

## **METHODS FOR IMPROVING VIRUS-LIKE PARTICLE PRODUCTION PER CELL**

### **Cross-Reference to Related Applications**

[0001] This application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Application Nos. 63/608,085, filed December 8, 2023; 63/640,415, filed April 30, 2024; 63/642,346, filed May 3, 2024; and 63/654,447, filed May 31, 2024. The contents of each of these applications are incorporated herein by reference in their entirety.

### **Technical Field**

[0002] The present disclosure relates to a method and device for improving the production of virus-like particles or protein nanoparticles per cell by, for example, using electroporation, DNase, sodium butyrate, and temperature shifting the cells during cell culture.

### **Background**

[0003] Delivery systems for purposes of gene editing, gene expression changes, exon skipping, imaging, and other varied cell, tissue, organ, and system modulation for research and treatments have traditionally employed the use of viral vectors. While useful, the use of viral vectors is not always ideal as these are infectious and can lead to long term expression of the vectors, leading to undesired mutations, other off-target effects, and immune response complications. virus-like particles ("VLPs") or protein nanoparticles can help overcome these challenges as they are not infections and can be employed transiently, limiting mutations and other off-target effects.

[0004] VLPs are similar to viral vectors as they are covered by a protein shell that can self-assemble and contain a cargo for delivering into target cells or systems. Contrary to viral vectors, however, VLPs do not contain viral genetic material, which results in VLPs being non-infectious as they cannot replicate after they are inserted into a cell. VLPs can have bio-active molecules, such as lipids, proteins, and RNA that are ready to perform a specific function once

delivered inside of a cell. This allows VLPs to shortcut the reverse transcription, transcription, and translation that are usually necessary for a viral vector to effectuate its function.

[0005] Traditional methods and devices to produce VLPs include chemical transductions. These protocols for producing VLPs involve the use of expensive chemical mediated expression in VLP producer cells or employ the use of toxic chemicals that may lead to non-ideal levels of VLP produced or reduced levels of viable VLP. Together, these hindrances lead to increased cost, excessive use of reagents, and scientist time that results in slower scientific progress.

[0006] Therefore, there is a need for methods and devices to increase the production of VLPs per cell.

#### Summary

[0007] Consistent with some disclosed embodiments, methods of producing virus-like particle production using electroporation are disclosed. In one embodiment, the method comprises cultivating a population of cells in a log growth phase in a cell culture vessel using media, cultivating the population of cells to a desired level of confluency, removing the media from the cell culture vessel and adding a transfection buffer.

[0008] The method further comprises adding to the cell culture vessel at least one vector, transfecting the cell population, culturing the cell population and seeding the cells located in the cell culture vessel. In some embodiments, the disclosed method may further include adding an endonuclease to the cell culture vessel, such as prior to the seeding step.

[0009] In some embodiments, the method may further include adding a compound that increases transcription efficiency. In some embodiments, the method may additionally or alternatively comprise subjecting the cell culture to a temperature shift for a desired period of time.

### Brief Description of the Figures

[0010] The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate some disclosed embodiments and, together with the description, serve to explain the disclosed embodiments. The particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the present disclosure. The description taken with the drawings makes apparent to those skilled in the art how embodiments of the present disclosure may be practiced.

[0011]

[0012] **Figures 1A-1B** illustrate 100-400 µg/mL as a range of DNA concentration used for electroporation for the production of VLPs in HEK293T cells.

[0013] **Figures 2A-2J** illustrate how electroporation is more effective than chemical transfection methods in producing better transfection and higher production of VLPs. The efficiency stemming, at least in part, because the introduction of DNA into the cells is faster than chemical transfection methods.

[0014] **Figures 3A-3B** illustrate that the addition of DNase after electroporation may enhance VLP producer cell survival.

[0015] **Figures 4A-4B** illustrate how high seeding density after electroporation negatively influences VLP yield and cell number of VLP producing cells.

[0016] **Figures 5A-5B** illustrate how sodium butyrate may improve the production of VLP per producer cell.

[0017] **Figures 6A-6B** illustrate how temperature shifting VLP producer cells after 24 hours of electroporation may improve the VLP production and yield per cell.

[0018] **Figures 7A-7C** illustrate how electroporation enables higher productivity per VLP producer cell compared to chemical transfection methods.

[0019] **Figure 8** shows that electroporation energy correlated with higher VLP activity in an energy-dependent manner.

[0020] **Figure 9** shows that electroporation enables the packaging of adenine base editor (ABE) RNPs into VLPs to target HEK3 loci produced from suspension HEK cells.

#### **Detailed Description**

##### **Definitions:**

[0021] As used herein, “virus-like particles” (“VLPs”) or “virus nanoparticles” refer to natural or artificial structures that closely resemble viruses by having, for example, a protein shell that envelops cargo material and protects said cargo from degradation from outside influences. As described herein, “VLPs” and “virus nanoparticles” include nanoparticles, such as a pseudovirus or pseudovirions (“PsV”), nanoblades, and quasi-virions (“QV”). VLPs are distinct from viruses in part because they have no genetic material as part of their cargo, making VLPs non-infectious as these cannot replicate. Their non-infectious feature may be preferable as it presents a lower biosafety risk and may be better suited for applications where a transient use of the cargo is desirable. Instead of having genetic material as their cargo, VLPs can have active biomolecules as cargo. For example, VLPs can contain lipids, proteins, and other organic compounds. Once the VLP is inside the cell, the cargo gets released and the active biomolecules can rapidly begin to exert their functions without the added steps of transcription, translation, and post translation modifications.

[0022] As used herein, “log growth phase” or “logarithmic growth phase” refers to the phase in which cells are actively proliferating and their cell density and or quantity increases exponentially. This is in contrast to the other growth phases, lag, stationary, decline, and senescence, in which the cells grow and proliferate at a slower rate, stop growing, or begin to decline in total number and or density.

[0023] As used herein, “cell culture vessel” or “vessel” refers to the vessel where cells are grown. The vessel may be made of glass, plastic, thermoplastic, polyolefins, polycarbonate, polystyrene, or other materials. The vessel can be petri dishes having a diameter of, for example, 10 mm, 11 mm, 12.7 mm, 35 mm, 60 mm, 94 mm, 100 mm, and 145 mm. The vessels can be cell culture bags with a culture area of less than, more than, or equal to 640 cm<sup>2</sup>. The vessels can be 6-, 12-, 24-, 48-, or 96- well plates. The vessel can be a flask of a volume including 25ml (12.5cm<sup>2</sup>), 50ml (25cm<sup>2</sup>), 250ml (75cm<sup>2</sup>), and 600ml (182cm<sup>2</sup>), or cell stack or cell factory vessel.

[0024] As used herein, “confluency” includes a value representing how much of the vessel surface area cells are occupying. For suspension cells, confluency refers to the density of cells per volume.

[0025] As used herein, “transfection buffer” includes buffers made for electroporation, for example, electroporation buffer (“EP buffer,” “EP,” or “EB”), lipofection, and chemical transfection, including but not limited to Opti-MEM<sup>TM</sup> Reduced Serum Medium.

[0026] As used herein, “vector” or “vectors” include distinct pieces of non-genomic genetic material that may be in a closed circular conformation, like a plasmid.

[0027] As used herein, “electroporation” is meant to encompass the process of facilitating the insertion of foreign materials, like genetic material, into cells by using a combination of electricity in combination with other reagents and solutions. Any electroporation system may be used to carry out the claimed invention including but not limited to the ExPERT ATx<sup>TM</sup>, the ExPERT GTx<sup>TM</sup>, the ExPERT STx<sup>TM</sup>, and the ExPERT VLx<sup>TM</sup>.

[0028] As used herein, “cell culture conditions” include standard cell culture conditions for cell lines. Although the following parameters can vary per cell line and other factors, these usually include standard cell culturing conditions of CO<sub>2</sub> at a range of 37% and humidity at about 80% culture density. Additionally, cells can be cultured at a temperature of 32 °C to 37

°C, with a CO<sub>2</sub> concentration of 4% to 7%, and a humidity of about 80% to 95%. The cell culture conditions can vary beyond the limitations described depending on the cell type, the downstream applications, and according to what a person of ordinary skill in the art would determine applicable under the circumstances.

[0029] As used herein, “transcription efficiency” refers to the efficacy of transcribing DNA into messenger RNA (“mRNA”).

[0030] As used herein, “endonucleases” are enzymes or other molecules that cleave DNA in a nonspecific manner, usually to cause degradation of undesired DNA or genetic material. Examples of reagents that can achieve these results are DNase, Pulmozyme®, and Benzonase®. As disclosed herein, where appropriate the amount of endonuclease used ranges from 0.1 units to 5 units per 20 µL of transfection volume. A “unit” is defined as a microliter of the prepared solution of endonuclease.

**Description of Embodiments:**

[0031] The following is a detailed description with several non-limiting embodiments and accompanying figures, and substitutions, additions, or modifications may be made to the components and steps provided.

[0032] Embodiments of the present disclosure are directed to methods of improving VLP production per cell by using, for example surface adhered or dissociated HEK293, HEK293T, or another HEK293-derived cell line. Embodiments of the present disclosure include using cell culture media that can be, but is not limited to, Dulbecco's Modified Eagle Medium (“DMEM”), Roswell Park Memorial Institute (“RPMI”) 1640 Medium, Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (“DMEM F12”), TexMACS™, Minimum Essential Medium (“MEM”), Iscove's Modified Dulbecco's Medium (“IMDM”), CTS™ OpTmizer™, Opti-MEM™ Reduced Serum Medium, or others. The present disclosure also includes supplementing with industry standard serum such as but not limited to, fetal bovine serum,

horse serum, human serum, or other synthetic serum, at an amount of, for example, 2%, 5%, 10%, 15%, or 20% or any value between those percentages, as well as above and below the maximum and minimum values disclosed, taking into account variations in cell line, upstream and downstream applications, as well as other variables. Furthermore, embodiments of the present disclosure include culturing the cells until they have achieved a logarithmic growth state. Determining a logarithmic growth state can be done by estimating the number of cells at a particular point (for example 24 hours after seeding), and comparing the number of cells at later time points (for example, 48 hours and 72 hours after seeding). If the change in cell growth has increased logarithmically and in a non-linear fashion, then the cells can be said to be in a logarithmic phase. Further, embodiments of the present disclosure involve removing the media by using, for example, a vacuum aspirator, decanting the media, a serological pipette, or a micropipette. Embodiments of the present disclosure also include adding a transfection buffer to the cell culture that can be an electroporation buffer, such as MaxCyte's® commercially available electroporation buffers, in an amount at least sufficient to cover the surface area of the vessel.

[0033] The present disclosure and embodiments include mixing the desired vectors in a tube, for example, an Eppendorf 1.5 mL tube, containing electroporation buffer described above. The amount of each vector may vary depending on the application and it requires taking into account the number of cells to be transfected, the cell line, the application, and the vessel used. Similarly, the ratios of each of the different vectors used to generate VLPs are not limited to the ratios of each vector disclosed herein and will vary depending on the application, cell type, and other relevant factors. Per mL, the total amount of vector can add to a total of 0.1 ug to 300 ug of vector. For example, per mL, the total amount of vector can be 0.1 µg, 10 µg, 50 µg, 100 µg, 150 µg, 200 µg, 250 µg, 300 µg, and any value in between the disclosed values as well as higher and lower than the maximum and minimum values disclosed. Taking into consideration

the vessel size, a much larger vessel may have a proportionally larger total of vector and a much smaller vessel may have a proportionally reduced amount of vector. For example, a 10 cm cell culture vessel may have a total of 25 µg of vector applied, while a 100 cm may have 100 µg of vector added. The number of distinct vectors can also vary from 1 vector to 10 different vectors. By different vectors it is understood that it can mean vectors that have the same or different backbones, genes, open reading frames, variants of a gene or genes, reporters, selection markers, and features of a particular vector. A non-limiting example of the identity of these vectors adding up to about 300 µg/mL of vector for transfecting  $15 \times 10^6$  cells, and the amount that can be used per vector is: (1) MLV-Gag (109 µg/mL or 10.9 µg in 100 µl of EP volume); (2) MLV-Gag-Cas9 (36 µg/mL or 3.6 µg in 100 µl of EP volume); (3) VSV-G (13 µg/mL or 1.3 µg in 100 µL of EP volume); and (4) sgRNA (142 µg/mL or 14.2 µg in 100 µL of EP volume).

[0034] In some embodiments, the present disclosure includes adding the desired combination and amount of vector to concentrated cells in electroporation buffer and subjecting the cells to a transfection device, like an electroporation device, and applying a transfection protocol to transfect the cells. The device and method can include using electricity at a particular voltage for a particular time, and other particular parameters and reagents to effectively transfect the cells. The present disclosure includes transferring the cells to, for example, an Eppendorf tube, a falcon tube, or culture vessel, after completing a transfection protocol adding an optional washing step with EP buffer, and adding fresh EP buffer to an appropriate volume. An appropriate volume can be, for example, an amount equivalent to the electroporation volume, such as, 100 µL of buffer when 100 µL of electroporation volume was used.

[0035] In additional embodiments, there is described gently resuspending the residual cells in the EP buffer and transferring them to an Eppendorf tube, a falcon tube, or culture vessel. Other examples of transferring the cells post-transfection and resuspending them should take into

account the total number of cells transfected. For example, for transfecting  $3 \times 10^6$  cells, resuspending the cells in 1000  $\mu\text{L}$  of media may be appropriate, while transfecting a much larger number of cells, for example,  $5 \times 10^{11}$  cells may require resuspending the cells up to 16.7 L of culture media.

[0036] In some embodiments, there is described a lower cell number range for transfection of about  $10 \times 10^5$  cells. The present embodiment also encompasses the following cell numbers for transfection:  $10 \times 10^6$  cells,  $10 \times 10^7$  cells,  $10 \times 10^8$  cells,  $10 \times 10^9$  cells,  $10 \times 10^{10}$  cells, and  $10^{11}$  cells. The present disclosure also envisions using for electroporation any number of cells in between the numbers described above as well as amounts that go above and below the number disclosed.

[0037] In some embodiments, there is disclosed a method of plating an appropriate amount of the electroporated cells in EP buffer into plates, for example, 10 cm, 15 cm, 100 cm, cell stack, cell factory vessel and providing the cells with a recovery period that can be 10 minutes, 20 minutes, 30 minutes, 40 minutes, 50 minutes, 60 minutes, 90 minutes, or 120 minutes or any amount of time in between the disclosed values as well as more and less amount of time than the maximum and minimum amounts of time disclosed.

[0038] In some embodiments, the present disclosure also encompasses adding an appropriate amount of cell culture media like, for example, DMEM media to the cell culture plates containing electroporated cells and EP buffer. The disclosed media can be supplemented with serum, for example fetal bovine serum, horse serum, human serum, or other synthetic serum. The level of serum supplementation in the media can be, for example, 2%, 5%, 10%, 15%, or 20% or any value between those percentages, as well as above and below the maximum and minimum values disclosed.

[0039] In some embodiments, the present embodiments and disclosure also includes adding compounds to the cell culturing process to increase transcription efficiency in the cells. These

compounds may include, but are not limited to, sodium butyrate and/or caffeine. Sodium butyrate may be added to increase transcription efficiency at a final concentration of 0.4 mM, 1.0 mM, 1.5 mM, 2 mM, 2.5 mM, 3.0 mM, 3.5 mM, 4.0 mM, 4.5 mM, or 5 mM, or any value between those disclosed as well as a concentration that goes below or above the disclosed minimum and maximum. The sodium butyrate can be substituted by caffeine at a concentration that includes 0.4 mM, 1.0 mM, 1.5 mM, 2 mM, 2.5 mM, 3.0 mM, 3.5 mM, 4.0 mM, 4.5 mM, or 5 mM, or any value between those disclosed as well as a concentration that goes below or above the disclosed minimum and maximum. The present disclosure also encompasses sodium butyrate combined with caffeine to increase transcription efficiency. The present disclosure encompasses the combination of sodium butyrate and caffeine wherein the concentration of each reagent is applied using any of the concentrations described above for each reagent. The present disclosure encompasses applying the combination of caffeine and sodium butyrate at a ratio of, for example, 1:1, 1:2, 1:3, 1:4, 1:5, or others that go above and below the disclosed ratios. Similarly, the present disclosure encompasses applying the combination of sodium butyrate and caffeine at a ratio of for example 1:1, 1:2, 1:3, 1:4, 1:5, or others that go above and below the disclosed ratios. In some embodiments, the present disclosure includes adding sodium butyrate or caffeine, or the combination or none, after about 1 hour after adding media to the electroporated cells. Instead of sodium butyrate or caffeine, other compounds known to increase transcription efficiency can be added to the cell culture in an amount and concentration sufficient to increase transcription efficiency in the cells.

[0040] In some embodiments, the present disclosure also includes adding butyrate or caffeine, or the combination for 1.5 hours, 2.0 hours, 3.0 hours, 4.0 hours, 8.0 hours, or 10 hours after media was added to the electroporated cells. The addition of sodium butyrate or caffeine, or the combination of the two can also be added at any value of time between those disclosed as well as at a time below or above the disclosed minimum and maximum.

[0041] In some embodiments, the present disclosure also encompasses incubating the electroporated cells cultured with media and sodium butyrate or caffeine (or both) for a period of time including but not limited to 18 hours, 20 hours, 24 hours, 30 hours, 38 hours, 48 hours as well as a number of hours that go above and below the numbers disclosed.

[0042] In some embodiments, the method contemplates subjecting the cells cultured with media and sodium butyrate or caffeine (or both or none) to a temperature shift that can include a delta of 15° C of a difference. For example, the temperature shift can change a starting temperature from 37° C to 28° C, from 37° C to 30° C, 37° C to 32° C, 37° C to 34° C, or 37° C to 36° C. The temperature shift can also change the starting temperature from 35° C to 28° C, from 35° C to 30° C, 35° C to 32° C, or 35° C to 34° C. The temperature shift can also change the starting temperature from 33° C to 28° C, from 33° C to 30° C, or 33° C to 32°. The temperature shift can also include a starting temperature above or below those explicitly disclosed above and a final temperature above or below those explicitly disclosed above. The present disclosure encompasses culturing the cells in the final temperature of the temperature shift 12 hours, 18 hours, 24, hours, 30 hours, 36 hours, 42 hours, 48 hours, 72 hours, or 96 hours. The amount of time culturing the cells at the final temperature of the temperature shift can also include any amount of time between the times disclosed, as well as more and less time than the maximum and minimum amounts of time disclosed.

[0043] The present disclosure encompasses collecting the VLPs released by the cells to the media using a combination of syringes and filters, wherein the filters include those with pores of 0.45 microns or larger. The present disclosure encompasses concentrating the collected VLPs using Lenti-X™, using standard protocols available, using for example 9 mL VLP + 3 mL Lenti-X™. In addition, the present disclosure encompasses using ultracentrifugation to concentrate the VLPs, with, for example, 100,000 g for 2 hours at 4° C, with 20% sucrose

weight by volume as a cushion. The present disclosure encompasses using the concentrated VLP solution for downstream applications or storing the VLP solution at 4° C.

[0044] The present disclosure includes embodiments that generate VLPs for the purpose of generating non-targeted gene editing with, for example, Cas9 WT protein or any other suitable gene editing technology. The efficiency of the non-targeted gene edits can be quantified with tools like Inference of CRISPR Edits ("ICE"), Tracking of Indels by Decomposition ("TIDE"), Tracking of Insertion, Deletions and Recombination events ("TIDER"), or others.

[0045] The present disclosure includes embodiments wherein the VLPs are generated for the purpose of producing targeted gene editing. For such embodiments, the vectors used to generate the VLPs may differ from those disclosed above. For example, instead of a non-specific sgRNA vector as the guide sequence for gene editing, the vector guide sequence could include a guide sequence ranging from 15 nucleotides to 30 nucleotides that target a specific region of the DNA meant to be target and or edited.

[0046] The present disclosure includes embodiments wherein the VLPs are generated in such a way that they target specific cell types by, for example, replacing the VSV-G vector described above with another vector that synthesizes a different VLP envelope that targets a specific cell type, such as rabies virus envelop ("RV-G") that targets neural cells, or a viral glycoprotein bearing a cell-specific ligand or single chain antibodies.

[0047] The present disclosure includes embodiments wherein the VLPs are generated to function as a reporter system or visualization or imaging system for target cells. Such embodiments would include the VLPs encapsulating green fluorescent protein, blue fluorescent protein, yellow fluorescent protein, red fluorescent protein, or other similar naturally occurring or altered fluorescent proteins. In another example, the protein encapsulated by the VLP is luciferase or a similar protein that allows measurements of light emitted by the protein acting on a particular target molecule.

[0048] The present disclosure includes embodiments wherein the VLPs are used to suppress transcription of a target sequence by, for example, replacing the Cas9 vector disclosed with a catalytically inactive Cas9 protein (*e.g.*, dCas9) and providing a corresponding guide sequence targeting the desired DNA sequence, such that the Cas9 protein blocks the transcription of corresponding RNA molecules, thus suppressing the resulting protein mRNA alone and, where applicable, the corresponding protein.

[0049] The present disclosure includes embodiments wherein the VLPs are used to activate transcription of a target sequence by, for example, replacing the disclosed vectors coding for the Cas9 protein and the vector containing the guide sequence and replacing it with a vector containing dCas9 fused with an activation domain that recruits transcription activation proteins (*e.g.*, VP64 or others alone or in combination) and a corresponding guide sequence that targets, for example, the promoter or enhancer region of the desired gene, to activate transcription of the desired gene.

[0050] The present disclosure includes embodiments wherein the VLPs are used to effectuate prime editing. Prime editing includes gene editing that introduces base to base conversions as well as small insertions or deletions without creating double-strand breaks in the process. The embodiments supporting the use of VLPs for prime editing include, for example, replacing the vector coding for the Cas9 protein disclosed above with a vector that codes for Cas9 nickase fused with a reverse transcriptase. Similarly, the embodiments supporting the use of VLPs for prime editing include replacing the guide sequence vector disclosed above with another guide sequence that is longer, for example a guide sequence that is more than 90 or more than 100 nucleotides in length.

[0051] The present disclosure includes embodiments wherein the VLPs are used to effectuate base editing. Base editing includes gene editing that introduces precise changes to nucleotides without creating double-strand breaks. The embodiments supporting the use of VLPs for base

editing include, for example, replacing the vector coding for the Cas9 protein discussed above with a vector that codes for an inactive Cas9 enzyme (*e.g.*, dCas9) fused with a base modifying enzyme. Suitable base modifying enzymes include cytosine base editors (*e.g.*, cytidine deaminase) and adenine base editors (*e.g.*, adenine deaminase).

[0052] The present disclosure includes embodiments wherein the VLPs are used to reprogram cells by, for example, replacing the vector coding for the Cas9 protein described above with vectors coding for the Yamanaka factors (*e.g.*, Oct3/4, Sox2, Klf4, c-Myc) or other combination of proteins or molecules that help induce pluripotency, multipotency, unipotency on target cells, or that can help differentiate a target cell into another cell type such as neurons and skeletal muscle cells.

[0053] The present disclosure includes embodiments wherein the VLPs are produced for the purpose of inserting a target molecule into a target cell, by replacing for example the Gag vector or vectors described above with another Gag vector fused to the genetic sequence of the protein of interest.

[0054] **Figure 1** tests ranges of DNA to use in electroporation experiments to maximize the yield of VLPs. Contrary to chemical transfection, electroporation allows researchers to experiment with different DNA concentrations and amounts to maximize results. If the DNA concentration or amount used is too low, the resulting transfection will be less efficient, however, if the DNA concentration is too high, then cell death and cytotoxicity will also render the transfection less effective. For Figure 1, the following experimental procedures were followed. Adherent cells were passaged one day before electroporation so that the cells were around 70% confluent on the day of electroporation. For the electroporation, adherent HEK293 cells were washed once with PBS, after removing the PBS, the cells were incubated with a thin layer of TrypLE™ Select Enzyme dissociation reagent at a 1.5 dilution, sufficient to cover the cells. The TrypLE™ Select Enzyme dissociation reagent was added carefully to avoid

disrupting the cell adherence by, adding the reagent slowly and to the wall of the cell culture plate. The plate was rocked and then the dissociation reagent was immediately aspirated. Cells were incubated at 37° C for 30 seconds and then resuspended in DMEM media containing high glucose and 10% FBS. Cells were then centrifuged at  $250 \times g$  for ten minutes at room temperature. After aspirating the media, the cells were resuspended with MaxCyte buffer (MaxCyte EP Buffer and Opti-MEM) to a final density of  $1.5 \times 10^8$  cells/mL. The resuspended cells were separated and mixed with different amounts of total DNA concentration, the total DNA composed of varying amounts of four different vectors (VSV-G, MMLVgag-pro, MMLgag-3xNES-Cas9, and HEK3 sgRNA). The total DNA concentrations used were 50 µg/mL, 100 µg/mL, 200 µg/mL, 300 µg/mL, and 400 µg/mL. The amount of each vector used for each sample is outlined in Table 1.  $1.5 \times 10^7$  cells were mixed with the appropriate DNA combination and amount to a total of 100 µL and placed in an OC-100 processing assembly. Cells were electroporated and transferred to a culture vessel. Cells were incubated for 30 to 40 minutes at 37° C (5% CO<sub>2</sub>, 80% humidity) to allow their recovery. After recovery, pre-warmed complete media was added to the cells (DMEM, 10% FBS). The cells were then seeded in a 10 cm culture vessel ( $1.5 \times 10^7$  cells or  $2.6 \times 10^5$  cells/cm<sup>2</sup>). After 24 hours, the cells were shifted from 37° C to 33° C. The media was harvested to collect VLPs after 48 hours of electroporation. To precipitate VLPs from the media, Lenti-X was used. Briefly, a 1:4 amount of Lenti-X concentrator solution was used (e.g., 9 mL of media +3 mL of Lenti-X concentrator). The tube was inverted multiple times to mix, and then incubated overnight at 4° C. The tube was then centrifuged at  $1,500 \times g$  for 45 minutes at 4° C to obtain a white pellet containing the VLPs. The pellet was resuspended in 100 µL of PBS and stored in aliquots at -80° C. To test the VLP activity, 40,000 HEK293 cells were seeded into each well of a 48-well plate and were inoculated with 0.1, 1, or 5 µL of concentrated VLPs. Figure 1A shows that 300 µg/mL of DNA used showed the highest editing activity and best balance between the cell number used and

active VLPs produced. The output for editing activity or editing efficiency is based on the total number of insertions and deletions caused by the VLP-mediated introduction of the wild type Cas9 protein (generated from the vector: MMLgag-3xNES-Cas9) coupled with the guide sequence (generated from the vector: HEK3 sgRNA) that led to the production of non-specific genetic changes throughout the genomic region of the target cells. The DNA of the target cells was sequenced using Sanger sequencing and the sequences were analyzed using ICE. Figure 1B shows the total VLP producer cell number measured 48 hours after electroporation, demonstrating that higher concentrations of DNA correlate with reduced VLP producer cell number. The highest DNA concentration of 400 µg/mL caused the lowest cell yield. Taken together, the data shows that, while the DNA concentration of 300 µg/mL had reduced cell number compared with other DNA concentrations, this concentration of DNA resulted in the highest VLP activity. Therefore, it can be interpreted that although there were fewer cells, more VLPs were being generated per cell.

[0055] **Figure 2** tests multiple electroporation parameters to show higher transfection efficiency and VLP yield than what can be obtained using chemical transfection facilitated by jetPRIME®. The mechanism of entry of DNA into the cells is different between chemical and electrical transfection (electroporation). jetPRIME® encapsulates the DNA so it can enter the cell through endocytosis, a long process for the cargo to get delivered into the nucleus and start expressing to produce VLPs. Since it is a prolonged process, there is a delay in the titer production when using chemical transfection with jetPRIME®. In contrast, with electroporation, DNA is immediately introduced into the cells, likely through the cytoplasm first, then making its way to the nucleus for expression. In sum, the advantage of electroporation is that the DNA is delivered into the cell immediately and VLP production can start earlier because of its delivery mechanism. For the electroporated samples, the procedure describing Figure 1 was used, with the following changes: 1 mM of sodium butyrate was added

to the cells 4 hours after electroporation. VLPs were concentrated 100x and resuspended in PBS. Varying volumes of VLPs were inoculated onto 40,000 HEK293T cells and the gene editing efficiency was assessed using Sanger sequencing and an analysis tool called ICE that quantifies the insertions and deletions from a Sanger sequence file. Figure 2A shows the percentage of indels (indels is meant to define a type of genetic variation in which a nucleotide sequence or sequences were added or deleted) in target cells following a chemical transfection using jetPRIME® under the manufacturer's transfection protocol. The graph shows the difference in gene editing efficiency when VLPs were harvested after 24 hours or 48 hours after transfection. The results of Figure 2A show an increase in gene editing efficiency directly proportional to the amount of VLP added to the target cells. However, the gene editing efficiency wherein the VLPs were harvested 24 hours after chemical transfection is markedly reduced compared to VLPs harvested 48 hours after chemical transfection. Figures 2B through 2F also show gene editing efficiencies as above but using varying electroporation protocols as the methods of producing VLPs. For Figures 2B through 2F, for all electroporation protocols tested, VLP production and activity were higher than chemical transfection on both day 1 and day 2. Importantly, it can be interpreted that electroporation enabled faster delivery of the DNA cargo which allowed for faster production of VLPs in comparison with chemical transfection. There was only a modest increase in VLP production from Day 1 to Day 2 with electroporation, indicating that a 1-day manufacturing process could be used to shorten the time by one day. In contrast, chemical transfection had very low yields and activity of VLPs generated by Day 1 after transfection. Finally, Figure 2G shows the difference in the activity and VLPs generated by electroporation versus the standard chemical transfection method without any sodium butyrate and harvested 2 days after transfection. Electroporated cells had 1 mM of sodium butyrate added 4 hours after electroporation. Depending on the titrated volume of VLPs, the fold increase in editing efficiency varied but indicated that electroporation resulted in between

a 20-40-fold increase in VLPs versus chemical transfection. Figure 2H illustrates ELISA results that demonstrate that electroporated cells produced roughly 4x higher MMLV p30 levels than chemically transfected cells. The VLP samples used to measure the p30 levels are from Figure 2G. Figure 2I illustrates ELISA results that demonstrate that electroporated cells produced higher MMLV p30 levels than chemically transfected cells when VLPs were harvested on Day 1 or Day 2. The chemically generated VLP samples used to measure the p30 levels are from Figure 2A while the electroporation generated VLP samples used to measure the p30 levels are from Figure 2F. Figure 2J illustrates Western Blot Data of Cas9 protein contained in VLPs generated by chemical transfection (Figure 2A) or electroporation (Figure 2F). 0.75  $\mu$ g p30 (VLPs) denatured in 1X LDS sample buffer at 70° C for 10 minutes and subjected to electrophoresis through a 4-12% Bis Tris polyacrylamide gel at 200V for 30 minutes. Proteins were immobilized onto a PVDF membrane and probed with mouse  $\alpha$ -Cas9 (CST 14697) antibody, diluted 1:1000. Proteins were detected with AnP-conjugated anti-mouse secondary antibody and BCIP/NBT substrate from the Invitrogen™ WesternBreeze™ Chromogenic kit. Results show that Cas9 is incorporated more efficiently in electroporation generated VLPs than chemically produced VLPs.

[0056] **Figure 3** shows how the addition of DNase after electroporation may enhance VLP producer cell survival. For Figure 3, adherent HEK293T cells were electroporated using a MaxCyte EXPERT STx™ electroporation system at a density of  $1.5 \times 10^8$  cells/mL with 300  $\mu$ g/mL of DNA. Following electroporation, cells were incubated with or without DNase (1 unit of DNase per 20  $\mu$ L of electroporation volume) for 35 minutes during cell recovery before media was added. No sodium butyrate was added. VLP producer cells were harvested 48 hours after transfection. Figure 3A shows a bright field image of electroporated untreated or DNase treated cells after electroporation. Figure 3B shows the viable cell density of the VLP producer cells 48 hours after electroporation, demonstrating a modest increase in cell number after the

cells were treated with DNase, and indicating that DNase could be used for improving the VLP producer cell recovery and number of cells.

[0057] **Figure 4** shows how high seeding density after electroporation will negatively influence VLP yield and VLP producer cell number. For this experiment, adherent HEK293T cells were electroporated using the ExPERT STx™ at a density of  $1.5 \times 10^8$  cells/mL. After electroporation, the cells were pulled and seeded onto a 10 cm plate at three densities ( $2.65 \times 10^5$  cells/cm<sup>2</sup>,  $5.29 \times 10^5$  cells/cm<sup>2</sup>,  $7.94 \times 10^5$  cells/cm<sup>2</sup>). Figure 4A shows indel activity induced in 40,000 target HEK293T cells with varying volumes of VLPs generated at the different seeding densities. Increasing from  $2.65 \times 10^5$  to  $5.29 \times 10^5$  cells/cm<sup>2</sup> resulted in lower VLP activity, while there was only a modest improvement in VLP activity at the highest seeding density of  $7.94 \times 10^5$  cells/cm<sup>2</sup>. Figure 4B shows a significant loss in cell number at the time of harvest compared when comparing the low seeding density with the higher seeding density. There was about 43% recovery with the lowest seeding density while a loss of up to 80% of cells was observed at the highest seeding density. These results suggest that one should avoid seeding at a very high cell density or else more cells would be lost, resulting in less VLP activity and yield.

[0058] **Figure 5** shows how sodium butyrate may improve the VLP productivity per cell. Sodium butyrate was added to electroporated cells, following the methods described above, 4 hours after electroporation at concentrations ranging from 0 mM to 4 mM. VLPs were harvested and concentrated 100x in PBS, as described above, and then inoculated onto 40,000 target HEK293T cells at varying volumes (0.1 µL, 1 µL, and 5 µL). The editing efficiency of the cells was evaluated by Sanger sequencing and analysis using a tool called ICE. Figure 5A shows there is not a large difference in the impact of sodium butyrate on the overall VLP activity and yield. Figure 5B shows the total VLP producer cell number measured 48 hours after electroporation and treatment with a range of sodium butyrate concentrations from 0 mM

to 4 mM. These data demonstrated that higher concentrations of sodium butyrate correlate with reduced VLP producer cell number, even though the overall VLP activity produced was the same. The combined results of Figure 5A and 5B suggest that although cell number decreases, a higher productivity of VLP activity is achieved per producer cell when adding increasing concentrations of sodium butyrate.

[0059] **Figure 6** shows how temperature shifting VLP producer cells after 24 hours may improve the VLP production per cell. For this figure, cells were electroporated as described above and plated onto 10 cm plates. 1mM of sodium butyrate was added 4 hours after cells were electroporated. VLPs were harvested and concentrated 100x in PBS and then inoculated onto 40,000 target HEK293T cells at 5  $\mu$ L as described above. Figure 6A shows the total VLP producer cell number calculated 48 hours after electroporation by trypan blue staining. Less cells were observed when the producer cells were shifted to 33° C after 24 hours compared with cells maintained at 37° C for 48 hours. Figure 6B shows the relative activity of indels generated per VLP producer cell, demonstrating that the productivity per producer cell is higher when the temperature shift from 37° C to 33° C is implemented.

[0060] **Figure 7** shows how electroporation enables higher productivity per VLP producer cell when compared with chemical transfection. For this figure, adherent HEK293T cells were transfected with a chemical transfection reagent according to the manufacturer's instructions, or electroporated with a MaxCyte ExPERT STx™ electroporation system. No sodium butyrate was added to the electroporated cells, but the cells were shifted to 33° C 24 hours after electroporation, and VLPs were harvested and concentrated 100x in PBS and then inoculated onto 40,000 target HEK293T cells using 5  $\mu$ L of concentrated VLPs, following the protocols described above. The editing efficiency of the cells was evaluated by Sanger sequencing and analyzed using a tool called ICE. Figure 7A shows a bright field image of HEK293T VLP producer cells 24 hours after electroporation. Figure 7B shows the activity of 5  $\mu$ L of VLPs

generated by either chemical or MaxCyte electroporation. The results show the VLP activity from electroporated VLPs compared with those produced from chemical transfection. Figure 7C shows the percentage of VLP activity per 1 million VLP producer cells generated by chemical and electroporation. The results show better VLP activity per million producer cells with electroporation compared with chemical transfection. The calculation was performed by dividing the percentage of indels by the VLP producer cell number at the time of VLP harvest, 48 hours after transfection.

[0061] **Figure 8** shows that electroporation energy correlated with higher VLP activity in an energy-dependent manner. These results demonstrate that VLP activity could be improved by increasing the electroporation energy.

[0062] **Figure 9** shows electroporation enables packaging of adenine base editor (ABE) RNPs into VLPs to target HEK3 loci produced from suspension HEK cells. 5  $\mu$ L of VLPs produced without sodium butyrate and titrated onto adherent HEK293T cells could induce 42% base substitution at A6 (A:T to G:C). Addition of sodium butyrate to production media improved editing, up to 71% at A6. Base editing at A8 occurred at 48% and 75%, without and with sodium butyrate, respectively. These results provide preliminary evidence to demonstrate the capacity for MaxCyte electroporation to enable production of VLPs loaded with ABE-RNPs that induce high rates of base substitutions. VLPs were produced in suspension HEK293 cells with chemical enhancer. To produce the data, VLPs were harvested from culture supernatant one day post-electroporation, purified, and titrated onto adherent HEK293 cells. Genomic DNA was isolated 3 days post-transduction, PCR amplified, and submitted for Sanger sequencing. Base edits are calculated using the EditR software.

[0063] Table 1 describes the total amount of DNA used for every experiment as well as how much of each vector was used for each experiment.

**Table 1.** Vectors and Amounts of DNA Used in Experiments.

| Plasmid            | 1 × 10 cm plate by Lipid Transfection (μg) | For an OC100- 50 μg/mL concentration total (μg) | For an OC100- 100 μg/mL concentration total (μg) | For an OC100- 200 μg/mL concentration total (μg) | For an OC100- 300 μg/mL concentration total (μg) | For an OC100- 400 μg/mL concentration total (μg) |
|--------------------|--------------------------------------------|-------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|
| VSV-G              | 0.4                                        | 0.2                                             | 0.4                                              | 0.9                                              | 1.3                                              | 1.7                                              |
| MMLVgag-pro        | 3.375                                      | 1.8                                             | 3.6                                              | 7.3                                              | 10.9                                             | 14.5                                             |
| MMLVgag-3xNES-Cas9 | 1.125                                      | 0.6                                             | 1.2                                              | 2.4                                              | 3.6                                              | 4.8                                              |
| HEK3 sgRNA         | 4.4                                        | 2.4                                             | 4.7                                              | 9.5                                              | 14.2                                             | 18.9                                             |
| Total              | 9.3                                        | 5                                               | 10                                               | 20                                               | 30                                               | 40                                               |

### Examples

[0064] What follows are a series of examples that illustrate the present invention in a non-limiting matter. Accordingly, the following detailed description is not limited to the disclosed embodiments and examples. Instead, the proper scope is defined by the appended claims.

[0065] **Example 1:** HEK293 cells were grown using standard cell culture conditions until the cells were in a log phase and the total cells was about  $2.08 \times 10^6$ . The cells were dissociated using EDTA. The cells were transferred in suspension into a capability tube of 80 mL of suspension. The cells were spun at a speed of 250 g for 10 minutes at room temperature. In parallel, the vectors were prepared and mixed in electroporation buffer (MaxCyte EP Buffer and Opti-MEM) in a separate vial. The vectors used, and the amount used per 10 cm plate were the following: (1) MLV-Gag 109 μg/mL or 10.9 μg in 100 μl of EP volume; (2) MLV-Gag-Cas9 36 μg/mL or 3.6 μg in 100 μl of EP volume; (3) VSV-G 13 μg/mL or 1.3 μg in 100 μl of EP volume; and (4) sgRNA 142 μg/mL or 14.2 μg in 100 μl of EP volume. 0.8 mL of electroporation buffer was mixed with the plasmid mix. The supernatant was removed from the cell pellet after centrifugation was finished. The cell pellet was resuspended with fresh electroporation buffer and the final volume was adjusted to 1.1 mL. The suspended pellet was transferred into an appropriate vessel and resuspend in either OC-100. The cells were

electroporated and transferred from OC-100 to a 1.5 mL Eppendorf tube. The OC-100 was washed with 10-20  $\mu$ L of electroporation buffer. The volume was adjusted to 1100  $\mu$ L using electroporation buffer. 100  $\mu$ L, 200  $\mu$ L, and 300  $\mu$ L of the mixture was transferred from the Eppendorf tube to a 10 cm plate (separately, respectively). The cells recovered at 37° C for 35 minutes. 10 mL of DMEM media supplemented with 10% fetal bovine serum was added and mixed. The mixture was incubated at 37° C for 4 hours. Sodium butyrate was added at a concentration ranging from 0.5 mM to 4 mM, to the 10 cm plates. Without being bound by any particular theory, a concentration of sodium butyrate of 4 mM was found to be effective and improved the production of VLP per producer cell. After 24 hours, the temperature was shifted from 37° C to 33° C and incubated for 48 hours. The VLPs were collected from media using a syringe and filter of 0.45 microns. The resulting solution was inverted numerous times. The solution with VLPs was stored overnight at 4° C. 40000 HEK293T cells were seeded in a 48-well plate and incubated for 24 hours. The cells were inoculated in each well of the 48-well plate with VLP solution using the following volumes: 0.1  $\mu$ L, 1  $\mu$ L, 5  $\mu$ L. Following culturing of the cells with the VLPs, editing of the cells was determined by harvesting the cells, collecting gDNA, amplifying the HEK3 loci, purifying the PCR products, and submitting the samples for Sanger sequencing. Indel analysis was conducted on the Sanger trace files using the Synthego ICE tool.

**Claims**

What is claimed is:

1. A method of producing virus-like particles ("VLP") using electroporation, comprising:
  - cultivating a population of cells in a log growth phase in a cell culture vessel using media;
  - cultivating the population of cells to a desired level of confluency;
  - removing the media from the cell culture vessel and adding a transfection buffer;
  - adding to the cell culture at least one vector in a desired concentration and a desired amount;
  - transfecting the cell population via electroporation;
  - culturing the cell population at a desired temperature for a desired period of time; and
  - seeding the cells at a desired density.
2. The method of claim 1, further comprising adding an endonuclease to the cell culture vessel prior to seeding.
3. The method of claim 1, further comprising adding a compound that increases transcription efficiency at a desired concentration and amount to the cell culture after the seeding.
4. The method of claim 1, further comprising subjecting the cell culture to a temperature shift for a desired period of time.
5. The method of claim 1, wherein the population of cells is comprised of the cell line HEK293.
6. The method of claim 1, wherein the population of cells is derived from the cell line HEK293.

7. The method of claim 1, wherein the media comprises cell culture media supplemented with serum.
8. The method of claim 1, wherein the cell culture vessel comprises a 6-well plate, a 10 cm plate, a 15 cm plate, a cell stack or cell factory vessel, or another vessel.
9. The method of claim 1, wherein the high level of confluence comprises at least 60%-80%.
10. The method of claim 1, wherein the transfection buffer is electroporation buffer.
11. The method of claim 1, wherein the desired final concentration of the at least one vector added to the cell culture comprises a range of 50 µg/mL to 400 µg/mL.
12. The method of claim 1, wherein the desired amount of the at least one vector added to the cell culture comprises a range from 0.1 µg to 300 µg.
13. The method of claim 1, wherein the desired amount of the at least one vector added to the cell culture comprises a range from 300 µg to 1000 µg.
14. The method of claim 1, comprising adding multiple desired vectors added to the cell culture vessel, which are not identical.
15. The method of claim 14, wherein the amount of each vector added is not identical.
16. The method of claim 14, wherein the desired vectors added to the cell culture comprise combinations of identical and non-identical vectors.
17. The method of claim 1, wherein the at least one vector added to the cell culture vessel ranges from about 1 to 10.
18. The method of claim 1, wherein transfecting the cell population is performed using an appropriate device that includes one or more electroporation machines.
19. The method of claim 1, wherein the desired temperature comprises a range from about 32° to 37° C.

20. The method of claim 1, wherein the seeding density after transfection comprises an amount equal to or less than  $3 \times 10^5$  cells per  $\text{cm}^2$  of surface area.
21. The method of claim 3, wherein the compound that increases transcription efficiency comprises sodium butyrate.
22. The method of claim 21, wherein sodium butyrate is found in a concentration ranging from 0.1 mM to 10 mM.
23. The method of claim 3, wherein the compound that increases transcription efficiency comprises caffeine.
24. The method of claim 23, wherein the caffeine is found in a concentration ranging from 0.1 mM to 10 mM.
25. The method of claim 3, wherein the compound that increases transcription efficiency comprises a combination of sodium butyrate and caffeine.
26. The method of claim 4, wherein the temperature shift comprises a reduction in temperature of about  $10^\circ\text{C}$ .
27. The method of claim 4, wherein the temperature shift comprises a reduction in temperature from about  $37^\circ\text{C}$  to about  $28^\circ\text{C}$ .
28. The method of claim 4, wherein the temperature shift comprises a reduction from a higher temperature to a lower temperature.
29. The method of claim 4, wherein the temperature shift comprises a reduction in temperature from about  $37^\circ\text{C}$  to about  $33^\circ\text{C}$ .
30. The method of claim 4, wherein the desired period of time for a temperature shift comprises about 18 to 72 hours.
31. The method of claim 2, wherein the endonuclease is DNase.