

PURIFICATION OF CLOSED-ENDED DNA MOLECULES

5 CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to EP Application No. 23307105.9, filed November 30, 2023; U.S. Patent Application No. 63/654,761, filed May 31, 2024; U.S. Patent Application No. 63/685,618, filed August 21, 2024; and U.S. Patent Application No. 63/711,988, filed October 25, 2024. The disclosures of the aforementioned priority
10 applications are incorporated by reference herein in their entirety.

BACKGROUND

[0002] Gene therapy is a cutting-edge technology for treating diseases caused by dysfunction in gene expression. Some approaches of gene therapy involve delivery of a
15 therapeutic gene that encodes a protein deficient in the patient. Viral vectors are commonly used for such delivery. However, viral vectors are often limited by their cargo capacity. For example, adeno-associated viral (AAV) vectors typically deliver a cargo of no more than about 5 kb in size. Additionally, viral vectors, which contain viral proteins, e.g., in the form of viral capsids, can provoke immune responses against the vectors in patients, limiting the
20 redosing potential of gene therapy.

[0003] Closed-ended DNA (ceDNA) circumvents the limitations of viral-based gene delivery. CeDNA is linear, double-stranded DNA (dsDNA) and is more resistant to nucleases, therefore more stable than conventional dsDNA and RNA. Further, ceDNA encapsulated in lipid nanoparticles (LNPs) presents many advantages over existing viral gene
25 delivery systems. Unlike viral capsids, LNPs typically do not encounter pre-existing antibodies against them in the patients, enhancing redosing potential of ceDNA-LNP therapy. Additionally, ceDNA-LNP has a much larger genetic capacity, able to accommodate more than 10 kb of genetic material, expanding the range of genetic diseases that can be targeted by gene therapy. Furthermore, the stability and self-replication of ceDNA will render a
30 single ceDNA dose sufficient for a much larger therapeutic window than existing AAV-based therapy.

[0004] Although ceDNA is a promising tool for gene therapy, its production remains challenging. CeDNA is prone to truncation, nicking, and folding. These problems create

hurdles for large-scale production. Thus, there remains a need for efficient and scalable means of producing and purifying large amounts of ceDNA molecules for clinical use.

SUMMARY

5 **[0005]** The present disclosure provides methods for obtaining a purified preparation of closed-ended DNA (ceDNA) from ceDNA-producing cells. In one aspect, the method comprises: incubating the cells in an alkaline buffer to lyse the cells to generate a cell lysate, wherein the alkaline buffer does not contain a detergent and has a pH of 10 or higher; and isolating the ceDNA from the lysate. In some embodiments, the method comprises, before
10 the isolating step, neutralizing the cell lysate with an acidic salt. In some embodiments, the method further comprises, before the isolating step, pre-clarifying the neutralized cell lysate by adding sodium bicarbonate and separating the resultant flocculants from the cell lysate to generate a pre-clarified cell lysate.

15 **[0006]** In another aspect, the present disclosure provides a method for obtaining a purified preparation of closed-ended DNA (ceDNA) from ceDNA-producing cells, comprising: obtaining a lysate of the cells; pre-clarifying the cell lysate by adding sodium bicarbonate and separating the resultant flocculants from the cell lysate to generate a pre-clarified cell lysate; and isolating the ceDNA from the lysate. In some embodiments, the lysate is obtained by incubating the cells in an alkaline buffer to lyse the cells, wherein the alkaline buffer does not
20 contain a detergent and has a pH of 10 or higher. In further embodiments, before the pre-clarifying step, the cell lysate is neutralized with an acidic salt.

[0007] In the present methods, the isolating step may be performed with, for example, anion exchange chromatography.

25 **[0008]** In some embodiments, the method of the present disclosure further comprises, before the isolating step, removal of RNA by calcium chloride precipitation, optionally wherein the removal of RNA is conducted by: subjecting the pre-clarified cell lysate to filtration to generate a clarified cell lysate, and subjecting the clarified cell lysate to calcium chloride precipitation to remove RNA; in further embodiments, the clarified lysate undergoes ultrafiltration before addition of calcium chloride, and/or the lysate undergoes ultrafiltration
30 and diafiltration after calcium chloride precipitation to reduce the concentration of calcium chloride. In other embodiments, the method of the present disclosure further comprises, after the isolating step, removal of RNA by calcium chloride precipitation; in further embodiments, the nucleic acid preparation undergoes diafiltration after calcium chloride

precipitation to reduce the concentration of calcium chloride. Calcium chloride may be added to achieve a concentration of, for example, about 1-3 M, optionally about 2 M.

[0009] In some embodiments of the present methods, the isolated ceDNA is polished (further purified) (e.g., after removal of RNA). The polishing step may be performed with one or both of (i) hydrophobic interaction chromatography (HIC), optionally wherein the HIC is performed with monolith or perfusive resin; or (ii) multimodal core shell resin, optionally wherein the multimodal core shell resin comprises resin beads with a size-exclusion outer shell, further optionally wherein the size-exclusion out shell has a molecular weight cutoff (MWCO) of 400 or 700 kDa.

10 [0010] In some embodiments of the present methods, the isolated ceDNA preparation undergoes viral filtration, e.g., with a 35 nm filter.

[0011] In some embodiments, the alkaline buffer for cell lysis contains sodium hydroxide, optionally at a final concentration of about 100-300 mM, further optionally at about 150 mM, after being added to the cells. In some embodiments, the incubating step for cell lysis does not last more than five minutes, e.g., lasts about 2.5 minutes or about 3.5 minutes. In some
15 embodiments, the incubating step for cell lysis is performed in a continuous in-line system.

[0012] In some embodiments, the acidic salt for neutralizing the cell lysate is potassium acetate, e.g., at about 2.5-3.5 M (e.g., about 3.1 M).

[0013] In some embodiments, the sodium bicarbonate is added in the pre-clarifying step to reach a concentration from about 5 to about 50 (e.g., about 10) g/L. In some embodiments,
20 the flocculants are removed by a filter with pore sizes of about 7.5-60 μ m. In some embodiments, the incubation time for pre-clarification is about two hours.

[0014] In some embodiments, the isolated ceDNA is subjected to tangential flow filtration, e.g., with an MWCO of 10 and/or 100 kDa.

25 [0015] In some embodiments, the ceDNA-producing cells are insect cells infected with a recombinant baculovirus expression vector. In some embodiments, the recombinant baculovirus expression vector comprises a heterologous nucleic acid sequence comprising a transgene flanked by inverted terminal repeats (ITRs). In some embodiments, the ITRs are parvoviral ITRs (e.g., ITRs from AAV such as AAV2). In some embodiments, the
30 heterologous nucleic acid encodes a therapeutic protein. In some embodiments, the ceDNA-producing cells are transgenic insect cells whose genome comprises a coding sequence for the ceDNA.

[0016] In some embodiments, the ceDNA preparation methods herein comprise in-process monitoring of ceDNA purity by measuring levels of ceDNA and impurities through ion

exchange ultra-performance liquid chromatography in a sample taken before, during, or after the isolating step.

[0017] Also provided herein are ceDNA preparations obtained by the present methods and the use of ceDNA for therapeutic purposes.

- 5 [0018] Other features, objectives, and advantages of the invention are apparent in the detailed description that follows. It should be understood, however, that the detailed description, while indicating embodiments and aspects of the invention, is given by way of illustration only, not limitation. Various changes and modification within the scope of the invention will become apparent to those skilled in the art from the detailed description.

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BRIEF DESCRIPTION OF THE FIGURES

[0019] FIG. 1 is a diagram illustrating a ceDNA molecule containing sequences derived from parvoviral (e.g., AAV) inverted terminal repeats (ITRs).

- 15 [0020] FIG. 2 is a diagram illustrating three insect cell systems for producing ceDNA containing parvoviral ITRs. In the diagram, the illustrated transgene encodes coagulation factor VIII. Other transgenes that do not encode factor VIII may also be incorporated into the cells in the same manner. "One-Bac": a system using one baculoviral vector. "Two-Bac": a system using two baculoviral vectors. "PCL": a producer cell line containing stably integrated copies of a transgene cassette (e.g., a FVIII expression cassette comprising parvoviral ITRs).

- 20 [0021] FIG. 3 is an agarose gel electrophoresis image showing the integrity of ceDNA isolated from ceDNA-producing insect (Sf9) cells lysed under the indicated lysis conditions. Agarose gel electrophoresis was run with ceDNA samples that were untreated or treated with T5 exonuclease, which degrades nicked ceDNA and has no impact on non-nicked ceDNA. The lysis methods were performed using either lysis buffer containing 66.7 mM NaOH and 0.33% SDS or lysis buffer containing 150 mM NaOH.

- 25 [0022] FIG. 4 is a bar graph comparing the integrity of ceDNA obtained from two lysis methods. The comparison was made by both densitometry of electrophoresis agarose gel image (as shown in FIG. 3) and quantitative PCR. Bars on the left in each group of two: ceDNA-producing Sf9 cells were resuspended in PBS. Bars on the right in each group of two: ceDNA-producing Sf9 cells were resuspended in a buffer containing 100 mM Tris and 10 mM EDTA.

[0023] FIGs. 5A-B are agarose gel electrophoresis images showing the integrity of ceDNA (in the presence or absence of T5 exonuclease) isolated from ceDNA-producing insect (Sf9) cells lysed under the indicated lysis conditions.

5 [0024] FIG. 6A is an agarose gel electrophoresis image showing the integrity of ceDNA (in the presence or absence of T5 exonuclease) isolated from ceDNA-producing insect (Sf9) cells lysed with 150 mM NaOH for the indicated lysis hold times (2.5 minutes, 5 minutes, 7.5 minutes, or 15 minutes).

10 [0025] FIG. 6B is a bar graph showing the titers of ceDNA (left bar), bacDNA (middle bar), and sf9DNA (right bar), and the percent purity of ceDNA isolated from ceDNA-producing insect (Sf9) cells lysed with 150 mM NaOH for the indicated lysis hold times (2.5 minutes, 5 minutes, 7.5 minutes, or 15 minutes).

[0026] FIG. 7 is a diagram showing a continuous in-line system for lysis and neutralization of the ceDNA-expressing cell paste.

15 [0027] FIG. 8 is an agarose gel electrophoresis image showing the integrity of ceDNA (in the presence or absence of T5 exonuclease) isolated from ceDNA-producing insect (Sf9) cells lysed with 150 mM NaOH in a continuous in-line system under the indicated conditions. The control was performed in batch mode using 150 mM NaOH lysis buffer with a hold time of 5 min.

20 [0028] FIG. 9 is an agarose gel electrophoresis image comparing the impact of ammonium hydrogen bicarbonate (AHC) and sodium hydrogen bicarbonate (NaHC) as a pre-clarification salt on the integrity of ceDNA after T5 exonuclease treatment.

[0029] FIG. 10A is a diagram showing a pre-clarification process using sodium hydrogen carbonate (NaHC).

25 [0030] FIG. 10B is a pair of photographs showing the separation of flocculants at 5 minutes and 2 hours after addition of NaHC at (from left to right in each photograph) 20 g/L, 15 g/L, 10 g/L, and 5 g/L.

[0031] FIG. 11 is a bar graph comparing the recovery, throughput, and turbidity across different filters used in the cell lysate clarification step. For each group of two, the left bar indicates % recovery, and the right bar indicates % throughput. Turbidity values are
30 associated with the bars with a line.

[0032] FIG. 12 is a pair of graphs for evaluating the impact of feed flux using NaHC-treated neutralized cell lysates on filter throughput.

[0033] FIG. 13A is a diagram showing the study design for evaluating the incorporation of calcium chloride precipitation into the ceDNA purification process to remove residual RNA (rRNA). UF: ultrafiltration. DF: diafiltration.

5 [0034] FIG. 13B is an agarose gel electrophoresis image showing the yield and percent purity of ceDNA isolated following several different calcium chloride precipitation methods as described in FIG. 13A.

[0035] FIG. 13C is a bar graph comparing the ceDNA yield (left bar) and the rRNA impurities (right bar) of the four different rRNA removal processes shown in FIG. 13A. The control in this experiment is absent any CaCl₂ treatment.

10 [0036] FIG. 14A is a chromatograph showing the elution peaks of the ceDNA product after application to a Sartobind® Q column with varying concentrations of NaCl in the load.

[0037] FIG. 14B is an agarose gel electrophoresis image of the eluted fractions obtained in the process of FIG. 14A.

15 [0038] FIG. 15 is a chromatograph showing the elution peaks of the ceDNA product after application to a Sartobind® Q column.

[0039] FIG. 16A is a chromatograph showing the profile of step elution of the ceDNA product after application to a C4 HLD monolith column.

[0040] FIG. 16B is an agarose gel electrophoresis image of the eluted fractions obtained in the process of FIG. 16A.

20 [0041] FIG. 17A is a chromatograph showing the elution profile of the ceDNA product after application to a C4 HLD monolith with a reverse ammonium sulfate step gradient operated in a bind-and-elute approach.

[0042] FIG. 17B is an agarose gel electrophoresis image of the eluted fractions obtained in the process of FIG. 17A.

25 [0043] FIG. 18A is a chromatograph showing the elution profile of the ceDNA product after application to a C4 HLD monolith operated in a flow-through mode.

[0044] FIG. 18B is an agarose gel electrophoresis image of the eluted fractions obtained in the process of FIG. 18A.

30 [0045] FIG. 19A is a bar graph showing the percent recovery and percent purity of the ceDNA product recovered from several different hydrophobic interaction chromatography (HIC) media. "HIC Load": % qPCR purity. "Poros Benzyl ultra": % ceSDS purity. "Poros Ethyl," "Poros Benzyl," and "HIC Monolith": bars from left to right are % recovery, % qPCR purity, and % ceSDS purity, respectively.

- [0046] FIG. 19B is an agarose gel electrophoresis image of the eluted fractions obtained in the process of FIG. 19A.
- [0047] FIG. 20 is a diagram showing the study design for using the POROS™ Benzyl Ultra HIC column in either a bind-and-elute mode or a flow-through mode.
- 5 [0048] FIG 21A is a set of chromatographs showing the elution profile of the ceDNA product after polishing using a POROS™ Benzyl Ultra HIC column in either a bind-and-elute mode or a flow-through mode as described in FIG. 20.
- [0049] FIG. 21B is an agarose gel electrophoresis image of the eluted fractions obtained in the process of FIG. 21A.
- 10 [0050] FIG. 22A is a chromatograph showing the elution profile of the ceDNA product after polishing using a Capto™ Core 400 or Capto™ Core 700 core shell resin.
- [0051] FIG. 22B is an agarose gel electrophoresis image of the filtrate obtained from applying the ceDNA product to core shell resins as in FIG. 22A.
- [0052] FIG. 23 is an agarose gel electrophoresis image of the filtrate obtained from
15 applying purified ceDNA to a Planova™ 35N viral removal filter.
- [0053] FIG. 24 is an agarose gel electrophoresis image comparing 10 kDa and 30kDa molecular weight cutoff (MWCO) tangential flow filtration (TFF) cassettes used to concentrate ceDNA material containing about 1.5-1.7 M ammonium sulfate.
- [0054] FIG. 25 is a schematic diagram illustrating a “three-column” ceDNA purification
20 process.
- [0055] FIG. 26 is a panel of agarose gel electrophoresis images and table showing the ce-SDS Lab Chip and agarose gel electrophoresis results of eluate, product, strip, wash, and regeneration fractions from all column chromatography runs in a three-column purification approach. Product fractions from each polishing column runs are star-marked.
- 25 [0056] FIG. 27 is a schematic diagram illustrating two polishing strategies employing different orders of Capto™ Core shell-based and HIC adsorbents.
- [0057] FIG. 28 is an agarose gel and table showing the presence and intensity of the ceDNA product band and impurities based on agarose gel densitometry or next-generation sequencing short-read sequencing analysis.
- 30 [0058] FIG. 29 is an agarose gel and table showing the purify of ceDNA product using a combination of Lab Chip, agarose gel densitometry, and next-generation sequencing short-read sequencing analyses.
- [0059] FIG. 30 is an overlaid ion exchange (IEX) chromatogram of four samples (“Load,” “FT,” “Wash,” and “Elution”) taken from the Sartobind® Q ceDNA purification step. rHCP:

residual host cell protein. RFP: red fluorescent protein (an introduced cell marker). rRNA: residual RNA. FT: flow-through.

DETAILED DESCRIPTION

- 5 **[0060]** The present disclosure provides a scalable and robust manufacturing process to purify ceDNA in eukaryotic cells (e.g., insect cells). The ceDNA may comprise a sequence of interest (e.g., a coding sequence for a therapeutic protein). Once purified, the ceDNA may be encapsulated in lipid nanoparticles for delivery in patients. The ceDNA preparations of the present disclosure have reduced amounts of nucleic acid
- 10 impurities, such as open-ended double-stranded DNA, and are expected to have an improved safety profile, including causing less anti-drug immune response when delivered to patients.
- 15 **[0061]** The present disclosure is based on discoveries associated with purifying ceDNA from eukaryotic cells such as insect cells. In general, the present purification process comprises: (i) harvesting and resuspending the producer cells to form a cell paste, optionally wherein the cells are harvested by continuous centrifugation; (ii) lysing the cells for a brief period of time in an alkaline buffer that does not contain a detergent (e.g., SDS) and contains, for example, sodium hydroxide, and neutralizing the cell lysate with an acidic salt (e.g., potassium acetate), where a continuous in-line system is used for the lysis and neutralization;
- 20 (iii) chemically pre-clarifying the neutralized cell lysate by adding in salt that does not include ammonium salts (e.g., ammonium bicarbonate) and contains, for example, sodium bicarbonate, and removing flocculants by filtration; (iv) removing RNA by salt precipitation (e.g., calcium chloride precipitation), rather than by using RNase; (v) purifying ceDNA from the lysate by anion exchange capture membrane chromatography and finer polishing
- 25 chromatography such as hydrophobic interaction chromatography (e.g., using resins such as perfusion resin or monolithic columns) and/or mixed mode such as core shell resin; and (vi) further downstream steps such as viral filtration and tangential flow filtration and diafiltration. The present process reduces DNA nicking and fragmentation, reduces nucleic acid and host cell protein impurities, and is suitable for environmentally friendly large-scale
- 30 manufacturing.

I. Production of ceDNA in Host Cells

[0062] CeDNA may be characterized by having no exposed ends and containing loop structures at its ends. In some embodiments, ceDNA has covalently linked ends, i.e., the 5'

end of the sense strand is covalently linked to the 3' end of the antisense strand, and the 3' end of the sense strand is covalently linked to the 5' end of the antisense strand. In other embodiments, the ceDNA contains self-annealed loop structures at both ends of its both strands.

- 5 **[0063]** In some embodiments, the ceDNA contains viral-derived inverted terminal repeat (ITR) sequences such that each end of the DNA strands is self-annealed into a hairpin-like structure (**FIG. 1**). In some embodiments, the ITR sequences are derived from parvoviruses such as adeno-associated viruses (AAV) and bocaviruses. The ITR sequences may be wildtype viral sequences, or contain mutations relative to wildtype viral sequences. In
- 10 particular embodiments, the ITR sequences may be from AAV2. The ITR sequences flank a cargo sequence such as a transgene expression cassette; for example, an expression cassette for a therapeutic protein (e.g., an enzyme, an antibody, a cell surface receptor, a transcription factor, a hormone, or a cytokine). The expression cassette may contain a promoter (e.g., constitutive or inducible) and other regulatory elements (e.g., enhancers, insulators,
- 15 polyadenylation sites, etc.) for directing expression of the coding sequence in the host cells. The promoter may be a promiscuous promoter that is active in multiple tissues, or may be a tissue-specific promoter. By way of example, the promoter may be specific for the liver, the lung, muscles, cells in the peripheral or central nervous system, cells in the cardiovascular system, cells in the ocular system, or cells in the immune system.
- 20 **[0064]** CeDNA such as ceDNA containing viral ITRs may be produced in recombinant eukaryotic host cells. In some embodiments, the ceDNA may be produced in mammalian host cells such as HEK293 cells, HeLa cells, and CHO cells. In other embodiments, the ceDNA may be produced in non-mammalian host cells such as insect cells. In further
- 25 embodiments, the ceDNA may be produced in insect cells such as Sf21, Sf9, S2, Tni-Hi5, Super9, and ExpressSF+. In certain embodiments, the insect cells derived from *Spodoptera frugiperda* such as Sf21 and Sf9 cells are free of rhabdovirus (Sf-rhabdovirus-negative).
- [0065]** In some embodiments, the ceDNA may be produced in stable cell lines (e.g., mammalian or insect cells) that are engineered to contain copies of a transgene expression cassette flanked by parvoviral ITRs. The ITR-specific replicase protein encoded by the *Rep*
- 30 gene recognizes the ITR sequences at the terminal resolution sites (TRS) and generates copies of the transgene-containing ceDNA in the producing cells. The *Rep* gene may be stably integrated into the genome of the host cells, or may be transiently expressed from an episomal vector such as a baculoviral vector in the case of insect producing cells.

[0066] In some embodiments, the transgenic host cell lines are derived from insect cells such as Sf21, Sf9, S2, Tni-Hi5, Super9, and ExpresSF+. A parvoviral (e.g., AAV) *Rep* gene may be introduced into the insect cells transiently via a baculoviral vector. In certain embodiments, the template for the ceDNA is carried on the same baculoviral vector (“one-bac” system). In other embodiments, the template for the ceDNA is carried on a separate baculoviral vector (“two-bac” system). In the “two-bac” system, the two baculoviral vectors may be introduced into the insect host cells simultaneously or sequentially. In yet another system, a producing cell line (PCL) is established with stably integrated copies of the ceDNA template, and a baculoviral vector carrying the *Rep* gene is introduced into the cell line transiently. These three exemplary systems of ceDNA production in insect cells are illustrated in **FIG. 2**.

[0067] The ceDNA purification process provided herein can be implemented across multiple scales, including shake flasks, mini bioreactors (e.g., 100-250 mL), benchtop bioreactors (e.g., 50 L), and large bioreactors (e.g., 500 L, 1000 L, and 10,000 L).

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II. Purification of ceDNA from Host Cells

[0068] In the aforementioned ceDNA production system, the host cell genome and baculoviral vector DNAs pose the major challenges for downstream purification of transgene-specific full-length ceDNA. This production system also includes the intermediate replicative species of ceDNA that are not full-length and potentially interfere with purification of the transgene-specific full-length ceDNA. Additionally, the process is complicated by the presence of RNA impurities, including those from baculovirus and viruses endogenous to the cell lines (e.g., rhabdovirus). The present disclosure provides an improved method of purifying ceDNA from producing host cells that is efficient and scalable for commercial production. The steps of this method are described in detail below.

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A. Harvest and Resuspension of Cells

[0069] In some embodiments, the ceDNA-producing cells are harvested by centrifugation, e.g., continuous flow centrifugation. In continuous flow centrifugation, large volumes of material are centrifuged at high centrifugal forces while the supernatant is simultaneously extracted through a drain line. By way of example, a fixed volume of cell-containing culture liquid is collected in a bowl while maintaining a pre-determined centrifugal speed and the supernatant continuously flows out of the bowl into a collection vessel. Once the fixed volume is pumped into the bowl, the concentrated cells are dispensed through a collection

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line for further processing while the supernatant is discarded. The number of cycles is determined according to the pre-established cell concentration factor needed for the process.

[0070] The concentrated cells may then be resuspended in a buffer to generate a cell paste. A variety of resuspension buffers may be used. The pH of the buffer may range from about 6.5 to about 8.5. The buffer may contain sodium salts, potassium salts, and/or buffering agents.

[0071] In some embodiments, the buffer contains Tris, EDTA, and a polyol (e.g., sucrose) may be used. For example, the buffer contains 100 mM Tris, 10 mM EDTA, and 50 mM sucrose, pH 8.

[0072] In some embodiments, the buffer is a phosphate-buffered saline (PBS). By way of example, the PBS may contain about 100-150 mM NaCl, about 1.5-3.0 mM KCl, and about 10-15 mM phosphate, pH 7.4. In further embodiments, the PBS contains about 135 mM NaCl, about 2.7 mM KCL, and about 11 mM phosphate (e.g., 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄), pH about 7.4.

B. Lysis and Neutralization of Cell Paste

[0073] There are a variety of methods to lyse eukaryotic cells. In the present process, the cell paste is lysed with an alkaline buffer that does not contain any detergent such as SDS, or contains a detergent at a very low concentration (e.g., SDS as a concentration no greater than 0.1%). The inventors have discovered that the presence of a detergent in the lysis buffer may cause nicking of the ceDNA, possibly by breaking down phosphodiester bonds.

[0074] In some embodiments, the lysis buffer comprises an alkaline agent such as NaOH. In certain embodiments, the lysis buffer comprises NaOH at a stock concentration of about 25 mM to 500 mM, e.g., about 25 mM, about 50 mM, about 75 mM, about 100 mM, about 125 mM, about 150 mM, about 175 mM, about 200 mM, about 225 mM, about 250 mM, about 275 mM, about 300 mM, about 325 mM, about 350 mm, about 375 mM, about 400 mM, about 425 mM, about 450 mM, about 475 mM, or about 500 mM. In further embodiments, the lysis buffer comprises NaOH at a stock concentration of about 300 mM. The effective concentration of NaOH decreases once the lysis buffer is added to the cells. In some embodiments, the effective NaOH concentration of the lysis buffer in the cell mixture is about 150 mM.

[0075] In some embodiments, the alkaline lysis buffer has a pH of about 9-14. For example, the lysis buffer comprises a pH of about 9.0, about 9.2, about 9.4, about 9.6, about 9.8, about 10.0, about 10.2, about 10.4, about 10.5, about 10.6, about 10.8, about 11.0, about 11.2, about 11.4, about 11.5, about 11.6, about 11.8, about 12.0, about 12.2, about 12.4, about

12.5, about 12.6, about 12.8, about 13.0, about 13.2, about 13.4, about 13.5, about 13.6, about 13.8, or about 14.0. In further embodiments, the lysis buffer comprises a pH of about 12.5 or higher.

[0076] The lysis buffer is applied to the cell paste for a predetermined amount of time.

5 This amount time is also termed herein “lysis hold time.” In some embodiments, the lysis hold time is no greater than ten minutes, e.g., no greater than five minutes. A longer lysis hold time results in a longer exposure to high pH and consequently irreversible denaturation of the ceDNA product. In some embodiments, the lysis hold time may be about 0.5 minutes, about 1 minute, about 1.5 minutes, about 2 minutes, about 2.5 minutes, about 3 minutes,
10 about 3.5 minutes, about 4 minutes, about 4.5 minutes, or about 5 minutes. In further embodiments, the cells are lysed in an alkaline lysis buffer comprising a NaOH concentration of about 300 mM, pH of about 12.5 or higher, and once the cell solution is mixed with the lysis buffer in equal volume, the effective NaOH concentration is halved to become about 150 mM; and the lysis hold time is about 2.5 minutes, about 3 minutes, about 3.5 minutes,
15 about 4 minutes, about 4.5 minutes, or about 5 minutes.

[0077] After alkaline lysis, the lysate is neutralized to return the pH of the lysate to achieve acidic conditions (e.g., a pH of about 5.0 to 6.5, or about 5.5 to 6.0). In some embodiments, the lysate is neutralized with an acidic salt such as potassium acetate. In further embodiments, the alkaline lysate is neutralized with potassium acetate by mixing with
20 a potassium acetate solution of about 0.5 M to about 5 M. For example, the acidic salt such as potassium acetate may be provided at a concentration of about 0.5 M, about 1.0 M, about 1.5 M, about 2.0 M, about 2.5 M, about 3.0 M, about 3.5 M, about 4.0 M, about 4.5 M, or about 5.0 M. In some embodiments, the potassium acetate is provided at a concentration of about 3.0 M (e.g., 3.1 M), with a pH of about 5.2.

25 **[0078]** To more precisely control lysis hold time, the cells may be lysed in an continuous in-line lysis operation. **FIG. 7** illustrates such an operation. In this operation, concentrated cells (cell paste) and the alkaline lysis buffer are continuously fed through the apparatus at predetermined rates and volume ratio (e.g., 1:1 volume ratio) by controlling pump speed for each buffer/lysate performed. The cells travel through a tube in which lysis occurs. The lysis
30 hold time is determined by the length of the tube and the feed rates. At the end of the lysis tube, the mixture is then mixed with a continuous stream of the neutralization buffer and the neutralized cell lysate continues into a collection tank. In some embodiments, continuous cell lysis may be used to process 10 L or more cell paste with a lysis hold time of about 2.5

minutes (e.g., using a static mixer). To reduce shearing of ceDNA and breakage of genomic DNA or viral DNA during lysis, a gentle mixing speed is preferred.

C. Chemical Pre-clarification of Neutralized Cell Lysate

[0079] Once the cell lysate is neutralized, it may be pre-clarified, e.g., chemically, to
5 remove impurities such as high molecular weight (HMW) genomic DNA, host cell proteins (HCP), and other cellular components. The present inventors have discovered that chemical pre-clarification demonstrates superior results over physical pre-clarification (e.g., by batch or continuous centrifugation).

[0080] In some embodiments, the neutralized cell lysate is treated with a pre-clarifying
10 salt. Conventionally, such operation is performed with ammonium bicarbonate. But ammonium bicarbonate generates noxious ammonia gas and may become an environmental hazard. The present inventors have discovered that sodium bicarbonate not only is environmentally friendly, but also generates satisfactory pre-clarification results.

[0081] In some embodiments, the pre-clarifying salt is sodium bicarbonate and may be
15 added to reach a concentration of about 5-50 g/L. For example, the pre-clarifying salt may be present at a concentration of about 5 g/L, about 7.5 g/L, about 10 g/L, about 12.5 g/L, about 15 g/L, about 17.5 g/L, about 20 g/L, about 22.5 g/L, about 25 g/L, about 27.5 g/L, about 30 g/L, about 32.5 g/L, about 35 g/L, about 37.5 g/L, about 40 g/L, about 42.5 g/L, about 45 g/L, about 47.5 g/L, or about 50 g/L. In preferred embodiments, the pre-clarifying salt is present
20 at a concentration of 10 g/L.

[0082] In some embodiments, the neutralized cell lysate is treated with the pre-clarifying
salt for a pre-determined amount of time. In some embodiments, the neutralized cell lysate is treated with the pre-clarifying salt for a period of about 0.5-8 hours. For example, the
neutralized cell lysate may be treated with the pre-clarifying salt for about 0.5 hours, about 1
25 hour, about 1.5 hours, about 2 hours, about 2.5 hours, about 3 hours, about 3.5 hours, about 4 hours, about 4.5 hours, about 5 hours, about 5.5 hours, about 6 hours, about 6.5 hours, about 7 hours, about 7.5 hours, or about 8 hours. In further embodiments, the neutralized cell lysate may be treated with the pre-clarifying salt for about 2 hours or about 4 hours. In some
embodiments, the neutralized cell lysate is mixed with the pre-clarifying salt through mild
30 agitation for efficient solubilization.

D. Clarification of the Pre-Clarified, Neutralized Cell Lysate

[0083] The pre-clarified, neutralized cell lysate may be clarified through depth filtration to remove cell debris and particles from feed stream. The choice of clarification filter may be driven by cell density, cell types, and harvest viscosities. Depth filtration may be performed

by using, e.g., a polypropylene filter, a cellulose filter, a silica filter, a polyacrylic filter, or a filter with mixed materials. In some embodiments, the filter may have pore sizes of 0.6-60 μm , e.g., 7.5-60 μm , or 0.6-8.0 μm . Examples of suitable depth filtration systems are Clarisolve® 60HX and D0HC filters (Millipore).

5 **E. Removal of RNA**

[0084] Conventional methods of removing RNA impurities from DNA products use RNase. However, RNase typically is sourced from animals, which not only increases costs, but also poses safety issues for therapeutic products. The present inventors have discovered that calcium chloride achieve excellent results in removing RNA as well as other impurities
10 such as genomic DNA fragments, baculoviral DNA, and HCP, from the cell lysate.

[0085] In some embodiments, the clarified cell lysate is treated with CaCl_2 , wherein the calcium chloride salt is present at a concentration of about 0.5-10.0 M. For example, the salt may be present at a concentration of about 0.5 M, about 1.0 M, about 1.5 M, about 2.0 M, about 2.5 M, about 3.0 M, about 3.5 M, about 4.0 M, about 4.5 M, about 5.0 M, about 5.5 M,
15 about 6.0 M, about 6.5 M, about 7.0 M, about 7.5 M, about 8.0 M, about 8.5 M, about 9.0 M, about 9.5 M, or about 10.0 M. In preferred embodiments, the salt may be present at a concentration of about 2 M.

[0086] In some embodiments, the clarified cell lysate undergoes ultrafiltration (e.g., TFF) before the calcium chloride treatment, to concentrate the lysate. For example, the clarified
20 cell lysate is concentrated by a factor of about 2-10X, e.g., by a factor of about 2X, about 3X, about 4X, about 5X, about 6X, about 7X, about 8X, about 9X, or about 10X. After the calcium precipitation, the lysate may undergo ultrafiltration and diafiltration in the presence of a buffer suitable for subsequent chromatography. By way of example, the diafiltration may be done in the presence of a buffer comprising Tris, EDTA, and sodium chloride, pH
25 about 8 (e.g., 50 mM Tris, 10 mM EDTA, and 0.3 M NaCl).

[0087] In some embodiments, the clarified cell lysate is treated with calcium chloride salt to remove RNA prior to the isolation of the ceDNA product from the lysate. In some
embodiments, the removal of RNA from the clarified lysate via calcium chloride treatment occurs after the ceDNA is isolated from the cell lysate (e.g., through anion exchange), prior
30 to polishing of the ceDNA product. When calcium chloride precipitation is implemented after the capture/isolation step, the material may not need to be filtered, and the capture eluate (isolation step product) may undergo diafiltration after calcium chloride precipitation and before going through polishing.

F. Isolation of ceDNA Product

[0088] The ceDNA product may be isolated from the cell lysate through chromatography. In some embodiments, the present method comprises one or more, two or more, or three or more chromatography steps of the same or different chromatography modes.

- 5 [0089] In some embodiments, the clarified cell lysate is treated with a load adjustment salt to remove impurities during chromatography. In some embodiments, the load adjustment salt is NaCl. In some embodiments, the load adjustment salt is present in the cell lysate load at a concentration of about 50-500 mM. For example, the clarified cell lysate load may contain NaCl at a concentration of about 100 mM, about 150 mM, about 200 mM, about 250 mM,
 10 about 300 mM, about 350 mM, about 400 mM, about 450 mM, about 500 mM, about 550 mM, or about 600 mM. In further embodiments, the clarified cell lysate load may comprise NaCl at a concentration of about 300 mM.

- [0090] In some embodiments, the ceDNA is isolated by anion exchange chromatography. Suitable anion exchange chromatography systems include strong basic anion ligand
 15 exchanger with quaternary ammonium ($R-CH_2-N^+(CH_3)_3$) or weak basic anion ligand exchanger with diethyl amino ethyl (DEAE) ($R-C_2H_4-N^+H(C_2H_5)_2$). For example, the anion exchange chromatography system may be the Sartobind® Q system (Sartorius), Natrix® HD Q (Millipore Sigma), CIMmultus® DEAE (Sartorius), CIMmultus® Q (Sartorius), Mustang™ Q (Pall), ReadytoProcess Adsorber Q (Cytiva), POROS™ 50D (Thermo Fisher),
 20 POROS™ 50HQ (Thermo Fisher), Sartorius STIC® PA (Sartorius), or an equivalent thereof. In some embodiments the capture chromatography media is a Membrane or Monolith or resin. In some embodiments, the anion exchange chromatography step comprises flow-through of impurities with a load adjustment of NaCl at 50-500 mM (e.g., 300 mM), a wash step to further remove trace impurities, and then elution of ceDNA with an elution buffer. In
 25 some embodiments, the wash buffer comprises a salt, such as NaCl, at a concentration of about 0.15-0.8 M. For example, the wash buffer may comprise a salt, such as NaCl, at a concentration of about 0.15 M, about 0.2 M, about 0.25 M, about 0.3 M, about 0.35 M, about 0.4 M, about 0.45 M, about 0.5 M, about 0.55 M, about 0.6 M, about 0.65 M, about 0.7 M, about 0.75 M, or about 0.8 M. In further embodiments, the wash buffer comprises a salt,
 30 such as NaCl, at a concentration of 0.6 M. In some embodiments, the elution buffer comprises a salt, such as NaCl, at a concentration of about 0.5-3 M. For example, the elution buffer may comprise a salt, such as NaCl, at a concentration of about 0.5 M, about 0.6 M, about 0.7 M, about 0.8 M, about 0.9 M, about 1.0 M, about 1.1 M, about 1.2 M, about 1.3 M, about 1.4 M, about 1.5 M, about 1.6 M, about 1.7 M, about 1.8 M, about 1.9 M, about 2.0 M,

about 2.1 M, about 2.2 M, about 2.3 M, about 2.4 M, about 2.5 M, about 2.6 M, about 2.7 M, about 2.8 M, about 2.9 M, or about 3.0 M. In further embodiments, the elution buffer comprises a salt, such as NaCl, present at a concentration of about 0.9 M. In some embodiments, the elution buffer comprises a different salt such as sodium sulfate, sodium acetate, or ammonium acetate.

G. Polishing of ceDNA Product

[0091] In some embodiments, the above isolated ceDNA preparation may be further polished by one or more additional chromatography steps that may be based on, e.g., hydrophobic interaction chromatography (HIC). The HIC media may be monolith columns or resins and may contain additional operation modes such as multimodal core shell resin-based purification.

[0092] In some embodiments, the ceDNA preparation is treated with an HIC load adjustment salt prior to the HIC polishing step. For example, the HIC load adjustment salt is ammonium sulfate (AS). In some embodiments, the HIC load adjustment salt is present at a concentration of about 1-4 M. For example, the clarified cell lysate may be treated with salt at a concentration of about 1.0 M, about 1.5 M, about 2.0 M, about 2.5 M, about 3.0 M, about 3.5 M, or about 4.0 M. In preferred embodiments, the clarified cell lysate is treated with an HIC load adjustment salt present at a concentration of about 3.0 M.

[0093] In some embodiments, the HIC step comprises an elution step selected from a linear gradient or a step gradient may be operated in a bind-and-elute mode or a flow-through mode. In some embodiments, the linear gradient elution step comprises a reverse salt gradient containing AS at a linear gradient of salt concentration from, e.g., about 4-0 M or about 3-0 M.

[0094] In some embodiments, the step gradient elution step comprises a step-wise reverse salt gradient. In some embodiments, the step-wise reverse salt gradient comprises AS at concentration from about 10-0 M, e.g., 2-0 M.

[0095] In some embodiments of the bind-and-elute elution step, the AS salt in the elution buffer may be present at a concentration of about 0.5 M, about 1.0 M, about 1.5 M, about 2.0 M, about 2.5 M, about 3.0 M, about 3.5 M, or about 4.0 M. In further embodiments, the bind-and-elute salt may be present at a concentration of about 1.74 M.

[0096] In some embodiments of the flow-through mode, the AS salt in the load may be present at a concentration of about 0.75-2.5 M. For example, the HIC flow-through purification step may be performed at an AS load concentration of about 0.75 M, about 1 M,

about 1.5 M, about 2 M, or about 2.5 M. In further embodiments, the AS concentration in the load is about 1.5 M.

[0097] In some embodiments, the HIC step comprises a channel. In some embodiments, the HIC channel has a size of about 0.5-10 μm . For example, the HIC channel size may be about 0.5 μm , about 1.0 μm , about 1.5 μm , about 2.0 μm , about 2.5 μm , about 3.0 μm , about 3.5 μm , about 4.0 μm , about 4.5 μm , about 5.0 μm , about 5.5 μm , about 6.0 μm , about 6.5 μm , about 7.0 μm , about 7.5 μm , about 8.0 μm , about 8.5 μm , about 9.0 μm , about 9.5 μm , or about 10.0 μm . In preferred embodiments, the HIC channel has a size of about 2.0 μm . In preferred embodiments, the HIC channel has a size of about 6.0 μm .

10 [0098] Examples of HIC systems include: C4 HLD Monolith resin (Sartorius), POROS™ Ethyl perfusive resin (Thermo Scientific), POROS™ Benzyl perfusive resin (Thermo Scientific), or POROS™ Benzyl Ultra perfusive resin (Thermo Scientific), Capto™ PlasmidSelect (Cytiva), Capto™ Phenyl (Cytiva), or Sartobind® Phenyl (Sartorius).

[0099] In some embodiments, the polishing step includes mixed-mode resins combining size exclusion chromatography with anionic and hydrophobic interaction chromatographic properties. In some embodiments, the ceDNA preparation is treated with a load adjustment salt, prior to application to the size exclusion column. In some embodiments, the load adjustment salt is NaCl. In some embodiments, the load adjustment salt is AS. In some embodiments, the load adjustment salt is present at a concentration of about 0.1-4.0 M. For example, the load adjustment salt may be present at a concentration of about 0.1 M, about 1.2 M, about 1.3 M, about 1.4 M, about 1.5 M, about 1.6 M, about 1.7 M, about 1.8 M, about 1.9 M, about 2.0 M, about 2.1 M, about 2.2 M, about 2.3 M, about 2.4 M, about 2.5 M, about 2.6 M, about 2.7 M, about 2.8 M, about 2.9 M, about 3.0 M, about 3.1 M, about 3.2 M, about 3.3 M, about 3.4 M, about 3.5 M, about 3.6 M, about 3.7 M, about 3.8 M, about 3.9 M, or about 4.0 M. In further embodiments, the load adjustment salt is NaCl present at a concentration of about 0.15-0.9 M. In other further embodiments, the load adjustment salt is AS present at a concentration of about 1.5-2.5 M. Examples of size exclusion chromatography systems include Capto™ Core 700 and Capto™ Core 400 multimodal resin (Cytiva), which operates by both size exclusion and HIC. In some embodiments, the size exclusion chromatography has a MWCO of 400 to 900 kDa, e.g., 750 kDa. In some embodiments, Capto™ Core 400 may be used for ceDNA products that are about 3 to 5 kb in length.

[0100] In some embodiments, the polishing step utilizes two different chromatography methods. For example, the ceDNA isolated by Sartobind® may be subject to HIC (e.g., C4 HLD HIC monolith in bind-and-elute mode, C4 HLD HIC monolith in flow-through mode,

POROS™ Benzyl Ultra HIC resin in bind-and-elute mode, or POROS™ Benzyl Ultra HIC resin in flow-through mode), and then subject to a multimodal core shell resin (e.g., Capto™ Core 400 or 700); or in a reverse order.

H. Further Filtration Steps

5 [0101] In some embodiments, the ceDNA preparation undergoes viral filtration to remove any viral contaminants. In some embodiments, viral filtration is performed using a filter with a surface area of about 0.001-1.0 m². For example, the filter may be present with a surface area of about 0.001 m², about 0.01 m², about 0.12 m², about 0.3 m², or about 1.0 m². In preferred embodiments, viral filtration is performed using a filter with a surface area of about
10 0.001 m². Examples of viral filtration systems include Planova™ 35N viral removal filter (Asahi Kasei Bioprocess).

[0102] In some embodiments, a purified ceDNA preparation is concentrated by a tangential flow filtration (TFF) cassette. In some embodiments, the TFF cassette may comprise a MWCO of about 1-100 kDa. For example, the TFF cassette may comprise a
15 molecular MWCO of about 1 kDa, about 5 kDa, about 10 kDa, about 20 kDa, about 30 kDa, about 40 kDa, about 50 kDa, about 60 kDa, about 70 kDa, about 80 kDa, about 90 kDa, or about 100 kDa. In further embodiments, the TFF comprises a MWCO of about 10 kDa.

[0103] In some embodiments, the ceDNA product is concentrated by a TFF by a factor of about 2-20X. For example, the ceDNA product may be concentrated by a tangential flow
20 filtration cassette by a factor of about 2X, about 3X, about 4X, about 5X, about 6X, about 7X, about 8X, about 9X, about 10X, about 11X, about 12X, about 13X, about 14X, about 15X, about 16X, about 17X, about 18X, about 19X, or about 20X. In preferred embodiments, the purified ceDNA product is concentrated by a TFF cassette by a factor of about 10X.

25 I. In-Process Monitoring of Purification Status

[0104] The compositions of intermediate products of the ceDNA manufacturing may be monitored. For example, during chromatography purification of ceDNA (e.g., purification by Sartobind® Q chromatography), samples taken from different stages of the purification process, such as samples from the loading material, the flow-through material, the wash
30 product, and the elution product, may be analyzed to assess amounts of the ceDNA and impurities (e.g., host cell proteins and DNA/RNA fragments) present in the samples.

[0105] In some embodiments, the analysis may be done by ion exchange (IEX) ultra-performance liquid chromatography (UPLC) to separate proteins and nucleic acids based on their charges. The levels of the various components in the analyzed sample may be

determined by spectrophotometry at wavelengths of 254 nm (for nucleic acids) and 280 nm (for proteins). Non-limiting examples of IEX columns are those comprising non-porous particles (e.g., polymethacrylate particles) coated by networks of ion exchange groups (e.g., sulfopropyl, carboxymethyl, and/or quaternary ammonium groups), such as Protein-Pak Hi Res Q by Water™ and TSKgel DNA-STAT by Tosoh.

[0106] In some embodiments, the ceDNA-producing cells may contain an exogenously introduced expression cassette for expressing a fluorescent protein (e.g., a red, blue, yellow, green, or cyan fluorescent protein). In those instances, fluorescence spectrophotometry may be additionally used to monitor the amount of the fluorescent protein in the samples as one indicator for the presence of host cell proteins.

[0107] Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Exemplary methods and materials are described below, although methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present disclosure. In case of conflict, the present specification, including definitions, will control. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. Throughout this specification and embodiments, the words “have” and “comprise,” or variations such as “has,” “having,” “comprises,” or “comprising,” will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers. All publications and other references mentioned herein are incorporated by reference in their entirety, as if each individual reference were specifically and individually indicated to be incorporated by reference in its entirety. Although a number of documents are cited herein, this citation does not constitute an admission that any of these documents forms part of the common general knowledge in the art. As used herein, the term “approximately” or “about” as applied to one or more values of interest refers to a value that is similar to a stated reference value. In certain embodiments, the term refers to a range of values that fall within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less in either direction (greater than or less than) of the stated reference value unless otherwise stated or otherwise evident from the context.

[0108] Headers herein are created for ease of organization and are not intended to limit the scope of the claimed invention in any manner.

[0109] In order that this invention may be better understood, the following examples are set forth. These examples are for purposes of illustration only and are not to be construed as limiting the scope of the invention in any manner.

5

EXAMPLES

[0110] These Examples below describe experiments that optimized the purification procedures of ceDNA from producing insect Sf9 cells. These procedures included resuspension of producing cells after harvesting, lysis, neutralization, pre-clarification, clarification, column purification, and analytics. The optimized procedures allow for a robust and scalable manufacturing process. In these experiments, the ceDNA was produced in Sf9 cells and carried a coding sequence for human factor VIII. The size of the ceDNA was 8.3 kb (FIG. 1).

Example 1: Evaluation of Resuspension Buffer

[0111] The ceDNA-producing Sf9 cells were harvested and centrifuged. The cell pellet was resuspended in a resuspension buffer. The initial process development runs were performed by resuspending the cells in a 50 mM Tris/10 mM EDTA buffer (pH 8), which is similar to conditions of commercial plasmid DNA purification kits. The resuspension buffer was modified by increasing the Tris concentration to 100 mM Tris (pH 8) and adding sucrose to a final concentration of 50 mM. The data show that inclusion of sucrose in the resuspension buffer prevented abrupt osmotic shock to the Sf9 cells and mitigated the loss of cell viability during the cell concentration phase using UniFuge®.

[0112] As an alternative to Tris buffer, a PBS-based resuspension buffer (with or without the addition of sucrose) was explored. It was hypothesized that having sucrose in the resuspension buffer could cause the concentrated Sf9 cells to clump together, which would prevent optimal lysis of the concentrated cells. In this study, concentrated Sf9 cells were resuspended in two different resuspension buffer conditions: resuspended in a buffer containing 100 mM Tris and 10 mM EDTA (pH 8) or in 1X PBS (pH 7.4), and then lysed in a lysis solution containing 66.7 mM NaOH and 0.33% SDS, or containing 150 mM NaOH. For all the conditions, the lysates were neutralized, clarified, and finally purified using an ion exchange column (Sartobind® Q). Titer assessment of ceDNA was performed using qPCR and nicking impact on ceDNA was evaluated by treating the purified ceDNA with T5 exonuclease and further running T5 non-treated and treated samples side by side on an agarose gel (FIG. 3). T5 treatment works on the principle that a nicked DNA will be completely digested by T5 exonuclease and would not show up as intact DNA on agarose gel

electrophoresis. T5 has no impact on non-nicked ceDNA. For T5 treatment, the DNA sample was incubated with the enzyme at 37°C for 30 minutes.

[0113] The densitometry analysis of the agarose gel shows that cells resuspended in the Tris buffer and the PBS buffer and lysed using 0.15 M NaOH exhibited similar amounts of intact ceDNA (FIG. 4). However, under the 66.7 mM NaOH+0.33% SDS lysis condition, cells resuspended in PBS exhibited higher ceDNA titer as well as more intact ceDNA than cells suspended in Tris buffer. Based on this result, the PBS-based buffer was selected as the resuspension buffer for further downstream studies.

10 Example 2: Evaluation of Lysis Buffer

[0114] The commercial plasmid DNA extraction kit from Invitrogen uses 0.1 M NaOH + 0.5% SDS as the lysis buffer recipe. A high throughput study was implemented to identify a suitable lysis condition for ceDNA-producing Sf9 cells. During this study, varying concentrations of NaOH (from about 0 mM to about 100 mM) and SDS (from about 0% to about 1%) were evaluated. A qPCR assay shows that 66.7mM NaOH+0.33% SDS resulted in high titers of ceDNA and less genomic DNA.

[0115] However, high titers of ceDNA may not translate into high quality of ceDNA. Subsequent runs showed that ceDNA purified using the 66.7mM NaOH +0.33% SDS lysis buffer resulted in significant product degradation, suggesting that deterioration of ceDNA product quality could result from a combination of nuclease activity and/or specific lysis conditions.

[0116] Investigation into lysis conditions was incorporated to identify specific root causes which led to nicking of ceDNA and find out optimal lysis conditions that mitigated ceDNA product quality fragmentation. Different lysis strategies were tested as described below.

25 A. Detergent-Based Lysis

[0117] Experiments using SDS-containing lysis buffers show that lysis performed using SDS alone (0.5% SDS and 1% SDS) resulted in complete nicking of ceDNA, as shown by agarose gel electrophoresis after T5 treatment. An alternative detergent called RIPA buffer, which is routinely used for protein extraction, was also evaluated, but ceDNA degradation was also observed. Next, we evaluated lysis buffers containing both NaOH and SDS (150 mM NaOH+0.05% SDS, or 150 mM NaOH+1% SDS). Nicking again was observed.

[0118] When we removed SDS from the 150 mM NaOH lysis buffer, ceDNA nicking was not observed. Based on this encouraging result, it was decided to evaluate lysis conditions having only NaOH without the presence of any detergent component.

B. pH-Based Lysis (NaOH only)

[0119] Lysis study was performed by changing the NaOH concentration from 50 mM to 200 mM within a pH range of 10-12.6. Arginine was used as one of the conditions corresponding to pH 10. The data show that all lysis conditions ranging from 50 mM to 200 mM NaOH resulted in fully intact and non-nicked ceDNA (**FIG. 5A**). Furthermore, using a lysis buffer containing only NaOH resulted in higher yields of ceDNA than using a lysis buffer containing a detergent (**FIG. 5B**). Based on this study, highly alkaline 150 mM NaOH was chosen as the final lysis buffer condition for further downstream studies. The 150 mM NaOH lysis condition demonstrated consistent and reproducible intact ceDNA across multiple harvests/runs. We hypothesized that the absence of SDS may mitigate nuclease activity, and the presence of highly alkaline condition (pH 10-12.6) might be beneficial towards denaturation of the nucleases present in the cell lysate.

Example 3: Impact of Lysis Hold Time

[0120] For the above experiments, the lysis hold time, i.e., the incubation time of the cells with a lysis buffer, was kept constant at 5 minutes. Due to potential irreversible denaturation of DNA structure at highly alkaline conditions, we explored the impact of various lysis hold times on ceDNA product homogeneity for lysis with 150 mM NaOH. T5 digestion was used to indicate the presence of nicks in ceDNA. The experiment was designed to evaluate different lysis time: 2.5, 5, 7.5, or 12.5 minutes in batch mode followed by purifying ceDNA using Sartobind® Q in high throughput format. Based on the agarose gel electrophoresis, the ceDNA band showed decrease in intensity and homogeneity with increase in time. The study shows that a lysis hold time of 2.5 to 5 minutes gave rise to the best yield of intact ceDNA (**FIG. 6A**) while reducing impurities such as baculovirus and insect cell DNA (**FIG. 6B**).

[0121] We next investigated different modes of mixing at lab scale to understand how the mode of mixing might affect product quality at a commercial scale. The modes were stirrer bar, overhead mixer and combination of both stirrer bar/overhead mixer. Since ceDNA is highly shear-sensitive, gentle mixing with stirrer bar provided the best result at bench scale. However, there were limitations when scaling to 500L, typically the minimum volume required for mixing in the production tank, such as large pumps required to add lysis/neutralization buffer and duration of addition of lysis/neutralization buffer. Thus, continuous in-line cell lysis, rather than batch lysis, was used to better control process parameters and to achieve efficient lysis (**FIG. 7**). Flowrates 1, 2, and 3 were the same; two flowrates were tested, 15 mL/min and 27 mL/min, dependent on shear rate – 165/sec and

308/sec. Multiple parameters were studied in the continuous in-line cell lysis system. The residence time of lysis duration tested was 3.7, 2.5, and 1.5 minutes. Different neutralization mechanisms were also tested: static mixer, T-mixer, and batch mode (in-bottle). The data show that continuous cell lysis was successfully scaled up to 10L with 2.5-minute lysis duration using static mixer and neutralization step using T-mixer (**FIG. 8**).

Example 4: Pre-clarification

[0122] We found that neutralization of the cell lysate using 3.1 M potassium acetate led to precipitation of HMW genomic DNA, host cell proteins and other cellular components and as a result, generated large flocculants. Thus, we incorporated an intermediate pre-clarification/pre-treatment step before the clarification step to remove large flocculants and extend the throughput of the clarification devices.

[0123] The initial pre-clarification approach was centered around removing the flocculants using UniFuge®/minifuge based centrifugation approach (Carr Biosystems). However, this approach resulted in shearing of the ceDNA product. This approach also did a poor job of separating the flocculants and instead resulted in much smaller flocculants, decreasing the clarification throughput to below 15L/m². While a batch centrifuge-based pre-clarification step produced intact ceDNA with a higher clarification throughput of 70-100 L/m², it would be challenging to scale up this method to manufacturing levels.

[0124] A salt-based pre-clarification approach was then developed for efficient removal of flocculants and improved filtration capacity. The initial attempt in this approach was performed by using ammonium hydrogen carbonate (i.e., ammonium bicarbonate (NH₄HCO₃); "AHC"). The use of this salt was based on the mechanism that after addition of ammonium hydrogen carbonate to the neutralized lysate, flocculants are lifted to the surface of solution by the release of carbon dioxide and ammonia. A range of AHC concentrations of 2.5 to 25 g/L was evaluated for optimal phase separation. 25 g/L of AHC was found to be the optimal salt concentration for efficient phase separation for ceDNA pre-clarification process, as compared to the 5g/L salt concentration highlighted in published literature for plasmid production in *E. coli* cells (Blom et al., *Vaccine* (2010) 29(1):6-10). AHC-based pre-clarification process also prevented the fragmentation of ceDNA product.

[0125] Although efficient phase separation was observed using AHC, this process runs the risk of producing ammonia gas, which is hazardous in large-scale manufacturing. To prevent the generation of harmful ammonia gas, an alternative salt, sodium hydrogen carbonate (i.e., sodium bicarbonate (NaHCO₃); "NaHC"), was evaluated as a possible substitution for AHC.

NaHC reacts with water to produce sodium chloride, carbon dioxide and water, and the CO₂ gas helps in the phase separation of flocculants. A comparison study was done to compare the effectiveness of cell debris separation using AHC and NaHC. Four arms were designed: 15 g/L AHC, 20 g/L AHC, 15 g/L NaHC, and 20 g/L NaHC. 900 mL of neutralized lysate was added into a 2L bag (i.e., with about 50% void space in bag) containing 15 or 20 g/L of AHC or NaHC. We observed that NaHC gave rise to less bloating as compared to AHC, while maintaining the cell debris separation from supernatant. Additionally, comparison of the ceDNA product quality via T5 exonuclease treatment indicated that the full-length ceDNA band integrity was maintained with the use of NaHC as a pre-clarification salt (**FIG. 9**).

[0126] We then evaluated whether we could change the mode of addition of salt, reduce the amount of salt and duration required for separation in a small scale. The results show that the addition of NaHC at 5 g/L or 10 g/L was sufficient to allow flocculate layer to rise to the surface, resulting in effective cell debris separation from supernatant containing ceDNA product (**FIGs. 10A-B**).

[0127] The salt-mediated phase separation approach for pre-clarification was able to be implemented on a 500 L scale with no product losses. Approximately 90% (w/w) of pre-clarified supernatant could be loaded onto the depth filter. This approach improved the depth filter load capacity from 15-20 L/m² to approximately 70-100 L/m², reduced the turbidity of the pre-clarified sample from approximately 6000 nephelometric turbidity units (NTU) to about 50 NTU for depth filter loading, and did not affect the quality of the product.

Example 5: Clarification

[0128] After separation of the flocculant phase and the liquid phase of the cell lysates, clarification was done to remove the cell debris and particles from feed stream. The choice of clarification filters was driven largely by cell density, viabilities, cell type, and harvest viscosities. The objective of this study was to identify filters for robust scalable ceDNA purification. The performance indicators included qPCR as well as filter throughput and turbidity. Table 1 below lists the filters tested.

Table 1. Filters for Clarification

Filters	Chemistry	Rationale
J100 HDC, Pall (Control)	Polypropylene, 10 µm	Current process (control)
D0HC, Millipore	Double layer, Cellulose,	PCL platform filter.

	Inorganic filter aid, 0.6-8.0 μm	plasmid clarification (Literature)
Clarisolve 60HX, Millipore	Polypropylene, Synthetics, 7.5-60 μm	Non-charged, single-stage and smaller footprint (Literature)
D0SP, Millipore	Double layer, Silica filter aid / Polyacrylic, non-woven, 7.5-60 μm	New version / technology, Synthetic
10SP02, ZetaPlus, 3M	Positively charged, crosslinking polymer, 0.8-5.0 μm	Alternative vendor

[0129] The comparative study shows that the Clarisolve 60HX filter (Millipore) and the D0HC filter (Millipore) provided satisfactory yields, throughputs, and turbidity (**FIG. 11**). Pre-clarification with NaHC was shown to increase the throughput of clarification filters.

- 5 Further, two feed fluxes were tested: 75 LMH and 150 LMH, with the max feed pressure tested being 15 psi. The maximum throughput obtained was 66 L/m² with 75 LMH and 48 L/m² with 150 LMH (**FIG. 12**).

Example 6: rRNA Removal

- 10 [0130] The changes made to the lysis buffer conditions appeared to have led to a significant amount of residual RNA (rRNA) released post lysis. Due to the concerns about using animal- derived (primarily from bovine pancreas) RNase in the ceDNA purification process, we investigated the use of CaCl₂ in removing rRNA. CaCl₂ was added to the sample to reach a final concentration of 0.5, 1, 1.5, or 2 M. We used purified material (Q eluate)
- 15 under two buffer conditions: (i) 1 M potassium acetate (pH 5.5); and (ii) 25 mM Tris (pH 8.0) + 10 mM EDTA + 0.9 M NaCl. Two total DNA concentrations were tested based on Nanodrop: 75 and 250 $\mu\text{g/mL}$. The data show that regardless of the DNA concentration and buffer condition, increased amounts of CaCl₂ led to decreased amounts of RNA in the sample. 2M CaCl₂ was chosen for subsequent studies.
- 20 [0131] In order for efficient binding to anion exchange resin (Sartobind® Q) to occur, the CaCl₂-treated cell lysate was concentrated and buffer-exchanged. To this end, we investigated different tangential flow filtration (TFF) cassette screens (T screen and J screen) and molecular weight cutoffs (MWCO; 10 kDa and 100 kDa) and encountered poor permeate flux due to viscosity issues. Hence, we split the process into two steps: one to concentrate
- 25 only (ultrafiltration or UF), treat with CaCl₂ and then perform buffer exchange/concentrate (UF/DF). The experimental design, with four different processes, is shown in **FIG. 13A**. Treatment with CaCl₂ resulted in higher percent ceDNA purity compared to samples lacking CaCl₂ treatment (**FIG. 13B**). Furthermore, the ceDNA band intensity was comparable

among the four processes (left bars of each of the groups of twos); however, significant removal was observed in samples treated with UF, followed by CaCl₂ precipitation and then UF/DF (Condition 3) (**FIG. 13C**).

[0132] We further investigated whether CaCl₂ treatment helps in removing impurities other than rRNA. This study compared CaCl₂-treated samples with or without added RNase A. The precipitates from the samples were solubilized to run onto agarose gel. The data show that calcium chloride addition resulted in about 70-90% of reduction in impurities, including DNA from the baculoviral vectors and the Sf9 genome, as well as fragmented ceDNA (below 5 kb) and rRNA.

Example 7: Evaluation of Capture Chromatography Media

[0133] Previous experiments utilized commercial gigaprep plasmid DNA purification kits to recover the ceDNA product. However, this process lacked selectivity in distinguishing between the ceDNA product and other nucleic acid impurities. To optimize ceDNA capture, we screened different chromatography media ranging from membrane, monoliths, and resins.

[0134] With regard to chromatography membranes, the Natrix® HDQ (Millipore Sigma), Mustang™ Q (Cytiva) and Sartobind® Q (Sartorius) membranes were evaluated. Natrix® HDQ and Sartobind® Q were both found to be capable of handling a high load challenge of 200 mL/MV and 178 mL/MV, respectively. However, Mustang™ Q fouled after loading 18 mL/MV of clarified ceDNA cell culture lysate. Natrix® HDQ and Sartobind® Q both exhibited a ceDNA eluate peak that was further verified by agarose gel electrophoresis.

[0135] With regard to chromatography monoliths, the CIM® DEAE (2 µm channel) anion-exchange monolith (BIA Separations) also ran into pressure issues similar to Mustang™ Q. However, the loading capacity was relatively higher at 64 mL/MV.

[0136] With regard to resins, the POROS™ 50D anion exchange resin (Thermo Scientific) did not exhibit fouling issues; however, no ceDNA peak was observed in the eluate. Similar behavior was seen for the HyperCel™ STAR AX resin (Sartorius).

[0137] The Sartobind® Q membrane was selected for further studies. After evaluating the elution profile and then spiking in different concentrations of NaCl in the loading material, we found that 150 mM NaCl and 300 mM NaCl in the load shifted the impurity peak (composed primarily of low molecular weight species) to the flow-through fraction (**FIGS. 14A-B**). Droplet digital PCR and qPCR titer assays confirmed that the addition of NaCl to the load samples did not affect the titer of ceDNA in the eluate fractions (**Table 2**). Optimizing the load conductivity can direct low molecular weight impurities to the

flowthrough or wash fractions. This strategy potentially increases the binding capacity for the target molecule by reducing chances of impurities competing for available binding sites on the membrane (**FIG. 15**).

Table 2. Recovery of ceDNA

Eluate Fractions	qPCR ceDNA Titer ($\mu\text{g/mL}$)	ddPCR ceDNA Titer ($\mu\text{g/mL}$)
Eluate from load with no added NaCl	9.6	6
Eluate from load with 150 mM NaCl	6.7	4.8
Eluate from load with 300 mM NaCl	7.8	5.4

5

[0138] The ceDNA capture process with the Sartobind® Q membrane was evaluated with two different upstream processes: the one-bac system (using a producing cell line (PCL) that stably expressed a factor VIII transgene and was transiently transfected with a baculoviral vector for expressing AAV Rep proteins); and the two-bac system (Sf9 cells transiently transfected with two baculovirus vectors encoding AAV Rep proteins and factor VIII, respectively).

10

[0139] For the two-bac systems, we tested different MOIs of Rep/FVIII transgene (0.01/0.1, 0.05/0.5, 0.2/2.0, or 0.3/3.0). We found that the two-bac system (0.2/2.0 MOI) yielded 4-5X higher ceDNA titer than the one-bac system. We also observed a reduction of host cell proteins (HCP) with a log reduction value (LRV) of about 3. Due to the high conductivity load (about 62-70 mS/cm) due to the presence of 0.75-1.0 M potassium acetate and 300 mM NaCl, most of the HCPs were able to flow through during the capture step. The final elution of ceDNA was done with 900 mM NaCl.

15

20 **Example 8: Evaluation of ceDNA Polishing Methods**

[0140] The capture purification using the Sartobind® Q membrane provided good separation of HCP and RNA impurities in the flow-through and wash fractions. Additionally, Sartobind® Q resulted in a 20-30% reduction of Sf9 DNA and baculoviral DNA. However, complete removal of nucleic acid impurities was not observed across the capture step; some RNA, Sf9 DNA, and baculoviral DNA co-eluted with the ceDNA product. The purified ceDNA from the capture step was also composed of ceDNA fragments (size < 4.5 kb).

25

[0141] To polish the Sartobind® capture product, we investigated the use of a one-column or two-column approach to further separate the ceDNA product from product-related impurities (ceDNA lower molecular weight fragments) and process-related impurities (Sf9 DNA, baculoviral DNA, and RNA).

30

[0142] Several polishing adsorbent candidates were reviewed (Table 3). Hydrophobic interaction chromatography (HIC) mode was selected for further studies due to its ability to resolve between different isoforms of DNA and separate DNA isoforms from more hydrophobic nucleic acid components such as RNA, genomic, and denatured DNA.

5 **Table 3. Candidate Chromatography Columns for Polishing ceDNA Product**

Polishing Candidate	Modality and Adsorbent Type	Ligand	Pore or Channel Size
C4 HLD Monolith (Sartorius)	HIC monolith	C4 aliphatic	2 μ m
POROS TM Ethyl (Thermo Scientific)	HIC perfusive resin	Ethyl	100 nm
POROS TM Benzyl (Thermo Scientific)	HIC perfusive resin	Benzyl	100 nm
POROS TM Benzyl Ultra (Thermo Scientific)	HIC perfusive resin	Benzyl ultra*	100 nm
Capto TM Core 700 (Cytiva)	Core shell resin with multimodal aspects	Octylamine	700 kDa MWCO

*Higher density of benzyl ligands per unit area of the resin.

A. Evaluation of C4 HLD Monolith Columns

[0143] The C4 HLD monolith columns (Sartorius) have high ligand density and are butyl-modified. They were evaluated as a potential polishing medium for ceDNA purification. Binding of DNA to C4 HLD HIC monolith columns requires a high amount of ammonium sulfate (AS). Thus, the HIC load was adjusted to 3 M AS to induce hydrophobic interactions. Elution was then achieved by performing a linear descending ammonium sulfate salt gradient from 3 M to 0 M over 60 CV. Under these conditions, it was observed that the ceDNA product eluted over a wide elution range (2.25 M to 0 M AS) with typical four-peak elution behavior. A size-based selectivity was observed between the full-length ceDNA and lower molecular weight (LMW) fragments with more full-length ceDNA observed in the early elution fractions (2.25 M to 1.5 M AS) and LMW ceDNA fragments eluting in the later fractions (1.5 M to 0 M AS). We observed that ceDNA fragments, baculoviral DNA, Sf9 DNA, and RNA bound more strongly to the column and could be efficiently separated from the ceDNA product. High strength of binding of these species could have resulted from the stronger interaction of LMW nucleic acid fragments with the C4 butyl ligand.

[0144] Based on the linear gradient results, a step elution process was implemented for the ceDNA polishing process. Since full length ceDNA was observed to elute between 2.25 M to 1.5 M AS, a step elution was performed at a 1.74 M AS concentration to recover pure and

concentrated full length ceDNA. The step gradient also included steps at 0.9 M, 0.45 M, 0.24 M and 0 M AS to capture ceDNA and other nucleic acid fragments. It was observed that implementing a step elution at 1.74 M AS resulted in recovery of roughly 77% of ceDNA product (FIGs. 16A-B and Table 4).

Table 4. Step Elution Results

Eluate Fraction	Conductivity (mS/cm)	% ceDNA (qPCR*)
E1 – 1.74 M (NH ₄) ₂ SO ₄	210	77
E2 – 0.9 M (NH ₄) ₂ SO ₄	120	33
E3 – 0.45 M (NH ₄) ₂ SO ₄	75	7
E4 – 0.24 M (NH ₄) ₂ SO ₄	45	3
E5 – 0 M (NH ₄) ₂ SO ₄	5	5

*qPCR mass balance: 125%.

[0145] The weaker binding of the full length ceDNA to the C4 aliphatic ligand makes it possible to operate the C4 HLD monolith polishing purification both in a bind-and-elute approach or a flow-through approach for ceDNA purification. We performed a head-to-head comparison of the two approaches.

[0146] For the bind-and-elute approach, the elution of ceDNA was performed at 1.74 M AS and LMW fragments were removed in the later strip fractions. This approach resulted in a ceDNA product recovery rate of 66%, and 84% reduction (0.8 LRV) of residual Sf9 DNA and 81% reduction (0.7 LRV) of residual baculoviral DNA (FIGs. 17A-B and Table 5).

Table 5. ceDNA Recovery in Bind-and-Elute Approach

Eluate Fractions	% ceDNA Recovery (qPCR)
1.74 M eluate	66
0.81 M strip	18
0.45 M strip	3
0 M strip	9

[0147] Since the full length ceDNA product eluted earlier, we investigated a flow-through approach to resolving ceDNA with nucleic acid impurities (e.g., ceDNA fragments, baculoviral DNA, Sf9DNA, and RNA). One advantage of the flow-through approach over the bind-and-elute approach is that more material can be loaded onto the monolith at one go without any significant impact to the impurity clearance. To optimize the flow-through approach, the Sartobind® Q eluate was adjusted to a 1.5 M AS concentration and loaded onto the monolith. Analyzing the column fractions by agarose gel electrophoresis indicated that the flow-through fraction contained purified ceDNA product while the strip fractions removed LMW species. This approach resulted in a ceDNA product recovery rate of 64%,

and 96% reduction (1.4 LRV) of residual Sf9 DNA and 97% reduction (1.5 LRV) of residual baculoviral DNA (FIGs. 18A-B and Table 6).

Table 6. ceDNA Recovery in Flow-Through Approach

Eluate Fractions	% ceDNA Recovery (qPCR)
1.5 M flow-through	64
1.2 M strip	7
1 M strip	5
0.8 M strip	3
0 M strip	13

- 5 [0148] The studies here show that the ceDNA purification process is amenable to both the bind-and-elute and flow-through modes of chromatography using the C4 HLD monolith columns.

B. Evaluation of Perfusive HIC Resins

- 10 [0149] As an alternative to the C4 HLD monolith for ceDNA polishing, POROS™ resins were evaluated for efficiency of product separation. POROS™ resins have wide pore sizes in the order between 100-400 nm, making them ideal resin candidates for large biomolecule purification without any significant impact on binding capacity. Three POROS™ HIC candidates were tested to evaluate if the nature of HIC ligands (ethyl or benzyl) combined with perfusive flow properties would provide improved resolution between linear ceDNA
15 product and process- and product-related impurities.

- [0150] Experiments were performed by loading the Sartobind® Q eluate adjusted to a 3 M AS concentration at a load challenge of 2 mg/mL-resin, and the retention behavior of linear ceDNA product and nucleic acid impurities was mapped by performing reverse linear gradient from 3-0 M AS over 60 CV. As a comparison, C4 HLD was also evaluated as a
20 reference candidate using the same chromatographic experiment protocol.

- [0151] Three distinct elution peaks were observed after overlaying the chromatographic elution profile of POROS™ Ethyl, POROS™ Benzyl, POROS™ Benzyl Ultra and C4 HLD Monolith. On analysis of the eluate fractions (E1, E2, and E3) from the four HIC adsorbent candidates on agarose gel, it was observed that the E1 elution profile consisted of the highest
25 purity of intact ceDNA, with the E2 and E3 fractions primarily composed of truncated ceDNA fragments, RNA impurities, and Sf9 and baculoviral DNA fragments. qPCR analysis exhibited that all four HIC candidates (POROS™ Ethyl, POROS™ Benzyl, POROS™ Benzyl Ultra, and C4 HLD Monolith) improved ceDNA process-related purity from 48% to above 80%. However, ceSDS lab chip analysis exhibited monomeric ceDNA purity between

25-35% with POROS™ Benzyl and C4 HLD Monolith (**FIGs. 19A-B**). The data show that all of the tested POROS™ resin candidates can be used as an alternative to C4 HLD monolith since all three resin candidates show sufficient resolution in separating the pure ceDNA intact product in first eluate fraction.

- 5 **[0152]** All three POROS™ HIC resins behaved similarly, however POROS™ Ethyl exhibited highest binding capacity compared to Benzyl or Benzyl Ultra, where it as able to accommodate a load challenge of 2.5 mg/mL resin. The POROS™ HIC adsorbents demonstrated an inverse relationship in binding capacities for ceDNA material as a function of increasing hydrophobic strength of the HIC ligands (Ethyl, Benzyl, and Benzyl Ultra).
- 10 Typically, a stronger HIC ligand is expected to exhibit higher binding capacities for biomolecules compared to a ligand with lower hydrophobicity strength. However, despite Benzyl Ultra displaying the highest hydrophobicity strength among the tested ligands, followed by Benzyl, with Ethyl being the least hydrophobic, it was intriguing to observe that the POROS™ Ethyl resin exhibited higher binding capacities for ceDNA compared to
- 15 POROS™ Benzyl and POROS™ Benzyl Ultra.

- [0153]** This disparity can be attributed to the grafting process used to attach the ligands to the resin matrix, which significantly influences the pore structure and accessibility. In this context, the grafting nature of hydrophobic interaction chromatography ligands results in a smaller effective pore size for POROS™ Benzyl Ultra compared to POROS™ Ethyl. The
- 20 narrow pore size of POROS™ Benzyl Ultra induces steric hindrance for large DNA biomolecules and introduces additional diffusion mass transfer limitations, thereby reducing the available surface area for binding and ultimately limiting the binding capacity. Conversely, the more open pore structure of POROS™ Ethyl facilitates the diffusion of DNA biomolecules into the pores, allowing access to the internal surface area for binding, which
- 25 enhances the binding capacity. The DNA binding capacities of HIC adsorbents evaluated for polishing purification in both bind-and-elute and flow-through modes is shown in Table 7.

Table 7. DNA Binding Capacities of HIC Adsorbents

Capacity of HIC Adsorbents (mg DNA /mL-column)		
Polishing Adsorbents	Bind-and-elute mode	Flow-through mode
C4 HLD	4 mg	4 mg
POROS™ Ethyl	2.5 mg	4 mg
POROS™ Benzyl	2 mg	4 mg
POROS™ Benzyl Ultra	0.7 mg	4 mg

[0154] To further optimize the use of a POROS™ HIC column for polishing the ceDNA elution product, a comparison experiment was performed to compare the use of the POROS™ Benzyl Ultra HIC resins in a bind-and-elute approach and a flow-through approach (FIG. 20). For the bind-and-elute approach, the load was first adjusted to a final concentration of 3 M AS. Elution from the POROS™ Benzyl Ultra HIC column was performed using a reverse gradient approach by reducing the AS concentration from 3 M to 0 M over 60 CV. The POROS™ Benzyl Ultra HIC column was observed to have a binding capacity with breakthrough observed close to 0.7 mg/mL-resin load challenge. The bind-and-elute approach exhibited greater than 90% reduction of baculoviral and Sf9 DNA, with ceDNA product recovery of 42% due to loss of ceDNA in flow-through as a result of column breakthrough (FIGs. 21A-B).

[0155] In comparison to the bind-and-elute column operation, the POROS™ Benzyl Ultra HIC column was also assessed for the efficiency of flow-through operation. In the experiments described above, ceDNA was observed to elute between 2 M-1.5 M during the linear gradient run on the POROS™ Benzyl Ultra HIC column. For the flow-through operation, the load was first adjusted to a final AS concentration of 1.8 M and loaded onto the POROS™ Benzyl Ultra HIC column. Under 1.8 M AS concentration, the ceDNA product was resolved in the flow-through section and only nucleic acid impurities like Sf9, baculoviral DNA, and RNA bound onto the column and was subsequently removed in a strip step performed with a buffer containing 50 mM Tris and 10 mM EDTA, pH 8, and no salts. The flow-through approach resulted in 92% ceDNA product recovery with 62% reduction in process-related impurities. Evaluation of the ceDNA purity via agarose gel indicates similar purity for both bind-and-elute and flow-through modes operation.

C. Evaluation of Capto™ Core Resin to Polish Purified ceDNA

[0156] We also evaluated the core shell resin from Cytiva (Capto™ Core 400 and Capto™ Core 700) for use in a polishing step. Capto™ Core resin is based on a core shell or core bead concept with each resin bead having a ligand activated core and an outer inactive layer without ligands. The outer layer has size exclusion properties which prevents large targets from entering the beads, whereas smaller protein and DNA impurities are able to enter into the core where they bind to the hydrophobic and positively charged octylamine ligands. Capto™ Core resin has not been previously used as a polishing approach in DNA purification process. The Capto™ Core 700 resin has a larger pore structure and a larger MWCO than the Capto™ Core 400 resin (750 kDa vs. 400 kDa).

[0157] Our studies show that the Capto™ Core 700 resin was more effective in reducing Sf9 and baculoviral DNA impurities (>70% reduction), as compared to the Capto™ Core 400 resin (<50% reduction) (**Table 8**).

Table 8. ceDNA Recovery with Capto™ Core Resin

Outcome	Capto™ Core 700 Flow Through	Capto™ Core 400 Flow Through
qPCR ceDNA Recovery (%)	76	96
Reduction in sf9DNA by qPCR (%)	82	45
Reduction in bacDNA by qPCR (%)	76	43
ceDNA Purity by qPCR (%)	59	38

5

[0158] Chromatographic overlay of the flow-through blocks shows a higher peak height for Capto™ Core 400 over 700, exhibiting lower removal of impurities in the flow-through product portion (**FIG. 22A**). Agarose gel analysis of the flow-through fraction from these columns exhibit less smearing of ceDNA product with Capto™ Core 700 resin compared to
 10 Capto™ Core 400 resin (**FIG. 22B**). Overall, the Capto™ Core 700 resin performed well in a polishing step in ceDNA purification.

Example 9: Feasibility of Viral Filtration

[0159] Baculovirus is endogenously present in Sf9 cells due to the use of baculoviral virus
 15 for transduction and protein expression. Additionally, the original Sf9 cell line is known to be contaminated with rhabdovirus. Thus, clearance of viral contaminants is necessary in the present ceDNA purification scheme. A challenge of using viral filtration for DNA purification is the size of the product. The radius of gyration of the ceDNA monomer (about 150 nm) makes it difficult to distinguish ceDNA from baculoviral DNA by size-exclusion
 20 separation due to the size limitation. To evaluate the feasibility of viral removal in ceDNA purification, we utilized the Planova™ 35N size-based virus removal filter.

[0160] To understand the feasibility of implementation of viral filtration in the ceDNA platform process, three trains were tested with the Planova™ 35N 0.001 m² viral filter using the PendoTECH normal flow filtration (NFF) filter screening system. The experimental
 25 trains comprised three different feed streams: the Sartobind® Q eluate (50 mM Tris pH 8.0, 10 mM EDTA, 0.9 M NaCl), the C4 HLD load (50 mM Tris pH 8.0, 10 mM EDTA, 0.45 M NaCl, 1.5 M AS) and the C4 HLD product (flow-through and wash volume) (50 mM Tris pH 8.0, 10 mM EDTA, 0.45 M NaCl, 1.5 M AS). The pressure was controlled at 12 psi, and the tested throughput was 50 L/m². The data show that the ceDNA recovery from the

Sartobind® Q train and the C4HLD product trains were each >50% based on A260 absorbance (**Table 9**). Additionally, analysis of ceDNA product purity by agarose gel electrophoresis show a reduction of contaminating species from the C4 HLD product train, indicating that the Planova™ 35N viral filter represented a feasible size-based viral removal step in ceDNA purification (**FIG. 23**).

Table 9. ceDNA Recovery After Viral Filtration

Condition	% ceDNA Recovery by qPCR
Train 1 – Sartobind® Q eluate	78
Train 2 –C4 HLD load	20
Train 3–C4 HLD flow-through	59

Example 10: Evaluation of Final Tangential Flow Filtration Parameters

[0161] The presence of 1.5 M AS in the final tangential flow filtration (TFF) load appeared to have significantly alter DNA conformation. To address this issue, we evaluated the efficiency of different TFF cassette screens (L screen, J screen, Repligen) and molecular weight thresholds (10 kDa and 30 kDa) for use in a final TFF step. The C4 HLD column was used in flow-through mode and concentrated 10X. The buffer was exchanged (about 6-8 DV) into 1X TE buffer. The retentate material post UF/DF TFF was then run on an agarose gel to understand the ceDNA product quality via band intensity. It was observed that the 30 kDa MWCO TFF membrane resulted in loss of ceDNA in the permeate. However, the 10 kDa MWCO TFF membrane, resulted in no loss of ceDNA product in permeate and obtained 100% recovery based on qPCR as well as band intensity on an agarose gel (**FIG. 24**). The 10 kDa MWCO TFF membrane could therefore be used as a final purification step when the polished ceDNA product contains high molar concentrations of ammonium sulfate.

Example 11: Development of a Three-Column Purification Process

[0162] A three-column purification approach was tested for ceDNA purification (**FIG. 25**). In this approach, ceDNA was purified using the Sartobind® Q capture column. The Sartobind® Q eluate was further polished using four different polishing approaches comprising of C4 HLD HIC monolith in bind-and-elute mode, C4 HLD HIC monolith in flow-through mode, POROS™ Benzyl Ultra HIC resin in bind-and-elute mode, and POROS™ Benzyl Ultra HIC resin in flow-through mode. Finally, the eluate from the bind-and-elute secondary column approach was combined and purified further using the Capto™ Core multimodal core shell resin. ceDNA product quality attribute for all these runs were analyzed using agarose gel electrophoresis along with ce-SDS lab chip.

[0163] The agarose gel and lab chip results are shown in **FIG. 26**. The data show that ceDNA intact purity from the initial purification process (Sartobind® Q – HIC polishing) ranged between 27-34%. The ce-SDS purity of intact ceDNA increased to 80% and greater after the incorporating CaCl₂ precipitation and two-column polishing process comprising of HIC and Capto™ Core polishing purification steps.

[0164] Both hydrophobic interaction chromatography adsorbents and Capto™ Core shell resins demonstrated significant potential in removing process- and product-related impurities. However, to achieve enhanced purity, a two-column polishing strategy was deemed essential. This approach combines the ability of HIC adsorbents to remove primarily lower molecular weight nucleic acid impurities (0.1 kb–4 kb) with the ability of Capto™ Core resins to remove mid-sized nucleic acid impurities (0.1 kb–6 kb).

[0165] Two polishing strategies were evaluated by altering the order of polishing columns to enhance the quality of intact closed-ended DNA (ceDNA) (**FIG. 27**):

Polishing Strategy 1: Capto™ Core followed by HIC adsorbent;

Polishing Strategy 2: HIC adsorbent followed by Capto™ Core resin

[0166] Additionally, the efficacy of calcium chloride (CaCl₂) precipitation of the clarified feed material in improving ceDNA product quality was assessed. This was achieved by conducting a comparative purification study involving capture, intermediate, and final polishing steps using both non-CaCl₂ and CaCl₂-treated clarified feed material. For the non-CaCl₂ treated feed material, both polishing strategies were evaluated and designated as Train 1 and Train 2. For the CaCl₂-treated feed material, only the HIC followed by Capto™ Core sequence was evaluated, designated as Train 3.

[0167] Process conditions were optimized to facilitate the direct loading of eluate from the capture column onto the intermediate polishing column, and subsequently onto the final polishing column with minimal adjustments. This enables a fully integrated continuous approach for the capture, intermediate polishing, and final polishing steps.

[0168] For Train 1, where the polishing process involves Capto™ Core resin followed by HIC adsorbent, the Sartobind® Q eluate in 50 mM Tris, 10 mM EDTA, 0.9 M NaCl, pH 8.5, can be directly loaded onto the Capto™ Core column. The product from the Capto™ Core intermediate polishing step in the same buffer matrix can then be loaded onto the final HIC polishing adsorbent after adjusting to 3 M or 1.5–2 M ammonium sulfate (AS) in 50 mM Tris, 10 mM EDTA, 0.45 M NaCl, pH 8, depending on whether the HIC step is operated in bind-and-elute or flow-through mode. The final purified ceDNA material is recovered in the

eluate (for bind-and-elute mode) or the flow-through and wash fractions (flow-through mode) in 1.5–2 M AS in 50 mM Tris, 10 mM EDTA, 0.45 M NaCl, pH 8.

[0169] For Train 2 and Train 3, where HIC adsorbent is used as the intermediate polishing step followed by Capto™ Core resin, the Sartobind® Q capture eluate in 50 mM Tris, 10 mM EDTA, 0.9 M NaCl, pH 8.5, is adjusted to 3 M or 1.5 M AS in 50 mM Tris, 10 mM EDTA, 0.45 M NaCl, pH 8, and loaded onto the HIC adsorbent operated in either bind-and-elute or flow-through mode. Following the HIC polishing step, the ceDNA product, within the 1.5–2 M AS range, is then loaded onto the Capto™ Core column. The final purified ceDNA product is obtained in the flow-through and wash fractions in the same buffer matrix as the Capto™ Core loading condition.

[0170] The recovery results from this study are presented in Table 10, providing a comparative overview of ceDNA step recovery at various stages. Additionally, the product quality attributes of ceDNA were examined using various orthogonal assays, including qPCR, gel densitometry (Agarose gel), capillary gel electrophoresis (LabChip), and next-generation sequencing (NGS) short-read sequencing. Product-related purity, indicating the percentage of intact full-length ceDNA, was assessed by gel densitometry and capillary gel electrophoresis. Process-related purity, indicating the percentage of ceDNA transgene, was more accurately determined by NGS short-read sequencing and qPCR analysis. The product quality metrics for the two polishing approaches using non-CaCl₂ and CaCl₂ treated feed material are further presented in FIG. 28 and FIG. 29.

Table 10. Estimated Process Recovery Using Two Polishing Approaches for Non-CaCl₂ and CaCl₂-Treated Clarified Feed Material

Unit Operation	Train 1 (n=1)	Train 2 (n=1)	Train 3 (n=2)
Lysis-Neutralization	50-60%		
Clarification	60-80%		
Precipitation	non CaCl ₂		CaCl ₂ Treated
CaCl ₂ & TFF			20-40%
Sartobind® Q Capture	50-60%		90-100%
Order of Polishing	Capto™ Core>HIC	HIC>Capto™ Core	HIC>Capto™ Core
Intermediate Polishing	65-80%	50-60%	60-80%
Final Polishing	50-60%	40-50%	40-50%
Final Recovery	5-14%	3-9%	2-8%

Example 12: Exemplary Purification Process

[0171] An exemplary process using the aforementioned tested steps is further described below.

A. Cell Harvest and Resuspension

[0172] Sf9 insect producer cells previously infected with two baculoviral expression vectors were concentrated by a factor of 4-6X using a continuous centrifugation system at 2000 x g with a flow rate of 3 L/min (Minifuge/UniFuge®, CARR Biosystems UniFuge®).

- 5 The concentrated Sf9 cells were resuspended in 1X PBS, pH 7.4 (2.7 mM KCl, 11 mM phosphate, 135 mM NaCl) wash buffer to produce a cell paste.

B. Cell Lysis and Neutralization

- [0173] The resuspended cell paste was immediately lysed through alkaline lysis by addition of 0.3 M NaOH to the suspension for a final concentration of 150 mM NaOH. The
10 cell paste was lysed via continuous in-line lysis with a flow rate of 15 mL/min. The cell paste was lysed under alkaline conditions for no more than five minutes, preferably no more than 2.5 minutes.

- [0174] In continuation of the continuous in-line system, the highly alkaline cell lysate (pH >12.5) was immediately neutralized by the addition of 3.1 M potassium acetate, pH 5.2,
15 until the lysate pH reached pH 5.5-5.6. Neutralization of the cell using potassium acetate led to the precipitation of HMW genomic DNA, HCP, and other cellular components, resulting in the generation of large flocculants in the cell lysate.

C. Pre-Clarification of Cell Lysate

- [0175] As a pre-clarification step prior to clarification and filtration, the flocculant cell
20 lysate was treated with 10 g/L of sodium bicarbonate and stirred for ~30 seconds. The reaction then was allowed to proceed for about two hours. This pre-clarification treatment caused a phase separation between the flocculants and the cleared cell lysate.

D. Clarification and Concentration of the Cell Lysate

- [0176] To remove the cell debris and particles generated during the neutralization and pre-clarification steps, the neutralized cell lysate was passed through either a 23 cm² Clarisolve 60HX (Millipore) or 23 cm² D0HC (Millipore) depth filter at a flow rate of 50 L/m²/hr. To
25 reduce the volume, the clarified cell lysate was subjected to a TFF system to concentrate the cell lysate six- to ten-fold. The tangential flow filtration step was conducted in 50 mM Tris, pH 8.0, 10 mM EDTA, 0.3 M NaCl.

- 30 **E. Precipitation of Impurities**

[0177] To precipitate contaminating RNA and DNA impurities from the clarified cell lysate, the clarified cell lysate was supplemented with 5 M CaCl₂ for a final concentration of 2 M CaCl₂ in solution. The precipitation reaction was allowed to proceed at room temperature for 30 minutes. The clarified cell lysate was passed through a second tangential

flow filtration system to exchange the buffer into 50 mM Tris, pH 8.0, 10 mM EDTA, 0.3 M NaCl. The buffer was exchanged six times.

F. Isolation and of ceDNA Product

[0178] To purify the target ceDNA, the clarified cell lysate was passed through a Sartobind® Q anion exchange chromatography column. After binding of the ceDNA to the column, the column was washed with 50 mM Tris, pH 8.0, 10 mM EDTA. The column was washed a second time with 50 mM Tris pH 8.0, 10 mM EDTA, 0.6 M NaCl. Following the washing steps, the ceDNA was eluted from the Sartobind® Q column with 50 mM Tris pH 8.0, 10 mM EDTA, 0.9 M NaCl. The anion exchange chromatography step proceeded with a flow rate of 3 matrix volumes/min.

G. Polishing

[0179] To polish the Sartobind® Q eluted ceDNA, the eluate was applied to an HIC chromatography column. The HIC column was either a C4 HLD Monolith 2 µm channel column (Sartorius) or POROS™ perfusive resin (Thermo Scientific). Using the Monolith C4 HLD column, the ceDNA product was isolated via either a bind-and-elute or flow-through method. In the bind-and-elute method, the Sartobind® Q-eluted ceDNA was first supplemented to 3M ammonium sulfate to induce hydrophobic interactions. After binding of ceDNA, the C4 HLD monolith was washed with 50 mM Tris pH 8.0, 10 mM EDTA, 0.45 M NaCl, 1.5 M ammonium sulfate. Following the wash step, the bound ceDNA was eluted from the column by a step-wise reverse salt gradient of ammonium sulfate (3 M, 1.74 M, 0.9 M, 0.45 M, 0.24 M, 0 M) in 50 mM Tris pH 8.0, 10 mM EDTA. In the flow-through method, the Sartobind® Q-eluted ceDNA was first supplemented to 1.5 M ammonium sulfate. The unbound ceDNA product was recovered from the column in the flow-through pool using 50 mM Tris pH 8.0, 10 mM EDTA, 0.45 M NaCl, 1.5 M ammonium sulfate. Some additional ceDNA product was removed during the wash step using 50 mM Tris pH 8.0, 10 mM EDTA, 0.45 M NaCl, 1.5 M ammonium sulfate.

[0180] Alternatively, using the POROS™ perfusive HIC resin in the bind-and-elute method, the Sartobind® Q-eluted ceDNA was first supplemented to 3 M ammonium sulfate. Following sample loading, the POROS™ HIC resin was washed with 50 mM Tris pH 8.0, 10 mM EDTA, 0.45 M NaCl, 1.5 M ammonium sulfate. The bound ceDNA was eluted from the column using a reverse linear salt gradient of 3-0 M ammonium sulfate in 50 mM Tris pH 8.0, 10 mM EDTA. In the flow-through method using the POROS™ perfusive HIC resin, the Sartobind® Q-eluted ceDNA was first supplemented to 1.8 M AS. The unbound ceDNA was recovered from the column in the flow-through pool using 50 mM Tris pH 8.0, 10 mM

EDTA, 0.9 M NaCl, 1.8 M ammonium sulfate. Some additional ceDNA product was recovered during the wash step using 50 mM Tris pH 8.0, 10 mM EDTA, 0.45 M NaCl, 1.5 M ammonium sulfate.

[0181] To further polish the eluted ceDNA product, Capto™ Core-based shell multimodal resin-based purification was implemented to remove trace amounts of nucleic acid fragments from the ceDNA product. Capto™ Core can be employed as either a secondary polishing chromatography column subsequent to the Sartobind® Q capture step, or it can alternatively serve as a tertiary polishing column following the second column polishing step involving Hydrophobic Interaction Chromatography (HIC). The Capto™ Core polishing purification process was optimized to facilitate direct loading of the eluate from the preceding column purification step onto the Capto™ Core resin without necessitating any supplementation or adjustments to the load conditions.

[0182] For instance, in the scenario where Capto™ Core functions as the secondary column after the capture step, the eluate from Sartobind® Q, which contained 50 mM Tris (pH 8.0), 10 mM EDTA, and 0.9 M NaCl, was loaded directly onto the Capto™ Core resin. Subsequently, ceDNA product was removed from the flow-through and wash fractions using the same buffer conditions of 50 mM Tris (pH 8.0), 10 mM EDTA, and 0.9 M NaCl.

[0183] In the case of Capto™ Core being employed as the tertiary column after the HIC second polishing step, the ceDNA product obtained from the HIC step was directly loaded onto the Capto™ Core resin under conditions characterized by ammonium sulfate concentrations ranging from 1.7 M to 2 M in 50 mM Tris (pH 8.0) and 10 mM EDTA. Subsequently, the resin was subjected to a washing step using conditions identical to the loading condition, namely 1.7-2 M ammonium sulfate in 50 mM Tris (pH 8.0) and 10 mM EDTA.

25 H. Viral Filtration

[0184] To remove contaminating viral DNA from the purified ceDNA composition, the Sartobind® Q eluate in 50 mM Tris pH 8.0, 10 mM EDTA, 0.9 M NaCl composition or the HIC polishing product in 50 mM Tris pH 8.0, 10 mM EDTA, 0.45 M NaCl, 1.5 M ammonium sulfate composition was filtered through a 0.001 m² Planova® 35N viral filter (Asahi Kasei Bioprocess) in dead-end filtration mode. The filtration was performed under constant operating pressure of 12 psi with a target throughput of 50 L/m². After filtration, a post-recovery flush of 10 L/m² was performed with the ceDNA product composition buffer

(50 mM Tris (pH 8.0), 10 mM EDTA, 0.9 M NaCl, or 50 mM Tris (pH 8.0), 10 mM EDTA, 0.45 M NaCl, 1.5 M ammonium sulfate).

I. TFF

[0185] To prevent conformational changes in the isolated ceDNA product and concentrate the ceDNA product, the purified ceDNA composition was passed through two stages of TFF, both with a MWCO of 10 kDa. Both TFF stages were operated at a constant TMP of 8 psi, cross flow flux of 240 LMH; Stage 1 had a throughput of 20-30 L/m² and Stage 2 had a throughput of 40 L/m². Stage 1 had an ultrafiltration step to concentrate the ceDNA product 10-20 times, followed by a diafiltration step where buffer was exchanged ten times. This tangential flow filtration step exchanged the ceDNA product buffer into 10 mM Tris (pH 8.0), 1 mM EDTA. Stage 2 only had an ultrafiltration step to further concentrate ceDNA product 10 times to the desired concentration of 2 mg/mL.

Example 13: In-Process Monitoring of ceDNA Quality and Quantity

[0186] Given the complexity of ceDNA production, we have developed an ionic exchange (IEX) ultra-performance liquid chromatography (UPLC) method that could separate all process impurities from ceDNA and quantify them and the ceDNA at any given stage of the entire purification process.

[0187] Four samples taken from different stages of the Sartobind® Q purification step were used to evaluate the capability of the IEX method. The method creates separation of proteins and nucleic acids by leveraging a linear salt gradient. The ceDNA samples were directly injected or diluted with water and then injected into the IEX column. Sartobind® Q was the first purification step specific to ceDNA in the ceDNA manufacturing process. The load of the Sartobind® Q step contained many process impurities, such as host cell proteins (including, red fluorescence protein, an introduced cell marker), DNA and RNA fragments, and ceDNA. In this experiment, we chose a column containing quaternary ammonium anion exchange groups functionalized on 5 µm diameter of particles (Protein-Pak Hi Res Q (Waters™, Cat. No. 186004931).

[0188] We loaded IEX columns with the four samples, taken respectively from the load, the flow-through, the wash product, and the eluate of the Sartobind® Q purification step. The IEX columns were then run with a linear gradient of sodium chloride from 0.5 M to 2.8 M. The chromatogram traces for the four samples are overlaid in FIG. 30. As shown in the figure, the “load” and “flow-through” samples contained large peaks for host cell proteins, red fluorescent proteins, and nucleic fragments; and the “flow-through” sample contained a

very small amount of ceDNA. The “wash” sample contained just impurities (largely nucleic acid fragments and residual RNA) and did not contain a detectable amount of ceDNA. The “elution” sample contained predominantly ceDNA, with about 2500-fold reduction in the red fluorescent protein, a marker protein expressed by the ceDNA-producing cells.

- 5 [0189] The principle of IEX UPLC is to use the difference in charge property of molecules to achieve the separation. The IEX analysis here combines different wavelength of the detectors, UV and fluorescence. The above results show that IEX chromatography is a sensitive and convenient in-process method to monitor the contents of impurities and ceDNA during ceDNA purification.

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CLAIMS

1. A method for obtaining a purified preparation of closed-ended DNA (ceDNA) from ceDNA-producing cells, comprising:
 - incubating the cells in an alkaline buffer to lyse the cells to generate a cell lysate, wherein the alkaline buffer does not contain a detergent and has a pH of 10 or higher; and
 - isolating the ceDNA from the lysate.
2. The method of claim 1, further comprising, before the isolating step:
 - neutralizing the cell lysate with an acidic salt.
3. The method of claim 2, further comprising, before the isolating step:
 - pre-clarifying the neutralized cell lysate by adding sodium bicarbonate and separating the resultant flocculants from the cell lysate to generate a pre-clarified cell lysate.
4. A method for obtaining a purified preparation of closed-ended DNA (ceDNA) from ceDNA-producing cells, comprising:
 - obtaining a lysate of the cells;
 - pre-clarifying the cell lysate by adding sodium bicarbonate and separating the resultant flocculants from the cell lysate to generate a pre-clarified cell lysate; and
 - isolating the ceDNA from the lysate.
5. The method of claim 4, wherein the lysate is obtained by incubating the cells in an alkaline buffer to lyse the cells, wherein the alkaline buffer does not contain a detergent and has a pH of 10 or higher.
6. The method of claim 5, further comprising, before the pre-clarifying step:
 - neutralizing the cell lysate with an acidic salt.
7. The method of any one of claims 1-6, wherein the isolating step is performed with anion exchange chromatography.

8. The method of any one of claims 3-7, further comprising, before the isolating step, removal of RNA by calcium chloride precipitation, optionally wherein the removal of RNA is conducted by:
- subjecting the pre-clarified cell lysate to filtration to generate a clarified cell lysate,
 - and
 - subjecting the clarified cell lysate to calcium chloride precipitation to remove RNA.
9. The method of claim 8, wherein the clarified lysate undergoes ultrafiltration before addition of calcium chloride, and/or the lysate undergoes ultrafiltration and diafiltration after calcium chloride precipitation to reduce the concentration of calcium chloride.
10. The method of any one of claims 3-7, further comprising, after the isolating step, removal of RNA by calcium chloride precipitation.
11. The method of claim 10, further comprising subjecting the product from the isolating step to diafiltration after calcium chloride precipitation to reduce the concentration of calcium chloride.
12. The method of any one of claims 8-11, wherein the calcium chloride is at about 1-3 M, optionally at about 2 M.
13. The method of any one of claims 1-12, further comprising polishing the isolated ceDNA.
14. The method of claim 13, wherein the polishing step is performed with one or both of
- (i) hydrophobic interaction chromatography (HIC), optionally wherein the HIC is performed with monolith or perfusive resin; or
 - (ii) multimodal core shell resin, optionally wherein the multimodal core shell resin comprises resin beads with a size-exclusion outer shell, further optionally wherein the size-exclusion out shell has a molecular weight cutoff (MWCO) of 400 kDa or 700 kDa.
15. The method of any one of claims 1-14, further comprising, after the isolating step: viral filtration, optionally performed with a 35 nm filter.

16. The method of any one of claims 1-3 and 5-15, wherein
the alkaline buffer contains sodium hydroxide, optionally at a final concentration of
about 100-300 mM, further optionally at about 150 mM, after being added to the cells, and/or
17. The method of claim 16, wherein the incubating step does not last more than five
minutes,
optionally wherein the incubating step lasts about 2.5 minutes or about 3.5 minutes,
and
optionally wherein the incubating step is performed in a continuous in-line system.
18. The method of any one of claims 2, 3, and 6-17, wherein the acidic salt is potassium
acetate, optionally at about 2.5-3.5 M, further optionally at about 3.1 M.
19. The method of any one of claims 3-18, wherein the sodium bicarbonate is added to
reach a concentration from about 5 to about 50, optionally about 10, g/L, and/or the
flocclulants are removed by a filter with pore sizes of about 7.5-60 μm , optionally wherein the
incubation time is about two hours.
20. The method of any one of claims 1-19, wherein the isolated ceDNA is subjected to
tangential flow filtration, optionally with an MWCO of 10 and/or 100 kDa.
21. The method of any one of claims 1-20, wherein the ceDNA-producing cells are insect
cells infected with a recombinant baculovirus expression vector.
22. The method of claim 21, wherein the recombinant baculovirus expression vector
comprises a heterologous nucleic acid sequence comprising a transgene flanked by inverted
terminal repeats (ITRs), optionally wherein the ITRs are parvoviral ITRs or AAV ITRs,
optionally AAV2 ITRs.
23. The method of claim 22, wherein the heterologous nucleic acid encodes a therapeutic
protein.
24. The method of any one of claims 1-23, wherein the ceDNA-producing cells are
transgenic insect cells whose genome comprises a coding sequence for the ceDNA.

25. The method of any one of claims 1-24, further comprising in-process monitoring of ceDNA purity by measuring levels of ceDNA and impurities through ion exchange ultra-performance liquid chromatography in a sample taken before, during, or after the isolating step.