

SCALABLE PRODUCTION OF CLOSED-ENDED DNA

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority from EP Application No. 24305055.6, filed January 9, 2024; and U.S. Patent Application No. 63/662,482, filed June 21, 2024. The disclosures of the aforementioned priority applications are incorporated by reference herein in their entirety.

BACKGROUND OF THE INVENTION

[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 therapeutic gene that encodes a protein deficient in the patient. Viral vectors are commonly used for such delivery. However, viral vectors are often limited in 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 redosing potential of gene therapy.

[0003] Closed-ended DNA (ceDNA) circumvents the limitations of viral-based gene delivery. ceDNA is a closed-ended, 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 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 compared to AAV, able to accommodate more than >5 kb of genetic material, expanding the range of genetic diseases that can be targeted by gene therapy. Furthermore, the enhanced stability of ceDNA provides advantages compared to therapies based on other types of nucleic acids such as mRNA and plasmid DNA. The ability to re-dose ceDNA may enable an expanded duration of transgene expression, thus avoiding the waning efficacy sometimes observed with AAV-mediated gene therapies.

[0004] ceDNA can be produced by using a baculovirus expression vector system (BEVS). Baculoviruses infect arthropods including many insect species such as *Spodoptera frugiperda*. The BEVS platform has primarily been used to produce therapeutic proteins and

vaccine antigens. For ceDNA expression, a desired ceDNA template may be integrated into a baculovirus vector, which is then used to infect insect cells. Once infected, the insect cells produce the ceDNA from the vector. Use of the BEVS platform in ceDNA expression has safety benefits as baculoviruses are unable to replicate in human cells and the majority of human viruses are unlikely to replicate within insect cells. Consequently, there is a reduced risk associated with adventitious agents that are capable of infecting humans. In spite of these benefits, technical challenges remain for production of large quantities of ceDNA in insect cells, including high costs and long production time.

SUMMARY OF THE INVENTION

[0005] The present disclosure provides a method of producing closed-ended DNA (ceDNA), comprising (a) infecting a starting population of insect cells in a bioreactor with a first baculoviral vector comprising a parvoviral Rep gene and a second baculoviral vector comprising a template for the ceDNA, wherein the viable cell density at the time of the infection is about 4-6 million cells, optionally about 5 million cells, per mL, and wherein the multiplicity-of-infection (MOI) ratio of the first and second baculoviral vectors is 1:5 to 1:15, optionally 1:10; (b) culturing the infected cells under conditions that allow the production of the ceDNA in the cells; and (c) harvesting the cultured infected cells.

[0006] In some embodiments, the MOI of the first baculoviral vector is about 0.2 and the MOI of the second baculoviral vector is about 2.0.

[0007] In some embodiments, the power density of the bioreactor is about 25-35 W/m³, optionally about 30 W/m³.

[0008] In some embodiments, the culture medium in the bioreactor comprises about 30-60%, optionally about 30%, dissolved oxygen.

[0009] In some embodiments, the pH of the culture medium in the bioreactor is between 6.1 and 6.5, optionally about 6.4, optionally wherein the pH is adjusted by CO₂ gas or phosphoric acid and/or sodium carbonate.

[0010] In some embodiments, the temperature of the bioreactor is 27.5°C.

[0011] In another aspect, the present disclosure provides a method of producing closed-ended DNA (ceDNA), comprising (a) culturing a population of insect cells in a bioreactor at a power density of about 25-35 W/m³, optionally about 30 W/m³, wherein the culture medium in the bioreactor (i) comprises about 30-60%, optionally about 30%, dissolved oxygen, and (ii) has a temperature of 27.5°C; (b) infecting the cultured insect cells with a baculoviral vector comprising a parvoviral Rep gene, wherein the cultured insect cells

comprise a template for the ceDNA; (c) culturing the infected cells under conditions that allow the production of the ceDNA in the cells; and (d) harvesting the cultured infected cells. In some embodiments, the culture medium in the bioreactor has a pH of 6.1 to 6.5, optionally about 6.4, optionally wherein the pH is adjusted by CO₂ gas or phosphoric acid and/or sodium carbonate. In some embodiments, the viable cell density at the time of the infection is about 4-6 million cells, optionally about 5 million cells, per mL.

[0012] In some embodiments, the ceDNA template is stably integrated in the genome of the cultured insect cells.

[0013] In some embodiments, the ceDNA template is introduced to the cultured insect cells through a baculoviral vector comprising the template, optionally wherein the baculoviral vector comprising a parvoviral *Rep* gene and the baculoviral vector comprising the ceDNA template are two different vectors and optionally simultaneously introduced into the cultured insect cells. In some embodiments, the multiplicity-of-infection (MOI) ratio of the baculoviral vector comprising a parvoviral *Rep* gene and the baculoviral vector comprising the ceDNA template is 1:5 to 1:15, optionally 1:10. In some embodiments, the MOI of the baculoviral vector comprising a parvoviral *Rep* gene is about 0.2 and the MOI of the baculoviral vector comprising the ceDNA template is about 2.0.

[0014] In some embodiments, the bioreactor has two impellers and optionally has a volume of 5 L, 50 L, 500 L, or 2000 L.

[0015] In some embodiments, the ceDNA comprises a transgene flanked by inverted terminal repeats (ITRs), optionally wherein the ITRs are parvoviral ITRs or AAV ITRs, optionally AAV2 ITRs. In some embodiments, the transgene encodes a therapeutic protein (e.g., a recombinant protein that supplements an activity deficient in a patient, such as an coagulation activity or a lysosomal enzymatic activity).

[0016] In some embodiments, the cultured infected cells are harvested *via* continuous centrifugation.

[0017] In some embodiments, the baculovirus(es) for the infecting step are produced by four passage cycles. In some embodiments, each passage cycle comprises (a) inoculating insect host cells in a bioreactor with a baculovirus, wherein the viable cell density at the time of the infection is about 1 million cells per mL, and wherein the MOI of the baculovirus is about 0.1; (b) culturing the infected host cells under conditions that allow the replication of the baculovirus in the host cells; (c) harvesting the baculovirus-containing supernatant to obtain produced baculovirus; and (d) if needed, repeating steps (a)-(c) in the next passage cycle, using the produced baculovirus for step (a).

[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.

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).

[0020] **FIG. 2** is a diagram illustrating two 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. “Two-Bac”: a ceDNA production system using two baculoviral vectors. “PCL” or “One-Bac”: a ceDNA production system using a producer cell line containing stably integrated copies of a transgene cassette (e.g., a FVIII expression cassette comprising parvoviral ITRs) and one baculoviral vector for expressing a parvoviral *Rep* gene.

[0021] **FIG. 3** is a diagram depicting the growth of Sf9 insect cells in media and the production and harvest of ceDNA in an insect cell system. “BR”: bioreactor. “CCI”: cell concentration at infection (i.e., infection density).

[0022] **FIG. 4** is a set of line graphs showing the viable cell density (VCD, left) and percent viability (right) over time of insect cells infected with either a single baculovirus in a producer-like cell line (PCL) or a two-baculovirus system (Two-Bac).

[0023] **FIG. 5** is a bar graph depicting the ceDNA titers upon harvest of insect cells infected in either a PCL or Two-Bac system and grown in various sized cultures.

[0024] **FIG. 6** is a set of line graphs showing the VCD (left) and percent viability (right) over time of insect cell cultures grown at various power densities (P/V) and multiplicities of infection (MOI).

[0025] **FIG. 7** is a bar graph showing the ceDNA titers upon harvest of insect cell cultures grown at various power densities and multiplicities of infection.

[0026] **FIG. 8** is a set of line graphs depicting the VCD (left) and percent viability (right) over time of insect cell cultures grown in bioreactors configured with either one or two impellers in the bioreactor vessel.

[0027] FIG. 9 is a bar graph showing the ceDNA titers upon harvest of insect cell cultures grown in bioreactors configured with either one or two impellers in the bioreactor vessel.

[0028] FIG. 10 is a set of line graphs showing the VCD (left) and percent viability (right) over time of insect cell cultures grown with a dissolved oxygen (DO) setpoint of either 30% or 60%.

[0029] FIG. 11 is a bar graph showing the ceDNA titers upon harvest of insect cell cultures grown with a dissolved oxygen (DO) setpoint of either 30% or 60%.

[0030] FIG. 12 is a line graph showing the VCD over time of insect cell cultures grown with at a variety of temperatures and pH values.

[0031] FIG. 13 is a bar graph showing the ceDNA titers upon harvest of insect cell cultures grown at a variety of temperatures and pH values.

[0032] FIG. 14 is a set of line graphs depicting the VCD (left) and percent viability (right) over time of insect cell cultures grown at different pH values and with pH controlled via carbon dioxide gas (CO₂) or phosphoric acid (phos).

[0033] FIG. 15 is a bar graph showing the ceDNA titers upon harvest of insect cell cultures grown at different pH values and with pH controlled via carbon dioxide gas or phosphoric acid.

[0034] FIG. 16 is a bar graph showing the ceDNA titers upon harvest of insect cell cultures with a multiplicity of infection ratio of either 1:5 or 1:10 (*Rep*:FVIII transgene) grown at various VCD ranges and MOI levels.

[0035] FIG. 17 is a bar graph showing the ceDNA titers upon harvest of insect cell cultures grown with various multiplicity of infection ratios (*Rep*:FVIII transgene) and *Rep* MOI levels.

[0036] FIG. 18 is a set of bar graphs depicting the ceDNA titers upon harvest of insect cell cultures grown with various multiplicity of infection ratios (*Rep*:FVIII transgene) and *Rep* multiplicity of infection levels (left) or grown in different sizes of culture (right) at an MOI ratio of 1:10 with different *Rep* MOI levels.

[0037] FIG. 19 is a set of bar graphs showing the ceDNA titers upon harvest of insect cell cultures grown in either a shake flask (SF) or bioreactor (Ambr® 250) at various temperatures.

[0038] FIG. 20 is a line graph showing the VCD over time of insect cells grown and infected in different sizes of culture during ceDNA production.

[0039] FIG. 21 is a set of line graphs showing the VCD (left) and percent viability (right) over time of insect cells grown in different sizes of culture.

[0040] FIG. 22 is a bar graph depicting the doubling times and ceDNA titers upon harvest of insect cells grown in various sizes of culture.

[0041] FIG. 23 is a bar graph showing the viability changes between cultured insect cells and concentrated insect cells when concentrated by continuous centrifugation to various concentration factors.

[0042] FIG. 24 is a diagram depicting a process for producing baculovirus stock using Sf9 insect cell culture.

[0043] FIG. 25 is a diagram depicting the infection of insect cells with dilutions of baculovirus stock to measure baculovirus titer. This protocol is provided with BacPAK™ Baculovirus Rapid Titer kit (Takara Bio).

[0044] FIG. 26 is a bar graph showing the ceDNA titers upon harvest of insect cells infected with baculovirus after four baculovirus passage cycles (P4) or five baculovirus passage cycles (P5).

[0045] FIG. 27 is a bar graph showing the ceDNA titers upon harvest of insect cells infected with baculovirus after four baculovirus passage cycles (P4) or five baculovirus passage cycles (P5) and grown in various sizes of culture. For each pair of bars, the bar on the left is titer from P4 and the bar on the right is titer from P5. “SF”: shake flask. Ambr250: 250 mL culture.

DETAILED DESCRIPTION OF THE INVENTION

[0046] The present disclosure provides a scalable process to manufacture closed-ended DNA (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 targeted delivery to tissues of interest in patients. The methods of insect cell growth and infection described in the present disclosure improve insect cell viability and ceDNA product titers. The methods also are highly scalable, easing the cost and time required for large-scale manufacture of ceDNA product for medicinal purposes.

I. Production of ceDNA in Host Cells

[0047] 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.

[0048] 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 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, 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.

[0049] 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 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).

[0050] 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* 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.

[0051] 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 (“Mono-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 (“PCL” or “One-Bac” system). Exemplary systems of ceDNA production in insect cells are illustrated in **FIG. 2**.

[0052] The ceDNA production process provided herein can be implemented across multiple scales, including shake flasks, mini bioreactors (e.g., 100-250 mL), benchtop bioreactors (e.g., 5 L and 50 L), and large bioreactors (e.g., 500 L, 1000 L, 2000 L and 10,000 L).

II. Growth and Infection of Insect Cells

[0053] The present disclosure provides improved methods of producing ceDNA using host insect (e.g., Sf9) cells. These methods are efficient and scalable for commercial production while yielding improved ceDNA product titers. The methods are described in detail below.

A. Bioreactor Process Parameters

[0054] The present disclosure provides optimal parameters of cell culture conditions that help to produce the highest titers of ceDNA. The parameters of cell culture conditions are termed “process parameters” herein and may be applied throughout the ceDNA production process, including cell cultures before, during, and after the baculoviral infection.

[0055] One of the process parameters is power density applied to the bioreactor. Power density is indicative of the energy transferred from the impellers (stirrers) to the cell culture, defined in power/volume (P/V) units. It is a parameter used to describe mixing, gas dispersion, gas hold-up and mass transfer. In some embodiments, the power density applied to the cell culture is about 10-100 W/m³, e.g., about 10 W/m³, about 20 W/m³, about 30 W/m³, about 40 W/m³, about 50 W/m³, about 60 W/m³, about 70 W/m³, about 80 W/m³, about 90 W/m³, or about 100 W/m³. In further embodiments, the power density applied to a cell culture comprising a Two-Bac system is about 30 W/m³.

[0056] Another process parameter is tip speed. Tip speed is a measure of how fast the impellor tip is moving through the cell culture liquid in the bioreactor. It is a parameter used to describe shear to the cells and mixing. The diameter of the impellor primarily affects the tip speed. In some embodiments, the tip speed applied is about 0.2-1.2 m/s, for example, about 0.2 m/s, about 0.3 m/s, about 0.4 m/s, about 0.5 m/s, about 0.6 m/s, about 0.7 m/s, about 0.8 m/s, about 0.9 m/s, about 1.0 m/s, about 1.1 m/s, or about 1.2 m/s. In further embodiments, the tip speed applied is about 0.6 m/s for bioreactors with a maximum volume of about 250 mL. In some embodiments, the tip speed applied is about 0.7 m/s in bioreactors with a maximum volume of about 5 L. In further embodiments, the tip speed applied is about 0.96 m/s in bioreactors with a maximum volume of about 50 L.

[0057] Insect cell systems require a high volumetric oxygen transfer coefficient (kLa) to maintain sufficient cell oxygen intake. The volumetric oxygen transfer coefficient may be impacted by the bioreactor impellor, or agitator. In some cases, the cell bioreactor may have at least one impellor within the same working volume, *e.g.*, at least one impellor, at least two impellers, at least three impellers, or at least four impellers. In further embodiments, the cell bioreactor may have two impellers within the same working volume.

[0058] In some embodiments, the dissolved oxygen (DO) setpoint within the cell bioreactor may be set at about 15-75% DO. For example, the DO setpoint within the bioreactor may be about 15%, about 20%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, or about 75%. In certain embodiments, the DO setpoint within the cell bioreactor may be about 30%.

[0059] In some embodiments, the temperature of the culture is maintained at about 25-30°C, *e.g.*, the temperature of the culture is maintained at about 25°C, about 25.5°C, about 26°C, about 26.5°C, about 27°C, about 27.5°C, about 28°C, about 28.5°C, about 29°C, about 29.5°C, or about 30°C. In further embodiments the temperature of the culture is maintained at about 27.5°C.

[0060] In some embodiments, the pH of the cell culture is maintained at about 5.9 to 6.7. For example, the pH of the cell culture may be maintained at about 5.9, about 6.0, about 6.1, about 6.2, about 6.3, about 6.4, or about 6.5. In some embodiments, the pH of the culture is maintained at about 6.1 to about 6.5. In further embodiments, the pH of the culture is maintained at about 6.2 to about 6.4. In certain embodiments, the pH of the culture is maintained at about 6.4.

[0061] In some embodiments, the pH of the culture may be controlled through the addition of an acidic solution or gas, *e.g.*, carbon dioxide (CO₂), phosphoric acid (H₃PO₄), or

hydrochloric acid (HCl). In some embodiments, the pH of the culture may be controlled through the addition of a basic solution, *e.g.*, sodium carbonate (Na₂CO₃), sodium bicarbonate (NaHCO₃), or sodium hydroxide (NaOH). In certain embodiments, the acidic solution is phosphoric acid. In certain embodiments, the basic solution is sodium carbonate.

B. ceDNA Production Phase Parameters

[0062] The present disclosure provides optimal parameters of insect cell infection conditions that help to produce the highest titers of ceDNA. The parameters of infection conditions are termed “production parameters” or “production phase parameters” herein.

[0063] To maximize the production of baculovirus-carried ceDNA in the insect producer cells, the insect cells are grown to a desired cell density in the bioreactor before being infected with baculovirus. In some embodiments, the viable cell density (VCD) at the time of baculovirus infection may be about 1 million viable cells per milliliter (VC/mL) to about 8 million VC/mL, *e.g.*, the viable cell density at the time of baculovirus infection may be about 1 million VC/mL, about 1.25 million VC/mL, about 1.5 million VC/mL, about 1.75 million VC/mL, about 2 million VC/mL, about 2.25 million VC/mL, about 2.5 million VC/mL, about 2.75 million VC/mL, about 3 million VC/mL, about 3.25 million VC/mL, about 3.5 million VC/mL, about 3.75 million VC/mL, about 4 million VC/mL, about 4.25 million VC/mL, about 4.5 million VC/mL, about 4.75 million VC/mL, about 5 million VC/mL, about 5.25 million VC/mL, about 5.5 million VC/mL, about 5.75 million VC/mL, about 6 million VC/mL, about 6.25 million VC/mL, about 6.5 million VC/mL, about 6.75 million VC/mL, about 7 million VC/mL, about 7.25 million VC/mL, about 7.5 million VC/mL, about 7.75 million VC/mL, or about 8 million VC/mL. In further embodiments, the viable cell density at the time of baculovirus infection may be about 5 million VC/mL.

[0064] Upon reaching the desired viable cell density, a cell culture is inoculated with the desired baculovirus or baculoviruses. In some embodiments, the cell culture is inoculated with at least one baculovirus, *e.g.*, at least one, at least two, at least three, or at least four baculoviruses.

In some embodiments, a cell culture is inoculated with two baculoviruses (Two-Bac system). In further embodiments, the Two-Bac system comprises a first baculovirus bearing a parvoviral (*e.g.*, AAV) *Rep* gene and a second baculovirus carrying a template for the ceDNA. In some embodiments, the starting insect cell population is inoculated with the two baculoviruses at a multiplicity-of-infection (MOI) ratio of about 1:2, about 1:5, about 1:10, about 1:20 between the first baculoviral vector and the second baculoviral vector. In further embodiments, the ratio of the first and second vectors may be about 1:10. In some

embodiments, the first (*Rep*) vector and the second vector (ceDNA template) have respective MOIs of about 0.01 and about 0.1; about 0.03 and about 0.3; about 0.05 and about 0.5; about 0.1 and about 1.0; about 0.2 and about 2.0; or about 0.3 and about 3.0.

C. Cell Harvesting

[0065] 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 (G-force) 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 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. Examples of suitable continuous centrifugation systems include UFMini® benchtop systems (CARR Biosystems) and UniFuge® Pilot systems (CARR Biosystems).

[0066] In some embodiments, the cells are concentrated via continuous centrifugation at a G-force of about 500-3000xg. For example, the cells may be concentrated at a G-force of about 500xg, about 1000xg, about 1500xg, about 2000xg, about 2500xg, or about 3000xg. In some embodiments, the cells are concentrated by a factor of about 2-15X, *e.g.*, 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, or about 15X. In preferred embodiments, the harvest cells are concentrated by a factor of about 6X.

[0067] In some embodiments, the processes in Section A and Section B described above are conducted in the same bioreactor, thus streamlining the ceDNA manufacturing process.

D. Exemplary Protocol

[0068] In some embodiments, the process for producing insect cells that produce ceDNA is shortened to about seven days, where the process includes growth and infection of insect cells in a bioreactor and subsequent harvest of the ceDNA-producing cells by continuous centrifugation. The process is schematized in **FIG. 3**. On T-1 day, a single-use bag is set-up with all required connections followed by addition of media amounting to about 30-40% of the final working volume during infection. On Day 0, insect cells from source culture are added at about one-fourth of the target infection density and about 50% working volume. Insect cells typically have 21-28 hours of doubling time. Followed by two days of growth in

the bioreactor, fresh media may be added to supply the culture with nutrients and to ensure that cells are in an early or middle exponential phase of expansion at the time of infection. On Day 3 (*e.g.*, 25-28 hours after media addition), the cells may be simultaneously infected with two different baculoviruses as in the Two-Bac system described herein. Three days (*e.g.*, about 60-68 hours) post infection (Day 6), the infected cells may be harvested using continuous centrifugation, which utilizes a single-use continuous centrifuge system, to concentrate the cells by 3-9 (*e.g.*, 6) fold. ceDNA may then be isolated from the concentrated cells and then purified.

III. Production of Baculovirus Stock

[0069] The present disclosure also provides improved methods of producing baculovirus that carries the template for ceDNA. These methods utilize host insect (*e.g.*, Sf9) cells and are efficient and scalable for commercial production while yielding improved baculovirus titers. The methods are described in detail below.

A. Bioreactor Process Parameters

[0070] To improve baculovirus yield, the bioreactor for growing the insect host cells may have parameters as described above in Section II.A. In some embodiments, the power density applied to the bioreactor is about 10-100 W/m³. In further embodiment, the power density is about 10 W/m³, about 20 W/m³, about 30 W/m³, about 40 W/m³, about 50 W/m³, about 60 W/m³, about 70 W/m³, about 80 W/m³, about 90 W/m³, or about 100 W/m³. In further embodiments, the power density applied to a cell culture is about 30 W/m³.

[0071] In some embodiments, the tip speed applied to the bioreactor is about 0.2-1.2 m/s, for example, about 0.2 m/s, about 0.3 m/s, about 0.4 m/s, about 0.5 m/s, about 0.6 m/s, about 0.7 m/s, about 0.8 m/s, about 0.9, m/s, about 1.0 m/s, about 1.1 m/s, or about 1.2 m/s. In further embodiments, the tip speed applied is about 0.6 m/s for bioreactors with a maximum volume of about 250 mL. In some embodiments, the tip speed applied is about 0.7 m/s in bioreactors with a maximum volume of about 5 L. In further embodiments, the tip speed applied is about 0.96 m/s in bioreactors with a maximum volume of about 50 L.

[0072] In some cases, the cell bioreactor may have at least one impellor within the same working volume, *e.g.*, at least one impellor, at least two impellors, at least three impellors, or at least four impellors. In further embodiments, the cell bioreactor may have two impellors within the same working volume.

[0073] In some embodiments, the dissolved oxygen (DO) setpoint within the cell bioreactor may be set at about 15-75% DO. For example, the DO setpoint within the

bioreactor may be about 15%, about 20%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, or about 75%. In certain embodiments, the DO setpoint within the cell bioreactor may be about 30%.

[0074] In some embodiments, the temperature of the culture is maintained at about 25-30°C, e.g., the temperature of the culture is maintained at about 25°C, about 25.5°C, about 26°C, about 26.5°C, about 27°C, about 27.5°C, about 28°C, about 28.5°C, about 29°C, about 29.5°C, or about 30°C. In further embodiments the temperature of the culture is maintained at about 27.5°C.

[0075] In some embodiments, the pH of the cell culture is maintained at about 5.9 to 6.7. For example, the pH of the cell culture may be maintained at about 5.9, about 6.0, about 6.1, about 6.2, about 6.3, about 6.4, or about 6.5. In some embodiments, the pH of the culture is maintained at about 6.1 to about 6.5. In further embodiments, the pH of the culture is maintained at about 6.2 to about 6.4. In certain embodiments, the pH of the culture is maintained at about 6.4.

[0076] In some embodiments, the pH of the culture may be controlled through the addition of an acidic solution or gas, e.g., carbon dioxide (CO₂), phosphoric acid (H₃PO₄), or hydrochloric acid (HCl). In some embodiments, the pH of the culture may be controlled through the addition of a basic solution, e.g., sodium carbonate (Na₂CO₃), sodium bicarbonate (NaHCO₃), or sodium hydroxide (NaOH). In certain embodiments, the acidic solution is phosphoric acid. In certain embodiments, the basic solution is sodium carbonate.

B. Baculovirus Production Phase Parameters

[0077] To maximize the production of baculovirus in the insect host cells, the insect cells are grown to a desired cell density in the bioreactor before being inoculated with baculovirus. In some embodiments, the viable cell density (VCD) at the time of baculovirus infection may be about 500,000 viable cells per milliliter (VC/mL) to about 5 million VC/mL, e.g., the viable cell density at the time of baculovirus infection may be about 500,000 VC/mL, about 750,000 VC/mL, about 1 million VC/mL, about 1.25 million VC/mL, about 1.5 million VC/mL, about 1.75 million VC/mL, about 2 million VC/mL, about 2.25 million VC/mL, about 2.5 million VC/mL, about 2.75 million VC/mL, about 3 million VC/mL, about 3.25 million VC/mL, about 3.5 million VC/mL, about 3.75 million VC/mL, about 4 million VC/mL, about 4.25 million VC/mL, about 4.5 million VC/mL, about 4.75 million VC/mL, about 5 million VC/mL. In further embodiments, the viable cell density at the time of baculovirus infection may be about 1 million VC/mL.

[0078] Upon reaching the desired viable cell density, a cell culture is inoculated with the desired baculovirus, e.g., a baculovirus bearing a parvoviral (*e.g.*, AAV) *Rep* gene, or a baculovirus carrying a template for the ceDNA. In some embodiments, the starting insect cell population is inoculated with the baculovirus at a multiplicity-of-infection (MOI) level of about 0.01 to 1.0, *e.g.*, the baculovirus MOI level is about 0.01, about 0.05, about 0.1, about 0.15, about 0.2, about 0.25, about 0.3, about 0.35, about 0.4, about 0.45, about 0.5, about 0.55, about 0.6, about 0.65, about 0.7, about 0.75, about 0.8, about 0.85, about 0.9, about 0.95, or about 1.0. In some embodiments, the baculovirus MOI level is 0.1.

C. Supernatant Harvesting

[0079] In some embodiments, the baculovirus-producing cells are harvested by centrifugation, *e.g.*, continuous flow centrifugation, as described in Section II.C above. The supernatant is the baculovirus product stock and the cell paste may be discarded. In some embodiments, the cells are concentrated via continuous centrifugation at a G-force of about 500-3000xg. For example, the cells may be concentrated at a G-force of about 500xg, about 1000xg, about 1500xg, about 2000xg, about 2500xg, or about 3000xg. In some embodiments, the cells are concentrated by a factor of about 2-15X, *e.g.*, 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, or about 15X. In preferred embodiments, the harvest cells are concentrated by a factor of about 6X.

[0080] Following production of baculovirus, the harvested baculovirus may be used to subsequently infect another insect cell culture, referred to here as a baculovirus passage cycle. In some embodiments, production of the baculovirus stock may comprise one to eight baculovirus passage cycles, *e.g.*, two, three, four, five, or six baculovirus passage cycles. The quality and stability of baculovirus may be reduced or lost as a result of a high number of passages, which in turn leads to reduced ceDNA yield. In some embodiments, production of the baculovirus stock comprises four baculovirus passage cycles. In other embodiments, production of the baculovirus stock comprises five baculovirus passage cycles. The process comprising a baculovirus passage cycle may be performed as described in Section II.D below.

D. Exemplary Protocol

[0081] An exemplary process for producing insect cells that produce baculovirus is schematized in **FIG. 24**. On T-1 day, a single-use bag is set-up with all required connections followed by addition of media amounting to about 30-40% of the final working volume during infection. On Day 0, approximately 1×10^5 to 10^7 VC/mL (*e.g.*, 1×10^6 VC/mL) of

insect cells from a source culture are added to about 30-60% (e.g., about 50%) working volume. After about 24 hours of growth (Day 1), fresh media may be added to supply the culture with nutrients and to ensure that cells are in an early or middle exponential phase of expansion at the time of infection. Following media addition on Day 1, the cells may be infected with the desired baculovirus, wherein the viable cell density is about 1×10^5 to about 10^7 VC/mL (e.g., about 1×10^6 VC/mL) and the MOI upon infection is 0.05 to 0.5 (e.g., about 0.1). Four days post infection (Day 5), the infected cells may be concentrated and the supernatant harvested. The harvested supernatant may be subsequently used to infect another bioreactor seeded with insect cells. Infection of insect cell cultures and harvest of baculovirus-containing supernatant constitutes a baculovirus passage cycle. The exemplary baculovirus production protocol may comprise four or five baculovirus passage cycles.

[0082] 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.

[0083] According to the present disclosure, back-references in the dependent claims are meant as short-hand writing for a direct and unambiguous disclosure of each and every combination of claims that is indicated by the back-reference. Further, headers herein are

created for ease of organization and are not intended to limit the scope of the claimed invention in any manner.

[0084] 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.

EXAMPLES

Example 1: Platform Selection for ceDNA Production

[0085] For optimal expression of ceDNA from a baculovirus system, we evaluated two approaches: a dual-baculovirus infection system (Two-Bac) and a single baculovirus infection system (One-Bac). The Two-Bac system uses two baculoviral vectors for infection, one carrying a parvoviral (e.g., AAV) *Rep* gene while the other carried a therapeutic transgene (e.g., a recombinant FVIII-encoding transgene), flanked by parvoviral (e.g., AAV) ITRs (FIG. 2, top). The One-Bac system uses one baculoviral vector (FIG. 2, bottom); it requires that the parental insect cell be engineered to have a transgene (e.g., a recombinant FVIII expression cassette flanked by parvoviral ITRs) stably integrated into the host cell genome.

[0086] On comparison of the two approaches by culturing the appropriate cell line, we observed better insect cell growth using the Two-Bac approach (FIG. 4, left). The cell growth was comparable between the two approaches until Day 3, Day 3 being the day of infection (FIG. 4, right). Post infection, the cell growth seemed to arrest sooner in the Two-Bac approach with a deeper crash in cellular viability by the time of harvest. This enabled us to shorten our process timeline. Additionally, dual virus infection outperformed in titer compared to the One-Bac approach at several different culture volumes (FIG. 5).

[0087] In conclusion, we observed that a two-baculovirus system displayed superior cell culture performance in terms of growth, scalability, and productivity when compared to the single-baculovirus system, with 4X increase in product titer. Significant changes in titers were also observed with passage numbers of the baculoviruses used to infect insect cells. Thus, we adopted a Two-Bac approach for further studies.

Example 2: Evaluation of Process Parameters

A. Power Density

[0088] Shear stress that arises as a result of mixing and oxygen transfer has a critical impact on cell culture. To facilitate cell growth and ceDNA production, we evaluated

different power densities of the bioreactor, namely, 30, 60 and 90 W/m³, for our process at Ambr® 250 scale (Sartorius) with two different multiplicities of infection (MOI) levels, 0.01/0.1 or 0.2/2.0 (*Rep/transgene*).

[0089] Similar growth profiles were observed during the production phase of the process among the 30, 60 and 90 W/m³ power densities (**FIG. 6**, left). However, significant differences were observed in the viability at harvest among 30, 60 and 90 W/m³ power densities. Lower viability was observed in higher power densities indicating an effect on the cell performance due to greater shear stress (**FIG. 6**, right).

[0090] We also evaluated the ceDNA titers at these three different power densities. While the titers for 60 and 90 W/m³ were comparable, they were slightly lower than 30 W/m³, with the titer being about 20 µg/mL vs. 26 µg/mL (**FIG. 7**). Therefore, we selected 30 W/m³ as the power density for further experiments.

B. Bioreactor Configuration

[0091] Typically, a high volumetric oxygen transfer coefficient (kLa) is required for insect cell culture systems as the oxygen uptake rate tends to be higher for insect cells, especially following viral infection. Therefore, we evaluated different methods of changing and improving the volumetric oxygen transfer coefficient of the bioreactors. One of the parameters tested was configuring the vessels with one or two impellers with the same working volume and design. We found that although viable cell density profiles were comparable, viability was slightly lower for the two-impellor system (**FIG. 8**). However, that did not seem to impact the titers and therefore, we decided to implement a two-impellor configuration for our subsequent experiments (**FIG. 9**).

C. Dissolved Oxygen Level

[0092] The dissolved oxygen (DO) setpoint is typically about 30-40% for growth of insect cells in bioreactors at multiple scales. Based on our preliminary observations, ceDNA production required high oxygen gassing and therefore we evaluated a higher setpoint for the DO, i.e., 60% at Ambr® 250 scale and 5 L glass benchtop reactors. At Ambr® 250 scale, we found comparable growth profiles and viability at harvest for both 30% and 60% DO setpoints. In addition, ceDNA titers were comparable for 30% and 60% DO setpoints at harvest. At 5 L scale, we observed similar trends as Ambr® 250 scale, where the growth profiles and the ceDNA titers were comparable for 30% and 60% DO setpoints (**FIG. 10** and **FIG. 11**). Since we did not find differences in cell culture performance at Ambr® 250 and 5 L scales, a 30% DO setpoint was selected because the higher oxygen required to maintain the higher 60% DO setpoint did not benefit process performance.

D. Culture pH and Temperature

[0093] To improve process performance, we evaluated the ceDNA production process with and without pH control and at temperatures between 26.5°C and 28.5°C. We observed that changes to the culture temperature had comparable effects on cell growth, though culture pH of 6.2 and 6.4 both showed improved cell growth compared to pH 6.0 (**FIG. 12**). Additionally, ceDNA titers were greatly decreased when the pH of the culture was decreased to 6.0, regardless of temperature. Additionally, holding the temperature at 27.5°C yielded higher titers compared to 26.5°C and 28.5°C at both pH 6.2 and 6.4 (**FIG. 13**). We observed increased FVIII ceDNA titers by implementing pH control between pH 6.1 to 6.5 at a temperature of 27.5°C, with titers in the range of 9.8 µg/mL to 11.8 µg/mL. The culture pH was controlled by using carbon dioxide gas to increase acidity and 1 M sodium carbonate solution for base addition.

[0094] As a potential alternative to CO₂ gas for pH control, phosphoric acid was evaluated to determine if process feasibility could be achieved without CO₂ gas. Reducing CO₂ gas in the process would be useful when scaling the processes up to 2000 L. We found that elevated CO₂ levels in insect cell cultures had a detrimental impact on cellular growth and metabolism. At the same pH levels, cultures using CO₂ for pH control showed reduced growth rate during the cell growth phase compared to cultures using phosphoric acid, and this effect was more prominent as the acid requirement to maintain the pH increased (**FIG. 14**). Since the process pH setpoint is higher than the media pH, we compared the two control strategies at two pH levels (6.2 and 6.0). For the production phase, we observed comparability in FVIII ceDNA titer across both control strategies, including conditions at lower pH (**FIG. 15**). In the case of a pH excursion event, we expect this alternative to demonstrate improved process robustness by minimizing the negative impact of elevated carbon dioxide levels on insect cells.

Example 3: Evaluation of ceDNA Production Parameters

A. Infection Density

[0095] To assess production-specific parameters, viable cell densities (VCD) at the time of baculovirus infection were evaluated in terms of ceDNA titer and cell viability at the time of harvest on Day 3 post infection. VCD was determined by using Vi-CELL BLU cell viability analyzer (Beckman Coulter), which uses trypan blue dye exclusion to quantify viable cells.

[0096] VCD ranges between 1.25 million viable cells (VC)/mL and 7.5 million VC/mL were assessed at various *Rep* MOI levels and MOI ratios (*Rep*/transgene, 1:5, 1:10) in the Two-Bac system. We observed productivity levels ranging widely from 0.31 µg/mL to 12.1 µg/mL. We saw an increase in titers up to 16.3X, compared to the then process parameter of 0.01/0.1 MOI at 5 million VC/mL. Based on these data, we chose 5 million VC/mL as the VCD at the time of infection (FIG. 16).

B. MOI and MOI Ratio

[0097] As another process parameter, we evaluated the MOI ratios and levels for the Two-Bac system, specifically with FVIII BEV and *Rep* BEV stocks. We tested MOI ratios and levels at a seeding density of 5 million VC/mL. Using the Ambr® 15 high-throughput system as the screening platform for this parameter, *Rep*:FVIII MOI ratios of 1:5, 1:10, 1:20, 1:1, 2:1, 3:1, 5:1, 10:1, 20:1 at various MOI levels were screened. The data show that 1:5 and 1:10 were comparable at lower MOI while 1:10 resulted in better titers at higher MOIs (FIG. 17). The ratios of 1:1, 2:1, 3:1, 5:1, 10:1, 20:1 yield poor titers.

[0098] Based on these data, a second Ambr® 15 study was performed to compare MOI ratios of 1:5 and 1:10 at *Rep* MOI levels of 0.01, 0.03, 0.05, 0.1, 0.2, and 0.3 (FIG. 18). An Ambr® 250 study was also performed to compare MOI ratios of 1:5 and 1:10 at *Rep* MOI levels of 0.01, 0.03, 0.05, and 0.2. We found that an MOI ratio of 1:10 produced high ceDNA titers overall at this higher scale (FIG. 18, left). In addition, we observed 4-5X improvement in ceDNA titers as we increased the MOI level. A validation study was carried out in 5 L using the same *Rep* MOI levels as the Ambr® 250 study but only for MOI ratio 1:10. The scaled-up process showed similar ceDNA titers as Ambr® 250 scale (FIG. 18, right).

[0099] Through all four studies, higher ceDNA titers were observed for MOI levels between 0.03/0.3 – 0.3/3.0 compared to the control condition of 0.01/0.1. The MOI level of 0.2/2.0, at the MOI ratio of 1:10 was found to produce the highest product titers (18-25 µg/mL) across all scales.

C. Production Temperature

[0100] Studies of insect cell growth and ceDNA production were carried out between 24°C and 28°C. We observed that lower temperatures increased process duration to achieve comparable titer and viability at harvest at both shake flask (SF) and Ambr® 250 scale (FIG. 19). Therefore, we decided to continue with 27.5°C as the production temperature with harvest 3 days post-infection.

D. Robustness and Scalability of ceDNA Production Process

[0101] We evaluated the scalability of this process by measuring cell growth over time across cultures ranging in volume from Ambr® 250 to 500 L. Across scales, comparable cell growth performance was observed throughout the six-day process, indicating successful scalability of the process (**FIG. 20**). We evaluated the ceDNA growth and production process that showed 4-5X improvement in titers up to 5 L benchtop glass bioreactors. We scaled up the process to 50 L and 500 L scale and demonstrated repeatability and robustness of the process during four pilot-scale runs where we observed comparable cell growth and viability throughout the six-day process (**FIG. 21**).

[0102] Representative experiments were selected for comparison for each scale of evaluation: shake flask (SF), Ambr® 250, 5 L bioreactor, and pilot scale (50 L). Doubling times during the growth phase of the process were comparable across scales ranging from 19-23 hours without affecting the process performance (**FIG. 22**). The viable cell densities were also comparable throughout the process during the growth phase. The ceDNA titers were comparable across bioreactor scales. Ambr® 250, 5 L bioreactor and pilot scale titers were found to be in the 22-28 µg/mL range that we had seen at process development scale. These results indicate that the current process would not have scalability challenges.

E. Continuous Centrifugation to Harvest Cells

[0103] Post infection, we evaluated a novel way of harvesting the cell product from the infected cell culture. Given the intracellular location of ceDNA, employing traditional centrifugation as a harvest step presented challenges since it is time, labor and cost intensive. In addition, it also presented scalability and feasibility challenges. In our efforts to tackle some of these challenges that are unique to ceDNA production in insect cells, we evaluated a continuous centrifugation type cell concentration process using bench (Minifuge)/pilot scale (UniFuge® systems; CARR Biosystems) at a G-force of 2000xg. The flow-rate within the continuous centrifugation system was dependent upon the scale of the operation. We evaluated various concentration factors for our process, namely 3X, 6X, 9X, and 12X. We observed that 3X and 6X concentration factors recipes showed minor lower drops in viability compared to 9X and 12X conditions (**FIG. 23**). We decided to move forward with 6X concentration factor for the harvest process. This method also made large scale runs feasible and cut down the processing time by 10-fold at large scale, thus accelerating product manipulation downstream of cell culture activities.

Example 4: Exemplary Cell Growth and ceDNA Production Process

[0104] An exemplary process using the aforementioned tested steps is further described below.

A. Inoculation with Sf9 Cells and Media Addition

[0105] One day prior to seed (D-1), a single-use bag was prepared followed by addition of insect cell culture media to about 30-40% of the working volume (WV). On D0, cells from a source culture (shake flasks and/or bioreactor depending on the scale of operations) were added at about one-fourth of the target cell concentration at the point of infection (CCI) and about 50% of WV. The inoculated insect cell culture was grown for two days at 27.5°C in the bioreactor at 30 W/m³ and 30% dissolved oxygen, with the pH held at 6.1-6.5 using an acidic component such as CO₂ gas or phosphoric acid, and a basic component such as 1M NaHCO₃. The bioreactor was configured such that the vessel comprised two impellers within the same WV. After two days of growth in the bioreactor (D0-D2), fresh media was added to ensure that cells remained in early to mid-exponential phase at the time of infection. The volume of fresh media added to the bioreactor was sufficient to reach the target WV and about one-half of the target CCI.

B. Baculovirus Infection

[0106] On D3, the bioreactor culture reached the target infection density. The cells in the bioreactor were infected with two different baculovirus expression vectors, respectively carrying an AAV *Rep* gene and the desired ceDNA transgene (e.g., encoding a recombinant FVIII). These baculoviruses were added simultaneously into the bioreactor. At the time of infection, the viable cell density (VCD) was about 5x10⁶ VC/mL and the MOI levels of the *Rep* vector and the transgene vector were 0.02 and 0.2 respectively (i.e., a ratio of 1:10 between the two vectors). The infected culture was grown for three days at 27.5°C after infection.

C. Cell Harvest

[0107] Three days (60-68 hours) after baculovirus infection (D6), the baculovirus-infected insect cells were harvested using continuous centrifugation, which utilized a single-use module continuous centrifuge system. The insect cells were concentrated by a factor of 4-6X using a continuous centrifugation system at 2000 x g with a flow rate of 300-750 ml/min (Minifuge®, CARR Biosystems) and 1-3 L/min (UniFuge®, CARR Biosystems). The concentrated cells were then lysed and ceDNA was isolated and purified from the cell culture.

Example 5: Evaluation of Passaging Effect of Baculovirus Production

[0108] To improve the overall efficiency and yield of ceDNA, the effect of baculovirus passaging was evaluated. Baculovirus was produced from Sf9 insect host cells according to the scheme depicted in **FIG. 24**. Bacmid DNA was used to infect a small culture of Sf9 insect cells (P0). Following cell growth, the culture was passaged six times with low MOI upon each repeated passage. The baculovirus titer was measured using a BacPAK™ Baculovirus Rapid Titer Kit (Takara Bio) according to the manufacturer's instructions (**FIG. 25**). The ceDNA titers resulting from cell cultures infected with baculovirus of Passage 4 and Passage 5 (P4 and P5, respectively) were measured. The data show that ceDNA titers resulting from P5 baculovirus were consistently low, ranging from 5-8 µg/mL. However, the ceDNA titer obtained from P4 exhibited ceDNA titers of 20-25 µg/mL (**FIG. 26**). Additionally, P4 baculovirus led to higher ceDNA titers compared to P5 baculovirus across several different production scales, indicating that the ceDNA high titers observed in P4 are scalable (**FIG. 27**). Moreover, this indicated poor quality of baculovirus starting with P5 and therefore P5 was not ideal for ceDNA production.

Example 6: Exemplary Baculovirus Stock Production Process

[0109] An exemplary process for producing baculovirus stock is described below.

A. Inoculation with Sf9 Cells and Media Addition

[0110] One day prior to seed (D-1), a single-use bag was prepared followed by addition of insect cell culture media to about 30-40% of the working volume (WV). On D0, cells from a source culture (shake flasks and/or bioreactor depending on the scale of operations) were added at about 1×10^6 VC/mL and about 50% of WV. The inoculated insect cell culture was grown for one day at 27.5°C in the bioreactor at 30 W/m³ and 30% dissolved oxygen, with the pH held at 6.1-6.5 using an acidic component such as CO₂ gas or phosphoric acid, and a basic component such as 1 M NaHCO₃. The bioreactor was configured such that the vessel comprised two impellers within the same WV. After one day of growth in the bioreactor (D0-D1), fresh media was added to ensure that cells remained in early to mid-exponential phase at the time of infection. The volume of fresh media added to the bioreactor was sufficient to reach the target WV.

B. Baculovirus Infection

[0111] On D1, following the fresh media addition, the bioreactor culture reached the target infection density. The culture media contained 10% fetal bovine serum (FBS). The cells in the bioreactor were infected with baculovirus. At the time of infection, the viable cell

density (VCD) was about 1×10^6 VC/mL and the MOI level of the baculovirus was 0.1. The infected culture was grown for four days (about 96 hours) at 27.5°C after infection.

C. Cell Harvest and Baculovirus Passaging

[0112] Four days (about 96 hours) after baculovirus infection (D5), the baculovirus-infected insect host cells were harvested using continuous centrifugation, which utilized a single-use module continuous centrifuge system. The insect cells were concentrated by a factor of 4-6X using a continuous centrifugation system at 2000 x g with a flow rate of 300-750 ml/min (Minifuge®, CARR Biosystems) and 1-3 L/min (UniFuge®, CARR Biosystems). The supernatant was harvested as the baculovirus stock product. The preceding protocol constituted a single baculovirus passage cycle. The baculovirus stock product was used to subsequently infect a new bioreactor culture of insect cells according to the above protocol. Four baculovirus passage cycles were completed to produce the final P4 baculovirus stock product.

CLAIMS

1. A method of producing closed-ended DNA (ceDNA), comprising
 - (a) infecting a starting population of insect cells in a bioreactor with a first baculoviral vector comprising a parvoviral *Rep* gene and a second baculoviral vector comprising a template for the ceDNA, wherein the viable cell density at the time of the infection is about 4-6 million cells, optionally about 5 million cells, per mL, and wherein the multiplicity-of-infection (MOI) ratio of the first and second baculoviral vectors is 1:5 to 1:15, optionally 1:10;
 - (b) culturing the infected cells under conditions that allow the production of the ceDNA in the cells; and
 - (c) harvesting the cultured infected cells.
2. The method of claim 1, wherein the MOI of the first baculoviral vector is about 0.2 and the MOI of the second baculoviral vector is about 2.0.
3. The method of claim 1 or 2, wherein the power density of the bioreactor is about 25-35 W/m³, optionally about 30 W/m³.
4. The method of any one of claims 1-3, wherein the culture medium in the bioreactor comprises about 30-60%, optionally about 30%, dissolved oxygen.
5. The method of any one of claims 1-4, wherein the pH of the culture medium in the bioreactor is between 6.1 and 6.5, optionally about 6.4, optionally wherein the pH is adjusted by CO₂ gas or phosphoric acid and/or sodium carbonate.
6. The method of any one of claims 1-5, wherein the temperature of the bioreactor is 27.5°C.
7. A method of producing closed-ended DNA (ceDNA), comprising
 - (a) culturing a population of insect cells in a bioreactor at a power density of about 25-35 W/m³, optionally about 30 W/m³, wherein the culture medium in the bioreactor
 - (i) comprises about 30-60%, optionally about 30%, dissolved oxygen, and
 - (ii) has a temperature of 27.5°C;

- (b) infecting the cultured insect cells with a baculoviral vector comprising a parvoviral *Rep* gene, wherein the cultured insect cells comprise a template for the ceDNA;
- (c) culturing the infected cells under conditions that allow the production of the ceDNA in the cells; and
- (d) harvesting the cultured infected cells.

8. The method of claim 7, wherein the ceDNA template is stably integrated in the genome of the cultured insect cells.
9. The method of claim 7, wherein the ceDNA template is introduced to the cultured insect cells through a baculoviral vector comprising the template, optionally wherein the baculoviral vector comprising a parvoviral *Rep* gene and the baculoviral vector comprising the ceDNA template are two different vectors and optionally simultaneously introduced into the cultured insect cells.
10. The method of claim 9, wherein the multiplicity-of-infection (MOI) ratio of the baculoviral vector comprising a parvoviral *Rep* gene and the baculoviral vector comprising the ceDNA template is 1:5 to 1:15, optionally 1:10.
11. The method of claim 9 or 10, wherein the MOI of the baculoviral vector comprising a parvoviral *Rep* gene is about 0.2 and the MOI of the baculoviral vector comprising the ceDNA template is about 2.0.
12. The method of any one of claims 7-11, wherein the culture medium in the bioreactor has a pH of 6.1 to 6.5, optionally about 6.4, optionally wherein the pH is adjusted by CO₂ gas or phosphoric acid and/or sodium carbonate.
13. The method of any one of claims 7-12, wherein the viable cell density at the time of the infection is about 4-6 million cells, optionally about 5 million cells, per mL.
14. The method of any one of claims 1-13, wherein the bioreactor has two impellers and optionally has a volume of 5 L, 50 L, 500 L, or 2000 L.

15. The method of any one of claims 1-14, wherein the ceDNA comprises a transgene flanked by inverted terminal repeats (ITRs), optionally wherein the ITRs are parvoviral ITRs or AAV ITRs, optionally AAV2 ITRs.
16. The method of any one of claims 1-15, wherein the cultured infected cells are harvested *via* continuous centrifugation.
17. The method of any one of claims 1-16, wherein the baculovirus(es) for the infecting step are produced by four passage cycles.
18. The method of claim 17, wherein each passage cycle comprises:
- (a) inoculating insect host cells in a bioreactor with a baculovirus, wherein the viable cell density at the time of the infection is about 1 million cells per mL, and wherein the MOI of the baculoviral vector is about 0.1;
 - (b) culturing the infected host cells under conditions that allow the replication of the baculovirus in the host cells;
 - (c) harvesting the baculovirus-containing supernatant to obtain produced baculovirus;
- and
- (d) if needed, repeating steps (a)-(c) in the next passage cycle, using the produced baculovirus for step (a).