, 28, 32, 47, 66, 75, 83, 90, 95, 98, 110, 124, 125, 134, 139, 145, 151, 152 or 157 may be modified to comprise a combination of one or more insertions, deletions or transpositions while still maintaining adequate affinity to a toxin produced by *C. difficile* to have utility in a diagnostic assay.

[0087] In various embodiments, the aptamer to a toxin produced by *C. difficile* includes a sequence of contiguous nucleotides that are identical to a sequence of contiguous nucleotides included in any of the nucleotide sequences shown in Table 4, 6, 8 or 10. In various embodiments, the sequence of contiguous nucleotides in the aptamer to a toxin produced by *C. difficile* can include any number of nucleotides that are identical to the same number of nucleotides in a sequence of contiguous nucleotides included in any of the sequences shown in Table 4, 6, 8 or 10. In various embodiments, the sequence of contiguous nucleotides in the aptamer to a toxin produced by *C. difficile* includes a sequence of from about 4 to about 30 contiguous nucleotides that are identical to a sequence of from about 4 to

about 30 contiguous nucleotides included in any of the sequences shown in Table 4, 6, 8 or 10. In an exemplary embodiment, the aptamer to a toxin produced by *C. difficile* includes a sequence of 40 contiguous nucleotides that are identical to a sequence of 40 contiguous nucleotides included in any of the sequences shown in Table 4, 6, 8 or 10 which has 40 or more contiguous nucleotide. In an exemplary embodiment, the aptamer to a toxin produced by *C. difficile* includes a sequence of 30 contiguous nucleotides that are identical to a sequence of 30 contiguous nucleotides included in any of the sequences shown in Table 4, 6, 8 or 10. In another exemplary embodiment, the aptamer to a toxin produced by *C. difficile* includes a sequence of 20 contiguous nucleotides that are identical to a sequence of 20 contiguous nucleotides included in any the sequences shown in Table 4, 6, 8 or 10. In yet another exemplary embodiment, the aptamer to a toxin produced by *C. difficile* includes a sequence of 8 contiguous nucleotides that are identical to a sequence of 8 contiguous nucleotides included in any of the sequences shown in Table 4, 6, 8 or 10. In yet another exemplary embodiment, the aptamer to a toxin produced by *C. difficile* includes a sequence of 4 contiguous nucleotides that are identical to a sequence of 4 contiguous nucleotides included in any of the sequences shown in Table 4, 6, 8 or 10.

[0088] In one embodiment, the aptamer to toxin A is selected from the group consisting of SEQ ID NOS: 1-4, 6-10, 12-14, 16-22, 24-27, or 29-31. In yet another embodiment, the aptamer to toxin A is derived from a consensus sequence selected from any one of SEQ ID NOS: 5, 11, 15, 23 or 28. In one embodiment, the aptamer to toxin A is at least about 95% identical, at least about 90% identical, at least about 85% identical, at least about 80% identical, or at least about 75% identical to any of SEQ ID NOS: 1-31. In another embodiment, the aptamer to toxin A includes a sequence from any of SEQ ID NOS: 1-31 or fragments of any of these.

[0089] In one embodiment, the aptamer to toxin B is selected from the group consisting of SEQ ID NOS: 33-46, 48-65, 67-74, 76-82, 84-89, 91-94, 96-97, or 99-108. In yet another embodiment, the aptamer to toxin B is derived from a consensus sequence selected from any one of SEQ ID NOS: 32, 47, 66, 75, 83, 90, 95 and 98. In one embodiment, the aptamer to toxin B is at least about 95% identical, at least about 90% identical, at least about 85% identical, at least about 80% identical, or at least about 75% identical to any of SEQ ID NOS: 32-108. In another embodiment, the aptamer to toxin B includes a sequence from any of SEQ ID NOS: 32-108 or fragments of any of these.

[0090] In one embodiment, the aptamer to binary toxin A chain is selected from the group consisting of SEQ ID NOS: 109, 111-123, 126-133, 135-138, 140-144, or 146-150.

In yet another embodiment, the aptamer to binary toxin A chain is derived from a consensus sequence selected from any one of SEQ ID NOS: 110, 124-125, 134, 139, or 145. In one embodiment, the aptamer to binary toxin A chain is at least about 95% identical, at least about 90% identical, at least about 85% identical, at least about 80% identical, or at least about 75% identical to any of SEQ ID NOS: 109-150. In another embodiment, the aptamer to binary toxin A chain includes a sequence from any of SEQ ID NOS: 109-150 or fragments of any of these.

[0091] In one embodiment, the aptamer to binary toxin B chain is selected from the group consisting of SEQ ID NOS: 153-156, 158-162. In yet another embodiment, the aptamer to binary toxin B chain is derived from the consensus sequence of SEQ ID NOS: 151, 152 or 157. In one embodiment, the aptamer to binary toxin B chain is at least about 95% identical, at least about 90% identical, at least about 85% identical, at least about 80% identical, or at least about 75% identical to any of SEQ ID NOS: 151-162. In another embodiment, the aptamer to binary toxin B chain includes a sequence from any of SEQ ID NOS: 151-162 or fragments of any of these.

[0092] The aptamer to a toxin produced by *C. difficile* can contain any number of nucleotides in addition to the region which binds the *C. difficile* toxin. In various embodiments, the aptamer can include up to about 100 nucleotides, up to about 95 nucleotides, up to about 90 nucleotides, up to about 85 nucleotides, up to about 80 nucleotides, up to about 75 nucleotides, up to about 70 nucleotides, up to about 65 nucleotides, up to about 60 nucleotides, up to about 55 nucleotides, up to about 50 nucleotides, up to about 45 nucleotides, up to about 40 nucleotides, up to about 35 nucleotides, up to about 30 nucleotides, up to about 25 nucleotides, and up to about 20 nucleotides.

[0093] The aptamer to a toxin produced by *C. difficile* can be selected to have any suitable dissociation constant (K_d) for the respective toxin. In an exemplary embodiment, the aptamer to a toxin produced by *C. difficile* has a dissociation constant (K_d) for the respective toxin of about 10 nM or less. In another exemplary embodiment, the aptamer to a toxin produced by *C. difficile* has a dissociation constant (K_d) for the respective toxin of about 15 nM or less. In yet another exemplary embodiment, the aptamer to a toxin produced by *C. difficile* has a dissociation constant (K_d) for the respective toxin of about 20 nM or less. In yet another exemplary embodiment, the aptamer to a toxin produced by *C. difficile* has a dissociation constant (K_d) for the respective toxin of about 25 nM or less. A suitable dissociation constant can be determined with a binding assay using a multi-point titration and

fitting the equation $y = (\max - \min)(\text{Protein}) / (K_d + \text{Protein}) + \min$. It is to be understood that the determination of dissociation constants is highly dependent upon the conditions under which they are measured and thus these numbers may vary significantly with respect to factors such as equilibration time, *etc.* In other embodiments, the aptamer to a toxin produced by *C. difficile* is an aptamer with a K_d that is less than or equal to the K_d of an aptamer selected from the sequences disclosed in Tables 4, 6, 8 and 10.

[0094] Since the binary toxin is composed of an A chain and a B chain, more efficient binding may be achieved by using a dimeric or other multimeric form of aptamer. Thus, in another embodiment, the aptamer is a multimerization of any combination of the sequences of Table 8 and the sequences of Table 10. The same strategies could be applied to any aptamer sequence with the appropriate binding characteristics for binary toxin. In another embodiment, an aptamer for A chain could be used in conjunction with an aptamer for B chain to detect binary toxin in a sandwich assay.

Kits Comprising Aptamers to Toxins Produced by *C. difficile*

[0095] The present disclosure provides kits comprising any of the aptamers to toxins produced by *C. difficile* described herein. Such kits can comprise, for example, (1) at least one aptamer to a toxin produced by *C. difficile*; and (2) at least one diagnostic testing reagent, such as a solvent or solution. Additional kit components can optionally include, for example: (1) at least one container, vial or similar apparatus for holding and/or mixing the kit components; and (2) apparatus for collecting a sample to be tested for the presence of a *C. difficile* toxin.

Methods of Detecting Toxins Produced by *C. difficile*

[0096] The present disclosure provides methods of detecting the presence of a *C. difficile* toxin in a test sample comprising contacting said test sample with an aptamer that binds to a toxin produced by *C. difficile* wherein said toxin is selected from toxin A, toxin B, binary toxin A chain, and binary toxin B chain. Further disclosed are methods of detecting the presence of a *C. difficile* toxin comprising aptamers which comprise at least one pyrimidine modified at a C-5 position wherein said at least one pyrimidine modified at a C-5 position comprises a C-5 modification independently chosen from at least one of the modifications shown in Figure 9. Also disclosed are methods of detecting the presence of a *C. difficile* toxin comprising aptamers which comprise at least one pyrimidine modified at a

C-5 position wherein said at least one pyrimidine modified at a C-5 position comprises a C-5 modification independently chosen from benzylcarboxamide, naphthylmethylcarboxamide, tryptaminocarboxamide, tyrosylcarboxamide, 2-naphthylmethylcarboxamide and phenethyl-1-carboxamide. The methods of detecting a *C. difficile* toxin disclosed herein may comprise an aptamer which comprises at least one additional chemical modification, wherein said at least one additional chemical modification is a chemical substitution at one or more positions independently selected from the group consisting of a ribose position, a deoxyribose position, a phosphate position, and a base position. Further, the methods of detecting a *C. difficile* toxin disclosed herein may comprise at least one additional chemical modification is independently selected from the group consisting of a 2'-position sugar modification, a 2'-amino (2'-NH₂), a 2'-fluoro (2'-F), a 2'-O-methyl (2'-OMe) a modification at a cytosine exocyclic amine, a substitution of 5-bromouracil, a substitution of 5-bromodeoxyuridine, a substitution of 5-bromodeoxycytidine, a backbone modification, methylation, a 3' cap, and a 5' cap.

[0097] The present disclosure provides methods of detecting the presence of a *C. difficile* toxin in a test sample wherein the method of detecting is selected from a pull-down assay, dot blot assay, PCR assay or sandwich assay.

[0098] The present disclosure further provides methods of detecting the presence of *C. difficile* toxin A in a test sample comprising contacting said test sample with an aptamer comprising a sequence selected from the group consisting of SEQ ID NOS: 1-4, 6-10, 12-14, 16-22, 24-27, or 29-31 or a fragment thereof. Methods of detecting the presence of *C. difficile* toxin A in a test sample may comprise a pull-down assay, dot blot assay, PCR assay or sandwich assay. Sandwich assays used for detecting the presence of *C. difficile* toxin A may be selected from an aptamer-target-antibody assay, antibody-target-aptamer assay, and aptamer-target-aptamer assay. Methods of detecting the presence of *C. difficile* toxin A in a test sample may provide a quantitative measure of toxin A.

[0099] The present disclosure further provides methods of detecting the presence of *C. difficile* toxin B in a test sample comprising contacting said test sample with an aptamer comprising a sequence selected from the group consisting of SEQ ID NOS: 33-46, 48-65, 67-74, 76-82, 84-89, 91-94, 96-97, or 99-108 or a fragment thereof. Methods of detecting the presence of *C. difficile* toxin B in a test sample may comprise a pull-down assay, dot blot assay, PCR assay or sandwich assay. Sandwich assays used for detecting the presence of *C. difficile* toxin B may be selected from an aptamer-target-antibody assay, antibody-target-

aptamer assay, and aptamer-target-aptamer assay. Methods of detecting the presence of *C. difficile* toxin B in a test sample may provide a quantitative measure of toxin B.

[00100] The present disclosure further provides methods of detecting the presence of *C. difficile* binary toxin A chain in a test sample comprising contacting said test sample with an aptamer comprising a sequence selected from the group consisting of SEQ ID NOS: 109, 111-123, 126-133, 135-138, 140-144, or 146-150 or a fragment thereof. Methods of detecting the presence of *C. difficile* binary toxin A chain in a test sample may comprise a pull-down assay, dot blot assay, PCR assay or sandwich assay. Sandwich assays used for detecting the presence of *C. difficile* binary toxin A chain may be selected from an aptamer-target-antibody assay, antibody-target-aptamer assay, and aptamer-target-aptamer assay. Methods of detecting the presence of *C. difficile* binary toxin A chain in a test sample may provide a quantitative measure of binary toxin A chain.

[00101] The present disclosure further provides methods of detecting the presence of *C. difficile* binary toxin B chain in a test sample comprising contacting said test sample with an aptamer comprising a sequence selected from the group consisting of SEQ ID NOS: 153-156, 158-162 or a fragment thereof. Methods of detecting the presence of *C. difficile* binary toxin B chain in a test sample may comprise a pull-down assay, dot blot assay, PCR assay or sandwich assay. Sandwich assays used for detecting the presence of *C. difficile* binary toxin B chain may be selected from an aptamer-target-antibody assay, antibody-target-aptamer assay, and aptamer-target-aptamer assay. Methods of detecting the presence of *C. difficile* binary toxin B chain in a test sample may provide a quantitative measure of binary toxin B chain.

[00102] The present disclosure further provides methods of detecting the presence of a *C. difficile* toxin in a test sample comprising contacting said test sample with an aptamer comprising a consensus sequence selected from the group consisting of SEQ ID NOS: 5, 11, 15, 23, 28, 32, 47, 66, 75, 83, 90, 95, 98, 110, 124-125, 134, 139, 145, 151-152 and 157 or a fragment thereof. Methods of detecting the presence of a *C. difficile* toxin with an aptamer comprising such consensus sequence in a test sample may comprise a pull-down assay, dot blot assay, PCR assay or sandwich assay. Sandwich assays used for detecting the presence of a *C. difficile* toxin may be selected from an aptamer-target-antibody assay, antibody-target-aptamer assay, and aptamer-target-aptamer assay. Methods of detecting the presence of a *C. difficile* toxin in a test sample may provide a quantitative measure of such *C. difficile* toxin.

EXAMPLES

[00103] The following examples are provided for illustrative purposes only and are not intended to limit the scope of the invention as defined by the appended claims. All examples described herein should be considered in the context of standard techniques, which are well known and routine to those of skill in the art. Routine molecular biology techniques can be carried out as described in standard laboratory manuals, such as Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 3rd. ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (2001).

Example 1. SELEX with *C. difficile* Toxins A/B and Binary Toxin: Target Procurement

[00104] Target Procurement

[00105] Targets suitable for SELEX were prepared by PCR amplification of desired gene fragments from *C. difficile* genomic DNA, cloning in frame into vector pET-51b between the Strep-tag and His-tag sequences, and overexpression in *E. coli* Rosetta (Table 1). For toxin A, a recombinant polypeptide was obtained that consisted of the carboxy-terminal β -hairpin repeats 17-32. This toxin domain was chosen since a crystal structure has been published for a similar toxin A peptide of just five receptor-binding repeats (Ho, J.G., *et al.*, Proc. Natl. Acad. Sci. USA, 2005. 102(51): p. 18373-8). For toxin B, the amino-terminal catalytic domain was purified; a crystal structure of this domain has been obtained (Reinert, D.J., *et al.*, J. Mol. Biol., 2005. 351(5): p. 973-81). For binary toxin, the full-length CdtA subunit but without the predicted signal sequence was produced in recombinant form (crystal structure available (Sundriyal, A., *et al.*, J. Biol. Chem., 2009. 284(42): p. 28713-9)), and a CdtB fragment (amino acid residues 30-207) was produced that represents the so-called activation domain that has presumably been cleaved off the CdtB precursor protein (Perelle, S., *et al.*, Infect. Immun., 1997. 65(4): p. 1402-7).

Cloning and purification of all *C. difficile* toxins is shown in Figure 1A-1D, along with available crystal structures. The recombinant, double tagged proteins were purified via affinity chromatography on Ni-NTA agarose and streptactin agarose using standard protocols.

Table 1. PCR amplification of *C. difficile* toxin genes for cloning and overexpression

PCR amplification			Target protein		
Gene	PCR primers	Amplicon (nucleotides)	Protein (UniProt)	Domain (residues)	Size (kDa) (tagged)
<i>tcdA</i>	<i>tcdA-6 + tcdA-2</i>	6751-8127	Toxin A (TOXA_CLODI)	2250-2709	57.1
<i>tcdB</i>	<i>tcdB-5 + tcdB-7</i>	1-1638	Toxin B (TOXB_CLODI)	1-546	68.8
<i>cdtA</i>	<i>cdtA-1 + cdtA-2</i>	103-1387	Binary Toxin A (O32738_CLODI)	35-463	54.7
<i>cdtB</i>	<i>cdtB-15 + cdtB-16</i>	90-622	Binary Toxin B (O32739_CLODI)	30-207	26.0
SEQ ID NO:	Primer	Primer Sequence (restriction sites for cloning underlined)			
163	<i>tcdA-6</i>	GCGCA <u>AAGCTT</u> CTTCAAAATGGATATATTACTATTGAAAG			
164	<i>tcdA-2</i>	GCGCGAGCTCCATATATCCCAGGGGCTTTTAC			
165	<i>tcdB-5</i>	GCGCA <u>AAGCTT</u> ATGAGTTTAGTTAATAGAAAACAGTTAG			
166	<i>tcdB-7</i>	GCGCGAGCTCCATCTTCACCAAGAGAACCTTC			
167	<i>cdtA-1</i>	GCGCA <u>AAGCTT</u> CAAGACTTACAAAGCTATAGTG			
168	<i>cdtA-2</i>	GCGCGAGCTCCAGGTATCAATGTTGCATCAAC			
169	<i>cdtB-15</i>	GCGCA <u>AAGCTT</u> CAAAC TAGTACAAGTAATC			
170	<i>cdtB-16</i>	GCGCGAGCTCGGTCAAAGAAATTGTTATTTGGG			

Example 2. SELEX with *C. difficile* Toxins A/B and Binary Toxin: SELEX and Pool Affinities

SELEX with the purified His-tagged proteins was performed using Dynabeads® (Talon® or His-Tag) partitioning. Libraries of 40mer random sequences were used that contained one of the six modified nucleotides, 5-tyrosylcarboxyamide-dU (TyrdU), 5-benzylcarboxyamide-dU (BndU), 5-naphthylmethylcarboxyamide-dU (NapdU), 5-tryptaminocarboxyamide-dU (TrpdU), 5-(2-naphthylmethyl)carboxyamide (2NapdU), or 5-phenethyl-1-carboxyamide (PEdU) instead of dU. Seven or eight rounds of selection were carried out, and a kinetic challenge with dextrane sulfate was applied in rounds 2-8. The aptamer pools obtained after the last round of SELEX were tested for affinity to their targets in filter binding assays, and the K_d 's and plateaus were determined (Table 2).

[00106] The aptamer pools obtained after the last round of SELEX were tested for affinity to their targets in filter binding assays, and the K_d 's and plateaus were determined (Table 2). For toxin A, the aptamer pool 4943 (TrpdU) had good affinity with a K_d =2.42 nM. Pools 4936 (TyrdU) and 4939 (NapdU) were active, with K_d 's of 11.5 and 10.8 nM, respectively. Pools 5564 (2NapdU) and 5577 (2NapdU) were also active, with K_d 's of 4.63 and 6.40 nM. For toxin A, the aptamer pool 5570 (PEdU) was the best, with a K_d =1.61 nM.

[00107] For toxin B, aptamer pools TyrdU, BndU, NapdU, TrpdU, 2NapdU and PEdU demonstrated excellent affinities with K_d 's in the range of 0.11-1.11 nM.

[00108] Binary toxin A chain was also successful in selecting high affinity aptamers and yielded pool 4758 (TrpdU) with K_d =0.40 nM; pool 5567 (2NapdU) with K_d =0.19 nM; and pool 5574 (PEdU) with K_d =0.30 nM. Binary toxin B chain was used to select active pool 5556 (2NapdU), with K_d =7.58 nM.

[00109] All pools with sufficient affinity (K_d of ~10 nM or below) were cloned and the sequences of at least 48 clones per pool were determined.

Table 2. SELEX with *C. difficile* toxins

Target	Library	MOD	Sample ID	Pool	K_d (nM)	Plateau
Toxin A	40N29.14	TyrdU,	S247-R8-S1	4936	11.50	42%
		BndU	S247-R8-S9	n/a ¹	70.30	40%
		NapdU	S247-R8-S17	4939	10.80	48%
		TrpdU	S247-R8-S25	4943	2.42	46%
	40N32.24	2NapdU	S270-R8-S1	5564	4.63	42%
		PEdU	S270-R8-S9	5570	1.61	21%
		2NapdU	S270-R8-S29	5577	6.40	23%
Toxin B	40N29.14	TyrdU,	S247-R8-S3	4937	0.57	43%
		BndU	S247-R8-S11	4938	1.11	24%
		NapdU	S247-R8-S19	4940	0.20	44%
		TrpdU	S247-R8-S27	4944	0.24	43%
	40N32.24	2NapdU	S270-R8-S5	5566	0.11	18%
		PEdU	S270-R8-S13	5573	0.12	50%
		2NapdU	S270-R8-S30	5578	0.79	16%

Target	Library	MOD	Sample ID	Pool	K _d (nM)	Plateau
Binary Toxin	40N58.50	TrpdU	S239-R7-S39	4758	0.40	50%
A Chain	40N32.24	2NapdU	S261-R8-S24	5551	0.22	36%
		PEdU	S261-R8-S32	5555	3.73	33%
		2NapdU	S270-R8-S6	5567	0.19	45%
		PEdU	S270-R8-S14	5574	0.30	65%
		2NapdU	S270-R8-S31	5579	0.33	63%
Binary Toxin	40N32.24	2NapdU	S261-R8-S33	5556	7.58	32%
B Chain						

¹ pool not cloned due to insufficient affinity

Example 3. Toxin A Aptamer Clones

[00110] Representative clones from SELEX pools 4936 (TyrpU), 4939 (NapdU), 4943 (TrpdU), 5564 (2NapdU), 5577 (2NapdU), and 5570 (PEdU) were evaluated for affinity to toxin A in filter binding assays. Nearly all clones had good affinity to the recombinant 57.1 kDa toxin A fragment that had been used for selection, however, only some of the clones demonstrated affinity for the native, 308 kDa toxin A. This is not surprising, since some of the epitopes on the smaller, recombinant protein may not be accessible for aptamer binding of the full-length, native toxin.

[00111] The affinities (K_d's) and plateaus of the binding curves are shown in Table 3 and the corresponding sequences are listed in Table 4, with the best clones shown in bold.

[00112] Clones from Pool 4936 (TyrpU): Clone 4936-4 represented 20% of the sequences in the pool and was the most active with K_d=3.8 nM for the recombinant toxin A domain and K_d=14.5 nM for native toxin A.

[00113] Clones from Pool 4939 (NapdU): Three unrelated sequences were found five times each in this pool. Clone 4939-280 was the most active clone in this pool, with K_d=2.34 nM for the recombinant protein and K_d=15.3 nM for native toxin A.

[00114] Clones from Pool 4943 (TrpdU): Four clones with good (low nanomolar) affinity for native toxin A were found in this pool. The best clone, 4943-51 (K_d=1.78 nM for native toxin A), made up 19% of all sequences in this pool. The other three clones had the common motif NNANAnnCNNNCnnCnN (N = TrpdU; n = A, G, C, or TrpdU). Clones 4943-50, 4943-60, and 4943-49 which possessed only 32 nucleotides instead of the usual 40

within the random region (K_d 's, 5.60 nM, 4.57 nM, and 7.91 nM for native toxin A respectively) showed good affinity.

[00115] Clones from Pool 5564 (2NapdU): The most active clone of this pool was 5564-49 (K_d =1.78 nM for native toxin A). Several other clones were also present in this pool, sharing one of the three sequence patterns NAAAGNAGGN, GNNRNCMKNCNGA (SEQ ID NO: 15), or CGGGNCGNGACAGANCGCA, respectively (N = 2NapdU; R = A or G; M = A or C; K = G or N).

[00116] Clones from Pool 5570 (PEdU): The leading clone, 5570-54, with K_d =0.12 nM for the recombinant toxin A and K_d =6.91 nM for native toxin A, was the most abundant sequence in this pool.

[00117] Clones from Pool 5577 (2NapdU): The active clones shared all or part of the pattern NACCGAACGNNnNCAGNCNGA (N = 2NapdU; n = A, G, C or 2NapdU). These sequences had been selected in a special SELEX, where a competing toxin A aptamer (4943-51) was present in twofold excess over the target protein concentration.

Table 3. Affinities of Aptamer Clones from SELEX with *C. difficile* toxin A.

Toxin A (TOXA_CLODI) Aptamer			Affinity to recombinant protein (SELEX Target)		Affinity to mature protein (native target)	
Clone ID	Aptamer-ID	MOD	K_d (nM)	Plateau	K_d (nM)	Plateau
247-8-1-1	4936-1_0	TyrdU	17.0	33%		
247-8-1-3	4936-3_0	TyrdU	23.9	27%		
247-8-1-4	4936-4_0	TyrdU	3.80	37%	14.5	29%
247-8-1-9	4936-9_0	TyrdU	33.3	11%		
247-8-1-13	4936-13_0	TyrdU	25.7	45%		
247-8-1-18	4936-18_0	TyrdU	74.5	36%		
247-8-1-32	4936-32_0	TyrdU	19.9	40%		
247-8-17-195	4939-195_0	NapdU	23.3	9%	>100	2%
247-8-17-196	4939-196_0	NapdU	8.63	34%	61.6	37%
247-8-17-194	4939-194_0	NapdU	20.5	8%		
247-8-17-280	4939-280_0	NapdU	2.34	28%	15.3	30%
247-8-17-281	4939-281_0	NapdU	1.35	24%	>100	0%
247-8-17-209	4939-209_0	NapdU	11.4	27%	202	55%
247-8-17-202	4939-202_0	NapdU	13.2	34%	104	50%
247-8-17-246	4939-246_0	NapdU	12.5	34%	58.4	31%
247-8-25-49	4943-49_0	TrpdU	1.39	49%	7.91	43%
247-8-25-50	4943-50_0	TrpdU	2.81	36%	5.60	54%
247-8-25-51	4943-51_0	TrpdU	1.23	43%	1.78	54%

Toxin A (TOXA_CLODI)			Affinity to recombinant protein (SELEX Target)		Affinity to mature protein (native target)	
Aptamer						
Clone ID	Aptamer-ID	MOD	K _d (nM)	Plateau	K _d (nM)	Plateau
247-8-25-60	4943-60_0	TrpdU	2.65	46%	4.57	46%
247-8-25-71	4943-71_0	TrpdU	6.26	18%		
247-8-25-73	4943-73_0	TrpdU	0.82	13%		
247-8-25-91	4943-91_0	TrpdU	0.43	10%		
270-8-1-49	5564-49_0	2NapdU	1.13	30%	1.78	11%
270-8-1-50	5564-50_0	2NapdU	6.99	31%	>100	0%
270-8-1-52	5564-52_0	2NapdU	1.86	41%	19.30	20%
270-8-1-58	5564-58_0	2NapdU	6.29	43%	46.00	25%
270-8-1-65	5564-65_0	2NapdU	2.80	20%	>100	1%
270-8-1-84	5564-84_0	2NapdU	2.87	46%	>100	1%
270-8-1-89	5564-89_0	2NapdU	3.40	34%	11.90	17%
270-8-1-66	5564-66_0	2NapdU	7.17	41%	>100	2%
270-8-1-161	5564-161_0	2NapdU	2.71	67%	6.15	12%
270-8-9-50	5570-50_0	PEdU	4.87	23%	>100	0%
270-8-9-54	5570-54_0	PEdU	0.12	24%	6.91	8%
270-8-29-1	5577-1_0	2NapdU	1.59	48%	4.97	21%
270-8-29-3	5577-3_0	2NapdU	1.73	39%	5.52	22%
270-8-29-12	5577-12_0	2NapdU	6.48	53%	12.90	19%

Table 4. *C. difficile* Toxin A Aptamer Clones. Lead sequences have their “Aptamer ID No.” bolded and consensus sequences are underlined and appear under the label “Sequence Pattern”. Base capital “N” represents the modified nucleotide as indicated for the particular pool (TyrdU, NapdU, TrpdU, 2NapdU, or PEdU). Base lower case “n” in the consensus sequence indicates a variable base within a motif which is selected from A, G, C or the modified nucleotide for the particular pool (TyrdU, NapdU, TrpdU, 2NapdU, or PEdU). IUPAC nucleotide ambiguity codes were used: M = A or C; R = A or G; K = G or N (N representing the pool-specific modified dU), and a cut-off of 90% representation was used to define consensus.

Clones from Pool 4936 (TyrdU)				
SEQ ID NO.	Aptamer ID No.	Count	Pct	
1	4936-4	8	20%	AANNCCNANCCNANNANCACNNNCNNAGANNANNCNANG
Clones from Pool 4939 (NapdU)				
SEQ ID NO.	Aptamer ID No.	Count	Pct	
2	4939-196	5	3%	GNCANNNGGCCCCACGNCANNANCNGACNNCGACNAACGA
3	4939-280	5	3%	ACNNGNAGNAGCCCNNAANNGGGNNGCGNCGGCANNANGG
4	4939-246	5	3%	CNCGNNAGGGNNNANCCAANACCGNGGNGCCNNAACNAAA

Clones from Pool 4943 (TrpdU)				
SEQ ID NO.	Aptamer ID No.	Count	Pct	Sequence Pattern
				<u>NANAnnCnNNCnnCnN</u>
6	4943-50	6	7%	AGCANNAANANAGACNNNCGNCANCGNCCCCNNCGGN
7	4943-60	6	7%	ANCNCNCACANNANAGACNNNCCNANGGNCNCNGAGA
8	4943-49	4	5%	NGCCNAAACCNANAACNNNCCACGNGNACNN
9	4943-51	15	19%	GNANANCAACNCGNCANGGNAAGGNAANNCNAGCNNAGA
Clones from Pool 5564 (2NapdU)				
SEQ ID NO.	Aptamer ID No.	Count	Pct	Sequence Pattern
10	5564-49	6	7%	CCNGACGGGCGAGGNNCCAACNNACNNCCGNCACNANNGC
				<u>NAAAGNAGGN</u>
12	5564-52	15	17%	CGCANGNGNNCNGANACACNGGCCNAAAGNAGGNCNGAG
13	5564-57	1	1%	CCGNNCNAANACCAANGGANAAAGNAGGNAGGAGCNCGCA
14	5564-82	1	1%	NACCAGANAGNNANAANACGCGNGGCNAAAGNAGGNACNAA
				<u>GNNRNCMKNCNGA</u>
16	5564-89	2	2%	CNAAANGAAGNNGNCAGNCNGACGCCAGNGCGNACCGN
17	5564-54	1	1%	NGCGNACCCGCGNNGNCAGNCNGAGAGANCGGCNAAAGAA
18	5564-59	1	1%	CNAGCNGCANACCCACGNNNGNCAGNCNGAGCGCCACN
19	5564-164	1	1%	NGCCNGCCACCGNNGNCAGNCNGAGAGCNANCCAAACA
20	5564-167	1	1%	CANGCCNGCANACCACAGNNGNAGNCNGAGGGNNAGG
21	5564-67	4	5%	GGNACCNAACCCGAGNGNANACNNCNGACCGGACNNNN
22	5564-58	4	5%	CNGNNANCCGNCNGACACCNACCNAACCGGAGNAAGANCC
				<u>CGGGNCGNGACAGANCGCA</u>
23				<u>CGAGCGGGNCGNGACAGANCGCAGAGCGAAGGCNNACNAC</u>
24	5564-161	4	5%	
25	5564-152	2	2%	NCNGANGGGCCAACAAANGNCCGGGNCNGACAGANCGCA
Clones from Pool 5570 (PEdU)				
SEQ ID NO.	Aptamer ID No.	Count	Pct	Sequence Pattern
26	5570-54	10	23%	GCNGAGGCCGNGCNCANNAANNGAACNNAGAAANANCCNA
Clones from Pool 5577 (2NapdU)				
SEQ ID NO.	Aptamer ID No.	Count	Pct	Sequence Pattern
27	5577-1	4	9%	ACCGCNAAAGNAGGNCACGNNCNAANACCCNGGGAGGNGN
				<u>NACCGAACGNNnNCAGNCNGA</u>
28				
29	5577-3	3	7%	GCNGCCNACCGAACGNNNGNCAGNCNGAGCGANCGAACNNG
30	5577-12	3	7%	AGCCACGNACANACCGAACGNNANCAGNCNGACGCGNNGA
31	5577-16	1	2%	CCGNGCANACCCCGNNGNCAGNCNGACGCCAGACAC

Example 4. Toxin B Aptamer Clones

[00118] The affinities of aptamers for toxin B were generally very good and correlated well between the 68.8 kDa amino-terminal catalytic domain of *C. difficile* toxin B fragment that had been used in SELEX and the 270 kDa native, full-length toxin B. Aptamer clones with sub-nanomolar K_d 's for toxin B were isolated from all of the pools with TyrdU, BndU, NapdU, TrpdU, 2NapdU and PEdU modified nucleotides (Table 5); the sequences of *C. difficile* toxin B aptamers are listed in Table 6, with the best clones shown in bold.

[00119] Clones from Pool 4937 (TyrdU): Alignments of the TyrdU aptamers indicated the presence of two distinct sequence patterns (YNNSSNGAAW (SEQ ID NO: 32), YGAAWN (SEQ ID NO: 47)), (N = TyrdU; W = A or N; S = C or G; Y = C or N), as well as one orphan sequence.

[00120] Clones from Pool 4938 (BndU): This pool contained three unrelated sequences, all were present in multiple copies.

[00121] Clones from Pool 4940 (NapdU): The most abundant sequences, including four of the leading clones, contained all or part of the pattern KSGANNGGRW (SEQ ID NO: 66) (N = NapdU; R = A or G; W = A or N; S = C or G; K = G or N). In addition, three unrelated orphan sequences were present.

[00122] Clones from Pool 4944 (TrpdU): The majority of the sequences contained the pattern NnCYnnnNCNNnAARWNMAMSYN (SEQ ID NO: 75); two other sequences shared a different pattern, CNnGnANCNGGAAAN, (N = TrpdU; n = A, G, C or TrpdU; M = A or C; R = A or G; W = A or N; S = C or G; Y = C or N), and four orphan sequences were also present.

[00123] Clones from Pool 5566 (2NapdU): Pattern AnCnNNNAAGNGAACNNNnAnnnnnnnnnGnGNNnANA (N = 2NapdU; n = A, G, C or 2NapdU) was found in a couple of clones. This pool contained two additional unrelated sequences in multiple copies.

[00124] Clones from Pool 5573 (PEdU): Two patterns were identified, GCCNNNCNNGNNAACGNCNNNGANGGCAGCGNN and AGNNNGANCCC (N = PEdU). Six additional, unrelated clones were present.

[00125] Clones from Pool 5578 (NapdU): Two active clones were present in multiple copies. These sequences had been selected in a special SELEX, where a competing toxin B aptamer (4940-23) was present in twofold excess over the target protein concentration.

[00126] The highest-affinity aptamers were clones with NapdU or TrpdU modified nucleotides. Five aptamers demonstrated very low K_d 's of <0.1 nM for native toxin B.

Table 5. Affinities of Aptamer Clones from SELEX with *C. difficile* toxin B.

Toxin B (TOXB_CLODI) Aptamer			Affinity to recombinant protein (SELEX Target)		Affinity to mature protein (native target)	
Clone ID	Aptamer-ID	MOD	K_d (nM)	Plateau	K_d (nM)	Plateau
247-8-3-49	4937-49_0	TyrdU	0.19	23%	0.16	6%
247-8-3-50	4937-50_0	TyrdU	0.36	26%	0.27	4%

Toxin B (TOXB_CLODI) Aptamer			Affinity to recombinant protein (SELEX Target)		Affinity to mature protein (native target)	
Clone ID	Aptamer-ID	MOD	K _d (nM)	Plateau	K _d (nM)	Plateau
247-8-3-51	4937-51_0	TyrdU	0.60	31%	1.07	14%
247-8-3-55	4937-55_0	TyrdU	0.15	39%	0.15	15%
247-8-3-57	4937-57_0	TyrdU	0.15	35%	0.12	11%
247-8-3-66	4937-66_0	TyrdU	0.63	34%	0.59	14%
247-8-3-67	4937-67_0	TyrdU	0.11	21%	0.23	16%
247-8-3-81	4937-81_0	TyrdU	0.23	19%	0.28	13%
247-8-3-85	4937-85_0	TyrdU	0.27	32%	0.72	25%
247-8-3-94	4937-94_0	TyrdU	0.27	27%	0.36	12%
247-8-11-1	4938-1_0	BndU	0.84	23%	2.03	20%
247-8-11-6	4938-6_0	BndU	1.02	16%	1.52	17%
247-8-11-17	4938-17_0	BndU	0.36	32%	0.50	16%
247-8-19-1	4940-1_0	NapdU	0.04	24%	0.06	14%
247-8-19-3	4940-3_0	NapdU	1.04	27%	0.67	12%
247-8-19-6	4940-6_0	NapdU	0.05	15%	0.43	7%
247-8-19-8	4940-8_0	NapdU	0.19	33%	0.27	10%
247-8-19-19	4940-19_0	NapdU	0.11	23%	0.14	8%
247-8-19-23	4940-23_0	NapdU	0.07	37%	0.09	13%
247-8-19-27	4940-27_0	NapdU	0.10	34%	0.09	12%
247-8-27-1	4944-1_0	TrpdU	0.20	26%	0.13	10%
247-8-27-4	4944-4_0	TrpdU	0.10	29%	0.16	11%
247-8-27-5	4944-5_0	TrpdU	0.08	33%	0.09	10%
247-8-27-9	4944-9_0	TrpdU	0.18	36%	0.14	9%
247-8-27-11	4944-11_0	TrpdU	0.14	24%	0.22	6%
247-8-27-14	4944-14_0	TrpdU	0.24	20%	0.70	9%
247-8-27-20	4944-20_0	TrpdU	0.12	25%	0.18	7%
247-8-27-30	4944-30_0	TrpdU	0.06	28%	0.08	9%
247-8-27-34	4944-34_0	TrpdU	0.07	35%	0.41	10%
270-8-5-53	5566-53_0	2NapdU	0.02	5%	0.22	15%
270-8-5-74	5566-74_0	2NapdU	0.04	6%	0.25	21%
270-8-5-77	5566-77_0	2NapdU	NT	NT	0.23	6%
270-8-13-2	5573-2_0	PEdU	0.03	33%	0.11	15%
270-8-13-3	5573-3_0	PEdU	0.04	52%	0.25	12%
270-8-13-4	5573-4_0	PEdU	0.03	54%	0.08	12%
270-8-13-5	5573-5_0	PEdU	0.06	49%	0.55	14%
270-8-13-9	5573-9_0	PEdU	0.01	45%	0.14	13%
270-8-13-11	5573-11_0	PEdU	6.82	36%	2.70	8%
270-8-13-14	5573-14_0	PEdU	0.04	21%	0.33	9%
270-8-13-23	5573-23_0	PEdU	1.49	35%	0.55	9%
270-8-13-24	5573-24_0	PEdU	0.02	33%	0.11	10%
270-8-30-66	5578-66_0	2NapdU	0.98	14%	NT	NT
270-8-30-73	5578-73_0	2NapdU	1.62	18%	NT	NT

Table 6. *C. difficile* Toxin B Aptamer Clones. Lead sequences have their “Aptamer ID No.” bolded and consensus sequences are underlined and appear under the label “Sequence Pattern”. Base capital “N” represents the modified nucleotide as indicated for the particular pool (TyrdU, BndU, NapdU, TrpdU, 2NapdU, or PEdU). Base lower case “n” in the

consensus sequence indicates a variable base within a motif which is selected from A, G, C or the modified nucleotide for the particular pool (TyrdU, NapdU, TrpdU, 2NapdU, or PEdU). IUPAC nucleotide ambiguity codes were used: M = A or C; R = A or G; W = A or N; S = C or G; Y = C or N; K = G or N (N representing the pool-specific modified dU), and a cut-off of 90% representation was used to define consensus.

Clones from Pool 4937 (TyrdU)				Sequence Pattern	
SEQ ID NO.	Aptamer ID No.	Count	Pct		
				<u>YNNSSNGAAW</u>	
33	4937-50	6	8%	NANNNCCNNAAGC	NNGGNGAAAACCGC
34	4937-51	3	4%	NGGACCACNANCCN	CCCCACNNNNCGNGAAC
35	4937-53	2	3%	NGGANCACGNAN	CCCCACNNCCNGAA
36	4937-54	4	5%		ACNNGGNGAAANN
37	4937-56	2	3%	NNGGCACGAAGN	ANNGACNNNCGCC
38	4937-57	5	6%	NGGACACCNAN	NACAGNCNNCGG
39	4937-61	2	3%	GNGCNGCCC	ANCCNCCNNA
40	4937-63	2	3%	NCCANNCCAC	CGCGGCCACAG
41	4937-74	2	3%	NCCNANCC	CNCNNCGNGA
42	4937-78	2	3%	NCACAAAC	NANCCGNNCC
43	4937-81	4	5%	NACNANCA	CGCNCNNGG
44	4937-86	1	1%	AGCGGG	CNCNCANANCC
45	4937-87	2	3%	GNGACCA	ACNANGNN
46	4937-94	4	5%	CACACNAN	CCCNACCANG
				<u>YGAAWN</u>	
48	4937-85	1	1%	CNNACNGA	ANACNNGAG
49	4937-61	2	3%	GNGCNGCC	CANANCCN
50	4937-74	2	3%	NCCNANCC	CNCNNCGNGA
51	4937-87	2	3%	GNGACCA	ACNANGNN
52	4937-55	12	16%	AACCCNG	NANCCACAC
53	4937-52	2	3%	ACCANGN	ANNAACCC
54	4937-81	4	5%	NACNANCA	CGCNCNNGG
55	4937-49	4	5%	NCNANNC	CCCCGAGNC
56	4937-56	2	3%	NNGGCAC	GAAGNAN
57	4937-78	2	3%	NCACAA	CNANCCG
58	4937-73	2	3%	NCNACCG	GNNGC
59	4937-96	1	1%	GAGCNA	ANNGAAG
60	4937-53	2	3%	NGGANCA	CGNANNC
61	4937-66	4	5%	GGNCNAN	CGACAA
62	4937-67	1	1%	GGGCN	CAGNANCG
					(orphan)
Clones from Pool 4938 (BndU)				Sequence Pattern	
SEQ ID NO.	Aptamer ID No.	Count	Pct		
63	4938-1	5	10%	NNGGCGCC	GNNNGCGGN
64	4938-6	4	8%	AGNGCNAG	CGACNCCG
65	4938-17	3	6%	NANAAAG	ANCNNNG
Clones from Pool 4940 (NapdU)				Sequence Pattern	
SEQ ID NO.	Aptamer ID No.	Count	Pct		
				<u>KSGANNGGRW</u>	
67	4940-1	9	20%	NCCNNNG	CGAANC
68	4940-23	4	9%	AGGCNCA	ANGGNG
69	4940-19	2	5%	GCGCNC	AGNNGG
70	4940-31	1	2%	NNGGNC	CAAGN
71	4940-6	2	5%	CCNGCG	CNGANN
72	4940-3	1	2%	NCCANCG	GGACCA
73	4940-8	1	2%	NANCA	GACNCC
74	4940-27	1	2%	NANNNG	NCNCC
					(orphan)

Table 6. (cont'd)

Clones from Pool 4944 (TrpdU)				Sequence Pattern	
SEQ ID NO.	Aptamer ID No.	Count	Pct		
76	4944-1	2	4%	NnCYnnnnNCNNnAARWNMAMSYN	
77	4944-13	4	9%	CANGNCNCAANCNNNAAGANAACGNNGACCGCGAGNACCG	
78	4944-40	1	2%	NGCNGACAGACACANGNCCCCNCNCNNAAAGANAACGNNG	
79	4944-14	4	9%	ANCACCCNCNNNAAGANAACGNNCCGGACCGCGGANA	
80	4944-23	1	2%	NCNGCNANGNCNNNAAGANCAACCNAAGAGANGCANGANA	
81	4944-11	5	11%	GNNGGAGCGNNGGCCNNACCCNACNGGANCNNGAACCCNC	
82	4944-34	10	22%	GNCGANCCNCAAANNANGNACGANNGACCNAAACANGGNAC	
				NGGNNAGCACNNCANNCCANGGACCANANAACNCNAGNNNA	
				Sequence Pattern	
83				CnNnGnANCNGGAAAN	
84	4944-17	2	4%	ACNNNNCGCACCCGGCCNNANGCCNNGCANCNGGAAANGG	
85	4944-4	1	2%	NNNNCGGAAGCCGCNNANCCGCCACNCGGANCNGGAAAN	
86	4944-5	1	2%	NGNCGAGNAAACGGCGACCGNNNCCCNGNAGNAACNACA	(orphan)
87	4944-9	1	2%	NGNNNCAACNANGAANCCAGCNACCGNGCAACCAANGNA	(orphan)
88	4944-30	1	2%	AGNGNAANAGNAACCCNAGACNANGCCCNNGGNGNACGG	(orphan)
89	4944-20	1	2%	NGCGGCNGAAGAAGCANGCAAGNCANCGGNCNGNNGNAN	(orphan)
Clones from Pool 5566 (2NapdU)				Sequence Pattern	
SEQ ID NO.	Aptamer ID No.	Count	Pct		
90				AnCnNNNAAGNGAACNNNnAnnnnnnnnnGnGNNnANA	
91	5566-53	3	7%	CCAGCANNNAAGNGAACNNNAAGGAAGGGAGGAGNNCANA	
92	5566-90	1	2%	AGACCGNNNAAGNGAACNNNCAACGGGANGCGNNGNAANA	
93	5566-74	4	9%	AGNGGCGNNAANGCANNNAACGAGCACNGAGGCGNNAANA	
94	5566-77	3	7%	CNNNNNACCGCNGCANGACNNNAGCGGCAGNCGNGNGNG	
Clones from Pool 5573 (PEdU)				Sequence Pattern	
SEQ ID NO.	Aptamer ID No.	Count	Pct		
95				GCCNNNCNNGNNAACGNCNNGANGGCAGCGNN	
96	5573-23	2	5%	GAACGN	GCCNNNCNNGNNAACGNCNNGANGGCAGCGNN
97	5573-25	1	2%	AACNCG	GCCNNNCNNGNNAACGNCNNGANGGCAGCGNN
				Sequence Pattern	
98				AGNNNGANCCC	
99	5573-3	7	16%	NACGGCANNNGGNGGCAAGNNNGANCCNCCGAGCCNAN	
100	5573-5	2	5%	CGANCACANCGCACANNAGNCAGNNNGANCCANNAANCA	
101	5573-2	14	32%	NCAGGNNAACCCAGNGNAGGAAAACGNGNACGNNCCGAN	
102	5573-4	8	18%	AANNNANGNGANCAANNAGGACGACCGCCANNNGACNNCG	
103	5573-14	3	7%	GGNGGNGGAAANNNGGCAAGNGNANGNGGNNACGCCGNAN	
104	5573-24	2	5%	NGCGNCNGANCCGNAAAACANNNCAAGCNACCAANGNNNA	
105	5573-9	2	5%	CGCCGNNNCCGNCGGCCACAANNNAAGNACAANNNGGAN	
106	5573-11	2	5%	NGNCCGCCGACCANNNNCNGNANAGCCNCNNGNAANNAGN	
Clones from Pool 5578 (2NapdU)					
SEQ ID NO.	Aptamer ID No.	Count	Pct		
107	5578-66	3	7%	GAAAGCNNCGNACGNAGNNGNGAGAGGNCNCNGCCNCNN	
108	5578-73	4	9%	ANNAAGCNNNGGCGNGGAGCNGACAGCCAGGGANNCCGA	

Example 5. Binary Toxin (A Chain) Aptamer Clones

[00127] SELEX with the recombinant binary toxin A chain (CdtA) yielded active aptamers with TrpdU, 2NapdU, and PEdU modified nucleotides (Table 7).

[00128] The sequences and common sequence patterns of CdtA aptamers are shown in Table 8.

[00129] Cloning of pool 4758 (TrpdU) revealed clone 4758-6 which comprised 18% of the sequences in that pool and showed good affinity ($K_d=0.86$ nM) to CdtA binary toxin.

[00130] Twenty sequences from 2NapdU pools were obtained, most of them with subnanomolar affinity, and several sequence patterns shared between these 2NapdU clones were identified: GAANANnNCCGNGAnGNAANGnnANANNs (SEQ ID NO: 110), ANNRCNnCCNGGCS (SEQ ID NO: 124), WAWNNANNA (SEQ ID NO: 125), and GGANNGCAGGNNCMC (SEQ ID NO: 134) (N = PEdu; n = A, G, C or PEdu; M = A or C; W = A or N; S = C or G; R = A or G).

[00131] PEdu pools contained five active aptamers; they were present in multiple copies, and three of the sequences shared the pattern NAAAWGNNN (SEQ ID NO: 145) (N = PEdu; W = A or N).

Table 7. Affinities of Aptamer Clones from SELEX with *C. difficile* binary toxin A chain.

Binary Toxin A Chain (CdtA_CLODI) Aptamer			Affinity to recombinant CdtA protein (SELEX Target)	
Clone ID	Seq-ID	MOD	K_d (nM)	Plateau
239-7-39-6	4758-6_0	TrpdU	0.86	18%
261-8-24-49	5551-49_0	2NapdU	0.31	9%
261-8-24-50	5551-50_0	2NapdU	0.09	30%
261-8-24-52	5551-52_0	2NapdU	0.14	26%
261-8-24-60	5551-60_0	2NapdU	5.79	60%
261-8-24-81	5551-81_0	2NapdU	0.54	32%
261-8-32-6	5555-6_0	PEdu	0.62	21%
261-8-32-15	5555-15_0	PEdu	1.72	18%
261-8-32-39	5555-39_0	PEdu	0.34	12%
270-8-6-1	5567-1_0	2NapdU	0.15	24%
270-8-6-2	5567-2_0	2NapdU	6.81	25%
270-8-6-10	5567-10_0	2NapdU	0.03	17%
270-8-6-13	5567-13_0	2NapdU	0.03	22%
270-8-6-18	5567-18_0	2NapdU	0.16	10%
270-8-6-34	5567-34_0	2NapdU	0.09	14%
270-8-6-46	5567-46_0	2NapdU	0.05	12%
270-8-14-49	5574-49_0	PEdu	0.16	57%
270-8-14-56	5574-56_0	PEdu	2.92	55%
270-8-31-5	5579-5_0	2NapdU	2.24	13%
270-8-31-7	5579-7_0	2NapdU	2.44	44%
270-8-31-8	5579-8_0	2NapdU	0.53	41%

Binary Toxin A Chain (CdtA_CLODI) Aptamer			Affinity to recombinant CdtA protein (SELEX Target)	
Clone ID	Seq-ID	MOD	K _d (nM)	Plateau
270-8-31-10	5579-10_0	2NapdU	0.15	36%
270-8-31-11	5579-11_0	2NapdU	0.07	33%
270-8-31-12	5579-12_0	2NapdU	0.97	53%
270-8-31-21	5579-21_0	2NapdU	0.24	35%

Table 8. *C. difficile* Binary Toxin A Chain Aptamer Clones. Lead sequences have their “Aptamer ID No.” bolded and consensus sequences are underlined and appear under the label “Sequence Pattern”. Base capital “N” represents the modified nucleotide as indicated for the particular pool (TrpdU, 2NapdU, or PEdU). Base lower case “n” in the consensus sequence indicates a variable base within a motif which is selected from A, G, C or the modified nucleotide for the particular pool (TrpdU, 2NapdU, or PEdU). IUPAC nucleotide ambiguity codes were used: M = A or C; R = A or G; W = A or N; S = C or G; (N representing the pool-specific modified dU), and a cut-off of 90% representation was used to define consensus.

Clones from Pool 4758 (TrpdU)				
SEQ ID NO.	Aptamer ID No.	Count	Pct	
109	4758-6	8	18%	GAAGACTTTTAATTCTGACATGGTGTCCAATGGCGCGCGAG
Clones from Pools 5551, 5567, 5579 (2NapdU)				
SEQ ID NO.	Aptamer ID No.	Count	Pct	Sequence Pattern
				<u>GAANANnNCCGNGAnGNAANGnnANANNS</u>
111	5567-30	1	1%	GAANCNGNCCGNGACGNAANG AANANNC
112	5579-21	5	4%	GAANCNGNCCGNGACGNAANGCCANANNCGGAGGGGAN
113	5579-28	1	1%	GAANCNGNCCGNGAAGNAANGCCANANNCGCANG
114	5567-1	11	9%	GAANANGNCCGNGAAGNAANGCCGANANNC
115	5567-41	1	1%	GAANANGNCCGNGAAGNAANGCCANANNCGNCCACGNGGG
116	5551-77	1	1%	CGGGNCACCGCANNCCGNGACGNAANGACANANNCGGN
117	5551-60	2	2%	AACCCCGCGGCAANNANCCGNGAAGNAANG AANANNC
118	5579-48	1	1%	ACAGAGGCANNCCGNGANGNAANGCAANANNCGCCGN
119	5567-2	4	3%	NGCAACNANCCGNGANGNAANGCAANANNCACANGNGC
120	5567-26	1	1%	GGACNACNCCGNGANGNAANGCGAAANNCCAGANGNA
121	5551-81	4	3%	NCGAANGANAACANGNAACNCCGNGANNACANCGAANAGN
122	5579-7	7	6%	CNAAGCNCCGAGGCNNACNCCGNGANGCGCANGGNNNAACC
123	5579-12	4	9%	NCGAGCAACGAGNAACNCCGNGANNACAANCGANAGANGA
Sequence Pattern				
				<u>ANNRGCN CCNGGCS</u> <u>WAWNNANNA</u>
126	5579-11	10	11%	NNGCNACCCAANNAGCN CCNGGCGG GNNAANNANNAGACA
127	5551-64	1	1%	CANCCAANNAGCNCCNGGCGA NGNAANNANNANGGCACN
128	5551-78	1	1%	NCGNANACCGAANNAGCNGCCNGGCGA CCNAANNANNACA
129	5551-50	2	2%	CCNGCCNCAANNAGCN CCNGGCGCCCNNAANNANNAACN
130	5579-45	1	1%	GACCNANCANNGGCN CCNGGCG GNNAANNANNACCACC
131	5567-13	3	2%	NAGAGAAANNGGCNGCGNGGCCACCCNAANNANNAGAGCA
132	5567-34	3	2%	CNCAAGGCNANNGGCN GCNGGCAGA NNAANNANNAAGNC
133	5567-10	8	6%	ANNGGCN CCNGGCGGANAANNANNANACCAGNGAGNGAA

Clones from Pools 5551, 5567, 5579 (2NapdU)				
SEQ ID NO.	Aptamer ID No.	Count	Pct	Sequence Pattern
				<u>GGANNGCAGGNNCMC</u>
135	5579-10	2	2%	NAGNCACGGNGAACNGGANNGCAGGNNCCCCCNGGCNA
136	5551-52	13	15%	GGNCAGCNGGANNGCAGGNNCCCCCNGANAGGACGGNNN
137	5551-59	2	2%	GNAGNCGGANNGCAGGNNCCCACCAAACACNNNGGNAGA
138	5567-46	2	2%	CNGGAGACNGGNCAGAACAGCCGGGANNGCAGGNNACGG
				Sequence Pattern
139				<u>GAANNNGNNCCG</u>
140	5579-5	3	3%	GNNGAANNNGNNCCGCCGCCNNNCNGNCCGGGGNNGCNGN
141	5579-34	1	1%	NGNCAGAAANNNGNNCCGANAGGGNNGCNGCCACNGANAN
142	5551-49	5	6%	GCCNNNNGGCGAGGNGAGNNNNCCAGNCNGANGAAGCNN
143	5579-8	5	6%	CGGAGCCCGAAGGNNAAAGCGGNNCACCANNANACGANACG
144	5567-18	2	2%	CNCCGNANNGCGNCCNGGGCAGNNAANCNANNAGAAGCCA
Clones from Pools 5555, 5574 (PEdU)				
SEQ ID NO.	Aptamer ID No.	Count	Pct	Sequence Pattern
				<u>NAAAWGNNN</u>
146	5555-6	9	14%	GNGNGNCAGCGCANNANACGCGNAANNAAANGNNNAGAGA
147	5555-15	8	12%	GCGNGNCNGNANNAAGNNNGCGGAGGGGNNCCCCGGNAC
148	5574-49	13	20%	NNNCGAGAANAANGNNNGANACANNACNNANAANGGN
149	5555-39	3	5%	AGCCGGNGNGNGNANNAACNNNNCGGCNNNCCNCCGCA
150	5574-56	11	17%	CNNGNGNAAACCGNGCGNNAGNANNGGAGANAGCNGACAN

Example 6. Binary Toxin (B Chain) Aptamer Clones

[00132] SELEX with recombinant binary toxin B chain (CdtB) yielded active aptamers with 2NapdU modified nucleotides (Table 9). The sequences and common sequence patterns of CdtB aptamers are shown in Table 10. The most active clone, 5556-51, contained all of these patterns, NNARASCS (SEQ ID NO: 151), NNNGGCNNNACG (SEQ ID NO: 152), and AGCCNNNGRCNN (SEQ ID NO: 157) (N = 2NapdU; R = A or G; S = C or G), some of which were present in other sequences. Three additional clones had unrelated sequences.

Table 9. Affinities of Aptamer Clones from SELEX with *C. difficile* binary toxin B chain.

Binary Toxin B Chain (CdtA_CLODI) Aptamer			Affinity to recombinant CdtB protein (SELEX Target)	
Clone ID	Seq-ID	MOD	K _d (nM)	Plateau
261-8-33-51	5556-51_0	2NapdU	1.68	38%
261-8-33-57	5556-57_0	2NapdU	11.60	37%
261-8-33-60	5556-60_0	2NapdU	12.30	48%
261-8-33-67	5556-67_0	2NapdU	2.16	45%
261-8-33-83	5556-83_0	2NapdU	7.62	33%

Table 10. *C. difficile* Binary Toxin B Chain Aptamer Clones. Lead sequences have their "Aptamer ID No." bolded and consensus sequences are underlined and appear under the label

“Sequence Pattern”. Base capital “N” represents 2NapdU. IUPAC nucleotide ambiguity codes were used: R = A or G; S = C or G; and a cut-off of 90% representation was used to define consensus.

Clones from Pool 5556 (2NapdU)				
SEQ ID NO.	Aptamer ID No.	Count	Pct	Sequence Pattern
				<u>NNARASCS</u>
153	5556-51	5	15%	<u>NNNGGCNNNACG</u>
154	5556-87	1	3%	AAGNNAAACCGAGACGCGGCCGGAAGCCNNNGGCNNNACG
155	5556-94	1	3%	GNNAAACCCCGGGGGGCCAAGCGCANNNGGCNNNACGAA
156	5556-69	1	3%	CAACGNNAAANNAGAGCCNNNGNCCNAACAAANNACGCANG
				AANCGGAGCCCNANAAACCCNNAACCCNNNANACCAANN
				<u>Sequence Pattern</u>
				<u>AGCCNNNGRCNN</u>
158	5556-51	5	15%	AAGNNAAACCGAGACGCGGCCGGAAGCCNNNGGCNNNACG
159	5556-60	1	3%	GNNAANNAGAGCCNNNGACNNGAACAGGNNACGCANNAC
160	5556-57	4	12%	CNNGACNGNACCCNNNNCGACACAGAACAGCAAGACCNNC
161	5556-67	2	6%	GGACCGANGAANCNAGCNGNNAANAGCGNNGAGCNANCC
162	5556-83	2	6%	CACNNAGCAACCGACACAAGNNGNCCGNNANCCGNNANA

Example 7. Use of Aptamers for Toxin A/B and Binary Toxin as Diagnostic Reagents: Pull-down Assays

[00133] Aptamers were used to specifically pull-down their respective targets, toxin A, toxin B, or binary toxin, from spiked samples, thus affinity purification of these proteins is achieved, as needed.

[00134] In this assay, biotinylated aptamers were immobilized on MyOne streptavidin beads and mixed with their targets for 1 h to allow binding. The beads were then washed, and captured target was tagged with NHS-Alexa-647. After extensive washing, the captured targets were eluted with 20 mM NaOH, neutralized, analyzed by SDS-PAGE, and proteins were visualized using the cy5 channel.

[00135] Figure 2 illustrates the following pull down assay results: the toxin A aptamer pulled down toxin A (recombinant or native) with good specificity over the control proteins toxin B or BSA; the toxin B aptamer pulled down toxin B (recombinant or native), but not toxin A; the aptamer for binary toxin A subunit pulled down CdtA, but not CdtB; and no proteins were present in the pull-down fraction when a random aptamer was used.

Example 8. Use of Aptamers for Toxin A/B and Binary Toxin as Diagnostic Reagents: Dot Blot Assays

[00136] Aptamers were used for the detection of toxins in dot blot assays, *e.g.* using biotinylated aptamers and a signal amplifying enzyme such as alkaline phosphatase (AP) or horseradish peroxidase (HRP).

[00137] Toxin detection was demonstrated in simple dot blots of toxin A and B (Figure 3A and 3B). In this assay, 1 μ L of serially diluted target was spotted and air-dried onto nitrocellulose membranes. After blocking, individual biotinylated aptamers were added (1 nM), followed by SA-AP conjugate (200 ng/mL) and developed with NBT/BCIP substrate. Detection limits were \sim 1 fmole (300 pg) for either toxin A and B with aptamers. Monoclonal antibodies used for the same assay and at the same concentration (1 nM) were not as good, but were able to detect 2 fmoles (600 pg) of toxin A and 20 fMoles (6 ng) of toxin B.

Example 9. Use of Aptamers for Toxin A/B and Binary Toxin as Diagnostic Reagents: Catch 1&2 Assay with qPCR Detection

[00138] A Catch 1 – Catch 2 assay is illustrated at Figure 8. Toxins A and B were detected quantitatively via qPCR of catch 2 eluates (Figure 4A-B). In catch 1, spiked samples containing toxin (0.001 - 10 nM) and excess BSA (1.5 μ M) were equilibrated with aptamers comprising a photocleavable biotin-D spacer (10 nM) and captured on streptavidin agarose beads (relatively clear beads which allow light to pass through). After wash removal of free protein, the toxin (target) of the catch 1 samples was tagged with NHS-biotin, aptamers were photocleaved off the streptavidin agarose beads and complexes were captured on MyOne streptavidin beads (catch 2). Since after photocleavage the photocleavable biotin-D spacer could no longer mediate binding to the MyOne streptavidin beads, in catch 2 the binding to the MyOne streptavidin beads was mediated by the NHS-biotin on the toxin (target). After wash removal of free aptamers, the target-bound aptamers were eluted at high pH and used for qPCR; standard curves for the aptamers were run side-by-side. Quantitative results for toxin A and B were obtained at concentrations of >0.1 nM only. There was non-specific background at the lower target concentrations, and pPCR curves reached a plateau after less than 12 cycles. This was most likely due to considerable carry-over of free aptamers during catch 1 and 2, and due to a rather high (10 nM) aptamer concentration.

Example 10. Use of Aptamers for Toxin A/B and Binary Toxin as Diagnostic Reagents:

Aptamer-Target-Antibody Sandwich Assay

[00139] Toxins were detected in streptavidin plate sandwich assay, using biotinylated aptamers and monoclonal antibodies (Figure 5A-B).

[00140] Toxin detection was demonstrated for toxins A and B. Biotinylated aptamers (1 pmole/well) were immobilized on a streptavidin plate, and target proteins were added (1 nM, 100 pM, 10 pM, no protein), corresponding to 100 fMoles (30 ng), 10 fMoles (3 ng), 1 fMole (300 pg), and no protein control. The plates were washed and monoclonal antibodies to Toxin A or B (2 nM each) were added and allowed to bind for 1 h with shaking at RT. Complexes were detected with goat-anti-mouse HRP conjugate and TMB as HRP substrate (Figure 5A and 5B).

[00141] The sandwich assay produced robust results with target concentration-dependent signals and low background. All four toxin A aptamers were able to detect 10 pM toxin A (1 fMole, 300 pg) regardless of their K_d and did not cross-react with toxin B. Aptamers for toxin B, in spite of better K_d 's, had poor sensitivity in this assay, possibly due to overlapping binding sites of the aptamer and antibody. One of the toxin B aptamers (4937-49) did cross-react with toxin A, which is consistent with data from pull-down experiments.

Example 11. Use of Aptamers for Toxin A/B and Binary Toxin as Diagnostic Reagents:

Antibody-Target-Aptamer Sandwich Assay

[00142] Aptamers were used for the detection of toxins in antibody-aptamer sandwich assays, *e.g.* in a dipstick-type assay format, using biotinylated aptamers and monoclonal antibodies (Figure 6).

[00143] Monoclonal antibodies to toxin A and B were spotted separately on a small (0.6 cm x 2.5 cm) strip of nitrocellulose and air-dried. After blocking with SB18T+1% BSA, the strips were placed upright in a deep well plate and 0.6 mL samples containing toxin A and/or toxin B or controls were added. After shaking for 1 h at RT, the strips were washed 3x. Biotinylated aptamers (1 nM) were added (0.6 mL) and allowed to bind for 1 h at RT. The strips were washed again and developed with 1 nM streptavidin-alkaline phosphatase conjugate (1 h) and NBT/BCIP (Figure 6).

[00144] The toxin A aptamer 4943-51 detected toxin A accurately in all samples containing only toxin A or both toxins A and B (1000 fMoles or 100 fMoles), and did not

cross-react with toxin B. Similarly, toxin B aptamers were able to detect toxin B. Background was high especially in the toxin B spot, even when no protein was present in the sample and when random control aptamers were used, suggesting non-specific binding of the streptavidin-alkaline phosphatase conjugate to the toxin B monoclonal antibody.

Example 12. Use of Aptamers for Toxin A/B and Binary Toxin as Diagnostic Reagents:
Aptamer-Target-Aptamer Sandwich Assay

[00145] Aptamers were used for the detection of toxins in aptamer-aptamer sandwich assays, *e.g.* in a bead-based assay format using a pair of aptamers, without the need of any antibodies (Figure 7).

[00146] Capture beads were prepared by attaching the first, biotinylated aptamer (clone 4758-6) to MyOne™ streptavidin beads. A sample containing target protein (CdtA) in serial dilutions was added, and CdtA was allowed to bind to these capture beads. After washing the beads, a second, radiolabeled CdtA aptamer clone was added for equilibrium binding. Then the mixture was filtered through MAHVN plates (0.22 μ), using the capture beads themselves for partitioning. This method will detect only labeled aptamers that are bound in sandwich-type format to the streptavidin-aptamer 4758-6-CdtA complex.

[00147] The results of the capture bead assay for CdtA aptamer pairs are shown in Figure 7. Suitable pairs of aptamers produced target concentration-dependent signals. No signals were produced if the same aptamer (binding to the same epitope) or a control aptamer (binding to a different target) was used.

[00148] This assay can be used to screen aptamers for binding to distinct epitopes on the target, that is, each aptamer binding to a separate site, as opposed to competing for the same epitope.

WHAT IS CLAIMED IS:

1. An aptamer that binds to a toxin produced by *C. difficile* wherein said toxin is selected from toxin A, toxin B, binary toxin A chain, and binary toxin B chain.
2. The aptamer of claim 1, comprising at least one pyrimidine modified at a C-5 position.
3. The aptamer of claim 2, wherein said at least one pyrimidine modified at a C-5 position comprises a C-5 modification independently chosen from at least one of the modifications shown in Figure 9.
4. The aptamer of claim 2, wherein said at least one pyrimidine modified at a C-5 position comprises a C-5 modification independently chosen from benzylcarboxamide, naphthylmethylcarboxamide, tryptaminocarboxamide, tyrosylcarboxamide, 2-naphthylmethylcarboxamide and phenethyl-1-carboxamide.
5. The aptamer of claim 2, wherein said aptamer further comprises at least one additional chemical modification, wherein said at least one additional chemical modification is a chemical substitution at one or more positions independently selected from the group consisting of a ribose position, a deoxyribose position, a phosphate position, and a base position.
6. The aptamer of claim 5, wherein said at least one additional chemical modification is independently selected from the group consisting of a 2'-position sugar modification, a 2'-amino (2'-NH₂), a 2'-fluoro (2'-F), a 2'-O-methyl (2'-OMe) a modification at a cytosine exocyclic amine, a substitution of 5-bromouracil, a substitution of 5-bromodeoxyuridine, a substitution of 5-bromodeoxycytidine, a backbone modification, methylation, a 3' cap, and a 5' cap.
7. The aptamer of claim 4, wherein the aptamer comprises a K_d for *C. difficile* toxin A of 30 nM or less.
8. The aptamer of claim 4, wherein the aptamer comprises a K_d for *C. difficile* toxin B of 30 nM or less.
9. The aptamer of claim 4, wherein the aptamer comprises a K_d for *C. difficile* binary toxin A chain of 30 nM or less.
10. The aptamer of claim 4, wherein the aptamer comprises a K_d for *C. difficile* binary toxin B chain of 30 nM or less.
11. A method of detecting the presence of a *C. difficile* toxin in a test sample comprising contacting said test sample with an aptamer that binds to a toxin produced by *C.*

- difficile* wherein said toxin is selected from toxin A, toxin B, binary toxin A chain, and binary toxin B chain.
12. The method of claim 11, wherein said aptamer comprises at least one pyrimidine modified at a C-5 position.
 13. The method of claim 12, wherein said at least one pyrimidine modified at a C-5 position comprises a C-5 modification independently chosen from at least one of the modifications shown in Figure 9.
 14. The method of claim 12, wherein said at least one pyrimidine modified at a C-5 position comprises a C-5 modification independently chosen from benzylcarboxamide, naphthylmethylcarboxamide, tryptaminocarboxamide, tyrosylcarboxamide, 2-naphthylmethylcarboxamide and phenethyl-1-carboxamide.
 15. The method of claim 12, wherein said aptamer further comprises at least one additional chemical modification, wherein said at least one additional chemical modification is a chemical substitution at one or more positions independently selected from the group consisting of a ribose position, a deoxyribose position, a phosphate position, and a base position.
 16. The method of claim 15, wherein said at least one additional chemical modification is independently selected from the group consisting of a 2'-position sugar modification, a 2'-amino (2'-NH₂), a 2'-fluoro (2'-F), a 2'-O-methyl (2'-OMe) a modification at a cytosine exocyclic amine, a substitution of 5-bromouracil, a substitution of 5-bromodeoxyuridine, a substitution of 5-bromodeoxycytidine, a backbone modification, methylation, a 3' cap, and a 5' cap.
 17. The method of claim 11, wherein the method of detecting is selected from a pull-down assay, dot blot assay, PCR assay or sandwich assay.
 18. A method of identifying or producing a slow off-rate aptamer that binds to a toxin produced by *C. difficile* wherein said toxin is selected from toxin A, toxin B, binary toxin A chain, and binary toxin B chain, the method comprising:
 - (a) preparing a candidate mixture of nucleic acids, wherein the candidate mixture comprises modified nucleic acids in which one, several or all pyrimidines in at least one, or each, nucleic acid of the candidate mixture comprises a chemical modification at a C-5 position;
 - (b) contacting the candidate mixture with a target which is said toxin produced by *C. difficile* and exposing the candidate mixture to a slow off-rate enrichment process,

wherein nucleic acids having a slow rate of dissociation from the target relative to other nucleic acids in the candidate mixture bind the target, forming nucleic acid-target molecule complexes;

- (c) partitioning slow off-rate nucleic acids from the candidate mixture; and
 - (d) amplifying the slow off-rate nucleic acids to yield a mixture of nucleic acids enriched in nucleic acid sequences that are capable of binding to the target molecule with a slow off-rate, whereby a slow off-rate aptamer to the target molecule may be identified.
19. The method of claim 18, wherein said chemical modification at a C-5 position is independently chosen from at least one of the modification shown in Figure 9.
20. The method of claim 18, wherein said chemical modification at a C-5 position is independently chosen from benzylcarboxyamide, naphthylmethylcarboxyamide, tryptaminocarboxyamide, tyrosylcarboxyamide, 2-naphthylmethylcarboxyamide and phenethyl-1-carboxyamide.
21. The method of claim 18, wherein said aptamer further comprises at least one additional chemical modification, wherein said at least one additional chemical modification is a chemical substitution at one or more positions independently selected from the group consisting of a ribose position, a deoxyribose position, a phosphate position, and a base position.
22. The method of claim 21, wherein said at least one additional chemical modification is independently selected from the group consisting of a 2'-position sugar modification, a 2'-amino (2'-NH₂), a 2'-fluoro (2'-F), a 2'-O-methyl (2'-OMe) a modification at a cytosine exocyclic amine, a substitution of 5-bromouracil, a substitution of 5-bromodeoxyuridine, a substitution of 5-bromodeoxycytidine, a backbone modification, methylation, a 3' cap, and a 5' cap.
23. The method of claim 18, wherein said slow off-rate enrichment process is selected from incubation of a candidate mixture with a competitor molecule, dilution of a candidate mixture, or dilution of a candidate mixture in the presence of a competitor molecule.
24. A method of producing an aptamer having a slow rate of dissociation from a toxin produced by *C. difficile* wherein said toxin is selected from toxin A, toxin B, binary toxin A chain, and binary toxin B chain, said method comprising the step of preparing or synthesizing an aptamer based on a nucleic acid sequence identified by a process comprising the steps of:

- a) preparing a candidate mixture of nucleic acids, wherein the candidate mixture comprises modified nucleic acids in which one, several or all pyrimidines in at least one, or each, nucleic acid of the candidate mixture comprises a chemical modification at a C-5 position;
 - b) contacting the candidate mixture with a target which is said toxin produced by *C. difficile* and exposing the candidate mixture to a slow off-rate enrichment process, wherein nucleic acids having a slow rate of dissociation from the target molecule relative to other nucleic acids in the candidate mixture bind the target molecule, forming nucleic acid-target molecule complexes;
 - c) partitioning slow off-rate nucleic acids from the candidate mixture; and
 - d) amplifying the slow off-rate nucleic acids to yield a mixture of nucleic acids enriched in nucleic acid sequences that are capable of binding to the target molecule with a slow off-rate, whereby a slow off-rate aptamer to the target molecule is identified.
25. The method of claim 24, wherein said chemical modification at a C-5 position is independently chosen from at least one of the modification shown in Figure 9.
26. The method of claim 24, wherein said chemical modification at a C-5 position is independently chosen from benzylcarboxamide, naphthylmethylcarboxamide, tryptaminocarboxamide, tyrosylcarboxamide, 2-naphthylmethylcarboxamide and phenethyl-1-carboxamide.
27. The method of claim 24, wherein said aptamer further comprises at least one additional chemical modification, wherein said at least one additional chemical modification is a chemical substitution at one or more positions independently selected from the group consisting of a ribose position, a deoxyribose position, a phosphate position, and a base position.
28. The method of claim 27, wherein said at least one additional chemical modification is independently selected from the group consisting of a 2'-position sugar modification, a 2'-amino (2'-NH₂), a 2'-fluoro (2'-F), a 2'-O-methyl (2'-OMe) a modification at a cytosine exocyclic amine, a substitution of 5-bromouracil, a substitution of 5-bromodeoxyuridine, a substitution of 5-bromodeoxycytidine, a backbone modification, methylation, a 3' cap, and a 5' cap.
29. The method of claim 24, wherein said slow off-rate enrichment process is selected from incubation of a candidate mixture with a competitor molecule, dilution of a

candidate mixture, or dilution of a candidate mixture in the presence of a competitor molecule.

30. A method for producing a nuclease resistant aptamer that binds to a toxin produced by *C. difficile* wherein said toxin is selected from toxin A, toxin B, binary toxin A chain, and binary toxin B chain, the method comprising preparing or synthesizing said nuclease resistant aptamer based on a nucleic acid sequence identified by a process comprising:
- (a) preparing a candidate mixture of modified nucleic acids, wherein the candidate mixture comprises modified nucleic acids in which at least one pyrimidine in at least one, or in each, nucleic acid of the candidate mixture comprises a chemical modification at a C-5 position;
 - (b) contacting the candidate mixture with a target which is said toxin produced by *C. difficile*, wherein nucleic acids having an increased affinity to the target molecule relative to other nucleic acids in the candidate mixture bind the target molecule, forming nucleic acid-target molecule complexes;
 - (c) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and
 - (d) amplifying the increased affinity nucleic acids to yield a mixture of nucleic acids enriched in nucleic acid sequences that are capable of binding to the target molecule with increased affinity and that are nuclease resistant, whereby a nuclease resistant aptamer to the target molecule is identified.
31. The method of claim 30, wherein said chemical modification at a C-5 position is independently chosen from at least one of the modification shown in Figure 9.
32. The method of claim 30, wherein said chemical modification at a C-5 position is independently chosen from benzylcarboxamide, naphthylmethylcarboxamide, tryptaminocarboxamide, tyrosylcarboxamide, 2-naphthylmethylcarboxamide and phenethyl-1-carboxamide.
33. The method of claim 30, wherein said aptamer further comprises at least one additional chemical modification, wherein said at least one additional chemical modification is a chemical substitution at one or more positions independently selected from the group consisting of a ribose position, a deoxyribose position, a phosphate position, and a base position.
34. The method of claim 30, wherein said at least one additional chemical modification is independently selected from the group consisting of a 2'-position sugar modification,

- a 2'-amino (2'-NH₂), a 2'-fluoro (2'-F), a 2'-O-methyl (2'-OMe) a modification at a cytosine exocyclic amine, a substitution of 5-bromouracil, a substitution of 5-bromodeoxyuridine, a substitution of 5-bromodeoxycytidine, a backbone modification, methylation, a 3' cap, and a 5' cap.
35. A nuclease resistant aptamer that binds to a toxin produced by *C. difficile* wherein said toxin is selected from toxin A, toxin B, binary toxin A chain, and binary toxin B chain prepared by a method comprising preparing or synthesizing said nuclease resistant aptamer based on a nucleic acid sequence identified by a process comprising:
- (a) preparing a candidate mixture of modified nucleic acids, wherein the candidate mixture comprises modified nucleic acids in which at least one pyrimidine in at least one, or in each, nucleic acid of the candidate mixture comprises a chemical modification at a C-5 position;
 - (b) contacting the candidate mixture with a target which is said toxin produced by *C. difficile*, wherein nucleic acids having an increased affinity to the target molecule relative to other nucleic acids in the candidate mixture bind the target molecule, forming nucleic acid-target molecule complexes;
 - (c) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and
 - (d) amplifying the increased affinity nucleic acids to yield a mixture of nucleic acids enriched in nucleic acid sequences that are capable of binding to the target molecule with increased affinity and that are nuclease resistant, whereby a nuclease resistant aptamer to the target molecule is produced.
36. The aptamer of claim 35, wherein said chemical modification at a C-5 position is independently chosen from at least one of the modification shown in Figure 9.
37. The aptamer of claim 35, wherein said chemical modification at a C-5 position is independently chosen from benzylcarboxamide, naphthylmethylcarboxamide, tryptaminocarboxamide, tyrosylcarboxamide, 2-naphthylmethylcarboxamide and phenethyl-1-carboxamide.
38. The aptamer of claim 35, wherein said aptamer further comprises at least one additional chemical modification, wherein said at least one additional chemical modification is a chemical substitution at one or more positions independently selected from the group consisting of a ribose position, a deoxyribose position, a phosphate position, and a base position.

39. The aptamer of claim 38, wherein said at least one additional chemical modification is independently selected from the group consisting of a 2'-position sugar modification, a 2'-amino (2'-NH₂), a 2'-fluoro (2'-F), a 2'-O-methyl (2'-OMe) a modification at a cytosine exocyclic amine, a substitution of 5-bromouracil, a substitution of 5-bromodeoxyuridine, a substitution of 5-bromodeoxycytidine, a backbone modification, methylation, a 3' cap, and a 5' cap.
40. An aptamer comprising a sequence selected from the group consisting of SEQ ID NOS: 1-4, 6-10, 12-14, 16-22, 24-27, or 29-31 or a fragment thereof.
41. An aptamer comprising a sequence selected from the group consisting of SEQ ID NOS: 33-46, 48-65, 67-74, 76-82, 84-89, 91-94, 96-97, or 99-108 or a fragment thereof.
42. An aptamer comprising a sequence selected from the group consisting of SEQ ID NOS: 109, 111-123, 126-133, 135-138, 140-144, or 146-150 or a fragment thereof.
43. An aptamer comprising a sequence selected from the group consisting of SEQ ID NOS: 153-156, 158-162 or a fragment thereof.
44. A method of detecting the presence of *C. difficile* toxin A in a test sample comprising contacting said test sample with an aptamer comprising a sequence selected from the group consisting of SEQ ID NOS: 1-4, 6-10, 12-14, 16-22, 24-27, or 29-31 or a fragment thereof.
45. The method of claim 44, wherein the method of detecting is selected from a pull-down assay, dot blot assay, PCR assay or sandwich assay.
46. The method of claim 45, wherein the sandwich assay is an aptamer-target-antibody assay wherein the aptamer is immobilized on a substrate and the antibody is used to detect target bound to the immobilized aptamer.
47. The method of claim 45, wherein the sandwich assay is an antibody-target-aptamer assay wherein the antibody is immobilized on a substrate and the aptamer is used to detect target bound to the immobilized antibody.
48. The method of claim 45, wherein the sandwich assay is an aptamer-target-aptamer assay wherein a first aptamer is immobilized on a substrate and a second aptamer is used to detect target bound to the first, immobilized aptamer.
49. The method of claim 45, wherein the method of detecting provides a quantitative measure of a toxin produced by *C. difficile*.
50. A method of detecting the presence of *C. difficile* toxin B in a test sample comprising contacting said test sample with an aptamer comprising a sequence selected from the

- group consisting of SEQ ID NOS: 33-46, 48-65, 67-74, 76-82, 84-89, 91-94, 96-97, or 99-108 or a fragment thereof.
51. The method of claim 50, wherein the method of detecting is selected from a pull-down assay, dot blot assay, PCR assay or sandwich assay.
 52. The method of claim 51, wherein the sandwich assay is an aptamer-target-antibody assay wherein the aptamer is immobilized on a substrate and the antibody is used to detect target bound to the immobilized aptamer.
 53. The method of claim 51, wherein the sandwich assay is an antibody-target-aptamer assay wherein the antibody is immobilized on a substrate and the aptamer is used to detect target bound to the immobilized antibody.
 54. The method of claim 51, wherein the sandwich assay is an aptamer-target-aptamer assay wherein a first aptamer is immobilized on a substrate and a second aptamer is used to detect target bound to the first, immobilized aptamer.
 55. The method of claim 51, wherein the method of detecting provides a quantitative measure of a toxin produced by *C. difficile*.
 56. A method of detecting the presence of *C. difficile* binary toxin A chain in a test sample comprising contacting said test sample with an aptamer comprising a sequence selected from the group consisting of SEQ ID NOS: 109, 111-123, 126-133, 135-138, 140-144, or 146-150 or a fragment thereof.
 57. The method of claim 56, wherein the method of detecting is selected from a pull-down assay, dot blot assay, PCR assay or sandwich assay.
 58. The method of claim 57, wherein the sandwich assay is an aptamer-target-antibody assay wherein the aptamer is immobilized on a substrate and the antibody is used to detect target bound to the immobilized aptamer.
 59. The method of claim 57, wherein the sandwich assay is an antibody-target-aptamer assay wherein the antibody is immobilized on a substrate and the aptamer is used to detect target bound to the immobilized antibody.
 60. The method of claim 57, wherein the sandwich assay is an aptamer-target-aptamer assay wherein a first aptamer is immobilized on a substrate and a second aptamer is used to detect target bound to the first, immobilized aptamer.
 61. The method of claim 57, wherein the method of detecting provides a quantitative measure of a toxin produced by *C. difficile*.
 62. A method of detecting the presence of *C. difficile* binary toxin B chain in a test sample comprising contacting said test sample with an aptamer comprising a

- sequence selected from the group consisting of SEQ ID NOS: 153-156, 158-162 or a fragment thereof.
63. The method of claim 62, wherein the method of detecting is selected from a pull-down assay, dot blot assay, PCR assay or sandwich assay.
64. The method of claim 63, wherein the sandwich assay is an aptamer-target-antibody assay wherein the aptamer is immobilized on a substrate and the antibody is used to detect target bound to the immobilized aptamer.
65. The method of claim 63, wherein the sandwich assay is an antibody-target-aptamer assay wherein the antibody is immobilized on a substrate and the aptamer is used to detect target bound to the immobilized antibody.
66. The method of claim 63, wherein the sandwich assay is an aptamer-target-aptamer assay wherein a first aptamer is immobilized on a substrate and a second aptamer is used to detect target bound to the first, immobilized aptamer.
67. The method of claim 63, wherein the method of detecting provides a quantitative measure of a toxin produced by *C. difficile*.
68. An aptamer comprising a sequence selected from the group consisting of SEQ ID NOS: 5, 11, 15, 23 and 28 or a fragment thereof.
69. The aptamer of claim 68, wherein the aptamer binds to *C. difficile* toxin A.
70. The aptamer of claim 69, wherein the aptamer comprises a K_d for *C. difficile* toxin A of 30 nM or less.
71. An aptamer comprising a sequence selected from the group consisting of SEQ ID NOS: 32, 47, 66, 75, 83, 90, 95, and 98 or a fragment thereof.
72. The aptamer of claim 71, wherein the aptamer binds to *C. difficile* toxin B.
73. The aptamer of claim 72, wherein the aptamer comprises a K_d for *C. difficile* toxin B of 30 nM or less.
74. An aptamer comprising a sequence selected from the group consisting of SEQ ID NOS: 110, 124-125, 134, 139, and 145 or a fragment thereof.
75. The aptamer of claim 74, wherein the aptamer binds to *C. difficile* binary toxin A chain.
76. The aptamer of claim 75, wherein the aptamer comprises a K_d for *C. difficile* binary toxin A chain of 30 nM or less.
77. An aptamer comprising a sequence selected from the group consisting of SEQ ID NOS: 151-152 and 157 or a fragment thereof.

78. The aptamer of claim 77, wherein the aptamer binds to *C. difficile* binary toxin B chain.
79. The aptamer of claim 78, wherein the aptamer comprises a K_d for *C. difficile* binary toxin B chain of 30 nM or less.
80. A method of detecting the presence of a *C. difficile* toxin in a test sample comprising contacting said test sample with an aptamer of any one of claims 68, 71, 74 or 77.
81. The method of claim 80, wherein the method of detecting is selected from a pull-down assay, dot blot assay, PCR assay or sandwich assay.
82. The method of claim 81, wherein the sandwich assay is an aptamer-target-antibody assay wherein the aptamer is immobilized on a substrate and the antibody is used to detect target bound to the immobilized aptamer.
83. The method of claim 81, wherein the sandwich assay is an antibody-target-aptamer assay wherein the antibody is immobilized on a substrate and the aptamer is used to detect target bound to the immobilized antibody.
84. The method of claim 81, wherein the sandwich assay is an aptamer-target-aptamer assay wherein a first aptamer is immobilized on a substrate and a second aptamer is used to detect target bound to the first, immobilized aptamer.
85. The method of claim 81, wherein the method of detecting provides a quantitative measure of a toxin produced by *C. difficile*.

1/16

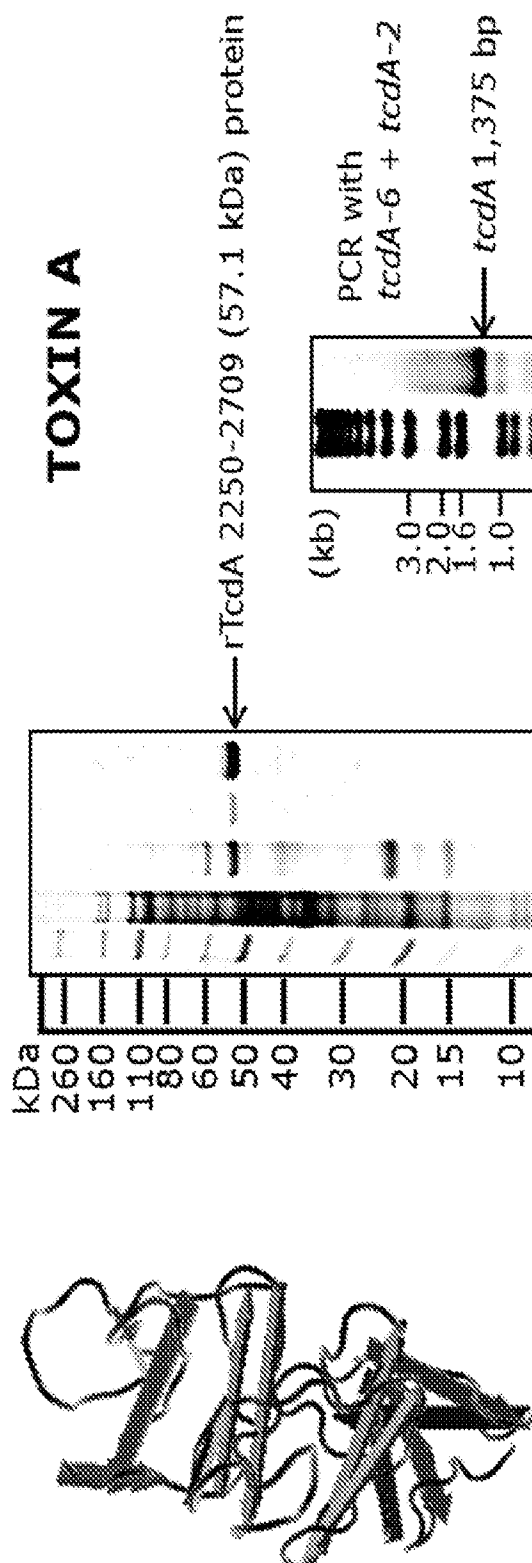


FIG. 1A

2/16

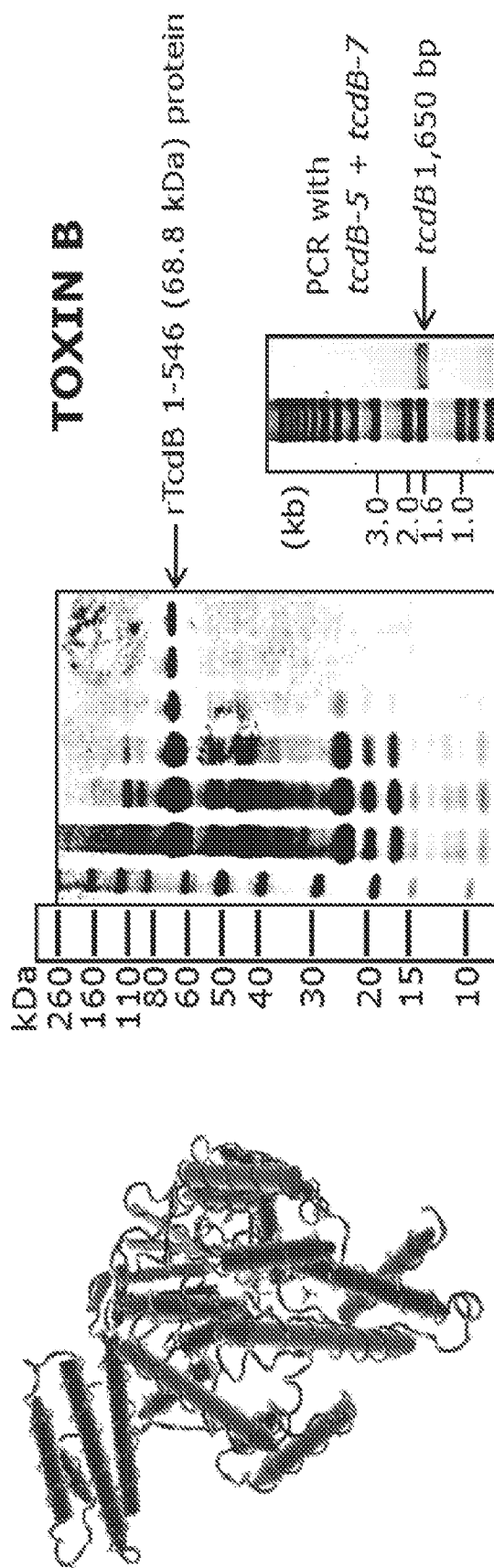


FIG. 1B

3/16



FIG. 1C



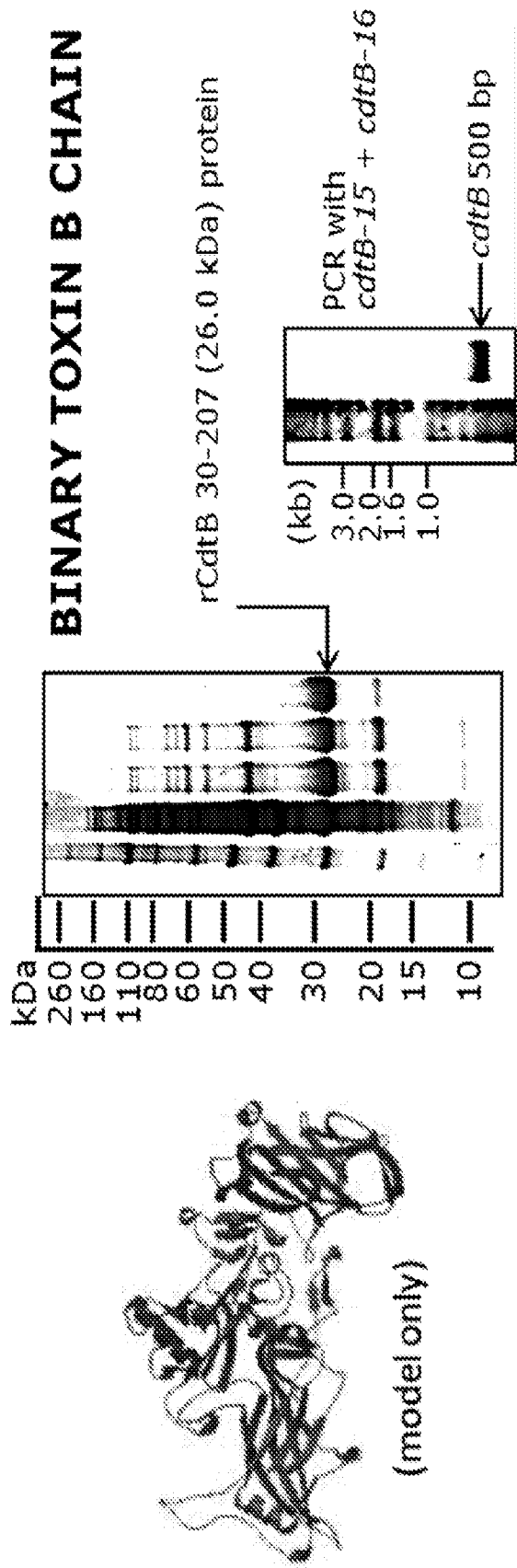


FIG. 1D

5/16

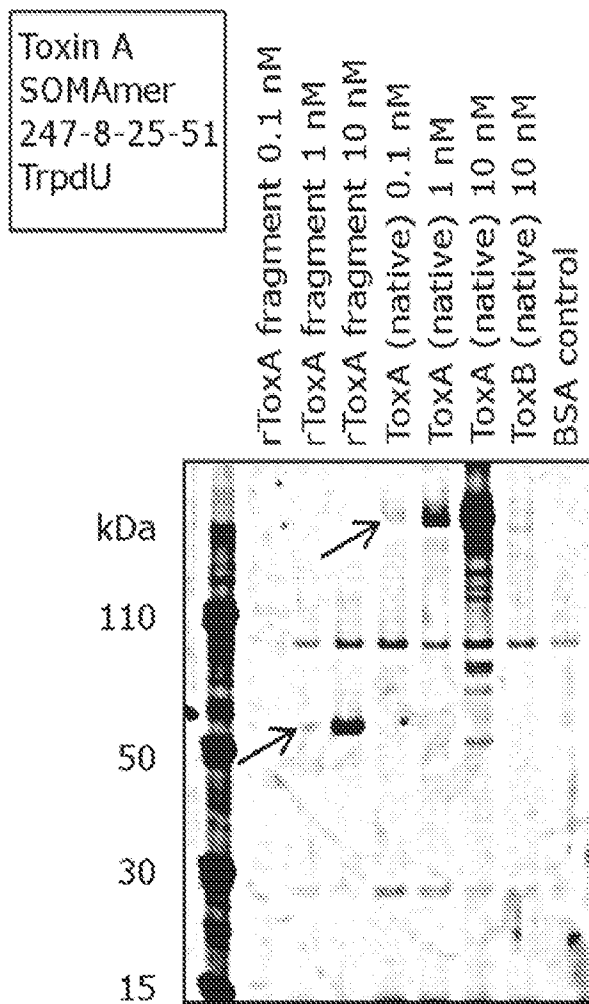


FIG. 2A

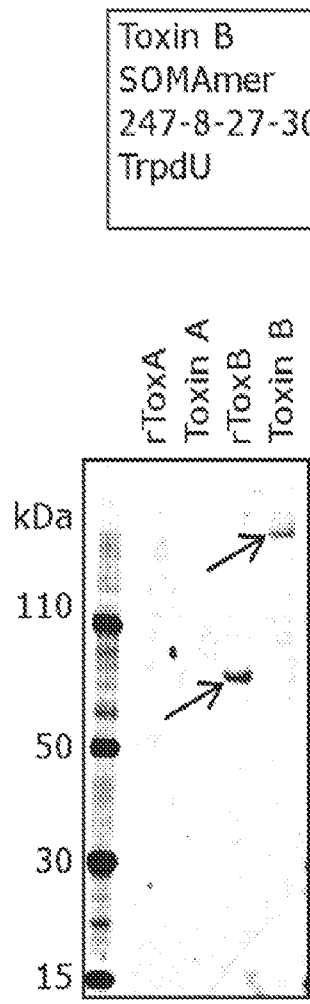


FIG. 2B

6/16

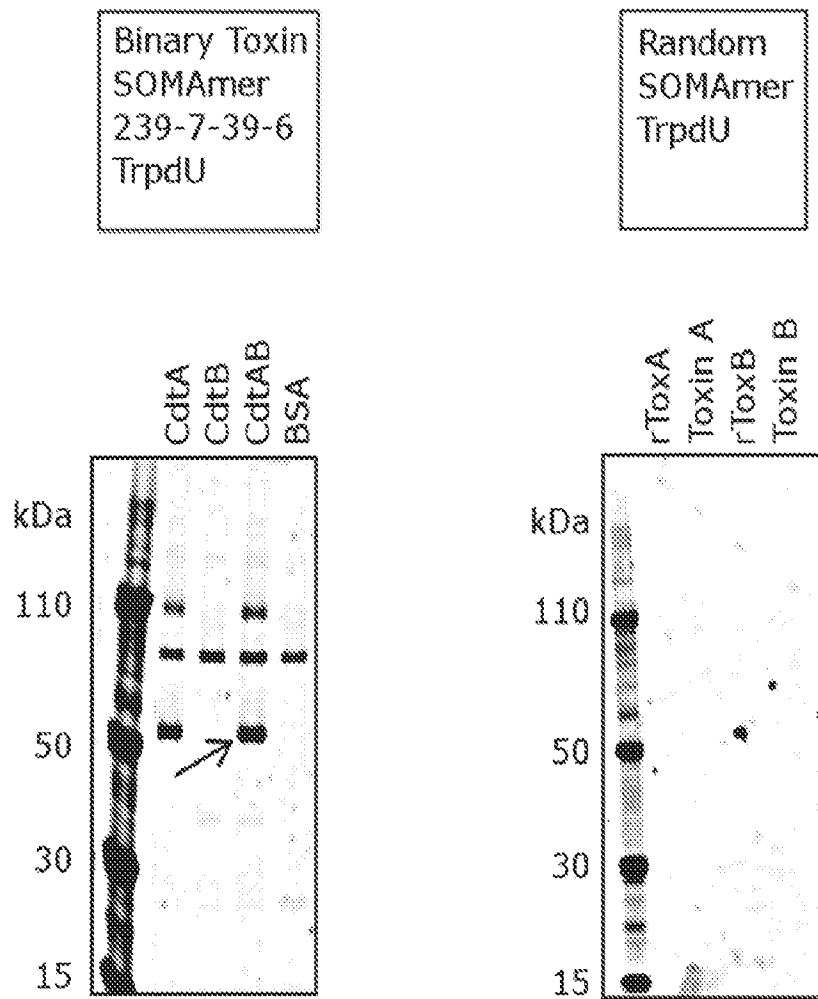


FIG. 2C

FIG. 2D

7/16

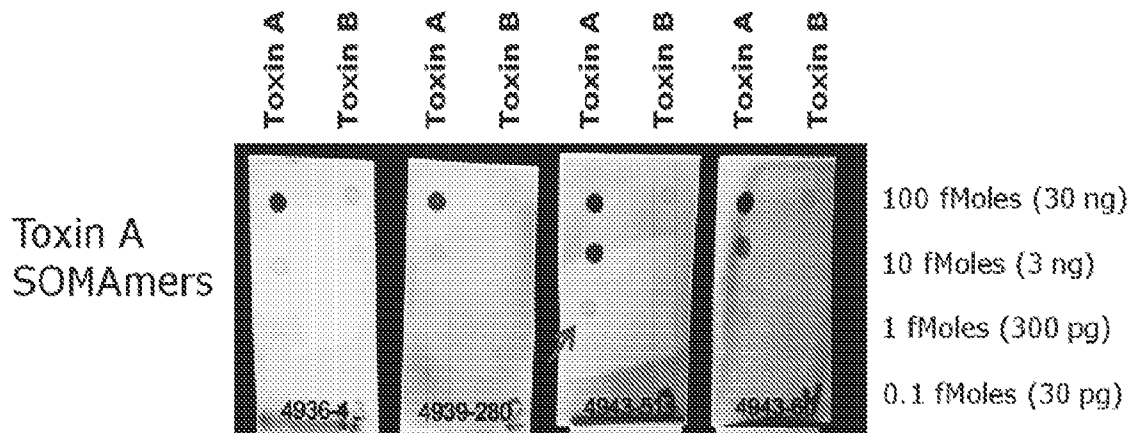


FIG. 3A

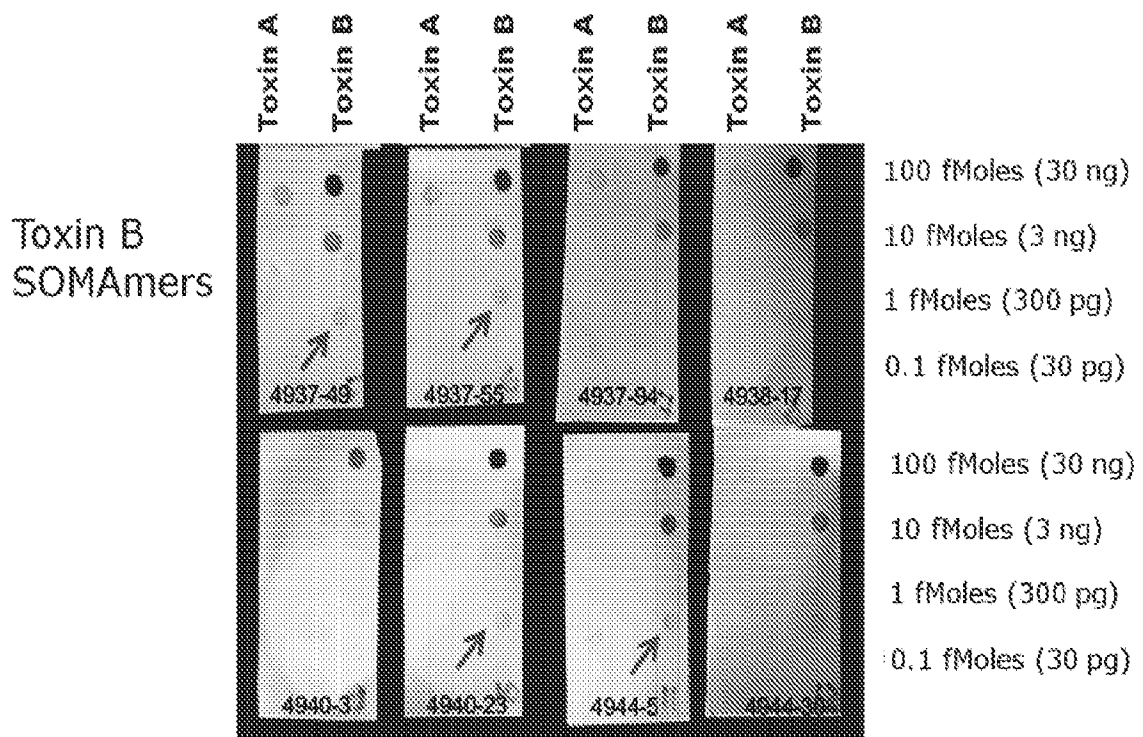


FIG. 3B

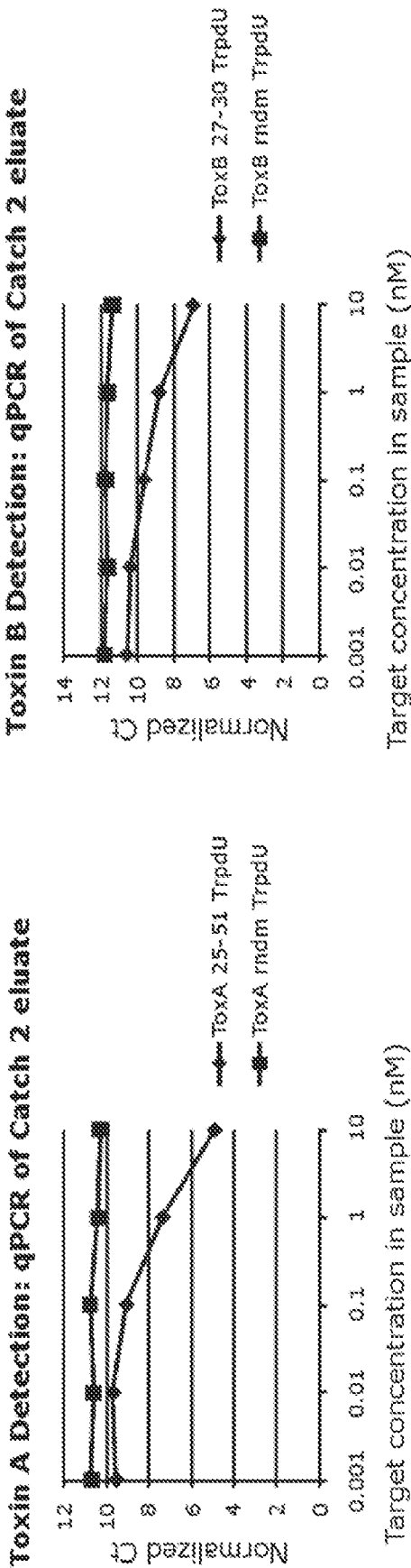


FIG. 4B

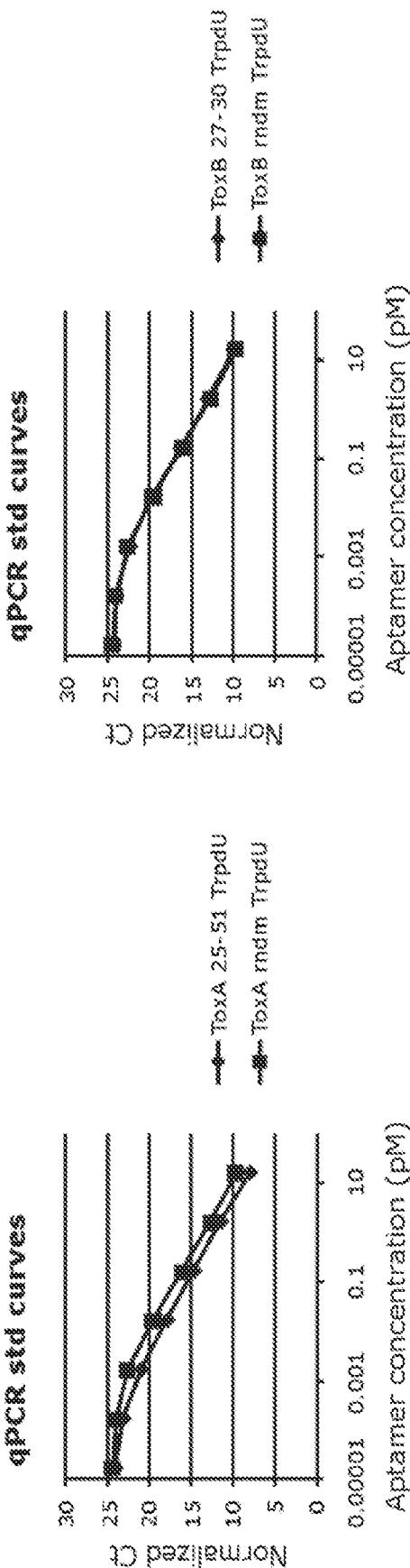


FIG. 4A

9/16

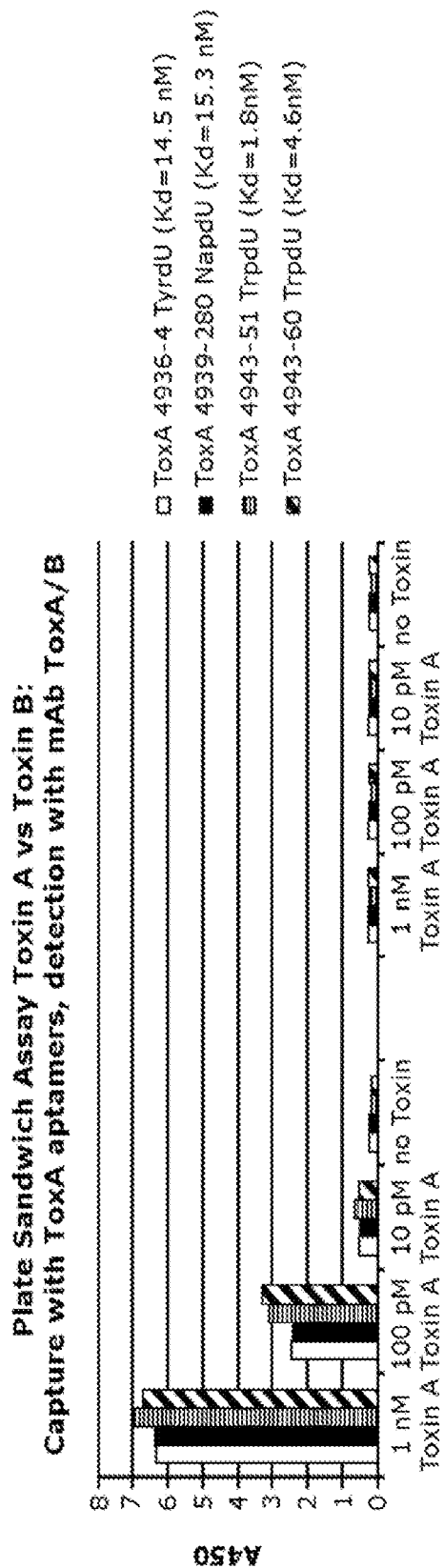


FIG. 5A

Plate Sandwich Assay Toxin A vs Toxin B:
Capture with ToxB aptamers, detection with mAb ToxA/B

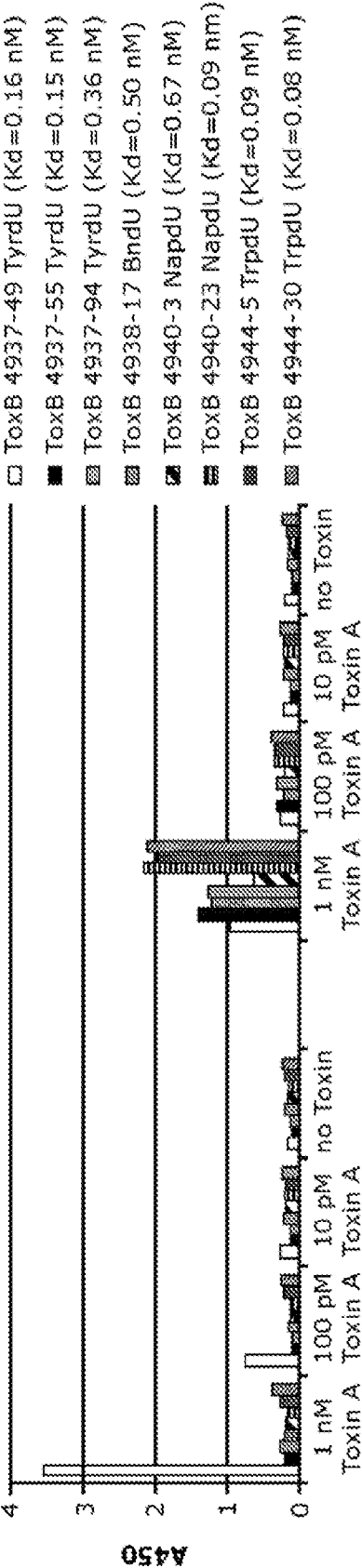
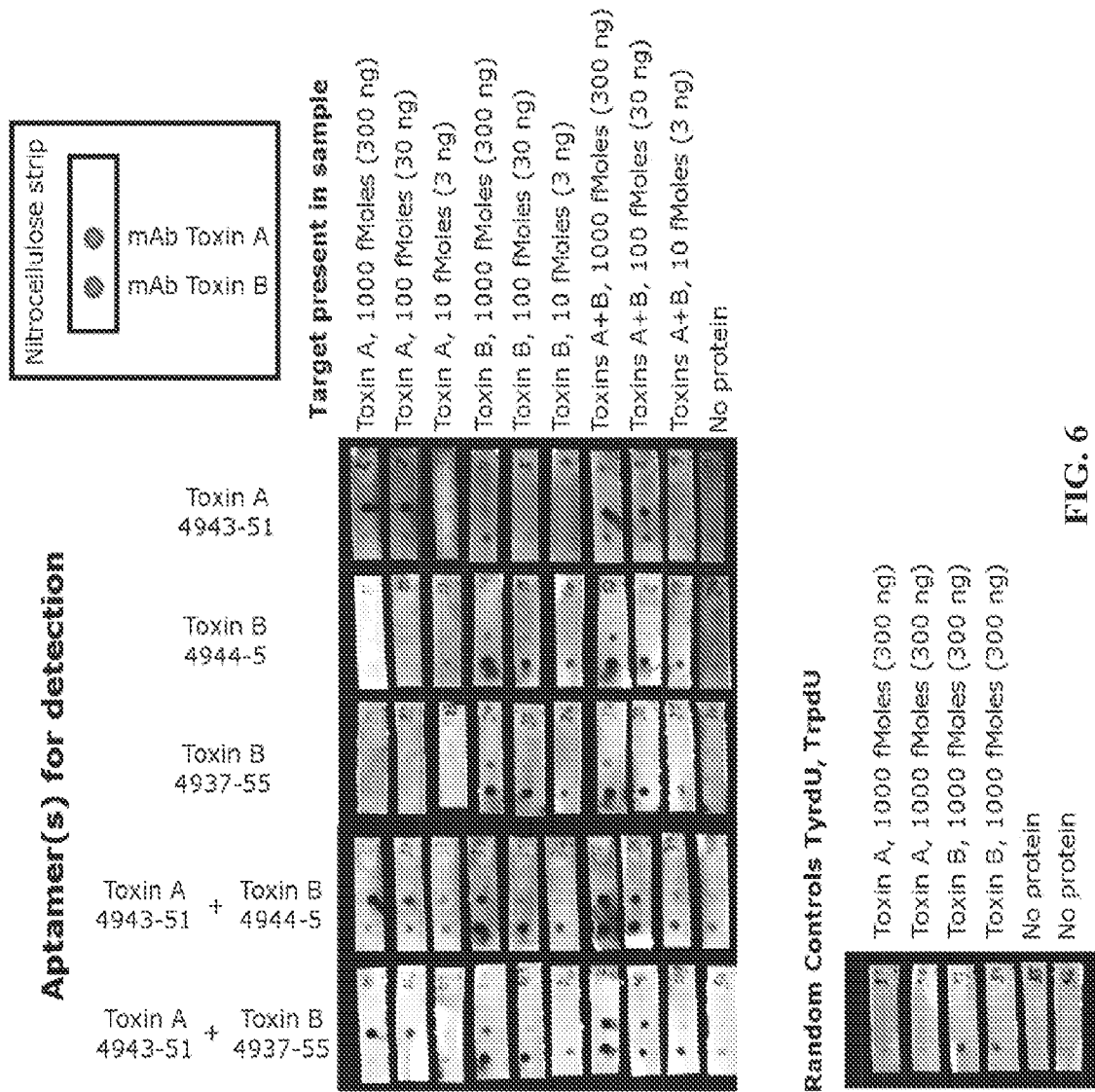


FIG. 5B

11/16



12/16

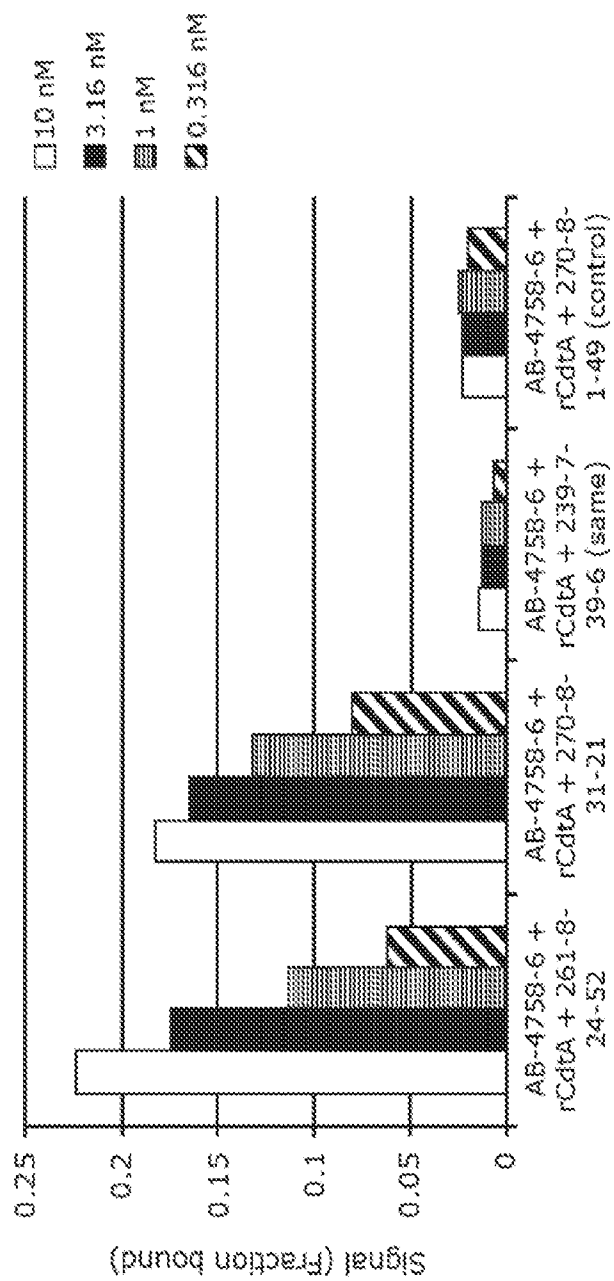


FIG. 7

13/16

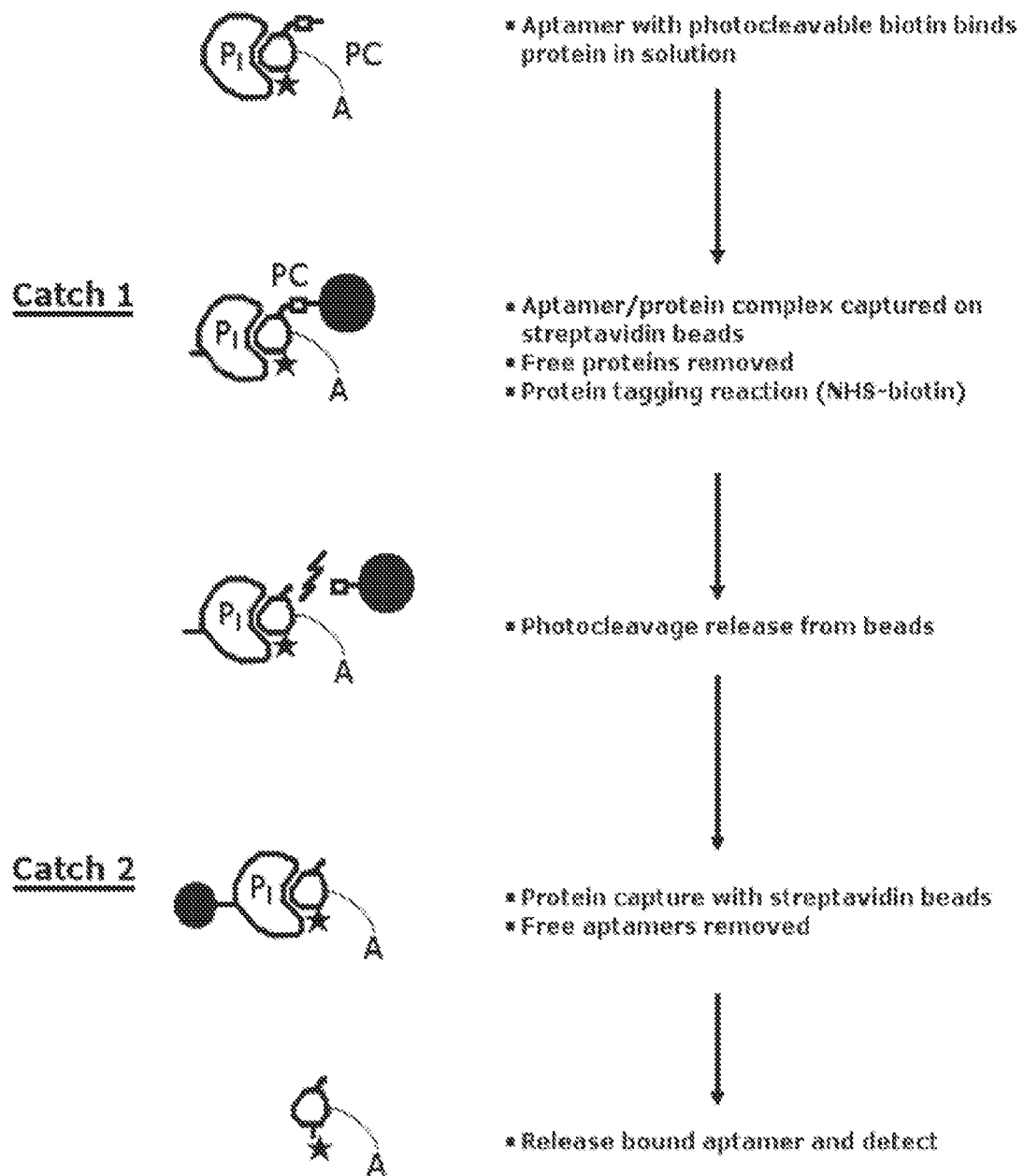
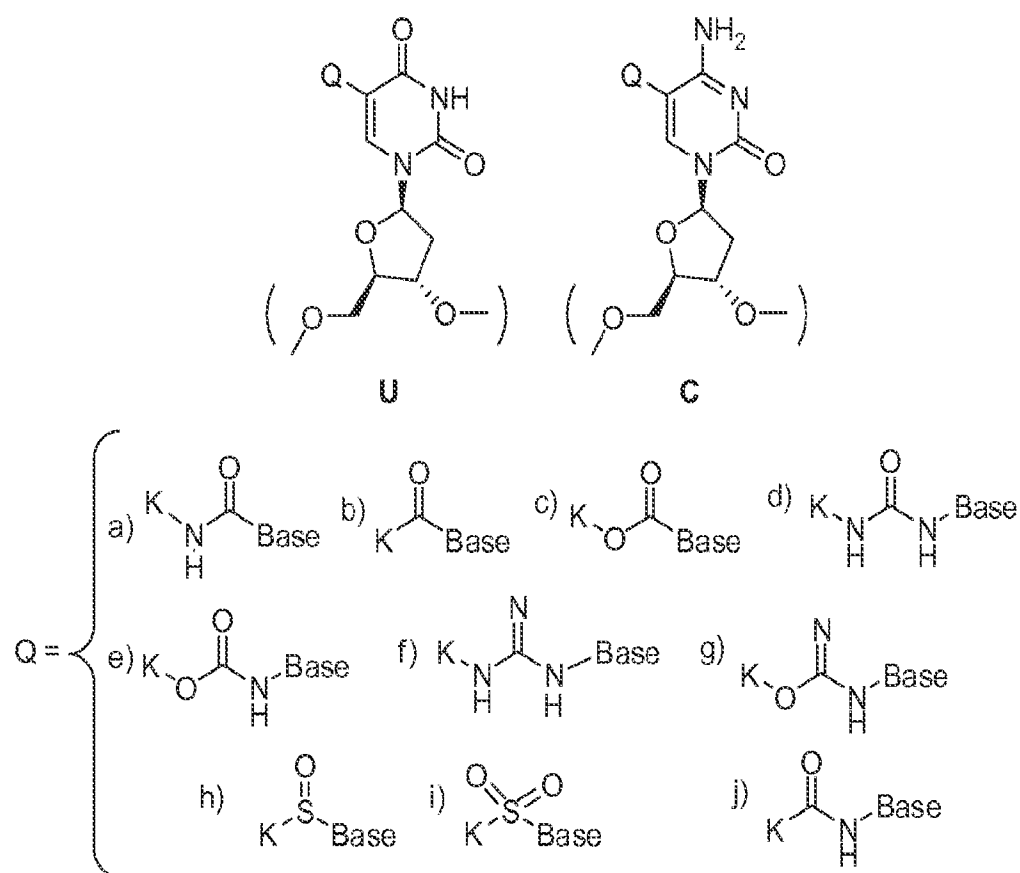


FIG. 8

14/16



Base = Uridine (U) or Cytidine (C) (attachment is to the 5-position)
 K = R' group plus $(\text{CH}_2)_n$ connecting group, where $n = 0-3$

FIG. 9

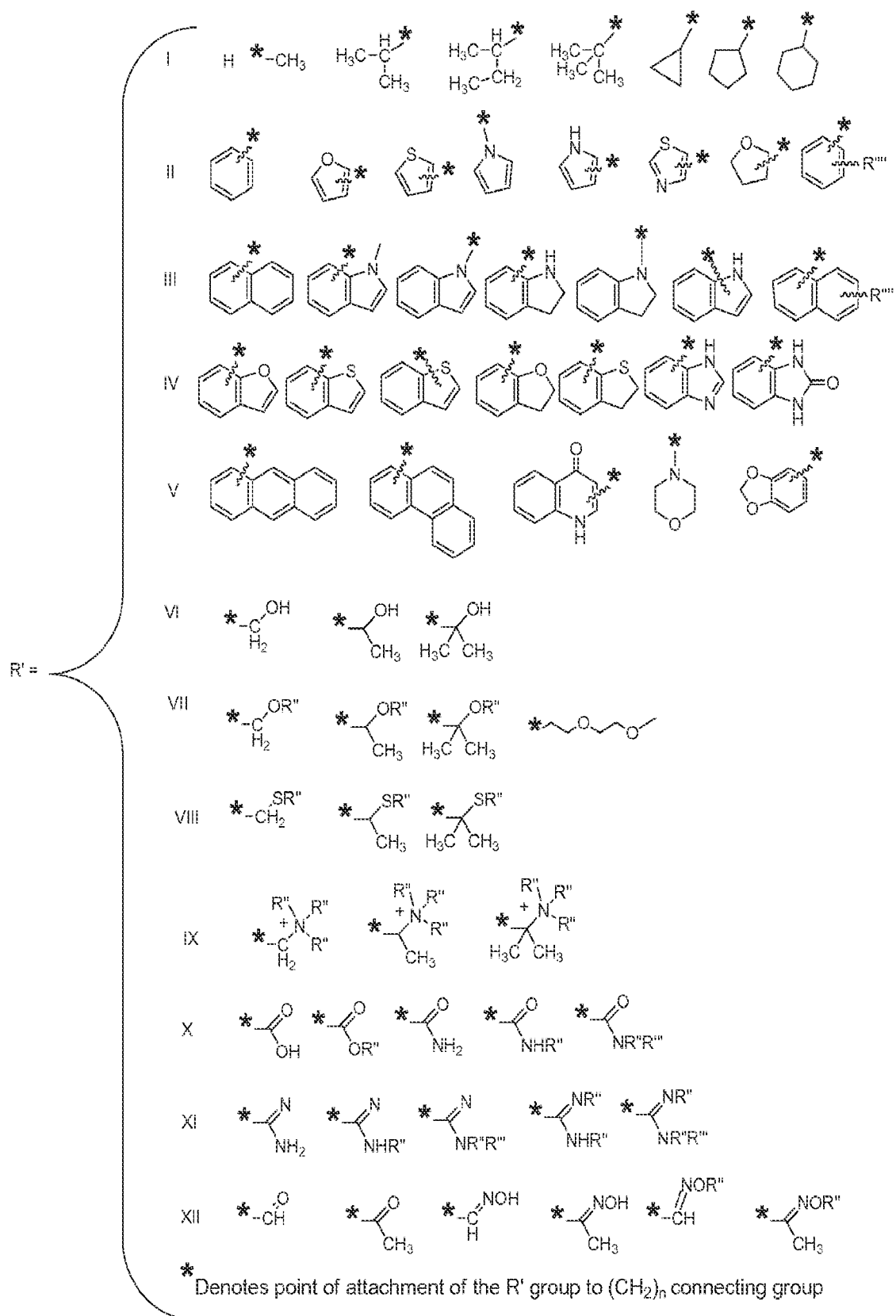


FIG. 9 continued

SUBSTITUTE SHEET (RULE 26)

16/16

wherein

R^{'''} is selected from the group consisting of a branched or linear lower alkyl (C1-C20); hydroxyl (OH), halogen (F, Cl, Br, I); nitrile (CN); boronic acid (BO₂H₂); carboxylic acid (COOH); carboxylic acid ester (COOR^{''}); primary amide (CONH₂); secondary amide (CONHR^{''}); tertiary amide (CONR^{''}R^{'''}); sulfonamide (SO₂NH₂); N-alkylsulfonamide (SONHR^{''});

wherein

R^{''}, R^{'''} are independently selected from a group consisting of a branched or linear lower alkyl (C1-C2)); phenyl (C₆H₅); an R^{'''} substituted phenyl ring (R^{'''}C₆H₄); wherein R^{'''} is defined above; a carboxylic acid (COOH); a carboxylic acid ester (COOR^{'''}); wherein R^{'''} is a branched or linear lower alkyl (C1-C20); and cycloalkyl; wherein R^{''} = R^{'''} = (CH₂)_n;

wherein n = 2-10.

FIG. 9 continued

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/28632

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07H 21/00; G01N 33/53 (2012.01)

USPC - 536/23.1; 436/501; 435/6.1

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)

IPC(8): C07H 21/00; G01N 33/53 (2012.01)

USPC: 536/23.1; 436/501; 435/6.1

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)

PubWEST(PGPB,USPT,USOC,EPAB,JPAB); Google Patents; Google Scholar, GenCore 6.3, search terms used: C. difficile toxin aptamer, pyrimidine modification aptamer, pyrimidine position 5 modification aptamer, ribose 2'-F 2'-NH2 modification aptamer, benzylcarboxamide aptamer, toxin aptamer nM affinity, C. difficile toxin aptamer nM affinity, C. dif

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	US 2010/0035247 A1 (Burton et al.) 11 February 2010 (11.02.2010), para [0006], [0008], [0046], [0049], [0057], [0058], [0062]	1, 11, 17 2-8, 12-16
Y	US 2010/0317723 A1 (Lee et al.) 16 December 2010 (16.12.2010), para [0002], [0004], [0018], [0019], [0024], [0025], Fig. 1	2-8, 12-16
Y	Hicke. "Escort aptamers: a delivery service for diagnosis and therapy" J. Clin. Invest.; 15 October 2000; Vol. 106, No. 8; pp. 923-928. entire document, especially pg 923, col 2, para 3, pg 924, col 2, para 2, pg 925, col 1 para 2, pg 931 col 1 para 3- col 2, para 2, Fig 1	5-8, 15-16
A	US 2009/0163536 A1 (Guiles et al.) 25 June 2009 (25.06.2009), para [0011]- [0013]	9, 10



Further documents are listed in the continuation of Box C.



* 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

3 August 2012 (03.08.2012)

Date of mailing of the international search report

14 AUG 2012

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/28632

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.:
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:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: claims 1-17, drawn to an aptamer that binds to a toxin produced by *C. difficile*.

- Please see extra sheet for continuation -

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.:
1-17

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.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/28632

Continuation of Box III: Lack of Unity of Invention

Group II: claims 18-39, drawn to a method of identifying or producing a slow off-rate aptamer that binds to a toxin produced by *C. difficile* wherein said toxin is selected from toxin A, toxin B, binary toxin A chain, and binary toxin B chain, the method comprising:

- (a) preparing a candidate mixture of nucleic acids, wherein the candidate mixture comprises modified nucleic acids in which one, several or all pyrimidines in at least one, or each, nucleic acid of the candidate mixture comprises a chemical modification at a C-5 position;
- (b) contacting the candidate mixture with a target which is said toxin produced by *C. difficile* and exposing the candidate mixture to a slow off-rate enrichment process, wherein nucleic acids having a slow rate of dissociation from the target relative to other nucleic acids in the candidate mixture bind the target, forming nucleic acid/target molecule complexes;
- (c) partitioning slow off-rate nucleic acids from the candidate mixture; and
- (d) amplifying the slow off-rate nucleic acids to yield a mixture of nucleic acids enriched in nucleic acid sequences that are capable of binding to the target molecule with a slow off-rate, whereby a slow off-rate aptamer to the target molecule may be identified.

Group III+: claims 40-85, drawn to an aptamer comprising a specified sequence, and a method of using said aptamer. The first invention is restricted to SEQ ID NO:1. Should an additional fee(s) be paid, Applicant is invited to elect an additional SEQ ID NO(s) to be searched.

The inventions listed as Groups I-III+ do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The inventions of Groups I-II do not include the inventive concept of an aptamer comprising a specified sequence, as required by Group III+.

The inventions of Group I do not include the inventive concept of a method of identifying or producing a slow off-rate aptamer that binds to a toxin produced by *C. difficile*, as required by Group II.

In addition, a method of identifying an aptamer that binds to a toxin produced by *C. difficile* would have been obvious to one of ordinary skill in the art over a publication "The Use of Phages and Aptamers as Alternatives to Antibodies in Medical and Food Diagnostics" by Mehta, et al. January 2011. [Retrieved from the Internet 24 May 2012: <<http://cdn.intechweb.org/pdfs/12836.pdf>>] (hereinafter "Mehta") in view of a publication titled "Detection of Clostridium difficile toxin: Comparison of enzyme immunoassays with results obtained by cytotoxicity assay" by Musher, et al. (J. Clin. Microbiol. 2007 [Retrieved from the Internet 24 May 2012: <<http://jcm.asm.org/content/early/2007/06/13/JCM.00686-07.full.pdf>>]) as follows:

Mehta discloses how to produce an aptamer to a target molecule (pg 449, "Aptamers are selected from synthetic nucleic acid libraries which can contain more than 10 to the 15 different sequences, by an in vitro selection procedure commonly called SELEX, an acronym for Systematic Evolution of Ligands by EXponential enrichment (Stoltenburg et al. 2007). This iterative process consists of binding and elution steps of aptamers in contact with the molecule or organism of interest, copying the resulting aptamers by using PCR and finally separating the two strands obtained after the polymerisation (Fig. 2). After each cycle, the pool is enriched and used for the next selection cycle, until the amount of eluted aptamers reaches 80 to 90% of the initial amount of the pool. Usually, eight to fifteen selection cycles are needed to isolate aptamers with a high binding potential").

A motivation to produce an anti-*C. Difficile* toxin aptamer is provided by Musher that discloses that "Clostridium difficile colitis has increased in prevalence and severity in hospitals throughout the developed world... Prompt, accurate diagnosis and early treatment shorten the duration of diarrhea which, in turn, further reduces spread of infection" (pg 2), and further discloses using *C. Difficile* toxins A and B for its detection (pg 2, enzyme immunoassay and cytotoxicity assay).

In addition, one of ordinary skill in the art would have been motivated to produce an anti-*C. Difficile* toxin aptamer to use it for detection of *C. difficile*, because Mehta further discloses that doing so would be advantageous due to aptamers High sensitivity and specificity for targets, Fast, inexpensive and animal-friendly production, Stability, Ease of modification, Reusability (pg 450).

A special technical feature of the inventions listed as Group III+ is the specific nucleic acid sequence recited therein. As no significant structural similarities can readily be ascertained among the sequences, the inventions do not share a special technical feature. Without a shared special technical feature, the inventions lack unity with one another.

Groups I-III+ therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.