(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 February 2007 (15.02.2007)

PCT

(10) International Publication Number
WO 2007/018744 A2

(51) International Patent Classification: Not classified

(21) International Application Number:
PCT/US2006/023439

(22) International Filing Date: 14 June 2006 (14.06.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/705,348 3 August 2005 (03.08.2005) US
PCTUS22000/045028
12 December 2005 (12.12.2005) US
60/762,172 25 January 2006 (25.01.2006) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

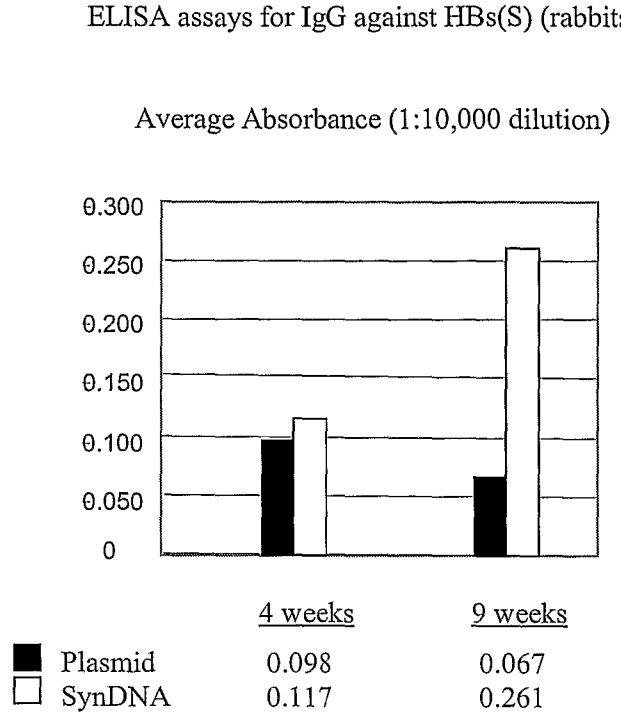
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

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(54) **Title:** CELL-FREE BIOSYNTHESIS OF NUCLEIC ACID



(57) **Abstract:** The invention provides for a process to make high quality DNA in a cell-free system, free of bacteria contaminants, and optimally free of flanking bacterial gene coding sequences which can minimize or silence gene expression when used for expression inside a target cell. The cell-free system herein is a rapid method that produces a high fidelity, cleaner end product suitable for therapeutic applications with less effort and expense, and can be adapted to amplification of plasmid-like templates lacking unnecessary bacterial plasmid gene sequences. This increases efficiency of the system and increases the effectiveness of the end product expression vector. The end product can be easily used as a DNA therapeutic due to low incidence of bacterial cell components and bacterial toxins. The invention also provides a method for the production of DNA for any research or therapeutic purpose that is essentially free of inherent bacterial cell contaminants and/or bacterial toxins, as well as for the end product therapeutics including DNA vaccines.

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CELL FREE BIOSYNTHESIS OF NUCLEIC ACID

FIELD OF THE INVENTION

[0001] The invention relates to a process for making research and high quality nucleic acids in a cell-free system, products made using this process, the use of these products in research and therapeutic applications, and an apparatus designed for making large batches of these products.

BACKGROUND OF THE INVENTION

[0002] The advent of DNA-based therapeutics in gene transfer, gene therapy and DNA vaccination has increased the demand for large-scale production of DNA that meets stringent quality criteria in terms of purity, potency, efficacy and safety. Because the efficacy and duration of gene expression in target tissue is relatively low, large amounts of DNA are typically needed and the need for high purity is critical in this context.

[0003] The current state of DNA therapeutic applications relies upon the use of plasmids grown in bacterial culture, requiring expensive purification techniques for the production of therapeutic quality nucleic acids. Typical plasmid purification procedures from bacteria and other cell sources include methods that use organic, mutagenic and toxic compounds including phenol, ethidium bromide and cesium chloride, and enzymes such as lysozyme, proteinase K and RNase A. All of these can constitute potential health hazards if injected as contaminants in a DNA-based therapeutic. Such procedures also carry a potential risk of incorporating unintended contaminating transposons and other foreign episomal DNA into the plasmid. There is also the potential for contamination by residual host cell nucleic acids, other cellular proteins and endotoxins. Such impurities can minimize the efficiency of DNA uptake and can lead to dose-related toxicity. To remove these impurities, accepted purification methods often use multiple chromatographic steps, including anion exchange, affinity, and size-exclusion. These purification procedures are costly.

[0004] In vitro isothermal amplification techniques have been used for years to study the mechanisms of DNA replication. In 1984, Blanco and Salas originally isolated the phage Phi29 DNA polymerase, which is a highly processive, strand displacement polymerase. Phi29 DNA polymerase can reliably reproduce DNA strands greater than 70 kilobases long (the full length of Phi29 genome) and can be used in DNA sequencing, DNA amplification, and for synthesis of DNA greater than 10 kilobases long. There are now modified forms of the enzyme, including an exonuclease-deficient form which is used to reduce the enzyme's inherent ability to remove the labeled bases useful in sequencing reactions. Such modified polymerases produce copies with lower overall fidelity and are primarily useful for only sequencing-type reactions where lower fidelity is preferred.

[0005] In nature, the replication of circular DNA molecules, including plasmid and some viral DNAs, frequently occurs by rolling circle amplification (RCA), whereby the circular DNA template

is replicated into a long concatamer of tandem repeats. The concatamer is subsequently packaged into a protein coat during viral replication. RCA has been used frequently for basic research applications including DNA sequencing, cloning, library construction, and screening. (Inoue-Nagata, et al., J. Virological Methods 116: 209-211, 2003). It has been streamlined for special applications such as for the determination of copy number for a target message in a given sample. This uses controlled cycle amplification which can be modified to detect specific mutant alleles of a target gene.

[0006] The method itself is adaptable for use with several different types of primers. Random primers as well as sequence specific primers have been used, offering flexibility for use of the process in multiple applications. For example, sequence specific primers designed to nest on newly synthesized DNA can be used to initiate secondary synthesis of already amplified product. Amplification can occur exponentially, providing an isothermal alternative to PCR for the detection of multiple targets simultaneously. Long terminal repeats have been used to facilitate these nested amplifications and to quickly identify the presence of a particular sequence in a sample. In a similar manner, the use of multiple primers enables the process to be used to identify specific target sequences, as well as for the synthesis and detection of address tags and oligonucleotides.

[0007] Different DNA polymerases have also been used to improve the performance of some of these common research applications, including sequencing, cloning, mapping, genotyping, probe generation and diagnostic screening. For instance, DNA polymerases lacking the normal corrective 3',5'-exonuclease activity have been designed and used to improve the rate of incorporation of labeled nucleotides for improving the efficiency of incorporation of labeled nucleotides during sequencing reactions.

[0008] Several different DNA polymerases have been shown to be effective for in vitro DNA amplification; however, the majority of RCA applications incorporate the use of the Phi29 DNA polymerase because it is highly processive, able to synthesize long concatamers of nucleic acid quickly and has a strand displacement activity which enables it to continuously synthesize new nucleic acid sequences while displacing any secondary primers it might encounter. Phi29 polymerase can produce large amounts of high fidelity nucleic acid in a relatively short period of time without thermal cycling and with an extremely low error rate of about 4 x 10-6.

[0009] Other polymerases such as bacterial DNA polymerases III (Pol III) and I (Pol I) can also be used in RCA. Different enzymes confer different advantages to the system. For example, Pol III reportedly has a clamp-like activity that provides an increased rate of DNA synthesis (about 700-800 nucleotides per second) which may be optimized by adding helicases or stabilizing proteins. Pol I can be used to amplify templates smaller than 100 bp because it uses predominantly single stranded templates; small circular templates can be readily formed without steric hindrance which is often

associated with extremely short double-stranded templates. A modified Pol I comprising a sequence derived from T7 DNA polymerase has been shown to increase its efficiency up to 500-fold while reducing its ability to discriminate between deoxy- and dideoxynucleotides and conferring an advantage for sequencing applications.

[0010] These reactions can be adapted to utilize either single- (ss) or double-stranded (ds) nucleic acid templates. Phi29 DNA polymerase can use either ss or ds templates, but Pol I can only use a ss template. Phi29 polymerase also recognizes both RNA and DNA templates and therefore has more flexibility for use in RCA and other similar amplification reactions. RCA has been adapted to produce RNA or DNA oligonucleotides (28-74 nucleotides long) using a small single-stranded circular DNA template.

[0011] Recent research efforts have turned to improving the use of nucleic acids for use in the production of vaccines. For example, Moreno's group developed minimalistic, immunogenically defined gene expression vectors (MIDGE vectors) (Vaccine, 22: 1709-1716, 2004) which minimally contain sequences needed for eukaryotic gene expression and induction of an immune response. They have also shown that end modification of the MIDGE expression cassettes to have hairpin loops increases their longevity and expression efficiency. However, the MIDGE vectors must first be cut out of bacterially grown plasmid and then modified; this is a complex, time consuming, and labor intensive process.

[0012] In a similar manner, Chen's group developed minicircle technology to remove bacterial sequences from the bacterial plasmid vector. They showed that cis-linked bacterial sequences can decrease or even silence eukaryotic gene expression (Human Gene Ther. 16: 126-131, 2005) whereby linear expression cassettes lacking bacterial genetic material, express 10-100 times more efficiently than comparable covalently closed circular plasmids and that such increased expression can last up to 90 days following transfection. The minicircle technology uses attB and attP sequences flanking the expression cassette to facilitate recombination within the plasmid and removal of unwanted bacterial sequences carried on the original plasmid vector. The appropriate host encodes for an inducible recombinase needed for the recombination event which can be induced after sufficient plasmid is produced inside the growing cell culture. The intracellular recombination generates two separate minicircles: one with the expression cassette and the other with bacterial genetic materials. This system again uses bacterially grown plasmid DNA which carries with it the problem of purifying away bacteria associated contaminants.

[0013] Therapeutic applications using DNA require strict adherence to safety and effectiveness standards and must be certified as Good Manufacturing Practice (GMP) (Prazeres, D.M. et al, Trends Biotechnol 17(4): 169-173, 1999). Plasmid DNA produced in large-scale facilities should be free of contaminating genomic DNA (<10 ng/dose), host proteins (<10 ng/dose), RNA (non-

detectable on 0.8% agarpse gel), and endotoxins (<1 Unit/kg body weight, or <0.1 EU/ug plasmid). In addition, the plasmid should be sterile and, in present practice, preferably in supercoiled form that can be more efficiently expressed. Other contaminants that require removal include purification reagents, such as ethidium bromide, chloroform, phenol, lysozyme, proteinase K, RNase A, and any potential contaminants that may leach from the purification columns such as quaternary amines from anion exchangers.

[0014] Any DNA therapeutic, whether for animal or human use, requires a high level of purity with such minimal, almost non-detectable levels of impurities. DNA products derived from bacterially grown plasmid are costly to produce because of the need to initially grow huge bacterial cultures in fermentation tanks and then to purify the product to eliminate contaminating bacterial cell products including proteins, DNA, RNA, toxins and endotoxins to meet the high standards of purity. Accepted purification methods primarily use multiple chromatographic procedures including a combination ofanion exchange, affinity, and size-exclusion chromatography purification steps. It is significant that the purification methods needed for therapeutic applications requires specialized equipment, expensive resins, extensive housing facilities and time.

[0015] Current costs for non-GMP (research quality) plasmid DNA range between $30,000–150,000 per gram of final product, and the costs for high quality GMP level DNA are approximately 2-3 fold higher. In short, the manufacture of therapeutic DNA using bacterially produced plasmid DNA can be prohibitively expensive.

[0016] Methods that focus on utilizing a cell free amplification would provide a significant cost and time savings because of the ability to avoid exposure to a multitude of bacterial contaminants. Everything in the cell free system is clearly defined. Providing that only high quality reagents and enzymes are used, only trace contaminants would be in the system. Thus, final purification can use simple procedures such as dialysis, ultrafiltration and/or gel filtration, and only small volumes of reaction mixture need be purified.

[0017] There remains a strong need for the efficient, affordable production of clean nucleic acid which is optimally free of the bacterial sequences required to reproduce the plasmids in fermentation and free of the contaminants associated with bacterially grown plasmids. To date, there are no developed methods optimized for the cell-free production of nucleic acid clean enough for use in gene therapy, DNA vaccines or other therapeutic applications. There are no developed cell-free methods for the large-scale production of high-quality research DNA which is virtually free of bacterial cell contaminants and extraneous bacterial gene sequences. Likewise, there are no DNA vaccines available on the market today which comprise DNA made using a cell-free system which, by virtue of the system of production, carry virtually "no" bacterial cell contaminants and "no" bacterial toxins. To the contrary, most of the literature suggests that the available in vitro DNA

synthesis systems have limited use for diagnostic and research purposes and even optimize these applications which tend to incorporate modifications and optimizations that work in opposition to the purpose intended herein. The current applications for in vitro DNA synthesis are primarily limited to: sequencing, genomic amplification, genomic analysis, tagging, cloning, PCR-type applications, library construction and other similar analytical applications.

BRIEF SUMMARY OF THE INVENTION

[0018] As described herein, high quality DNA can be made in a cell-free system, virtually free of bacteria contaminants, and optimally free of flanking bacterial gene coding sequences which can minimize or silence gene expression when used for expression inside a target cell. The cell-free system herein is a rapid method that produces a cleaner end product suitable for therapeutic applications, with less effort and expense, which can be designed to be more efficiently expressed in a target cell. The end product can be easily adapted for use as a DNA therapeutic due to affordable manufacture and lower levels of bacterial cell components and toxins.

[0019] The invention herein includes (1) a method optimized for the cell-free production of high quality nucleic acid, which may comprise an expression cassette, clean enough for use in gene therapy, DNA vaccines or other therapeutic applications and which may be free of unnecessary plasmid replication sequences; (2) a cell-free method for the production of DNA for any research or therapeutic purpose that is essentially free of inherent bacterial cell contaminants and/or bacterial toxins; (3) DNA vaccines comprising DNA made in a cell-free system which by virtue of the cell-free system of production, carry virtually "no" bacterial cell contaminants and "no" bacterial toxins; and (4) and apparatus for the large scale manufacture of DNA using the disclosed cell-free system.

[0020] One aspect of the invention relates to optimizing a cell free DNA amplification system for large-scale (e.g., > 1 mg) nucleic acid production, using streamlined expression cassette templates having a sequence of interest, sequence specific or random primers, high-fidelity polymerases, and a minimalistic buffer system. This system can be used to produce large amounts of nucleic acids, in small volumes, in short periods of time, with the need for only minimal and inexpensive purification procedures. Thus, the system can produce high-quality therapeutic grade nucleic acids for any basic analytical or research purpose, but also for therapeutic use.

[0021] The current invention combines several techniques for the purpose of affordably producing large amounts of high-quality nucleic acid for therapeutic, diagnostic and research applications. The method of the invention can produce 250-300 times more nucleic acids than what is produced in a comparable volume of bacteria culture. In the current invention, there is no contaminating source of endotoxin other than what is minimally contained in the reagents used. Additional advantages include: the capability of producing large fermentation-like quantities of product in a small laboratory flask; the requirement of only a minimal number of reagents; ability to

produce large amounts of product in a relatively short period of time; and streamlined purification procedures. Together, these advantages translate into an affordable way to produce large quantities of high-quality nucleic acids for therapeutic use.

[0022] In a preferred embodiment, a standard plasmid can be replicated quickly and affordably in a cell-free system, useful for both research and therapeutic applications. The advantage of producing a plasmid using this cell-free system is that the end product DNA is essentially free of any bacterial cell components and bacterial toxin. Although typical bacterially grown plasmid can be purified to acceptable levels for FDA applications as a therapeutic, these purification procedures are costly as well as time consuming, and the final product still has minimal levels of all sorts of bacterial contaminants that are not present in the end product of the current cell-free system. Many unknown contaminants may remain in even the highly purified DNA preparations that use traditional bacterially grown plasmid. Although at low levels, because of the method of production, there remains a risk of unidentified contamination which could contribute to unwanted interactions at the cellular level. DNA produced in a cell-free system is in a well defined environment and minimizes this risk significantly.

[0023] Any circular nucleic acid template can be used. In some embodiments, a template may simply be a circular expression cassette containing a sequence of interest flanked by genetic elements needed for expression and processing of the expressed product in a host (promoter, polyA, etc.). Streamlined templates having no extra genetic sequences offer multiple benefits: they eliminate any extraneous sequence that may silence the expression of the sequence of interest; the smaller constructs are more compact and can be more efficiently taken up by the target cell, leading to higher transfection efficiency; and they are more cost effective due to production of a larger quantity of an expression cassette with less material, a statistical increase in fidelity of the final product and no need for extensive purification.

[0024] Some embodiments use random primers, others use sequence-specific primers, some use a combination of the two. Sequence-specific primers are more efficient and economical in large scale amplifications but require pre-planned sequence analysis and primer synthesis. Primer sizes may range from four to greater than twenty nucleotides, and they may comprise modified bases and/or backbones for increased affinity, stability and prolonged storage. In one embodiment, a specific primer with phosphorothioate end-modification may be used to produce a large amount (about 1.5 mg in 1 ml) of nucleic acid.

[0025] The amplification step can use any specific polymerase providing buffer and temperature conditions are adjusted to accommodate the specific needs of that polymerase. Some embodiments use a thermocycling polymerase requiring multiple denaturation and annealing steps (ex, when using a high temperature taq-like polymerase). Others use processive, strand-displacing

polymerases such as Phi29-like polymerases, to efficiently amplify templates without thermal cycling. Preferred embodiments use Phi29 or Phi29-like polymerases, but other polymerases such as Pol I, Pol III, and T7 DNA polymerase, and their derivatives can also be used. The invention can also use other modified or chimeric polymerases designed to improve efficiency and/or fidelity.

[0026] Following amplification, the nucleic acid product may be further processed in a manner to facilitate its intended use. Research purposes, including detection, identification or sequencing, would typically only require shorter linear units (delivery unit) of the concatamer which may be attained by either restriction enzyme digestion or by physical or chemical methods such as shearing or induced cleavage at specific, photolabile nucleotide. Cellular transfections may be accomplished with a variety of forms, but higher efficiencies of uptake are typically attained with circular or supercoiled nucleic acid. However, as shown herein, linear forms can be used to produce a greater immune response than the comparable plasmid when used to effectuate immunity in an animal system. A preferred embodiment includes the use of a linear product made in the cell-free system as the active component of a DNA based therapeutic. Another embodiment incorporates a subsequent ligation step using DNA ligase to make circular nucleic acids (CNAs) from the linear forms. Another embodiment uses a recombinase or a similar enzyme to circularize the delivery unit into CNAs. Another includes the use of a DNA gyrase to supercoil the circular product to produce supercoiled CNA (sCNA).

[0027] If the product is intended for expression in eukaryotic cells, uptake by the cell is critical, whether in research (cell and culture) applications or in therapeutic applications. Transfections can be accomplished using circular, supercoiled CNA or specially designed linear forms which may be stabilized with modifications in the internal base and/or the ends of the linear unit. Such modifications include: blunting the ends by filling in with a Klenow fragment-like enzyme; phosphorothioating the ends of linear strands with appropriately modified bases; incorporating other modified bases either during the amplification process or following digestion of the concatamer, which stabilize or minimize degradation of the linear in vivo; and designing the expression cassette to comprise stabilizing sequences which facilitate rapid uptake and/or prolong longevity of expression of the cassette once inside the cell (Kay, M.A. et al., Molec. Ther. 3(3): 403-410, Mar. 2001). In effect, the linear can be stabilized during the amplification reaction by the random incorporation of chemically or structurally modified primers during replication. Such modification may incorporate components that are known to stimulate the immune response in a manner similar to the actions of an adjuvant.

[0028] The degree of modification or processing following the cell free amplification step is dependent upon the intended use for the product. The final processed product is then purified in order to eliminate reagents, contaminants, and/or any alternative forms of the product. Different forms of the product may include linear fragments, open circles, covalently closed circles

comprising monomers, dimers, trimers, etc., as well as supercoiled circles. The intended form is dependent upon the specific application and may alternate between any of the aforementioned forms. Depending upon the number of reagents used and on the degree of purity needed, the product can be subjected to chromatography, ultra filtration, dialysis, nucleic acid precipitation, or any other appropriate method known in the field. Those embodiments incorporating gel filtration and/or dialysis can provide high quality products for therapeutic applications. All forms of the DNA product made according to the method of the current invention are referred to herein as synDNA.

BRIEF DESCRIPTION OF THE DRAWINGS

[0029] FIG.1 shows multiple mechanisms for generating useful templates. For example, in addition to the use of normal circular plasmid, templates may also be produced by plasmid modification (restriction enzyme digestion with subsequent ligation, or intraplasmid recombination), PCR amplification, chemical synthesis, or cDNA synthesis.

[0030] FIG.2 shows a cell-free amplification process using a polymerase to synthesize a concatamer from the circular template. The concatamer can be later processed into smaller fragments, which may comprise at least one intact expression cassette having a sequence of interest. The end product may be used as short linear units (DUs), circularized nucleic acids (CNAs), or supercoiled circular nucleic acids (sCNAs).

[0031] FIG.3 shows an embodiment that separately amplifies the forward (A) and reverse (B) strands of a double-stranded template in separate reaction vessels. Each strand is separately amplified using a strand-specific primer and circularized into single-stranded circles. A second oligonucleotide comprising a sequence for a restriction site (OR1 or OR2) is then annealed to a pre-designed site in the single-stranded concatamer, whereby short segments of double-stranded templates are generated to enable digestion by a restriction enzyme. Following digestion but prior to denaturation, the double-stranded ends are circularized using a DNA ligase. Following ligation, the oligonucleotide is denatured to from single-stranded circles, which are then combined with the complementary single-stranded circles to form double-stranded circles that comprise only monomers. This method minimizes the formation of dimers, trimers and other multimer byproducts.

[0032] FIG.4 depicts the scale-up of the cell-free amplification process. The process involves sequential addition of template, primer, buffer components and enzymes at the designated times and shifting to the designated temperatures. This provides an efficient method for producing large amounts of product in a short period of time. Diluting the reaction volume prior to ligation favors the formation of monomeric circular product. Dilution, ligation and gyrase reactions are all optional.

[0033] FIG.5 depicts the design for an automated amplification apparatus. (A) represents a model where large numbers of individual reactions comprising volumes of less than 1 ml can be

used to amplify numerous individual templates simultaneously as for diagnostic purposes; (B) shows the use of a single vessel enabling the synthesis of large quantities of a single DNA product. (1) Programmable computer access to control reaction parameters; (2) monitor for evaluating and adjusting reaction parameters; (3) temperature controlled chamber for stock solutions including enzymes, buffers, and other components; (4) dispensing port for addition of reagents to the reaction vessels; (5) temperature controlled reaction vessel; (6) multiple-well dispenser; (7) multiple-well reaction vessel plates; (8) temperature controlled chamber for multiple-well plates.

[0034] FIG.6 schematically summarizes various mixing strategies for viscous reaction mixtures: (A) propeller-like mixing vessel; (B) perforated disk mixing vessel; (C) recycling mixing vessel using a peristaltic pump; (1) adjustable automated control and port for calibrated addition of reagents held in (2). The adjustable control (1) enables controlled mixing of reagent with a small stream of reaction mixture and supports the overall mixing of the reaction mixture by depositing the reagent modified reaction mixture back into the chamber at a position opposite the outlet port. Continued pumping without reagent facilitates thorough mixing.

[0035] FIG.7 depicts a process for intra-molecular ligation. Following amplification and digestion of DNA in vessel (B), the reaction mixture is added slowly to a second vessel (A) containing a ligation cocktail. Slow addition of the DNA into vessel (A) provides sufficient dilution of the DNA to facilitate monomeric circular nucleic acid (CNA) formation.

[0036] FIG.8 shows results of IgG antibody titers against gp160 produced in Balb/c mice after immunization with a plasmid, a short expression cassette (synDNA) produced in accordance with the invention, and a control solution. These results clearly show that the synthetic DNA is effective in inducing immune responses in mice.

[0037] FIG.9 shows results from immunization of rabbits using a plasmid or a synDNA (expression cassette), containing a sequence for the Hepatitis B virus small surface antigen (HBs(S)). These results clearly show that the synthetic expression cassette of the invention is effective in inducing immune responses in rabbits.

[0038] FIG.10 shows immunization results following the injection of BALBc mice against influenza H1N1 virus 8 weeks post-immunization; 2 weeks post last boost. Animals injected 3x, at weeks 0, 2 and 6. (1) 50 μg of Plasmid, pCMV-HA; (2) 32 μg of cell-free linear HA-DU; (3) mixture of 16 μg of cell-free linear HA-DU + 25 μg of control Plasmid without insert, pEV; (4) mixture of 10.6 μg of cell-free linear HA-DU + 16.7 ug of cytokine plasmid, pCMVi-GMCSF + 16.7 μg of cytokine plasmid, pCAGGSIL12; (5) mixture of 16.7 μg of Plasmid, pCMV-HA + 16.7 ug of cytokine plasmid, pCMVi-GMCSF + 16.7 μg of cytokine plasmid, pCAGGSIL12. The figure shows virus-neutralization titers recorded as the last dilution in which virus replication was inhibited for the various genetic immunization experiments.

[0039] FIG.11 shows immunization results following the injection of BALBc mice with a control plasmid or synDNA containing a sequence for a smallpox gene, B5R. Smallpox genetic immunization response: Plasmid vs B5R-DU.

[0040] (A) Antibody titers (ELISA) at week 3; (B) Number of INFγ producing T cells/ 106 CD8 + cells; (C) T-cell proliferation assay: rate of antigen specific T cell proliferation in vitro with addition of recombinant B5R antigen Antibody titers (A and C) and INFγ production (B) are higher in mice immunized with linear cell-free B5R-DU than in mice treated with plasmid DNA at equal molar amounts.

[0041] FIG.12 shows the expression of luciferase in mouse muscle at 24, 72 and 144 hours post-injection. Each mouse received a single injection of 50 μg of DNA (linear cell-free synDNA or intact plasmid DNA) in one leg. There was no significant difference (higher or lower) in expression of the luciferase enzyme over time between the linear Luc-SynDNA of the current invention and the standard circular luciferase plasmid DNA.

[0042] FIG.13 shows stability of the linear SynDNA made using two different modified primers (methyl phosphonate (MP) and phosphorothioate (P)). Following cell-free amplification of a standard plasmid carrying a lacZ marker, DNase was added to equivalent amounts of the MP-SynDNA and the P-SynDNA for various times; (A) Agarose gel; (B) Densitometry of gel bands.

DETAILED DESCRIPTION OF THE INVENTION

[0043] The invention includes methods for making cell-free nucleic acid, products made by this method which have fewer contaminants than traditional bacterially grown products, and an apparatus for producing large amounts of high-quality nucleic acids. Methods of the invention use a cell-free system to produce therapeutically useful and minimally contaminated nucleic acid products (Fig. 2).

[0044] "Composition" typically refers to a carrier (buffer or delivery vehicle) mixed with an active effector molecule which, in the context of this invention, is a high-quality nucleic acid molecule.

[0045] "Delivery" includes methods to administer an active compound or effector molecule to the target cell or organ, and may include injection (intramuscular, intravenous, intradermal), oral compositions, aerosol sprays, eyedrops, suppositories, topical ointments, skin patches and soaks, as well as surgically implanted devices.

[0046] "Delivery vehicle" in the context of this invention means any carrier suitable for transporting a nucleic acid effector molecule or sequence of interest to a site within the host (cell, animal, human, plant) which may or may not improve the uptake of the effector molecule by the target cell. Some delivery vehicles may target the effector molecule to a particular cell or organ, or

may diminish uptake by the cell in order to improve extracellular effector action. Typical delivery vehicles include viral packaging systems, topical ointments, aerosols, liposomes, microsomes, polymers, nanotubules, cell penetrating or receptor adhering peptides, and various oral carriers, but may be as simple as saline buffer or water.

[0047] "Expression cassettes" mean any combination of nucleic acid sequences that comprise the proper promoter, enhancer and/or termination sequences needed for expression of a particular sequence of interest. The invention is adaptable for the use of multiple expression cassettes where each cassette may contain a different sequence of interest.

[0048] "High quality" in the context of this invention primarily refers to nucleic acid products that have only defined components as contaminants and only trace levels of bacterial cell components, endotoxin or other bacterial toxins (contributed only by the addition of purified enzymes used in the cell-free process, which are themselves purified from the end synDNA product). Without bacterial cell components in the end product, the DNA so produced is easily and affordably purified for therapeutic and critical research applications where contaminating bacterial components can interfere with the efficiency and efficacy of the DNA application.

[0049] "Nucleic acid," "oligo," or "oligonucleotide," as used in the context of this invention, may be DNA or RNA, or an analog (e.g., phosphorothioate analog). Nucleic acids or oligonucleotides may also include modified bases (ex: phosphorothioates, morpholinos, methyl phosphonates, or other mimetic molecules), backbones, and/or ends. Synthetic backbones may include phosphorothioate (Pt), peptide nucleic acid (PNA), locked nucleic acid (LNA), xylose nucleic acid (XNA), or analogs thereof that confer stability and/or other advantages to the nucleic acids.

[0050] "Plasmid replication sequences" for this invention include origins of replication, antibiotic resistance genes, other marker or selection genes, and other bacterial specific sequences required for plasmid replication inside a prokaryotic cell.

[0051] "Protective response" as used herein means a beneficial response that a host elicits to counter a disease state caused by either a genetic aberration, an environmental inducer causing an aberrant expression pattern, or by a pathogen or toxic agent.

[0052] "Reporter construct" refers to a nucleic acid sequence useful for tagging and labeling which may compise antibiotic resistance genes and other common reporter or marker sequences including, but not limited to at least partial sequences of the B-galactosidase (LacZ), luciferase (Luc), secreted alkaline phosphatase (SEAP), green fluorescent protein (GFP), and chloramphenicol acetyltransferase (CAT) genes.

[0053] "Sequence of interest" (SOI) in the context of this invention, means any nucleic acid

sequence that is sufficient to elicit a cellular response in the targeted environment. The sequence can be as small as a typical oligonucleotide which may be as small as seven nucleotides in length, or as large as a polycistronic message comprising several genes, or genomic segment containing both exons and introns for the production of an unprocessed precursor protein. An SOI may be a nucleic acid, oligo or oligonucleotide with or without chemical modification which may contain a potential therapeutic SOI or a reporter or marker sequence for research purposes.

[0054] "Therapeutic compositions" as used in the context of this invention includes as an active component, at least one nucleic acid, oligo or oligonucleotide molecule which may be chemically modified. The active component will by virtue of the sequence used, be capable of acting prophylactically by eliciting a protective response (including cellular and/or an immune responses), remedial or growth inhibitory response inside an organism and may be applied in the appropriate composition for useful gene therapy, vaccinations, pathogen inhibition and other disease states when the composition is administered to a living organism.

[0055] "Research compositions" for this invention refer to any composition comprising at least one sequence of interest useful for research and/or pre-clinical purposes. In the context of this invention, a research composition can be used for any traditional research application which uses plasmids, viral and other similar molecular constructs, and can be effectively used as a tool to compare the efficacy of synDNA products produced according this invention to other traditional molecular transfer tools including plasmids and viruses.

[0056] As shown in FIG. 2, the process uses a polymerase to synthesize a concatamer from a circular template. The concatamer may be processed into smaller fragments, which may comprise at least one intact expression cassette. The synthesized product may be used as short linear units or these may be further processed to produce circularized nucleic acids (CNAs) or supercoiled circular nucleic acids (sCNAs).

[0057] The method can be adapted to use either DNA or RNA templates. The reactions starting with RNA templates would include a reverse transcriptase, such as the avian myeloblastosis virus reverse transcriptase, to make a cDNA template. Any method known in the art may be used to prepare a circular template for use in a method of the invention, as shown in FIG. 2. Some of these methods will be described in detail later with reference to FIG. 1.

[0058] Single-stranded binding proteins can be used to stabilize the templates and improve efficiencies of the amplifications for some polymerases. Additional enzymes can also be included in the amplification reaction to repair mistakes. Protein mediated error correction enzymes, such as the mutation splicing protein (MutS), can also effectively improve a polymerase's overall fidelity and may be used during or after the amplification reaction (Carr, P., et al., Nuc Ac Res 32(20): e162, 2004).

[0059] Depending upon the intended use, the DNA polymerases used in a method of the invention may be any known prokaryotic, fungal, viral, bacteriophage, plant or eukaryotic DNA polymerases and may include holoenzymes and any functional portions of the holoenzymes or any modified polymerase that can effectuate the synthesis of a nucleic acid molecule. Useful DNA polymerases include: bacteriophage phi29 DNA polymerase, other phi29-like polymerase (such as phage M2 DNA polymerase, phage B103 DNA polymerase, or phage GA-1 DNA polymerase), phage phi-PRD1 polymerase, VENT DNA polymerase, DEEP VENT DNA polymerase, KlenTaq DNA polymerase, DNA polymerase I, Klenow fragment of DNA polymerase I, DNA polymerase III holoenzyme, T5 DNA polymerase, T4 DNA polymerase holoenzyme, T7 DNA polymerase, Bst polymerase, rBST DNA polymerase, N29 DNA polymerase, TopoTaq DNA polymerase, and ThermoPhi™ DNA polymerase. Preferred embodiments of the invention use Phi29 polymerase, Phi29-like polymerase, or other high-fidelity polymerases (e.g., hybrid fusion polymerase).

[0060] Preferred embodiments of the invention use processive, strand-displacing polymerase to amplify DNA under conditions for high fidelity base incorporation. In the context of this invention, a high fidelity "DNA polymerase" is one that under recommended conditions, has an error incorporation rate equal to or lower than those (1.5 x 10-5 - 5.7 x 10-5) associated with commonly used thermostable PCR polymerases, such as Vent DNA Polymerase, KlenTaq DNA Polymerase, or T7 DNA Polymerase. Additional enzymes may be included in the reaction to minimize misincorporation events including protein mediated error correction enzymes, such as MutS, which effectively improves polymerase fidelity either during or following the polymerase reaction (Carr, P. et al, Nuc Ac Res 32(20):e162, 2004).

[0061] Similarly, a high fidelity "RNA polymerase" has an error incorporation rate equal to or lower than those of common RNA polymerases (Promega Technical Information). RNA polymerases useful in this invention include T7 RNA polymerase, SP6 RNA polymerase, T3 RNA polymerase, and their modified or chimeric versions.

[0062] During the amplification reaction, the circular template is replicated by a polymerase in the presence of deoxyribonucleoside triphosphates (dNTPs), ribonucleoside triphosphates (NTPs), or modified counterparts, forming a long concatamer comprising tandem repeats of the template. The concatamers are subsequently cleaved, e.g., by restriction enzyme cleavage or physical shearing, into smaller fragments referred to as "short expression cassettes" (SECs). An SEC contains a sequence of interest and may optionally contain eukaryotic expression sequences (or cassettes). Preferred embodiments use SECs that comprise at least one eukaryotic expression cassette. Unlike conventional, bacterially produced plasmids, an SEC of the invention consists solely of a sequence of interest flanked by the intended eukaryotic sequences, but no bacterial genetic material.

[0063] The "short expression cassette" may include: a eukaryotic promoter recognized by the targeted cell; the sequence of interest which may be an intact gene, a gene fragment, or a specific sequence of interest (SOI); and a transcription termination sequence. The short expression cassette may be flanked by additional sequences to facilitate ligation (e.g., making CAN) or to stabilize a linear fragment. The expression cassette, together with the desired flanking sequences, comprises a "delivery unit" (DU), and does not contain unnecessary genetic material which is solely used for selection and replication of a plasmid produced in bacterial culture. These unnecessary plasmid replication gene sequences include but are not limited to origins of replication, marker genes, and plasmid selection genes. By minimizing bacterial genetic material having no value inside a eukaryotic cell, it is possible to generate high concentrations of high quality, bio-active DNA molecules. The nucleic acid produced is smaller than a typical plasmid, frequently about half the size of a standard plasmid and is more efficiently transfected into a cell, and upon transfection, is more efficiently expressed inside the cell.

[0064] Enzymatic or chemical methods can be used to improve the homogeneity of the final products by eliminating DU with mismatched nucleotides resulting from errors in polymerization. For example, enzymes used in mutation detection (such as resolvases, T4 Endonuclease VII, or T7 Endonuclease I) or other enzymes used to detect gene mutations or polymorphism and in high-throughput screening of point mutations (such as TILLING) may be used to accomplish this goal.

[0065] As noted above, any method may be used to prepare a circular template for use with methods of the invention. FIG. 1 shows three commonly used methods for generating useful circular templates that include at least one sequence of interest (SEC or DU). One method involves enzymatic modification of an existing plasmid, whereby the DU including the eukaryotic expression cassette is selectively excised from a plasmid by restriction endonuclease digestion. The DU is free of the origin of replication or selectable marker genes, such as an antibiotic resistance mediator, which can silence expression of the SOI in vivo.

[0066] A preferred embodiment of the invention uses a template comprising an intact eukaryotic expression cassette with flanking sequences on either side of the cassette (Fig. 1) to enable circularization of the linear SEC into a CNA. The template can be any single- or double-stranded nucleic acid (DNA or RNA), which is converted into a circular template and includes plasmid as well as minicircle DNA. Pre-ligation reactions may be carried out as in the case of using padlock probes (Baner, J., et al., Nuc Ac Res 26(22): 5073-78, 1998).

[0067] Double-stranded templates may need to be denatured initially to optimize the polymerase reaction depending upon the polymerase used. In such reactions, both the forward and reverse strands can be simultaneously amplified in the same reaction. Subsequent processing may then require the addition of a restriction endonuclease, a ligase, and/or a gyrase. The products may

then be purified to yield DUs for therapeutic applications.

[0068] A second method for making the templates involves PCR amplification from a larger DNA template using specified oligonucleotides that flank the specific expression cassette to produce relatively short DUs for circularization.

[0069] A third method shown in FIG. 1 involves chemical synthesis of oligonucleotides (oligos) to make a single nucleic acid strand or complementary strands that are then circularized to produce a template containing a DU or expression cassette.

[0070] During amplification, the template may be freely suspended in solution or bound to a support, such as a chromosome or protein, or a solid support such as glass or polystyrene beads.

[0071] An alternative method is shown in FIG. 3. Each strand of a double-stranded template may be separately amplified using appropriately designed primers to produce single stranded concatamers of DUs. The separately amplified concatamers are individually mixed with oligos containing specific restriction sites and cleaved with the restriction enzymes. The temporarily double-stranded ends of these fragments are ligated to form circular single-stranded products (Dahl, F., et al., PNAS 101(13): 4548-53, 2004). The advantage of this method is that the single-stranded circles of each reaction can then be combined to form a single class of double-stranded monomeric circles, thus avoiding the need to purify the monomers away from other multimeric forms of the reaction. The monomeric circles can then be supercoiled with a DNA-gyrase or a similar enzyme to improve the efficiency of uptake and expression of the expression cassette.

[0072] Multiple embodiments use a circular, double-stranded DNA template with primers that specifically bind at designated sites to initiate concatamer synthesis. The primers can comprise any of the different variations of "nucleic acid" to improve stability, and may be of various lengths where the length is determined by the annealing temperatures of the DNA polymerase used. The primer sequences may comprise random or specific sequences, may be designed to have specific sequence alterations, or may include tags or detection sequences that are non-complementary to the template in order to facilitate manipulation or analysis of the amplified sequences. For example, in one embodiment, random hexamers are used to effectively amplify a DU, which upon processing and transfection into cells, would produce the desired effects. Other embodiments use specifically designed primers which enable the RCA reaction to be controlled by spacing out the initiation sites and by using primers of controlled affinity for optimizing the amplification reaction conditions. Sequence-specific primers, as short as a tetramer, may be used to effectively amplify a specific DU.

[0073] In most applications, the polymerases, restriction endonucleases, ligases, and other enzymes as used in this invention constitute soluble forms of the enzymes. However, solid phase amplification reactions or solid phase processing reactions including restriction digestion, ligation and supercoiling reactions may also be employed to streamline the amplification process. In

addition, fusion proteins comprising optimal regions of different enzymes (especially polymerases) which are designed to improve fidelity, efficiency, and processing or the final product may be used. Recombinant forms of the enzymes containing one or more affinity tags (such as 6XHis, S-Tag, Calmodulin-binding peptide, Protein A and others) expressed in bacteria, fungus, plants, insects, or animal cells may also be used. The advantage of using tagged enzymes is that they can be readily eliminated from the final product using affinity chromatography. Following purification, the recovered enzymes, immobilized on a solid matrix through the tag moiety, may be used in subsequent enzymatic reactions.

[0074] Following amplification, the concatamer is cleaved into short expression cassettes (SECs) comprising at least one DU, where a single SEC may comprise multiple copies of a DU and may be designed as such in order to optimize delivery and expression. The linear SECs may be directly administered as the linear fragments, circularized fragments (CNA), or supercoiled circularized fragments (sCNA) to facilitate uptake by the target cell. As such, the post-amplification processing would vary according to the intended use.

[0075] Processing of the SEC can include any one or more of the following: additional cutting of the SEC with other physical or enzymatic methods; filling in or processing the ends of the SEC either by enzymatic cleavage, as with Klenow, or by chemical methods; internally ligating the two ends of the SEC to produce a circularized CNA; supercoiling the CNA with gyrase-type enzymes including topoisomerase type II; enzymatically or chemically treating any of the forms to have modified internal bases or modified ends; ligating two or more SECs together; or ligating an SEC to a specific ligand to produce a functional conjugate. The term ligand as defined in the context of this invention includes: a nucleic acid, including DNA, RNA, PNA, LNA or modifications thereof; peptides, either to facilitate targeting and cellular uptake or to increase therapeutic efficacy; polypeptides that may be enzymatically active and/or physically functional; aptamers, nucleic acids that recognize, bind and modify a protein's function; bio-physical tags, including fluorescent, magnetic, and radiolabeled components; as well as polymers which facilitate either stabilization of the nucleic acid, or targeting of the product to the intended cell or tissue.

[0076] Therapeutic applications that can be successfully administered using DNA produced by the invention include several approaches to DNA therapy, including antibody production and gene silencing. For example, antibodies can be produced in vivo following successful administration of appropriate expression cassettes designed to prevent or treat a disease caused by a pathogen, such as influenza,HIV, hepatitis or smallpox viruses. For example, the sequence encoding the influenza haemagglutinin (HA) protein under the control of an eukaryotic promoter may be used to elicit a humoral and cellular immune response in animals targeted by influenza A virus. Similarly, the expression of a sequence encoding a truncated Human Immunodeficiency Virus (HIV) envelope protein can successfully induce an effective immunogenic response against HIV in mice. And the

expression of a sequence encoding a smallpox B2R gene which is amplified using the cell-free process described herein can induce the production of circulating antibody as well as stimulate a T-cell immune response.

[0077] It is generally believed that linear forms of DNA are not stable, are difficult to deliver to a cell, and are inefficiently taken up by cells in both in vitro and in vivo systems. In an attempt to circumvent this, Moreno S, et al. developed a series of linear form gene expression vectors (MIDGE) for producing DNA vaccines (Vaccine, 22: 1709-1716, 2004). However, the MIDGE approach involves a labor intensive enzymatic process to remove unnecessary bacterial sequences from bacterial grown DNA plasmids. In addition, the ends of the linear expression vectors must be subsequently modified with hairpin loops to increase gene expression efficiency.

[0078] The amplified nucleic acid of this invention, especially the linear form DNA, can be shown to induce immune response in several viral vaccine animal models, such as HIV, influenza, hepatitis and smallpox, indicating that linear form DNA can be delivered with or without carriers such as polyethyl-eneimine (PEI) both in vivo and in vitro and can be biologically active in an animal. These findings are contrary to the traditional views adhering to the notion that linear DNA is an inefficient component for nucleic acid-based therapeutics. These findings strongly suggest that cell-free linear forms as well as enzymatically digested bacterial grown plasmid are suitable for such therapeutic compositions, providing of course that the cutting sites occur outside of the sequence of interest or the gene expression cassette.

[0079] The amplified nucleic acid of this invention can also be shown to mediate targeted gene silencing in vivo. Herpes Simplex Virus (HSV), which causes painful blisters and sores on various parts of the body, and Herpes Zoster, which causes chicken pox (initial infection) and shingles (upon recurrence), are members of the same family of viruses which require the expression of both ICP4 and ICP47 proteins to effectuate a viral infection. Upon transfection in cell culture, amplified SECs expressing antisense oligos specific for ICP4 or ICP47 may be used to modulate these protein expression in vivo and can minimize further proliferation of the virus. Expression of an anti-ICP4 transcript in vivo successfully silences the ICP4 gene and blocks the production of ICP4 protein in the cell. This reduces the virus' ability to reproduce. A similar effect can be seen following expression of the ICP47 expression cassettes produced by this method.

[0080] ICP47 functions to inhibit the major histocompatibility complex (MHC) presentation pathway, which is critical for shielding the virus from host immunogenic attack. The gene product of ICP47 binds to a transporter protein involved in the presentation of antigens on the outside of an infected cell, thus blocking the major histocompatibility complex (MHC) class I antigen presentation pathway. Consequently, the HSV-infected cells are masked from immune recognition by cytotoxic T-lymphocytes. Thus, ICP47 plays an essential role in HSV-infection.

[0081] Transfecting the lung cancer cell line, A549, with an ICP47 SEC amplified according to this invention can effectively express antisense sequences and block production of the ICP47 protein as assayed by Western blot analysis. There are additional infected cell proteins (ICP's) in the herpes simplex genome that can be similarly silenced.

[0082] Other gene silencing targets include the respiratory viruses such as the rhinoviruses, coronavirus, adenovirus, influenza and para-influenza viruses, which are frequently associated with both upper and lower respiratory tract infections including the common cold, pneumonia, asthma, and chronic obstructive pulmonary disease (COPD). The human rhinovirus (HRV) has a single-stranded RNA genome that is approximately 7.2 kb in size with a single-open-reading frame that encodes for a capsid coat protein, an RNA polymerase and two viral proteases. Upon infection, the viral proteins effectively redirect the host machinery to manufacture thousands of viral particles which are eventually released when the cell lyses.

[0083] Most rhinoviruses make use of intercellular adhesion molecule I (ICAM-1) as a receptor to infect the cell. Expression of an amplified SEC encoding an antisense to the ICAM-1 message can effectively block expression of the ICAM-1 protein in vivo and may prove to be useful in minimizing viral infection. Other useful strategies for combating respiratory diseases include in vivo expression of antisense-like molecules (antisense, aptamers, triplex forming molecules, and similar molecules) to block activities of essential proteins that mediate infection, such as viral proteases that are required to process viral particles. Other approaches may include using the SECs to block mediators (e.g., bradykinin, prostaglandins, tachykinins, histamine, and various cytokines) of pathogen-induced tissue responses, or to block the cellular receptors that effectuate the physiological effect caused by these mediators.

[0084] Other targets for therapeutic applications of this invention include modulating infections caused by the human papilloma viruses (HPVs) which initially manifest infections as benign, non-cancerous warts but in some cases can progress into malignant growths. For example, genital HPVs can be passed from one person to another through sexual intercourse as well as through oral or anal sex. Virus-infected cervical cells can transition from an initial benign wart, into premalignant cells and eventually develop into a carcinoma. Cervical cancer is probably one of the best known examples of how infection with a virus can lead to cancer. In humans and animals, cell division is primarily regulated by Rb and p53. The E6 and E7 proteins of HPV can attach directly to Rb and/or p53, inhibit the tumor suppressor effects of the proteins and cause the infected cells to reproduce without control (Didelot, C. et al., Intl J Oncology 23:81-87, 2003). While the virus serves only as the initiating event, over time some of the wildly growing cells develop permanent changes in their genetic structure that cannot be repaired. By expressing antisense-like constructs designed to block E6 and E7, viral infections would be rendered ineffective.

[0085] Other types of HPV infections may manifest themselves as warts on or around the genitals and anus of both men and women and are also valid candidates for therapeutic antisense-like expression using the nucleic acid produced by this invention. In women, visible warts may also appear in the cervix. This type of a genital wart is known technically as Condyloma acuminatum and is generally associated with two HPV types, numbers 6 and 11. These warts rarely develop into cancer, and are considered to be "low-risk" viruses. Other sexually transmitted HPVs have been linked with genital or anal cancers in both men and women. These are called "high risk" HPV types and include HPV-16, HPV-18, HPV-31, HPV-45, as well as some others. High risk HPV types aren't usually contained in visible warts, but both high-risk and low-risk HPVs can cause the growth of abnormal cells in the cervix. Both types of HPV infections can be effectively controlled with an effective in vivo antisense-like expression topical therapeutic.

[0086] The amplification reaction of the invention can also be used to amplify either an intact plasmid comprising bacterial sequences, or a modified version of the plasmid to exclude these sequences. For example, a single-stranded DNA expression vector, pssXE, which includes: 1) a Mouse Moloney leukemia viral reverse transcriptase (MoMuLV RT) gene coding for a truncated but fully active RT; 2) a primer binding site (PBS) with flanking regions essential for reverse transcription initiation by MoMuLV RT; 3) a target gene coding sequence for the production of an antisense, an aptamer, a DNA enzyme, or a sequence that induces triplex formation; and 4) a stem-loop structure designed for the termination of the reverse transcription reaction, as an intact expression cassette, can be effectively amplified according to the invention. The amplified products can be transfected and used to effectively silence mammalian, viral, and bacterial genes. Upon expression inside the cell, the transfected RT subsequently uses an endogenous host tRNA (e.g., tRNAPro or tRNAVal) as a primer to bind to a primer binding site (PBS) at the 3' end of the RNA transcript and initiates ssDNA synthesis. After reverse transcription, ssDNA may be released when the mRNA template is degraded by RNase H or the RNase H activity of RT.

[0087] Delivery of the nucleic acid (SEC) can be accomplished by simple injection of a naked nucleic acid in stabilizing buffer into the targeted recipient. Embodiments of the invention may also use delivery vectors or other delivery vehicles which help target and delivery of the nucleic acid into the cell (Dias, N. Molec Cancer Ther 1: 347-355, 2002). Some embodiments use a viral vector system which may be an attenuated virus system, a viral packaging system that includes few or no immunogenic protein (Srivastava, I.K. and Liu, M.A. Ann Intern Med. 138: 550-559, 2003). Other embodiments include the use of neutral or cationic liposomes which either encapsulate the nucleic acids or bind the nucleic acid by electrostatic interactions. These embodiments may also use helper molecules (e.g., chloroquine or 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine) to prevent sequestering of the delivered nucleic acid in the endosomal compartments. Some of the commercially available liposomal vectors include Lipofectin, Eufectins, Cytofectin and

Lipofectamine.

[0088] Other methods of delivery include covalent coupling of the nucleic acids to cationic peptides (delivery vehicles), which may modulate the permeability of plasma membrane by physical interactions, receptor- or transporter-mediated mechanisms. Such coupling increases the effectiveness of the delivered nucleic acid which is delivered directly into the cytoplasm and is readily transported to the nucleus for expression (Luo, D. and Saltzman, W.M. Nature Biotech 18: 33-37, 2000). Still other embodiments use cationic polymers which interact electrostatically with the therapeutic nucleic acid to deliver nucleic acid to the cell. Cationic polymers, for example, include poly-L-lysine (PLL), polyethylene glycol (PEG), PEG-block-PLL-dendrimers, polyamidoamine (PAMAM) dendrimers, polyalkylcyano-acrylate nanoparticles, and polyethyl-eneimine (PEI) and its conjugates (such as mannose-PEI, transferin-PEI, linear PEI).

[0089] Aerosol delivery is a noninvasive mode of delivery to airway epithelium and pulmonary surfaces. For example, formulations comprising the delivery vehicle, PEI, and a nucleic acid can effectuate high level airway or pulmonary transfection upon delivery by nebulization. This application of PEI-nucleic acid complexes can effectuate higher levels of gene expression than many cationic lipid formulations, and exhibits a remarkably high efficiency (nearly 100%) of transfection into cells of the airway epithelium and lung parenchyma. In addition, repeated aerosol administrations of PEI-based formulations are associated with very low toxicity. This delivery method only minimally induces expression of tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1ß) as compared to intravenous injections of PEI-nucleic acid or aerosol delivery of cationic liposome-nucleic acid complexes.

[0090] A frequent problem of using bacterially produced plasmid DNA results from exposure of the host to unmethylated motifs inherent in bacterially processed DNA. Unmethylated DNA can induce a CpG-mediated cytokine response and the induction of pro-inflammatory cytokines which is a serious problem associated with lung toxicity and reduced efficiency of therapeutic applications. Consequently, the use of bacterially produced DNA has severely hampered many of the current gene therapy approaches used to date. Masking of the CpG response by PEI can facilitate the sustained expression of genes that are delivered via PEI-gene aerosol and, thus, the sustained therapeutic response achieved. When used in combination with the nucleic acid produced by the cell free amplification method of this invention, PEI-based aerosols can be extremely effective delivery systems for DNA therapeutics to lung and airway epithelium.

[0091] Some of the embodiments also use long-term release systems. Biocompatible controlled-release polymers such as poly(D,L-lactide-co-glycolide) (PLGA) microspheres and poly-(ethylene-co-vinyl acetate (EVAc) matrices can effectuate a controlled, adjustable and predictable release of the bioactive nucleic acid for up to several months, and both components have been

approved for therapeutic use by the U.S. Food and Drug Administration.

[0092] Physical delivery systems may also be used. Electroporation may be efficient for transferring therapeutics to skin cells, corneal endothelium and other tissues including muscle. Pressure-mediated or hydrodynamic injection can effectuate up to 50% efficiency in mammalian systems. Other methods include ultrasonic nebulization for delivery of DNA-lipid complexes in many different types of cells, including plants, and particle bombardment is also useful for plants. In the context of the invention, these physical delivery systems constitute additional delivery vehicles for effectuating the uptake of a therapeutic nucleic acid into a cell.

[0093] Scale-up of the cell free amplification process may be performed using a semi- or fully-automated platform, where sequential additions of salts, enzymes and nucleic acids, together with temperature and incubation times, can be tightly controlled for optimal efficiency (Fig. 4). In one embodiment, scale-up can be accomplished by increasing the number of reactions while keeping each reaction volume relatively small (< 1 ml) whereby the template(s) can be amplified simultaneously using multi-well plates in standard or custom built platforms (Fig. 5A). Alternatively, scale-up may involve larger volumes (e.g., 10 liters) to generate large quantities (kg amounts) of a single nucleic acid product in a single run using a fermenter-like vessel under environmental controls (Fig. 5B). Larger volumes may be used to produce larger yields of product. Multiple platforms of mixed capacities can be arranged in parallel within a confined space and can function in a coordinate manner as part of a larger bio-manufacturing facility that can meet various amplification scale requirements.

[0094] The production of large amounts of nucleic acid in a small volume presents the problem of mixing reagents into a highly viscous reaction mixture. The invention includes a reaction vessel that can be either a hardened pre-formed container or a flexible container such as a self contained plastic bag. In the preferred embodiment, the reaction vessel and all components that come in contact with the reaction mixture are clean, sterile and free of any contaminating nucleic acid sequences. The hardened pre-formed container contents are preferably mixed by a device that is contained inside the reaction vessel, but may involve a re-circulating device. The flexible vessel is preferably mixed by a re-circulating mechanism which could include the use of a peristaltic-like pump, or may incorporate an external mechanical device such as an automated squeezing apparatus or a low-energy pulsation device that avoids shearing of the nucleic acid product.

[0095] Internal devices can use several different mechanisms including propeller-like stirring devices with electronically controlled speeds and automated timing (FIG. 6A), or controlled liquid displacement processes using a perforated disk fixed to a shaft running from top to bottom within the reaction vessel's inner diameter (FIG. 6B). The disks are raised and lowered at various speeds within the liquid to provide adequate mixing of the reaction mixture. Both of these mixing

chambers can be equipped with a dispensing device which may comprise a small tube attached to the shaft of each mixer which delivers various stock components, which are chambered separately outside the mixing vessel, into the reaction mixture using a peristaltic pump to control the precise and sequential delivery of the various reagents.

[0096] Another embodiment implements a system where a steady constant flow of the reaction mixture is pumped from and then back into the chamber. For example, an outlet located at the bottom of the chamber enables a small stream of fluid to be combined with an added reagent and then channeled back through an entry port located at the top of the same reaction chamber to effectuate mixing (Fig. 6C). Peristaltic pumps and intake valves control and monitor the dispensing of various solutes and enzymes during the recycling process (Fig. 6C).

[0097] Yet another embodiment utilizes the thixotropic nature of the DNA mixture, wherein the mixture is cylindrically configured into an elongated form. Thixotropic compounds can change viscosity according to the degree of shear force applied to the compound. Typically, an increase in the shear force can decrease a thixotropic compound's viscosity. Once the shear force is removed, such a compound will begin to regress to its original viscosity. In this embodiment, the container holding the viscous reaction mixture has evenly spaced pores through which necessary chemicals are injected for processing. Elongation of the viscous reaction mixture through the small diameter cylinders therefore changes the viscosity sufficiently to promote localized mixing with reagents which are slowly infused into the small diameter cylinders and into the less viscous reaction mixture for a sufficiently long period in which to effectuate mixing.

[0098] The apparatus preferably includes one or more inline real-time monitoring of all relevant physical and biochemical parameters to verify product stability and maintain quality control and quality assurance, which are necessary to maintain certified good manufacturing practice (cGMP) required for a product acceptable for therapeutic applications. This may include a computer or similar means for monitoring viscosity, nucleic acid concentration, solution turbidity; conductivity; pH; temperature; protein content; endotoxin, bioburden, and/or chemical contaminants arising from degradable components of the system.

[0099] Processing of the linear SEC into a circular form requires that the ligation step favor an intramolecular (self-adhering) reaction over an intermolecular reaction. Traditional dilution of the final amplification product can be used to manipulate the molar ratio to favor intramolecular ligation. Preferred embodiments, however, minimize the overall reaction volume by mixing small amounts of the reaction mixture into a ligation cocktail containing the enzyme and buffer components. In one embodiment, the amplified product is added into a small stream of reaction mixture as shown in Fig. 6C, using very slow or pulsating pump rates. Other embodiments dispense the amplified reaction mixture drop-wise into a second vessel containing the ligation cocktail to

achieve dilution without generating large volumes of ligated reaction mixture (Fig. 7). Sufficient time is allowed between each aliquot addition to optimize the intramolecular ligation process for each new aliquot dispensed. Once ligation of the aliquot is complete, the circular DNA is no longer substrate for the enzyme and becomes part of the dilution mix. A second aliquot is then dispensed, and the cycle repeats until all the amplified DNA is dispensed and ligated. This process allows intramolecular ligation to occur without large dilutions of the initial amplification reaction and can incorporate multiple dispensing chambers to allow for simultaneous aliquots to be ligated and to minimize processing time.

[0100] Final purification of the product can be streamlined by using permeable membrane-based methods during the reaction process. These membranes permit low molecular weight molecules (salts, unincorporated primers, dNTPs, NTPs and other small molecules) in the amplified DNA reaction mixture to diffuse away while retaining the product. A modification of the hemodialysis process can be used to allow the selective retention of the amplified DNA over other reaction components. Once the reaction is complete, the amplification reaction is pumped from the vessel to a filter comprising membranes with specific molecular weight cut-offs. The DNA is at least partially purified when the smaller reagents diffuse from the reaction across the membrane of these small capillaries. Purified DNA is then either pooled, evaluated for quality and/or dispensed for end-use applications, or directly aliquoted and stored for analysis at a later time. Other embodiments utilize an ultrafiltration purification step which comprises a low-pressure membrane separation process to partition high molecular weight compounds from a feed stream to achieve the desired purification of the final RCA products.

[0101] The final product may be analyzed by traditional methods for size, form, contamination, and expression capacity. Gel electrophoresis, sequencing, and biochemical or HPLC analysis is routine. Expression of the final product is tested by transfection into appropriate cells, using standard techniques such as calcium phosphate treatments, electroporation or related techniques.

[0102] Administration of the amplified product as a therapeutic compound may include but is not limited to topical applications, intravenous, intramuscular and intra-tissue injections, nasal applications, suppository applications, injections using implanted reservoirs and/or pumps such as Omaya reservoirs, eye-drop applications, orally administered pharmaceuticals, and delivery using ultrasound techniques. In the context of this invention, all such mechanisms constitute a "delivery vehicle." Traditional delivery vehicles, including liposome-mediated or polymer-based transport vehicles as well as a wide variety of capsule or protein-targeting vehicles, and appropriate aerosol carriers for respiratory administration can also be used effectively.

EXAMPLES

EXAMPLE 1 - Synthesis and cell free production of β-galactosidase synDNA (LacZ-DU).

[0103] a) Plasmid-based Template. pSV-β-Galactosidase (LacZ) vector (Promega Corp. Madison, WI, USA) was partially digested with EcoR I and Pst I. A fragment of about 4.2 kb containing the CMV promoter, LacZ ORF and SV40 small T antigen termination sequences (LacZ-DU) [SEQ ID NO: 1] was isolated, blunt ended with T4 DNA polymerase and cloned into the SmaI site of pGEMTM-7Zf(+) (Promega Corp. Madison, WI, USA) creating the pGEM-LacZ-DU vector. The LacZ-DU was subsequently excised from pGEM-LacZ-DU with XbaI, gel purified, and circularized using T4 DNA ligase (New England Biolabs, Beverly, MA, USA) as per manufacturer recommendations.

[0104] b) PCR-based Template. LacZ-DU was amplified from the pVAXTM200-GW/lacZ vector (Invitrogen Carlsbad, CA) using forward (5'-CGGGATCCGACTCTTCGCGATG TAC-3') and reverse (5'-CGGGATCCCAGCATGCCTGC-3') primers containing the BamHI endonuclease recognition site. LacZ-DU was amplified in 50 µl reactions with 200 ng of each primer 10 ng pVAXTM200-GW/lacZ vector; 0.2 mM dNTPs; 1x Herculase buffer and 2.5 U HerculaseTM polymerase (Stratagene, La Jolla, CA, USA). Amplification was carried out in a RoboCycler Gradient 40 (Stratagene, La Jolla, CA, USA) under the following conditions: 2 min at 94oC; 5 cycles (30 sec 92oC; 30 sec 40oC, 5 min 72oC); 25 cycles (30 sec 92oC; 30 sec 55oC, 5 min 72oC) and 10 min 72oC. The ~4.2kb product was digested with BamHI, gel purified and circularized with T4 DNA ligase.

[0105] c) Amplification with random hexamers. Reactions containing 10 mM Tris pH 8, 10 ng of circular LacZ-DU and 200 pmol random hexamers (Integrated DNA Technologies, Inc. Coralville IA, USA) were heated to 95oC for 3 min and cooled to room temperature. Phi29 DNA polymerase (10 U, New England Biolabs, Beverly, MA, USA); 0.2 mM dNTPs and 100µg/ml BSA were added. Amplification was carried at 30oC in 50 mM Tris-HCl pH7.5; 10 mM MgCl2; 10 mM (NH4)2SO4, 4 mM DTT for 16 hr. Following amplification, the phi29 DNA polymerase was heat inactivated (5 min; 65oC) and the amplified LacZ-DU concatamer was ethanol/salt precipitated and digested with the appropriate endonuclease (XbaI or BamHI) as recommended by the enzyme manufacturer.

[0106] d) Amplification with specific primers. Using the same conditions as described above, two primers of defined sequence and of opposite complementarity were used to selectively amplify a 2788 bp DU. The defined primers were used at a concentration of 200 pmol each, and consisted of the following sequences: forward primer: 5'-CTGCCAACAAGGTACTCG-3'; reverse primer: 5'-AGCTGCTACTGGGTCTAG-3'. Amplification was examined by gel electrophoresis to assess successful amplification.

[0107] e) Amplification with a single sequence-defined hexamer. Reactions containing 400 pmol of hexamer 5'-GpGpApApApA-3' which anneals at 8 different sites on LacZ-DU (4 on the reverse DNA strand at positions 464, 1325, 2579 and 3911; 4 on the forward strand at positions 750, 2871, 3239, and 3260) and 10 ng of circular LacZ-DU were heated to 95oC for 3 min in 40 mM Tris-HCl pH 8; 10 mM MgCl2 and cooled to room temperature. Phi29 DNA polymerase (10U, New England Biolabs, Beverly, MA, USA); 1 mM dNTPs; 5% glycerol; 0.7 U yeast inorganic pyrophosphatase (Sigma, St.Louis, MO, USA) and 100μg/ml BSA were added. Amplification was carried out at 30oC in 50 mM Tris-HCl pH 7.5; 10 mM MgCl2; 10 mM (NH4)2SO4, 4 mM DTT for 16 hr. Following amplification, the phi29 DNA polymerase was heat inactivated (10 min; 65oC) and the amplified LacZ-DU concatamer was ethanol/salt precipitated and digested with the appropriate endonuclease (Xba I) as recommended by the enzyme manufacturer. After inactivation of the endonuclease (65oC for 20 min), circularization of linear LacZ-DU was carried out in ligation buffer (50 mM Tris-HCl pH 7.6; 5 mM MgCl2; 1 mM ATP; 1 mM DTT; 5% PEG-8000) with about 0.1 Unit/μL T4 DNA ligase (Invitrogen Carlsbad, CA, USA) per 100 fmol DNA for 16 hr at about 22oC (or slightly cooler). Circular LacZ-DU was then ethanol/salt precipitated and resuspended in 10 mM Tris-HCl pH 8.

[0108] f) Amplification with a single exonuclease-resistant sequence-defined hexamer. Using the same conditions as above, LacZ-DU was amplified using a defined hexamer with two thiophosphate linkages at the 3' terminal end (5'GpGpApApSApSA-3').

[0109] g) Amplification with a single sequence-defined pentamer. Using the same conditions as above, LacZ-DU was amplified using a sequence defined pentamer (5'GpGpApApA-3') which anneals to LacZ-DU at 19 different sites: 8 on the reverse strand at positions 465, 889, 1326; 1695, 2580, 3666 and 3912; 11 on the forward strand at positions 80, 119, 191, 602, 750, 912, 2871, 3239, 3606, 3815.

[0110] h) Amplification with a single exonuclease-resistant and sequence-defined pentamer. Using the same conditions as above, LacZ-DU was amplified using a sequence defined exonuclease resistant pentamer with thiophosphate linkages at the two 3' terminal nucleotides (5'GpGpApSApSA-3').

[0111] i) Amplification using a polymerase cocktail. Using the same conditions as described in section 1-e, LacZ-plasmid was amplified in the presence of phi29 DNA polymerase and T4 DNA polymerase at ratios ranging from 10:3 to 3:10 (Phi29 enzyme unit:T4 enzyme unit). Optimal amplifications conditions were also shown to work for other templates, i.e. Luciferase DU.

EXAMPLE 2 - Synthesis and cell free production of Luciferase synDNA (Luc-DU).

[0112] The pGL3 vector (Promega Corp. Madison, WI, USA) was digested with Sal I and Xho

l. A fragment of about 2.17 kb containing the SV40 promoter, Luciferase ORF and SV40 small T antigen termination sequences (Luc-DU) [SEQ ID NO: 3] was isolated, purified and re-circularized using T4 DNA ligase (Invitrogen, Carlsbad, CA) as recommended.

[0113] a) Cell Free Amplification. Reactions containing hexamers 5'-ApApTpTpSGpSC-3' and 5'-ApGpCpApSApST-3' at 400 pmol each and 10 ng/25 µl reaction of circular Luc-DU were heated to 95oC for 3 min in 40 mM Tris-HCl pH 8; 10 mM MgCl2 and cooled to room temperature. Phi29 DNA polymerase (10U, New England Biolabs, Beverly, MA); 1 mM dNTPs (25/25/25/25); 5% glycerol; 0.7 U yeast inorganic pyrophosphatase (Sigma, St.Louis, MO, USA) and 100µg/ml BSA were added. Amplification was carried out in 25 µl reaction at 30oC in 50 mM Tris-HCl pH 7.5; 10 mM MgCl2; 10 mM (NH4)2SO4, 4 mM DTT for 16 hr. Following amplification, the phi29 DNA polymerase was heat inactivated (10 min; 65oC) and the amplified Luc-DU concatamers were ethanol/salt precipitated and digested with endonuclease (BamH I) as recommended by the enzyme manufacturer. After inactivation of the endonuclease (65oC for 20 min), circularization of linear Luc-DU was carried out in ligation buffer (50 mM Tris-HCl pH 7.6; 5 mM MgCl2; 1 mM ATP; 1 mM DTT; 5% PEG-8000) with 0.1 Unit/µl T4 DNA ligase (Invitrogen Carlsbad, CA) per µg of DNA for 12-16 hr at 14oC. Circular Luc-DU was then ethanol/salt precipitated and resuspended in 10 mM Tris-HCl pH 8.

EXAMPLE 3 – Expression of Amplified δ-gal and Luciferase synDNA in human cells.

[0114] Human A549 lung carcinoma cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen Carlsbad, CA) and incubated at 37oC in 5% CO2 environment.

[0115] a) DNA Transfection. The day prior to transfection, A549 cells were seeded in 6-well plates at a density of 1 x 105 cells/ml. GenePORTER 2 transfection reagent (Gene Therapy System, San Diego, CA) was used for cell transfection as directed by manufacturer. Briefly, cell free amplified DU or parental plasmid DNA (Promega Corp. Madison, WI) were mixed with 2 µg of carrier pssXE DNA (Chen and McMicken, Gene Ther 10: 1776-1780, 2003) in 50 µl of DNA diluent B and incubated at room temperature for 5 min. DNA solution was then mixed with 7 µl of GenePORTER 2 reagent pre-diluted in 50 µl of serum/antibiotics-free DMEM and incubated at room temperature for an additional 5 min. Meanwhile, A549 cells were washed with PBS and topped with 0.9 ml of serum/antibiotic-free DMEM to which the DNA/GenePORTER solution was subsequently added. Following 4 hr incubation in normal growth environment, the cells were washed with PBS and transfection medium was replaced with normal growth medium supplemented with 10 µl/m of Booster 3 (Gene Therapy System, San Diego, CA). In experiments using LacZ as

reporter, transfections with 50-100 ng of LacZ-DU (~4.2 kb) were compared to transfections with 100 ng of parental pGEM-LacZ-DU plasmid (~7.2 kb). In other experiments using the luciferase enzyme as a reporter, 249 ng of Luc-DU (~2.17 kb) were compared to transfections with 570 ng of pGL3 parental vector (5.01 kb; Promega).

[0116] b) Detection of β-galactosidase activity in transfected cells. 24 hr post-transfection, A549 cells were rinsed with PBS and lysed in 200/250 ml of 0.1 M phosphate buffer pH 7.5; 0.02% Triton X-100 for 1 hr at room temperature. Cell debris was subsequently removed by centrifugation at 10-13,000 rpm for 5 min. Total protein concentration of cell lysates was determined spectrophotometrically at 280 nm or by modified Bradford assay. 50 μg of total protein was mixed with 0.01M phosphate buffer pH 7.5; 0.1 M MgCl2, 45 mMβ-mercaptoethanol and 0.01 mM (p-nitrophenyl β-D-galactopyronidase) in 1 ml reactions. After incubation for 1-16 hr at 37oC, absorbance at 410 nm was measured.

[0117] c) Detection of luciferase activity in transfected cells. 24 hr post-transfection, cells were processed as described above. Cell lysates were subsequently adjusted to reflect equal total protein concentration and mixed with an equal volume of 2x Bright-GloTM substrate (Promega Corp.). Light emission was immediately recorded using a Turner Biosystem 20/20n luminometer.

EXAMPLE 4 - Cell Free Production Reaction Optimization.

[0118] a) Amplification buffer. Glycerol concentration - Two amplification reactions using 10 ng of Luc-DU template each were set up as described in EXAMPLE 2. In one, addition of glycerol was omitted and replaced with water. Following amplification, DNA was ethanol/salt precipitated and subsequently digested with the appropriate restriction enzyme prior to spectrophotometric quantification at 260 and 280 nm wave lengths. In reactions where glycerol concentration was less than 4% w/v (carry over from the phi29 DNA polymerase and inorganic pyrophosphatase stock solutions) a 5.65% increase in amplification efficiency was observed.

[0119] Addition of molecular sponge: Two amplification reactions using 10 ng of Luc-DU template each were set up as described in EXAMPLE 2. In one, 5% w/v PEG-8000 was added. Following amplification, DNA was ethanol/salt precipitated and subsequently digested with the appropriate restriction enzyme prior to quantification at 260 and 280 nm wave lengths. No positive effect on amplification yields was recorded.

[0120] b) Template concentration. Amplification reactions containing Luc-DU template concentrations ranging from 1,156 nM to 29 nM were prepared as described in Example 2. Following amplification, DNA was ethanol/salt precipitated and subsequently digested with the appropriate restriction enzyme. Nucleic acid concentrations were determined spectrophotometrically at 260 and 280 nm wave length. A 670-fold amplification was observed using 578 nM template

under the amplification conditions delineated above.

[0121] c) Deoxyribonucleoside triphosphate (dNTP) concentration. Amplification reactions containing 578 nM DU were prepared as described in EXAMPLE 2. Reactions containing dATP, dCTP, dGTP, and dTTP (proportionate ratio of 25/25/25/25) concentrations ranging from 1 mM to 9 mM were tested. Following amplification, DNA was digested with the appropriate restriction enzyme and nucleic acid concentrations were determined spectrophotometrically. Amplification was about 3,000-fold in the presence of 6 mM dNTPs under the amplification conditions delineated above.

[0122] d) Customization of dNTP ratio to template. Amplification reactions containing 578 nM Luc-DU template were prepared essentially as described in EXAMPLE 2. dATP, dCTP, dGTP and dTTP were individually added to the amplification mix to a final concentration of 9 mM. The ratio of each dNTP with respect to the entire pool was tailored such as to reflect the composition of the luciferase template DNA unit i.e. 27.2% A, 22.3% C, 24.2% G and 26.3% T. Following amplification, DNA was ethanol/salt precipitated and subsequently digested with the appropriate restriction enzyme. Nucleic acid concentrations were determined spectrophoto-metrically at 260 and 280 nm wavelength. About a 2,780-fold amplification was recorded using 578 nM template under the amplification conditions delineated above.

[0123] e) Phi29 DNA polymerase concentration. Amplification reactions were prepared as described above in which various phi29 DNA polymerase (New England Biolabs) concentrations ranging from 1 to 20 U/578 nM DNA template were tested in the presence of 9 mM dNTPs. Following amplification, DNA was digested with the appropriate restriction enzyme and nucleic acid concentrations were determined spectrophotometrically. 1 U of phi29 polymerase/578 nM was sufficient to produce a 290-fold amplification, while 20 U of phi29 DNA polymerase amplified 10 ng of template DNA 3,985 times.

[0124] f) Sequence-defined exonuclease resistant hexamer concentration. Amplification reactions were prepared as described above in which various concentrations of sequence-defined exonuclease resistant hexamers of up to 800 pmol were tested. Following amplification, DNA was digested with the appropriate restriction enzyme and nucleic acid concentrations were determined spectrophotometrically. Increasing primer concentrations by 2 from the initial experimental conditions (Ex. 2) translated into a 1.25-fold increase in amplification yields.

[0125] g) One step amplification restriction enzyme digestion reaction. Amplification reactions containing 578 nM Luc-DU template were prepared as described in EXAMPLE 2. Following amplification, Phi29 DNA polymerase was heat inactivated at 65oC for 20 min and 6U of BamHI enzyme was directly added to the reactions. Following 2 hr at 37oC, the enzyme was heat inactivated and the DNA was ethanol/salt precipitated. Efficiency of DNA digestion was visually

assessed by agarose gel electrophoresis as described above.

[0126] h) Variable temperature. Using conditions established above (Example 1), amplification of LacZ-plasmid was carried out at temperatures varying from 25 to 34oC. The optimal temperature was determined based on DNA yields and quality. DNA yields were determined spectrophotometrically while DNA quality was assessed by the determination of error rate using a modified Kunkel method (Kunkel T.A; JBC 260:5787-5796,1985) described in Nelson, J.R. et al., BioTechniques 32:S44-S47, 2002) using full-length LacZ gene (3046 bp) as reporter. Amplifications carried out at 32oC resulted in a >3300-fold amplification with an error rate of 1.22 x 10-6 (a 2.5-fold decrease in the reported error rate of Phi29 DNA polymerase).

[0127] i) Amplification with variable reaction times. Using the conditions described in Example 1, amplification of LacZ-plasmid was carried out at 32oC for variable periods of time (reaction time) ranging from 1 to 16 hr. At each time point the DNA polymerases were heat inactivated at 65oC for 20 min and DNA was digested with appropriate amounts of restriction endonuclease of directly added to the reactions. The optimal reaction time was determined based on DNA yields and quality. The optimal reaction time resulted in a >3800-fold amplification with a polymerization error rate of 1.7 x 10-6.

[0128] j) Amplification with lower enzyme and template concentrations. Using the conditions described in Example 1, LacZ-plasmid and Luc-DU were amplified in reactions containing half the total enzyme concentration (including Phi29 DNA polymerase, T4 DNA polymerase and Inorganic pyrophosphatase) and 289nM DNA template. The amplification was carried out for 16 hr at 32oC. Following heat inactivation of the polymerases and subsequent endonuclease digestion of the amplification product, the DNA yields and quality were determined as described above. Half the enzymes and template concentrations from the initial experimental conditions (Example 1) translated into a >5000-fold in amplification yields with a polymerization error rate of 7.7 x 10-7 (about a 4 fold decrease in the reported error rate of Phi29 DNA polymerase).

[0129] k) Elimination/reduction of concatamer formation during RCA. Using the conditions described in Example 1, LacZ-plasmid was amplified in reactions containing 2U of methylation sensitive SexAI endonuclease in addition to the DNA polymerases. The reaction was carried out at 32oC for 16 hr. Following amplification/digestion, analysis of the synthesized DNA by agarose gel electrophoresis revealed the presence of discreet linear DNA units.

EXAMPLE 5 - Intramolecular ligation reaction optimization.

[0130] a) T4 DNA ligase. Following restriction enzyme digestion of cell free amplified DNA, heat inactivation of the restriction enzyme and ethanol/salt precipitation of the DNA, the intramolecular ligation (self-ligation) of linear DU was performed in 138 µl and 690 µl reactions respectively containing 700 fmol of DNA in 1x ligation buffer (5% PEG-8000; 50 mM Tris-HCl pH

7.5; 10 mM MgCl2; 1 mM DTT; 1 mM ATP). Various amounts of T4 DNA ligase (Invitrogen Carlsbad, CA) were then added (0.6-1.5 U) and ligations were carried out at 14oC for at least 1 hr. Ligation efficiency was subsequently visually determined by agarose gel electrophoresis of said DNA. 290 μU of T4 DNA ligase per fmol DNA in 690 μl reactions was deemed sufficient for driving the synthesis of monomeric circular DU.

[0131] b) Other DNA ligases. Following linearization of DNA with appropriate restriction enzyme and heat inactivation of said enzyme, intramolecular ligation (self-ligation) of linear DU was performed. Ligations were conducted with either E. coli DNA ligase (NEB) or Taq DNA ligase (NEB) following manufacturer recommendations at 14oC and 45oC respectively. Ligation efficiency was subsequently visually determined by agarose gel electrophoresis and compared with T4 DNA ligase products.

EXAMPLE 6 - Enrichment/Isolation of double stranded circular DNA.

[0132] T4 DNA ligation products were ethanol/salt precipitated and resuspended in 20 μl of Plasmid SafeTM DNase buffer (Epicenter) containing 5U of ATP-dependent DNase as per manufacturer recommendations. Following 30 min incubation at 37oC, DNase enzyme was heat inactivated at 65oC for 20 min. Reaction efficiency was visually determined by agarose gel electrophoresis revealing the presence of only circular dsDNA which can be re-digested to linear form with appropriate restriction enzymes.

EXAMPLE 7 - Expression of Luciferase synDNA in mouse lung (tail vein injection + carrier).

[0133] Various forms of Luc-DUs were prepared including: linear form, phosphorothioate modified linear form, circular form, circular form treated with Plasmid-Safe™ ATP-dependent DNase (Epicenter, Madison, Wisconsin). 1 μg of various forms of Luc-DUs were complexed with MAA-PEI at an N:P ratio of 15:1 in PBS at a final volume of 200 μL/mouse. Each group comprising 5 BALB/c mice was injected via tail vein without anesthesia with a single form of MAA-PEI-Luc-DU. Mouse lungs were harvested 24 hours following injection and homogenized in luciferase assay buffer. Luciferase gene expression was measured using Bright-Glo™ kits from Promega according to the manufacturer's instructions.

TABLE 1 - Expression of Luc-DU in Mouse lung (ng luc/lung; corrected for background).

	A circular	B circular + DNase	C linear	D control plasmid	E linear phosphorothioate
Cage 1	0.399	0.272	0.069	0.002	
Cage 2		0.281	0.036	0.169	0.058
Cage 3	0.299		0.098	0.269	0.125
Cage 4	0.313	0.257			0.147
Average	**0.34**	**0.27**	**0.07**	**0.15**	**0.11**
Std Dev	0.05	0.01	0.03	0.13	0.05

(A) circular Luc-DU; (B) circular Luc-DU treated with DNase; (C) linear Luc-DU; (D) control plasmid; (E) phosphorothioate modified linear. (average background: 0.05 ng/lung).

[0134] In other experiments without the carrier MAA-PEI complexed to the DNA, the expression cassette for the luciferase enzyme was changed to include a stronger promoter and the experiments delineated above were repeated. The results are summarized in the table below.

TABLE 2 - Expression of Luc-DU in mouse lung (ng luciferase/lung).

	Plasmid	Circular	Linear
Cage 1	369	194	392
Cage 2	457	280	407
Cage 3	475	283	587
Average	**433.6667**	**252.3333**	**462**
Std Dev	56.72154	50.54041	108.5127

[0135] Experiments using "naked" DNA were also conducted. In this experiment, 15μg of various DNAs expressing the luciferase gene were introduced into mice by intradermal injections without addition of any carrier. The upper and mid tail sections were chosen as sites of injection. 24 hr post-injection, the animals were sacrificed and the upper and mid tail sections were dissected, homogenized and assayed for expression of luciferase as described above. Results from these experiments are summarized below.

TABLE 3 - Expression of Luc-DU in mice skin (ng luciferase/injection site).

Mouse	**Plasmid**	**Circular**	**Linear**
1 Upper	121100	279100	75090
1 Mid	110100	187700	380400
2 Upper	378800	784000	992800
2 Mid	898000	169400	892400
3 Upper	1664000	190700	22310
3 Mid	68260	1279000	610800
4 Upper	233900	106500	381800
4 Mid	133600	11120	307400
Mean	**450,970**	**375,940**	**457,875**
SD	560,077	432,519	352,343

EXAMPLE 8 - Genetic immunization in mice against gp160 protein of HIV-1

[0136] A eukaryotic cassette expressing a modified form of human immunodeficiency virus (HIV-1) envelope protein gp160 (gp145ΔCF1; Chakrabarti et al., J. Virol. 2002; 76: 5357-68; Kong et al., J. Virol. 2003; 77: 12764-72) [SEQ ID NO: 5] was used as template to generate large quantities of linear gp145ΔCF1-DU expression cassette as described in Example 2.

[0137] All animal experiments were approved by the Institutional Review Board for Animal Studies (Baylor College of Medicine; BCM). Supercoiled plasmid DNA and cell-free amplified

linear DNA (devoid of plasmid backbone sequences) expressing the gp145ΔCF1 protein were diluted in sterile saline solution and injected into the anterior tibialis muscle of BALB/c mice. DNA concentrations were determined by fluorometry using The Quant-iT™ PicoGreen® dsDNA Assay Kit as per manufacturer recommendations (Invitrogen Corp.). Each mouse received injections of 50 μg in each leg at days 0, 14 and 28. Blood samples were collected at days 14 (2 weeks), 28 (4 weeks), 42 (6 weeks) and 56 (8 weeks). Groups of 5 mice were used for each DNA type in addition to a control group injected with saline only. The serum from each blood sample was then used in Enzyme-linked immunosorbant assays (ELISA) to assess the IgG antibody titers against gp160. Briefly, 96 well microtiter plates were coated with a solution of 12.5 ng/μL of purified recombinant HIV-1 IIIB gp160 (Advanced Biotechnologies Inc.) in 50 mM carbonate buffer pH 9.5. The wells were subsequently washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with a solution of 3% BSA in PBS-T. 100 μL of serially diluted mouse antisera (in 3% BSA) was then applied and plates were incubated overnight at 4oC. The plates were washed with PBS-T and filled with 100 μL of a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (Pierce). Following extensive washing, 50 μL of 3,3',5,5'-Tetramethybenzidine (Sigma) was added and the colorimetric reaction was stopped with 0.5 N H2SO4. The optical density reading was taken at 460 nm.

EXAMPLE 9 - Purification of processed synDNA product(s)

[0138] Once processed into the final form (linear, circular or other), the amplification product is purified by gel filtration chromatography using Sephacryl SF-1000 (GEHC). Briefly, DNA is added onto 1.7 m x 1.5 cm Econo-column (Bio-Rad) and eluted with 10 mM Tris Ph 8, 150 mM NaCl, 5 mM EDTA at a flow rate of 1 mL/3.6 min. The DNA content of each elution fraction is monitored by agarose gel electrophoresis and the desired fractions are pooled. The fractions are subsequently concentrated using Centriplus 300 cartridges (Millipore Corp.) are recommended by manufacturer.

[0139] Alternatively, anion exchange chromatography using Q sepharose column plumbed to an FPLC or HPLC system can be used. DNA in low salt buffer (LSB; 10 mM Tris-Cl pH 8) would be loaded onto columns. Columns are washed with 10 column volume of LSB. DNA is eluted from the resin with a linear gradient of 10-100% elution buffer (EB; LSB + 3 M NaCl) in 20 column volumes. The eluate is monitored at 254 nm and only the peak(s) containing the DNA is collected. Desalting by ultrafiltration using Millipore's Pellicon II UF membranes follows. The DNA quality/integrity is analyzed by agarose gel electrophoresis.

EXAMPLE 10 - Quality assessment of cell-free produced synDNA

[0140] Each production lot is assigned an identification number and undergoes a series of test to determine DNA concentration, purity and integrity. DNA concentration is determined my

photometric absorbance reading at 260 nm and/or fluorometry using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Corp.). DNA purity is determined using several methods. Photometric A260/280 ratio, real time PCR (Genomic DNA contamination); HPLC (RNA contamination); micro-BCA test (Protein content, Pierce kit) and LAL test (Endotoxin content, Cambrex kit). In addition a bioburden test is carried out to confirm the sterility of the end product. Each set of test needs to comply with the specification set by the therapeutic industry.

TABLE 4 - Quality Testing Assessment Profile

TEST	METHOD	SPECIFICATION
DNA concentration	Photometric A260 Densitometry Fluorometry	1.0 - 5.0 mg/ml
Purity	Photometric A260/280 ratio	1.80 -1.98
Appearance	Visual	Clear, colorless solution
Genomic DNA	Real time PCR	< 1% w/w
RNA	HPLC/ 1% agarose gel electrophoresis	< 1% w/w
Protein	Colorimetric (BCA Test)	< 100 ng/mg
Bioburden	Liquid LB medium (16-24 hrs, 37°C)	OD 600 = 0
Endotoxin	Chromogenic LAL test	< 5 EU/mg
Identity	Restriction Enzyme Analysis	Conforms to specified fragment length

EXAMPLE 11 - Genetic immunization in rabbits against Hepatitis B virus (HBV).

[0141] A eukaryotic cassette expressing the Hepatitis B small surface antigen (HBs(S); Davis et al., 1993; Human Mol. Gen. 2: 1847-1851.) was used as template to generate large quantities of linear HBs(S)-DU expression cassette [SEQ ID NO: 7] as described in Example 2. Groups of 3 NZ female albino rabbits were immunized via bilateral (hind limb) intramuscular injections on days 0, 28 and 56 with either a total dose of 400 μg of the plasmid each time or the gene equivalent quantity of cell-free amplified linear DNA. Sera from each sample were taken at days 0, 28, 42, 56 and 63 were analyzed with an established ELISA protocol to determine the extent of the humoral immune response. Figure 9 shows ELISA assay absorbance readings for sera taken from 3 rabbits immunized with either HBs(S) supercoiled plasmid or cell-free HBVs(S)-DU linear DNA for days 28 and 63 (normalized for day 0).

EXAMPLE 12 - Genetic immunization in mice against influenza H1N1 virus.

[0142] Five BALB/c mice were utilized in each experiment. All animal experiments were approved by the Institutional Review Board for Animal Studies (Baylor College of Medicine; BCM). Influenza A/Puerto Rico/8/34 (A/PR8; H1N1) was obtained from the Respiratory Pathogens Research Unit, BCM. The influenza virus comprises a family of related viruses with slightly

different lipid coat proteins on the outer surface. Two of the better characterized variable coat proteins involved in epidemics and pandemics of flu comprise hemaglutinin (HA; at least 15 types) and neuraminidase (NA; at least 9 types). The H1N1 is one of the earlier characterized viral forms and is used widely in researching influenza.

[0143] DNA immunization was conducted as described above using 50 μg of plasmid DNA or a gene equivalent amount of cell-free DNA in PBS. The influenza hemaglutinin open reading frame from viral strain A/PR8/34 (HA) was isolated from pCAG-HA-WPRE plasmid (Garg et al, 2004, J. Immunol. 173(1):550-8) and subcloned into pCMV-MCS (Stratagene) giving pCMV-HA. The CMV-HA expression cassette devoid of plasmid backbone (HA-DU) [SEQ ID NO: 9] was amplified as described in Example 2. Animals were given 3 injections at weeks 0, 2 and 6. Five different experiments were conducted. Mice were immunized with: (1) 50 μg of pCMV-HA; (2) 32 μg of HA-DU; (3) a mixture of 16 μg of HA-DU and 25 μg of plasmid DNA devoid of any expression cassette (Empty Vector, pEV); (4) a mixture of 10.6 μg of HA-DU and two cytokine-expressing plasmids (i.e. 16.7 μg of pCMVi-GMCSF and 16.7 μg of pCAGGSIL12) (Orson et al., 2006, J. Gene Med, Jan 3); or (5) a mixture of 16.7 μg of pCMV-HA and the two cytokine-expressing plasmids as above (16.7 μg of pCMVi-GMCSF and 16.7 μg of pCAGGSIL12).

[0144] Eight weeks post immunization, sera samples were taken from each animal and virus neutralization assays were conducted. Sera collected in immunization experiments were heat inactivated (56°C, 30 min) and then assessed in vitro for neutralization efficiency using a standardized microneutralization assay. Briefly, the serum samples were serially diluted 1:2 in duplicate in 96-well, round-bottom tissue culture plates (Falcon 3077) using MEM as the diluent. Then approximately 100 median tissue culture infectious doses (TCID50) of influenza A/PR8 virus was added to each well. A back titration of the test virus was also performed at this time. The plates containing the sera and virus were incubated at 37°C for 90 minutes, after which the contents of the round-bottom plate were transferred to new plates containing monolayers of Madin Darby canine kidney (MDCK) cells.

[0145] After overnight incubation at 37°C, the medium from each well was removed and replaced with MEM containing 2μg/ml of Worthington trypsin (Worthington Biochemical Corp., cat. no. 32C5468), the penicillin and streptomycin, but lacking any serum. Four days later, a 0.5% suspension of chicken red blood cells (rbc) washed and resuspended in PBS was added to each well. When the rbc in the serum control wells formed a tight button, the hemagglutination pattern in each well was read and recorded. Wells with a tight button of rbc were considered to be negative for FV, while those with a diffuse hemagglutination pattern were recorded as positive for virus. Figure 10 shows virus-neutralization titers recorded as the last dilution in which virus replication was inhibited for the various genetic immunization experiments.

EXAMPLE 13 - Genetic immunization in mice against Smallpox virus.

[0146] A eukaryotic cassette expressing the B5R gene encoding a type I membrane glycoprotein essential for the formation of the extracellular virion envelope; Hooper J.W. et al. 2004; J Virol. 78(9): 4433–4443) was used as template to generate large quantities of linear B5R-DU expression cassette [SEQ ID NO: 11] as described above. Groups of 5, 4 and 3 Balb/c mice were immunized via intramuscular injections with: 100 μg of B5R plasmid, 34 μg of B5R-DU (gene equivalent quantity), and 100 μg of pVax empty vector (naïve), respectively. Mice were immunized on weeks 0, 2, 4 and 6 and sera were collected on weeks 3 and 7. The immunogenic response was assessed by antibody titration (ELISA) and INFγ production by splenocytes (ELISPOT) - below. Figure 11 shows the results of the immune response analysis. These assays show that B5R-DU synthesized using our cell-free process is a superior immunogen than plasmid DNA. Indeed, antibody titers (Figure 11A and C), INFγ production (Figure 11B) are consistently superior in mice immunized with B5R-DU than in mice treated with plasmid DNA at equal molar amounts.

[0147] a) Protein ELISA: Serum samples are isolated from orbital bleeds. The blood is spun down at 1100 rpm for 5 minutes, and the serum carefully removed and stored at 4°C until ready for analysis. Nunc 96-well plates are coated with B5R protein (Viral Genomix) (1 microgram/well) in PBS at 4°C overnight. After three washes with PBS, nonspecific binding sites are blocked with 1% BSA in PBS solution for 2 hours at room temperature. Duplicate samples are loaded into the appropriate wells in a dilution series, and incubated for 2 hours at room temperature. After washing, anti-murine IgG, conjugated with horseradish peroxidase is added at a dilution of 1:1000. Using TMB as a substrate, bound antibody is measured in an ELISA reader at 405 nm.

[0148] b) IFNγ ELISPOT: ELISPOT 96-well plates (Millipore) are coated with anti-mouse IFN-γ capture antibody (MabTech, Sweden) and incubated overnight at 4°C. The plates are then washed and blocked for 2 hours with R10 media (blocked prior to loading of cells). Approximately 2 x 105 splenocytes from immunized mice are added to the ELISPOT plates and stimulated overnight at 37°C, 5% CO2, either in the presence of RPMI1640 (negative control), Concanavilin A (positive control), or B5R peptides. Following 24 hours of stimulation, the cells are washed and incubated overnight at 4oC in the presence of biotinylated anti mouse IFN-γ antibody (MabTech). The next day, the plates are washed and streptavidin-alkaline phoshaphatase (MabTech) is added to each well for 2 hours at room temperature. The plate is washed, and 5-Bromo-4-Chloro-3' Indolylphosphate p-Toluidine Salt (BCIP) and Nitro Blue Tetrazololium Chloride (NBT) (the Chromogen Color Reagent, Sigma) is added to each well for 15-20 minutes at room temperature in the dark. The plate is then rinsed with distilled water, and dried inverted at room temperature. Spots are quantified manually or by a specialized automated ELISPOT reader (CT LTD).

EXAMPLE 14 – Methods of Delivery for immunization using synDNA products.

[0149] a) Aerosol Delivery: Standard conditions for aerosol include a 10:1 N:P ratio (N: PEI

nitrogen; P: DNA phosphate) in water with a final concentration of DNA at 2 mg/10 mL for nebulization. Maximal gene expression is normally observed at 48h for the gene constructs used herein. Mice are enclosed in a standard mouse cage wherein each mouse is place in an individual wire mesh enclosure. The nebulization uses a standard clinical jet nebulizer with compressed 5% CO2 in air as the gas source at 10 L/min. The procedure is complete in about 25-30 minutes. Intermittent exposure to the aerosol for shorter or longer periods of time as well as the use of variable amounts of DNA can be adjusted as the particular construct demands.

[0150] b) Intravenous Injection: Standard conditions for intravenous (IV) delivery of DNA include the use of 1 ug of DNA complexed with MAA-PEI at an N:P ratio of 15:1 in PBS at a final volume of 200 uL/mouse. Maximal gene expression is normally observed between 24 and 48 h. Mice are injected via tail vein without anesthesia, usually over 20-30 seconds, although the time of injection is not critical to gene expression.

[0151] c) Intradermal (ID) /Intramuscular (IM) Injection: Naked DNA is used at a concentration of about 50 ug in 25 uL PBS with 2 injections per mouse, although some plasmids may express better at lower concentrations, which may have to be determined for a specific plasmid. IM injections are ordinarily done in the anterior tibialis muscle, while ID injections are done by injection at the base of the tail.

EXAMPLE 15 – Influenza virus challenge and determination of pulmonary virus levels.

[0152] The A/PR8/34 influenza virus strain was grown in MDCK cells. The medium and cells from the infected flasks were harvested when the infected monolayers exhibited approximately 90% virus-induced cytopathic effects (CPE). The medium was clarified by centrifugation and then filtered through a 0.4 μm filter. It was then stored at -70°C in 1 or 2 ml aliquots. A sample of each harvest was titered to determine its tissue culture infectious doses (TCID50), median mouse infectious (MID50) and median mouse lethal (MLD50) dose.

[0153] To inoculate mice, the animals were lightly anesthetized with Isoflurane (Abbot Laboratories, North Chicago) and 50 μL of medium containing approximately 100 MID50 of the A/PR8/34 virus was instilled into the nares of each mouse using a pipetting aid. Mice were sacrificed 4 days later; lungs were harvested, homogenized, serially diluted and tested for the flu virus (FV) levels. After 4 days of incubation at 37°C, the plates were removed from the incubator and a 0.5% suspension of chicken red blood cells (rbc) in PBS was added to each well. When the rbc in the tissue control wells formed a tight button, the hemagglutination pattern in each well was read and recorded. Wells with a tight button of rbc were considered to be negative for FV, while those with a diffuse hemagglutination pattern were recorded as positive for virus. Virus titers were recorded as the log10 of the reciprocal of the last dilution in which virus replication (CPE) was evident. Titers are shown as TCID50/lung (log10). Lower values represent greater protection by the

immunizing agent.

TABLE 5 - Protective Immunity Against Influenza A Challenge (viral titers in blood serum)

Treatment Group	(IM) Injection								(IV) Injection	
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
Mouse-1	4.3	6.3	6.8	1.8	1.8	1.8	3.3	4.8	4.3	3.3
Mouse-2	4.8	6.3	1.8	1.8	1.8	6.3	2.8	3.3	2.8	6.8
Mouse-3	4.8	6.3	1.8	1.8	3.8	3.8	3.8	5.3	3.3	6.8
Mouse-4	4.8	3.8	1.8	5.3	3.8	5.8	6.3	5.3	<2.3	5.8
Mouse-5	3.8	4.8	1.8	1.8	1.8	3.8	2.8	2.8	2.8	5.3
Mean	**4.5**	**5.5**	**2.8**	**2.5**	**2.6**	**4.3**	**3.8**	**4.3**	**3.3**	**5.6**

Letters represent different treatment groups (exp 051017, 051018);

A: (IM) -HA Plasmid alone;
B: (IM) -HA Linear alone;
C: (IM) -HA Linear + blank plasmid;
D: (IM) -HA Linear + plasmid GMCSF + Plasmid IL-12;
E: (IM) -HA Plasmid + Plasmid GMCSF + Plasmid IL-12;
F: (IM) -HIV Plasmid (control);
G: (IM) -HIV Linear (control);
H: CONTROL -No Injection;
I: (IV) -HA Plasmid;
J: (IV) -PBS.

[0154] These results show that synthetic HA and HIV DNA is effective in inhibiting viral proliferation in mouse challenge experiments. HA plasmid provides minimal protection alone but significant protection when injected with the two cytokine expression plasmids. HA linear synDNA provides significant protection in the presence of a carrier plasmid (blank or cytokine expression). HIV plasmid provides minimal protection while the HIV linear synDNA had a slightly better protective effect.

EXAMPLE 16 - Effect of time on expression of Luc-synDNA in vivo.

[0155] Most of the animal tests to date using synthetic DNA therapeutics as vaccines in accordance with the current invention demonstrate the induction of a strong immune response when measuring antibody titer and/or T-cell response. The level of immunity is typically more robust in animals receiving an IM injection of a vaccine comprising a synDNA construct than in animals receiving a traditional plasmid carrying the same DNA sequence. To see if this elevated response was caused by a longer expression profile of the immunogenic proteins/peptides the expression profile of the luciferase enzyme using either Luc-synDNA or its plasmid counterpart was followed over time following injection into the muscle of mice.

[0156] Linear Luc-DU synDNA was prepared using phosphorothioate primers according to the method of the current invention and supercoiled luciferase plasmid was prepared by growth in bacteria and purified using a Qiagen endofree kit; both were injected as naked DNA following dilution in sterile saline into the anterior tibialis muscle of mice (BALB/c). Each mouse received a single injection of 50 μg DNA in one leg. Groups of 5 mice were used for each DNA types. 24, 72 and 144 hr post-injection mice were sacrificed and tibialis muscles were dissected, ground in saline

and processed for luciferase activity assay.

[0157] As seen in Figure 12, there is no significant difference (higher or lower) in expression of the luciferase enzyme over time between the linear Luc-DU synDNA of the current invention and the standard circular luciferase plasmid DNA. The data suggest that reporter constructs made with the cell-free synDNA process are as effective as traditional plasmid for expressing in an animal system. This is significant because it is well known in the art that unprotected linear DNA is highly susceptible to exonuclease degradation (from either end). It is recognized that circular DNA is a more stable form of epigenetic material in cells since it does not have exposed sites susceptible to exonuclease degradation. However, the synDNA construct as shown here, despite its linear form, effectuates the same expression profile as its circular plasmid counterpart. This suggests that either the DNA itself is a more stable structure or it has a higher degree of expression efficiency as an inherent consequence of being produced by this cell-free method.

[0158] This also demonstrates that the Luc-DU reporter construct can be used as an experimental tool for testing the effectiveness and applicability of synDNA in existing research systems. Our in vitro amplification results using the LacZ-DU construct worked in vitro in a manner similar to Luc-DU so it would be expected to express in vivo in a similar manner. Likewise, it would be expected that any well characterized reporter gene such as secreted alkaline phosphatase (SEAP), green fluorescent protein (GFP), and chloramphenicol acetyltransferase (CAT) would be as useful as the Luc-DU to be used as a testing tool for the use of synDNA in any ongoing research application.

EXAMPLE 17 - SynDNA production using Methyl Phosphonate modified primers

[0159] The consistent increase in stimulation of the immune response observed using synDNA could be influenced by an extended half-life of the synDNA product inside the cell as a consequence of the modification of the primers. In a preferred embodiment of the invention, phosphorothioate modified random primers are used. Here, we demonstrate that other modifications to the primer can be used with the invention effectively which may confer additional benefits to the synDNA being produced. In this experiment the primers are modified with methyl phosphonate; the modified MP primers and compare the synDNA products containing the different modified primers.

[0160] Reactions containing 2400 pmol of the sequence specific hexamer 5'-GGAAAA-3' containing either two phosphothioate or methyl phosphonate linkages at the 3' end and 10 ng of circular plasmid DNA pGem-LacZ-DU were heated to 95oC for 3 min in 40 mM Tris-HCl pH 8; 10 mM MgCl2 and quick chilled on ice. Phi29 DNA polymerase (10 U, Fermentas, MD, USA); 1x Phi 29 reaction buffer [0.1% Tween-20; 33 mM TrisOAc pH 7.9; 10 mM Mg(OAc)2; 66 mM KOAc; 1 mM DTT as supplied by Fermentas]; 6 mM dNTP (Stratagene, CA, USA); 0.7 U yeast inorganic pyrophosphatase (Sigma, St.Louis, MO, USA); 3 U T4 DNA polymerase (Fermentas, MD, USA)

and 100μg/ml BSA were added. Amplification was carried out at 30oC for 16 hr. Following amplification, phi29 DNA polymerase was heat inactivated (20 min; 65oC), the amplified DNA concatamer digested with XhoI (New England Biolabs, MA) for 4 hr at 37oC, and heat inactivated at 65oC for 20 min.

[0161] To test for exonuclease resistance, 2.5 μg of each amplification product was incubated at 37oC with 2.5 U of Plasmid Safe™ DNAse (Epicenter Biotechnologies, WI, USA) in 50 μL of 1x buffer as recommended by manufacturer. 10 μL of reaction mix was taken at time 0, 7.5 min, 15 min, 22.5 min and 30 min. The reaction was stopped by addition of 0.5μL of 500 mM EDTA pH 8. Five μL of each time point was run through a 0.8% agarose gel and stained with ethidium bromide to determine the integrity of the 7.1 kb linear plasmid (Figure 13A). The gel was photographed and the Polaroid picture then scanned into Photoshop 7.0. The intensity of the 7.1 kb band was determined using ImageJ software (NIH). The decrease in 7.1 kb band intensity over time was determined using Microsoft Excel 2003 software (Figure 13B). This shows that the phosphorothioate amplified DNA (P-synDNA) is about 1.5 times more sensitive to exonuclease degradation than the methyl phosphonate-synDNA (MP-synDNA).

[0162] We have also demonstrated the exonuclease resistance phenomenon in vitro by using linear plasmid DNA and linear phosphorothioate synDNA in the presence of a DNase (ex: Plasmid Safe™) which in the presence of ATP degrades linear dsDNA. Our results indicate that phosphorothioate synDNA is approximately 1.6 times more resistant to degradation from its unprotected ends than linear plasmid DNA. Toxicity tests and protein expression patterns over time are currently in progress using MP-synDNA in mice.

[0163] The primary advantage of this technology is that it offers a method for rapidly making high quality DNA with almost no bacterial cell components or bacterial toxin (endotoxins). This cell-free amplification process can be streamlined for efficiency by the optional removal of unnecessary flanking sequences from the plasmid prior to amplification which can reduce the effectiveness of the DNA used as a target effector. The end product that is produced has lower levels of known and potentially unknown toxins which are common to bacterially grown plasmid preparations and their end products; purification requirements are reduced and costs are minimized. The method is faster, cleaner and less cumbersome to use. The end products can be easily adapted for use in DNA based therapeutics as vaccines, gene therapeutics, or as tools for down-regulating gene expression (triplex, antisense) or protein activity (aptamer).

CLAIMS

1. A process for producing high-quality nucleic acid in a cell free system, comprising:
   (a) combining a circular template in a reaction mixture with one or more primers, which are complementary to at least one strand of the circular template, to form a template-primer complex;
   (b) incubating the template-primer complex with at least one high-fidelity nucleic acid polymerase to produce a concatamer comprising tandem units of the circular template; and
   (c) cutting the concatamer into smaller fragments comprising at least one delivery unit having a sequence of interest.
2. The process according to claim 1, further comprising:
   (d) processing the smaller fragments by one or more of the following steps: filling in or removing the ends of the smaller fragments; ligating ends of the smaller fragments to produce circularized smaller fragments; and supercoiling the circularized smaller fragments.
3. The process according to claim 1 or 2, further comprising:
   modifying the smaller fragments or the circularized smaller fragments to produce modified ends and/or modified internal bases.
4. The process according to any of claims 1-3, further comprising:
   coupling the smaller fragments or the circularized smaller fragments with a peptide.
5. The process according to any of claims 1-4, wherein the delivery unit comprises one or more expression cassettes.
6. The process according to any of claims 1-5, wherein the high-fidelity nucleic acid polymerase is Phi29 DNA polymerase or a derivative thereof.
7. The process according to any of claims 1-5, wherein the high-fidelity nucleic acid polymerase is one selected from DNA polymerase I, DNA polymerase III, T3 DNA polymerase, T4 DNA polymerase, T5 DNA polymerase, T7 polymerase, and derivatives thereof.
8. The process according to any of claims 1-7, wherein the cutting of the concatamer is accomplished by using a restriction enzyme.
9. A composition comprising the delivery unit prepared by the process according to any of claims 1-8.
10. Use of the delivery unit prepared by the process according to any of claims 1-8 or the compositions of any of claims 9 or 21-61, in the manufacturing of medicament for preventing or treating a disease or genetic disorder of a human, animal or plant.

11. The use according to claim 10, wherein the disease is caused by a virus selected from HIV, influenza virus, parainfluenza virus, adenovirus, corona virus, herpes simplex virus, herpes zoster virus, papilloma virus, and rhino virus.

12. The use according to claim 10, wherein the disease is caused by bacteria, mycobacteria, eubacteria or fungi.

13. Use of the delivery unit prepared by the process according to any of claims 1-8 or the compositions of any of claims 9 or 21-61, or the composition of claim 9 in the immunization or protection of a human, animal or plant.

14. The use of the delivery unit or composition according to claim 13, wherein the delivery unit or the composition is delivered by injection (intramuscular, intravenous, intradermal), oral compositions, aerosol sprays, eyedrops, suppositories, topical ointments, skin patches and soaks, as well as surgically implanted devices,or as a virus derived particle with or without a carrier or delivery vehicle.

15. An apparatus for producing high-quality nucleic acid, comprising:

a continually verifiable cGMP-quality reaction vessel having at least one entry port and one exit port;

an input device attached to the entry port and which feeds at least one outside component from an outside holding chamber to the reaction vessel;

at least one outside holding chamber connected to the input device;

a means for pumping an outside component from the holding chamber, through the input device, into the reaction vessel;

an export device attached to the exit port of the reaction vessel;

at least one outside receiving chamber connected to the export device;

a means for regulating temperature of the reaction vessel;

a means for monitoring and controlling the progress of a reaction mixture within the reaction vessel; and

a means for mixing the reaction mixture.

16. The apparatus of claim 15 wherein the reaction vessel is made of either a flexible material wherein the means for mixing is applied to the outside of the vessel or a hardened pre-formed material wherein the means for mixing is applied inside the vessel.

17. The apparatus of claims 15 or 16 wherein the pump comprises a peristaltic pump.

18. The apparatus of claims 15, 16 or 17 wherein the input device can be connected to the export device to facilitate circulating the reaction mixture through the reaction vessel.

19. The apparatus of claims 15, 16 or 17 wherein the reaction vessel further comprises a second entry port and a second exit port and wherein a circulating device having a means for pumping the reaction mixture connects the second entry port to the second exit port.

20. The apparatus of claim 18 wherein the circulating device comprises a means for adding at least one outside component from an outside holding chamber to the reaction mixture contained within the circulating device.

21. A therapeutic or research composition comprising a high-quality nucleic acid made by a cell free process, comprising the following steps:

(a) combining a template in a reaction mixture with one or more primers, which are complementary to at least one strand of the circular template, to form a template-primer complex;

(b) incubating the template-primer complex with at least one high-fidelity nucleic acid polymerase to produce a concatamer comprising tandem units of the circular template; and

(c) cutting the concatamer into smaller fragments comprising at least one delivery unit having a sequence of interest.

22. A therapeutic or research composition according to claim 21, wherein the cell free process further comprises:

(d) processing the smaller fragments by one or more of the following steps: filling in or removing the ends of the smaller fragments; ligating the ends of the smaller fragments to produce circularized smaller fragments; and supercoiling the circularized smaller fragments.

23 A therapeutic or research composition according to either claim 21 or 22 wherein the primer is modified by the incorporation of phosphorothioate or methyl phosphonate nucleotides, a derivative or a combination thereof.

24 A therapeutic or research composition according to either claim 21 or 22 wherein the circular template comprises at least one sequence of interest.

25 A therapeutic or research composition according to claim 24 wherein the circular template comprising a sequence of interest is devoid of plasmid replication sequences.

26 A therapeutic or research composition according to claim 25 wherein the plasmid replication sequences include origins of replication, antibiotic resistance genes, marker genes, selectiion genes and other sequences needed for bacterial plasmid selection and replication.

27 A therapeutic or research composition according to any one of claims 24 to 26 wherein the circular template comprises a sequence of interest encoding for a molecule capable of eliciting a genetic improvement or a protective response when present in a host capable of eliciting a protective response.

28. A therapeutic or research composition according to claim 27 wherein the protective response involves the stimulation of an animal or human immune response.

29. A therapeutic or research composition according to claim 27 wherein the protective response is a disease protecting response in a plant.

30 A therapeutic or research composition according to claim 27 wherein the sequence of interest is derived from genes associated with a viral, bacterial, fungal or parasitic disease.

31. A therapeutic or research composition according to claim 30 wherein the sequence of interest is derived from the genes associated with a viral or bacterial disease.

32. A therapeutic or research composition according to claim 31 wherein the sequence of interest is derived from a gene associated with influenza.

33 A therapeutic or research composition according to claim 32 wherein the sequence of interest is derived from influenza hemaglutinin or neuraminidase.

34. A therapeutic or research composition according to claim 33 wherein the sequence of interest is derived from SEQ ID NOs: 9 or 10.

35 A therapeutic or research composition according to claim 31 wherein the sequence of interest is derived from a gene associated with Hepatitis B.

36. A therapeutic or research composition according to claim 35 wherein the sequence of interest is derived from Hepatitis B small surface antigen.

37. A therapeutic or research composition according to claim 36 wherein the sequence of interest is derived from SEQ ID NOs: 7 or 8.

38. A therapeutic or research composition according to claim 31 wherein the sequence of interest is derived from a gene associated with human immunodeficiency virus (HIV).

39. A therapeutic or research composition according to claim 38 wherein the sequence of interest is derived from HIV-1 envelope protein gp160.

40. A therapeutic or research composition according to claim 39 wherein the sequence of interest is derived from SEQ ID NOs: 5 or 6.

41. A therapeutic or research composition according to claim 31 wherein the sequence of interest is derived from a gene associated with smallpox.

42. A therapeutic or research composition according to claim 41 wherein the sequence of interest is derived from B5R membrane glycoprotein.

43. A therapeutic or research composition according to claim 42 wherein the sequence of interest is derived from SEQ ID NOs: 11 or 12.

44. A therapeutic or research composition according to any one of claims 21 to 43 further comprising a delivery vehicle.

45. A therapeutic or research composition according to claim 44 wherein the delivery vehicle is a topical ointment, aerosol, liposome, microsome, polymer, nanotubule, cell penetrating or receptor adhering peptide, an oral carrier, buffer or water..

46. A therapeutic or research composition according to claim 44 wherein the carrier comprises polyethyl-eneimine (PEI) or a derivative thereof.

47. A therapeutic or research composition comprising a high-quality nucleic acid wherein the nucleic acid is a linear form.

46. A therapeutic or research composition according to claim 47 wherein the linear form is produced in a cell-free amplification system.

49. A therapeutic or research composition according to claim 47 wherein the linear form is chemically modified or structurally designed to improve stability or efficiency in stimulating an immune response.

50. A therapeutic or research composition according to claim 49 wherein the linear form comprises chemically or structurally modified primers.

51. A therapeutic or research composition made according to any one of methods of claims 21-50 wherein the final composition comprises one or more modified bases to improve stability, accessibility, expression, therapeutic efficacy or a compination of thereof.

52. A therapeutic or research composition according to claim 51 wherein the modified bases comprise phosphorothioate, methyl phosphonate or morpholino groups.

53. A therapeutic or research composition according to any one of claims 21 to 52 further comprising at least one additional nucleic acid molecule.

54. A therapeutic or research composition according to claim 53 wherein the second nucleic acid molecule encodes for at least a portion of a cytokine molecule.

55 A therapeutic or research composition according to claim 54 wherein the cytokine is either GMCSF or IL-12.

56. A research or therapeutic reporter construct comprising a high-quality nucleic acid made by a cell free process, comprising the following steps:

(a) combining a template in a reaction mixture with one or more primers, which are complementary to at least one strand of the circular template, to form a template-primer complex;

(b) incubating the template-primer complex with at least one high-fidelity nucleic acid polymerase to produce a concatamer comprising tandem units of the circular template; and

(c) cutting the concatamer into smaller fragments comprising at least one delivery unit having a sequence of interest.

57. A therapeutic composition according to claim 1, wherein the cell free process further comprises:

(d) processing the smaller fragments by one or more of the following steps: filling in or removing the ends of the smaller fragments; ligating the ends of the smaller fragments to produce circularized smaller fragments; and supercoiling the circularized smaller fragments.

58. A research or therapeutic reporter construct according to claims 56 or 57, wherein the reporter construct's sequence of interest comprises at least a portion of the coding region of a reporter gene.

59. A research or therapeutic reporter construct according to claim 58 wherein the reporter gene encodes for B-galactosidase, luciferase, alkaline phsophatase, green fluorescent protein, or chloramphenicol acetyltransferase.

60. A research or therapeutic reporter construct according to claim 59 wherein the reporter gene encodes for B-galactosidase or luciferase.

61. A research or therapeutic reporter construct according to claim 60 wherein the sequence of interest is derived from SEQ ID NOs: 1, 2, 3, or 4.

Figure 1 Template Synthesis

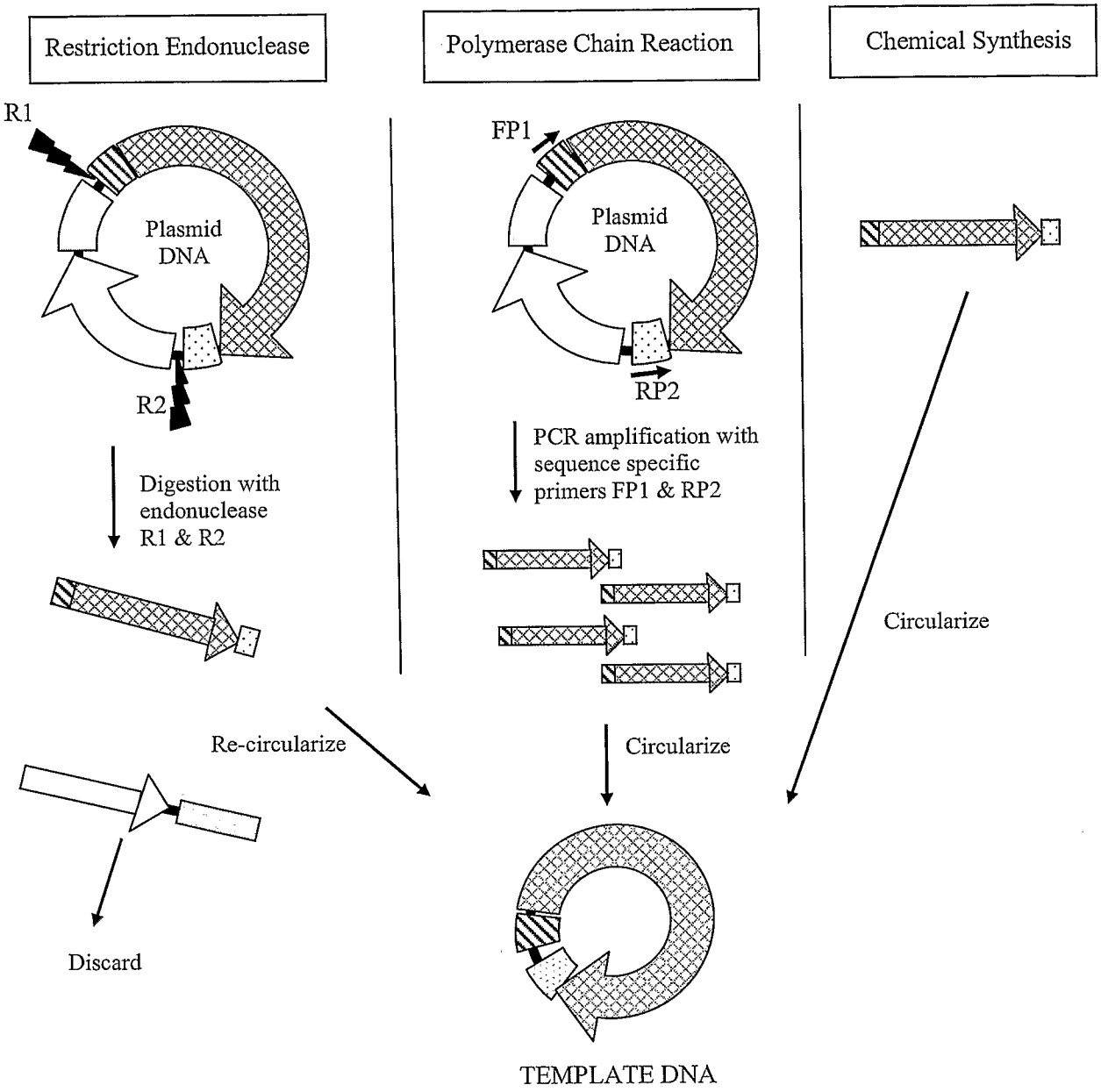


Figure 2 *In Vitro* Amplification Process

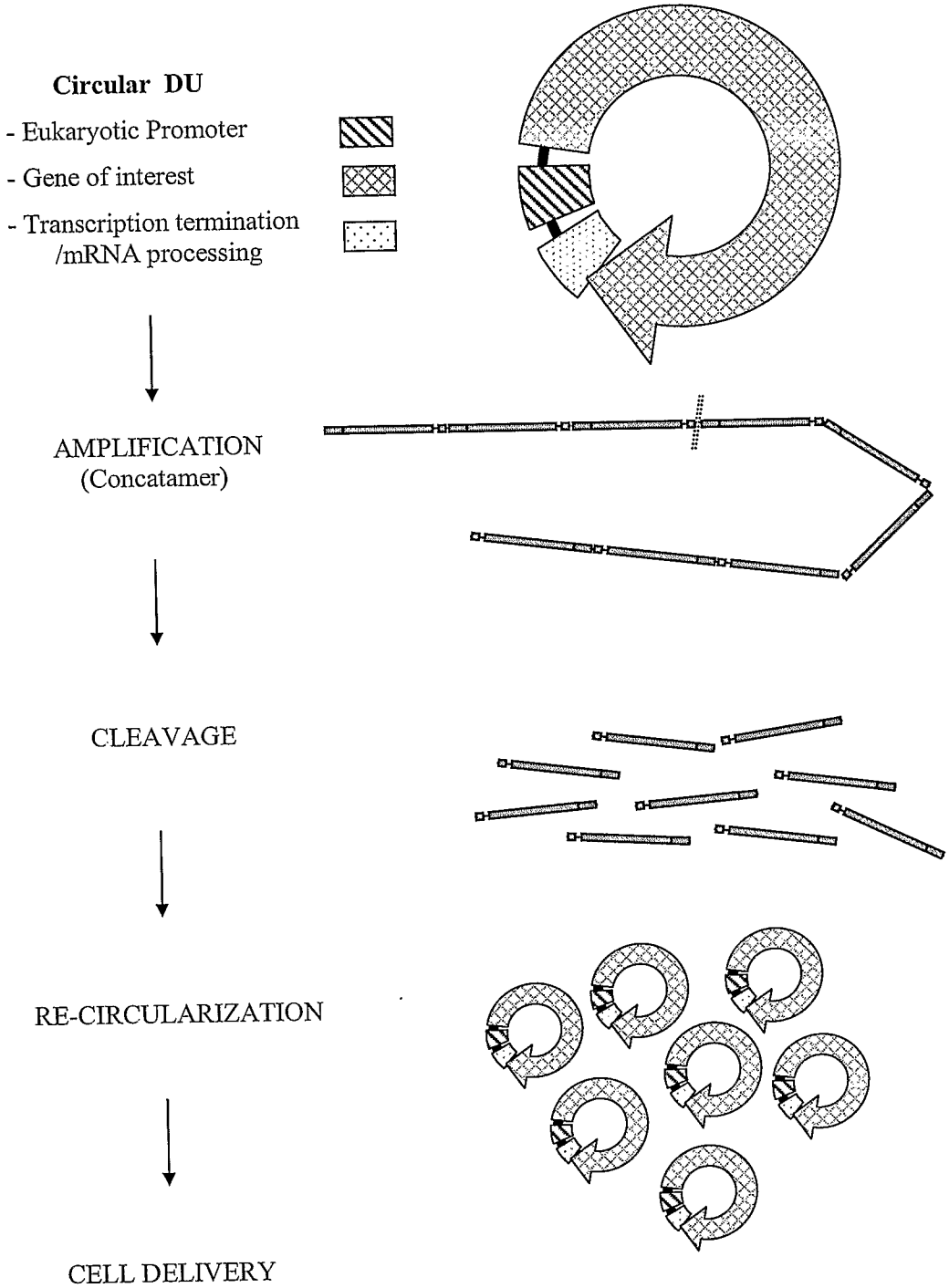


Figure 3 Production of Forward and Reverse DNA strands.

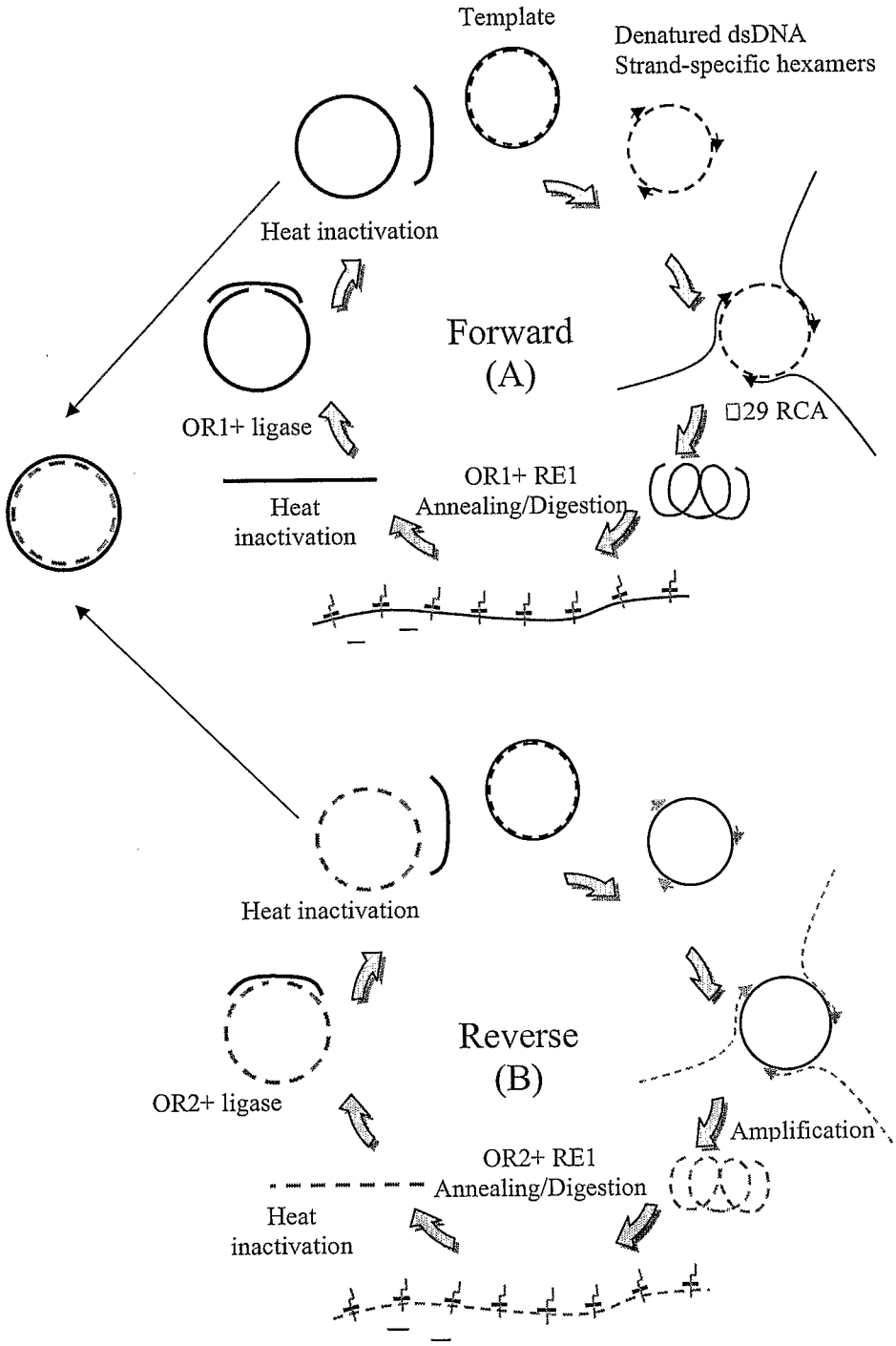


Figure 4 Scale Up Production of synDNA

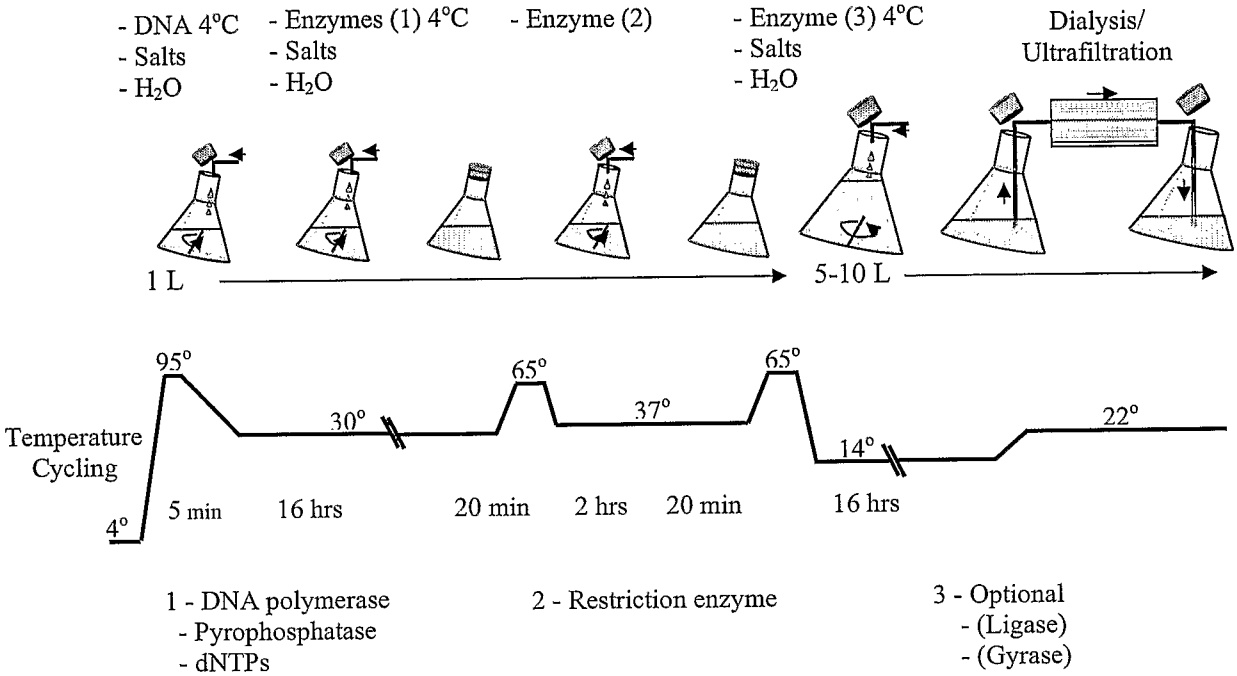


Figure 5 Automated Production

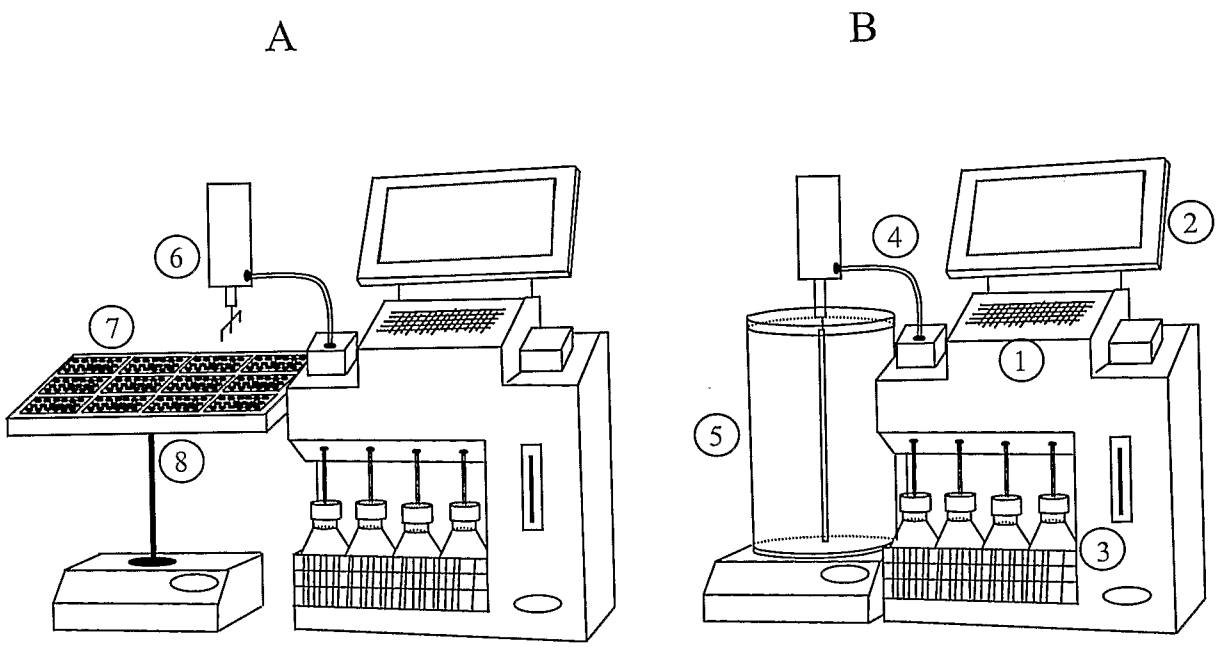


Figure 6 Mechanical Mixing

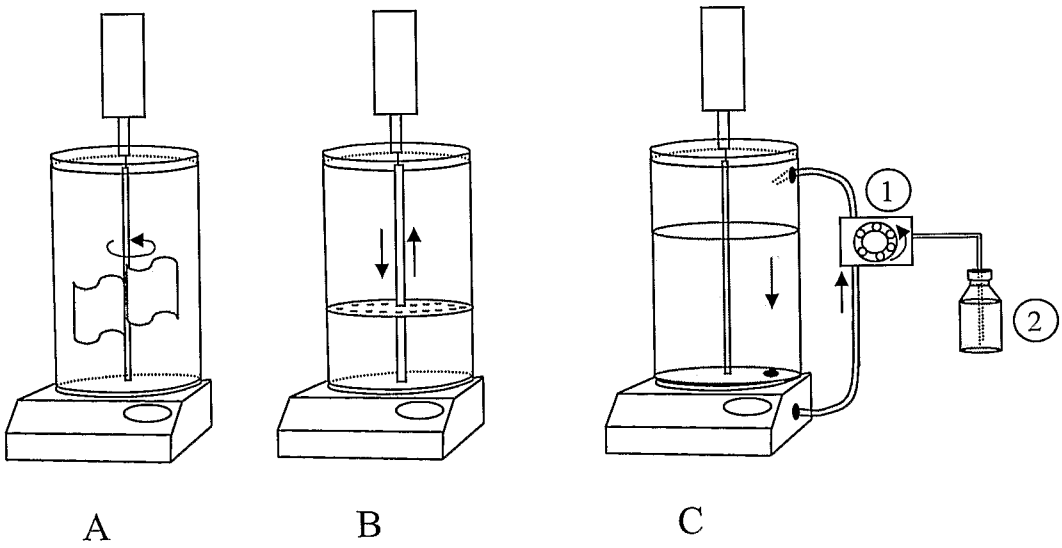


Figure 7 Intramolecular Processing with limited dilution

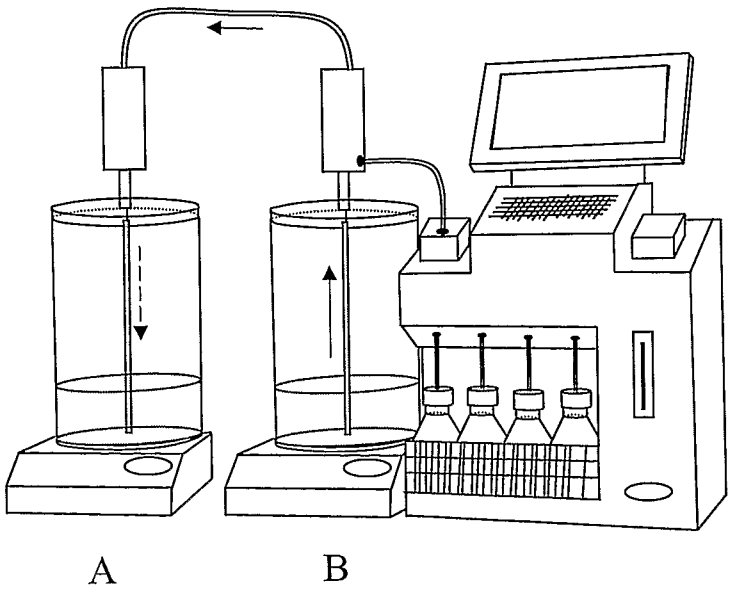
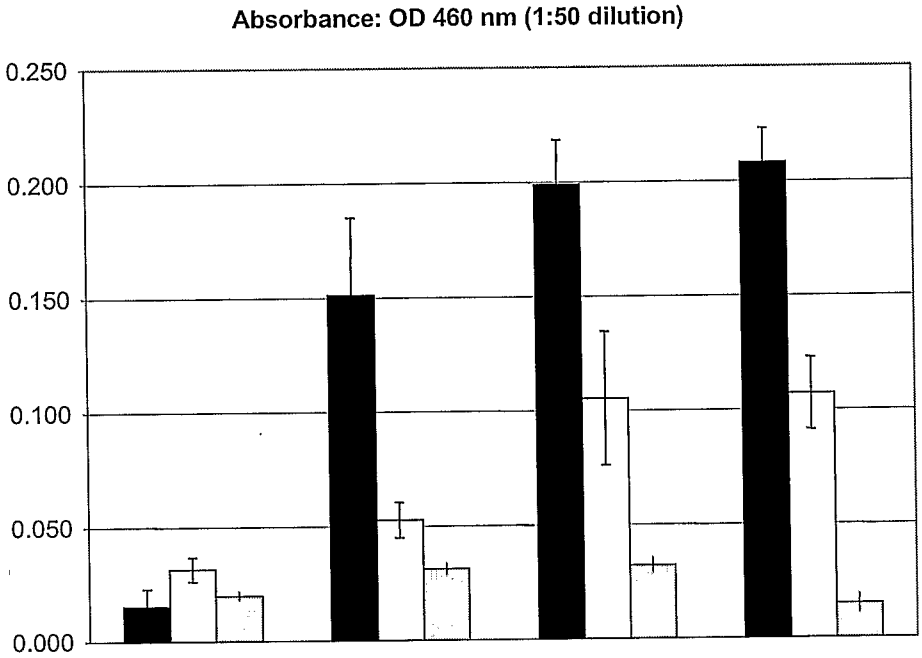
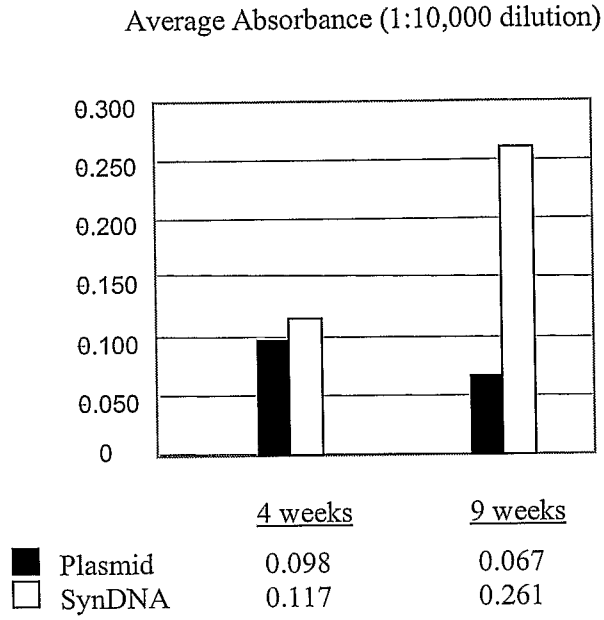


Figure 8 ELISA assays for IgG against gp160 of HIV-1 (mice)



	2 weeks	4 weeks	6 weeks	8 weeks
■ Plasmid	0.015	0.151	0.199	0.208
□ SynDNA	0.032	0.053	0.015	0.017
□ Control	0.020	0.031	0.032	0.015

Figure 9 ELISA assays for IgG against HBs(S) (rabbits)



	4 weeks	9 weeks
Plasmid	0.098	0.067
SynDNA	0.117	0.261

Figure 10 Influenza Virus Neutralization Assay (2 weeks following last boost)

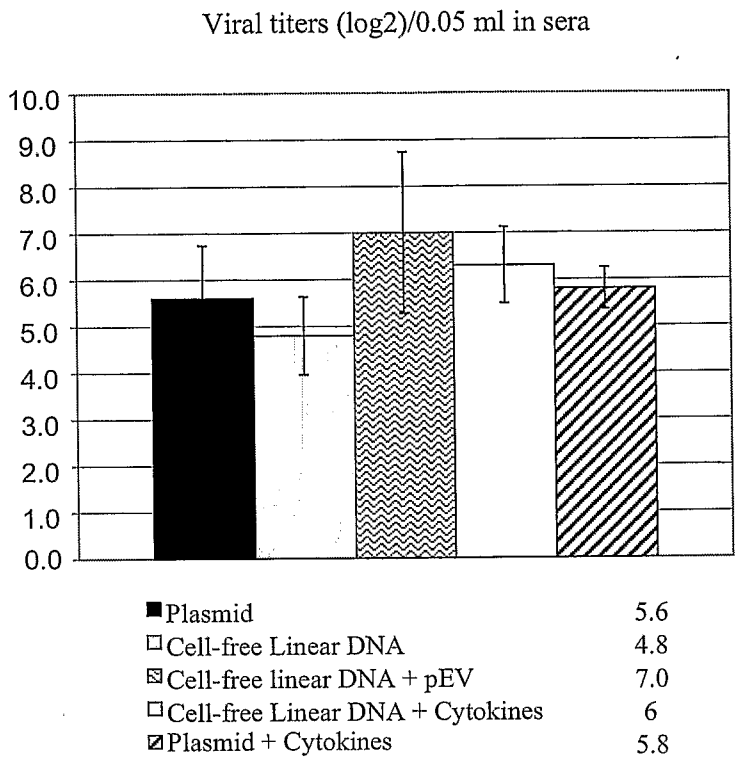
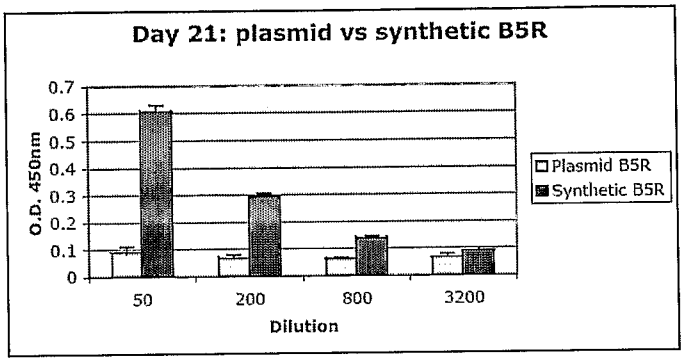
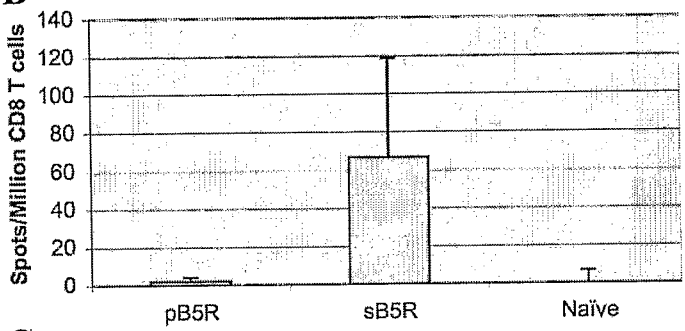


FIGURE 11 Smallpox Immunization Response

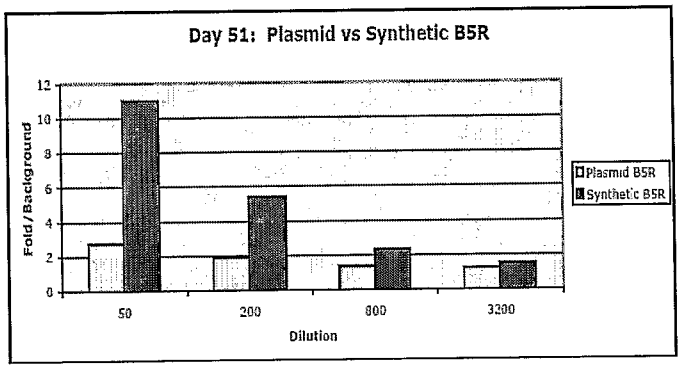
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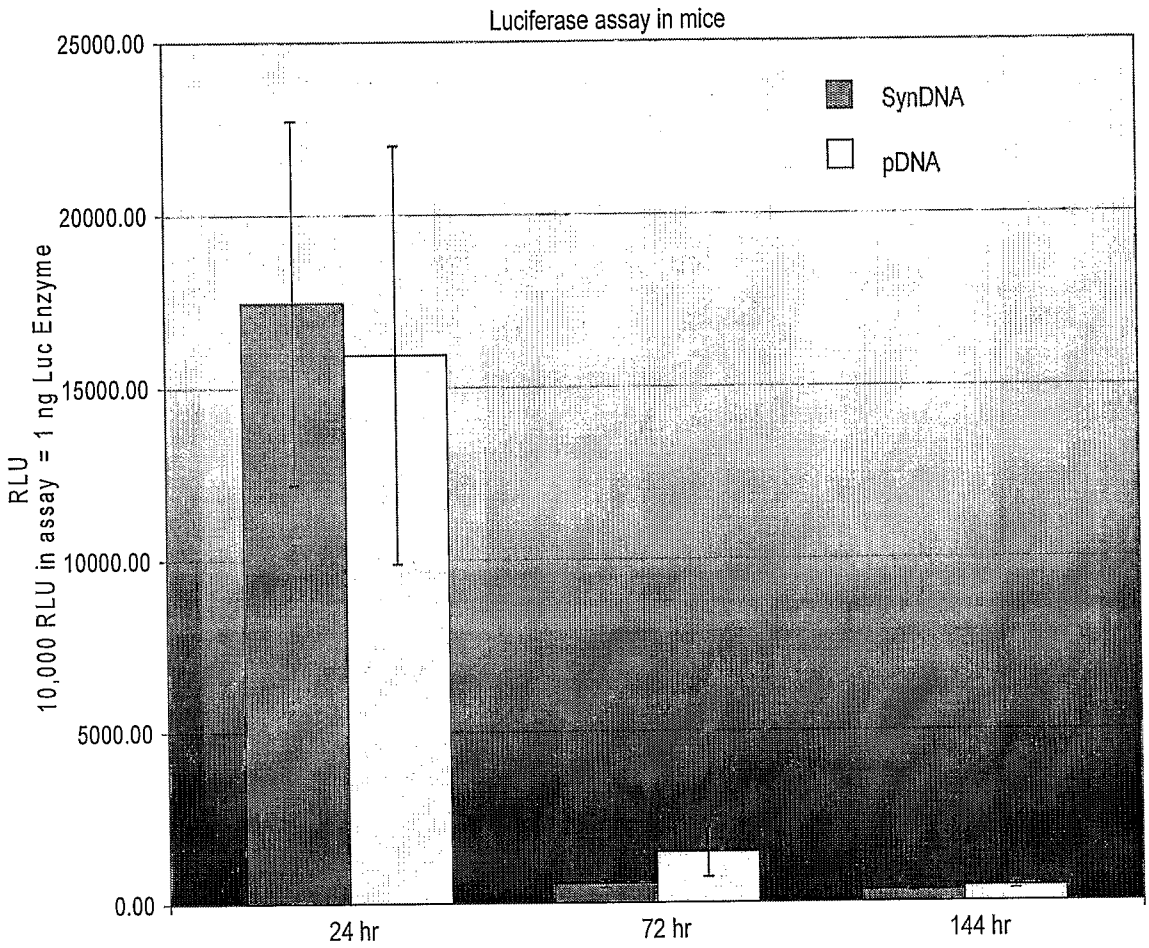


C



Smallpox genetic immunization response: Plasmid vs B5R-DU.
(**A**) Antibody titers (ELISA) in various serum dilutions at week 3.
(**B**) Number of INFγ producing T cells/ 10^6 CD8 + cells. (ELISPOT) 1 spot = 1 INFγ producing T cells.
(**C**) T-cell proliferation assay. Rate of antigen specific T cell proliferation *in vitro* upon addition of recombinant B5R antigen

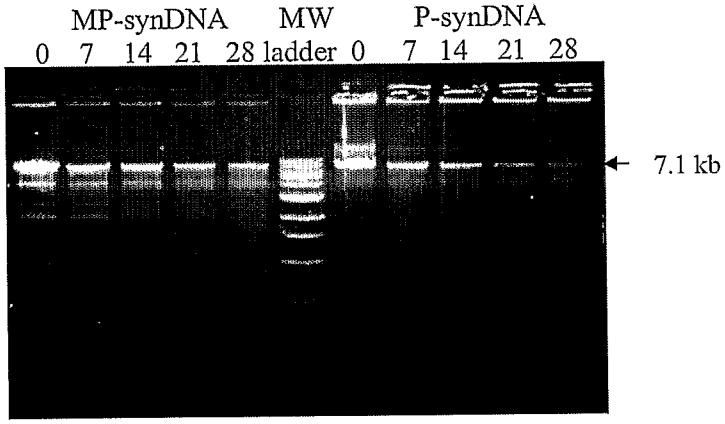
FIGURE 12 - Expression of Luciferase in mouse muscle



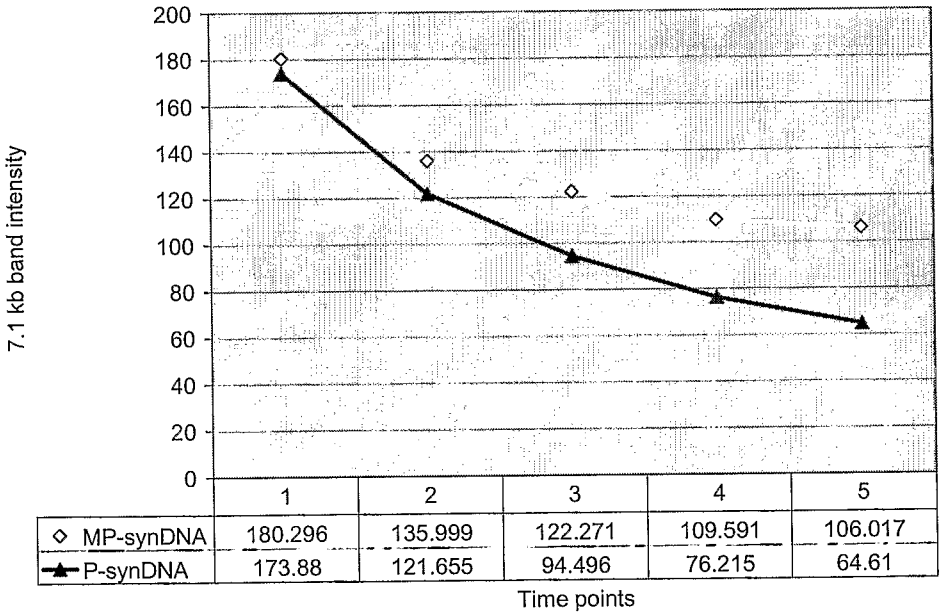
Expression of Luciferase in mouse muscle – samples taken at 24, 72 and 144 hr post injection. Five mice per group.

FIGURE 13 - Methyl Phosphonate v. Phosphorothioate Primer Stability

A. Ethidium Bromide Stained Agarose Gel



B. Densitometry



	1	2	3	4	5
◇ MP-synDNA	180.296	135.999	122.271	109.591	106.017
P-synDNA	173.88	121.655	94.496	76.215	64.61

CytoGenix, Inc.
PCT application – June 14, 2006

SEQUENCE LISTING

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 701 TTTGGCACCA AAATCAACGG GACTTTCCAA AATGTCGTAA CAACTCCGCC
 751 CCATTGACGC AAATGGGCGG TAGGCGTGTA CGGTGGGAGG TCTATATAAG
 801 CAGAGCTCGT TTAGTGAACC GTCAGATCGC CTGGAGACGC CATCCACGCT
 851 GTTTTGACCT CCATAGAAGA CACCGGGACC GATCCAGCCT CCGCGGCCGG
 901 GAACGGTGCA TTGGAACGCG GATTCCCCGT GCCAAGAGTG ACGTAAGTAC
 951 CGCCTATAGA CTCTATAGGC ACACCCCTTT GGCTCTTATG CATGCTATAC
1001 TGTTTTTGGC TTGGGGCCTA TACACCCCCG CTTCCTTATG CTATAGGTGA
1051 TGGTATAGCT TAGCCTATAG GTGTGGGTTA TTGACCATTA TTGACCACTC
1101 CCCTATTGGT GACGATACTT TCCATTACTA ATCCATAACA TGGCTCTTTG
1151 CCACAACTAT CTCTATTGGC TATATGCCAA TACTCTGTCC TTCAGAGACT
1201 GACACGGACT CTGTATTTTT ACAGGATGGG GTCCCATTTA TTATTTACAA
1251 ATTCACATAT ACAACAACGC CGTCCCCCGT GCCCGCAGTT TTTATTAAAC
1301 ATAGCGTGGG ATCTCCACGC GAATCTCGGG TACGTGTTCC GGACATGGGC
1351 TCTTCTCCGG TAGCGGCGGA GCTTCCACAT CCGAGCCCTG GTCCCATGCC
1401 TCCAGCGGCT CATGGTCGCT CGGCAGCTCC TTGCTCCTAA CAGTGGAGGC
1451 CAGACTTAGG CACAGCACAA TGCCCACCAC CACCAGTGTG CCGCACAAGG
1501 CCGTGGCGGT AGGGTATGTG TCTGAAAATG AGCGTGGAGA TTGGGCTCGC
1551 ACGGCTGACG CAGATGGAAG ACTTAAGGCA GCGGCAGAAG AAGATGCAGG
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1601 CAGCTGAGTT GTTGTATTCT GATAAGAGTC AGAGGTAACT CCCGTTGCGG
1651 TGCTGTTAAC GGTGGAGGGC AGTGTAGTCT GAGCAGTACT CGTTGCTGCC
1701 GCGCGCGCCA CCAGACATAA TAGCTGACAG ACTAACAGAC TGTTCCTTTC
1751 CATGGGTCTT TTCTGCAGTC ACCGTCGTCG ACACGTGTGA TCAGATATCG
1801 CGGCCGCTCT AGAGATATCG CCACCATGCG CGTGCGCGGC ATCCAGACCA
1851 GCTGGCAGAA CCTGTGGCGC TGGGGCACCA TGATCCTGGG CATGCTGGTG
1901 ATCTACAGCG CCGCCGAGAA CCTGTGGGTG GCCGTGTACT ACGGCGTGCC
1951 CGTGTGGAAG GACGCCGAGA CCACCCTGTT CTGCGCCAGC GACGCCAAGG
2001 CCTACGACAC CGAGGTGCAC AACGTGTGGG AGACCCACGC CTGCGTGCCC
2051 ACCGACCCCA ACCCCCAGGA GATCCACCTG GAGAACGTGA CCGAGGACTT
2101 CAACATGTGG CGCAACAACA TGGTGGAGCA GATGCACACC GACATCATCA
2151 GCCTGTGGGA CCAGAGCCTG AAGCCCTGCG TGAAGCTGAC CCCCCTGTGC
2201 GTGACCCTGG ACTGCAACGC CACCGCCAGC AACGTGACCA ACGAGATGCG
2251 CAACTGTTGC TTCAACATCA CCACCGAGCT GAAGGACAAG AAGCAGCAGG
2301 TGTACAGCCT GTTCTACAAG CTGGACGTGG TGCAGATCAA CGAGAAGAAC
2351 GAGACCGACA AGTACCGCCT GATCAACTGC AACACCAGCG CCATCACCCA
2401 GGCCTGCCCC AAGGTGAGCT TCGAGCCCAT CCCCATCCAC TACTGCGCCC
2451 CCGCCGGCTT CGCCATCCTG AAGTGCAAGG ACACCGAGTT CAACGGTACC
2501 GGCCCCTGCA AGAACGTGAG CACCGTGCAG TGCACCCACG GCATCCGACC
2551 GGTGATCAGC ACCCAGCTGC TGCTGAACGG CAGCCTGGCC GAGGAGGGCA
2601 TCCAGATCCG CAGCGAGAAC ATCACCAACA ACGCCAAGAC CATCATCGTG
2651 CAGCTGGATA AGGCCGTGAA GATCAACTGC ACCCGCCCCA ACAACAACAC
2701 CCGCAAGGGC GTGCGCATCG GCCCCGGCCA GGCCTTCTAC GCCACCGGCG
2751 GCATCATCGG CGACATCCGC CAGGCCCACT GCCACGTGAG CCGCGCCAAG
2801 TGGAACGACA CCCTGCGCGG CGTGGCCAAG AAGCTGCGCG AGCACTTCAA
2851 GAACAAGACC ATCATCTTCG AGAAGAGCAG CGGCGGCGAC ATCGAGATCA
2901 CCACCCACAG CTTCATCTGC GGCGGCGAGT TCTTCTACTG CAACACCAGC
2951 GGCCTGTTCA ACAGCACCTG GGAGAGCAAC AGCACCGAGA GCAACAACAC
3001 CACCAGCAAC GACACCATCA CCCTGACCTG CCGCATCAAG CAGATCATCA
3051 ACATGTGGCA GAAGGTGGGC CAGGCCATGT ACCCCCCCCC CATCCAGGGC
3101 GTGATCCGCT GCGAGAGCAA CATCACCGGC CTGCTGCTGA CCCGCGACGG
3151 CGGCAACAAC AGCACCAACG AGATCTTCCG CCCCGGCGGC GGCAACATGC
3201 GCGACAACTG GCGCAGCGAG CTGTACAAGT ACAAGGTGGT GAAGATCGAG
3251 CCCCTGGGCG TGGCCCCCAG CCGCGCCAAG CTTACCGCCC AGGCCCGCCA
3301 GCTGCTGAGC GGCATCGTGC AGCAGCAGAG CAACCTGCTG CGCGCCATCG
3351 AGGCCCAGCA GCACATGCTG AAGCTGACCG TGTGGGGCAT CAAGCAGCTG
3401 CAGGCCCGCG TGCTGGCCGT GGAGCGCTAC CTGAAGGACC AGCAGCTCGA
3451 GATCTGGGAC AACATGACCT GGCTGCAGTG GGACAAGGAG ATCAGCAACT
3501 ACACCCAGAT CATCTACAAC CTGATCGAGG AGAGCCAGAA CCAGCAGGAG
3551 AAGAACGAGC AGGACCTGCT GGCCCTGGAC AAGTGGGCCA GCCTGTGGAA
3601 CTGGTTCGAC ATCAGCCGCT GGCTGTGGTA CATCAAGATC TTCATCATGA
3651 TCGTGGGCGG CCTGATCGGC CTGCGCATCG TGTTCGCCGT GCTGAGCGTG
3701 ATCTGAACAC GTGGGATCCA GATCTGCTGT GCCTTCTAGT TGCCAGCCAT
3751 CTGTTGTTTG CCCCTCCCCC GTGCCTTCCT TGACCCTGGA AGGTGCCACT
3801 CCCACTGTCC TTTCCTAATA AAATGAGGAA ATTGCATCGC ATTGTCTGAG
3851 TAGGTGTCAT TCTATTCTGG GGGGTGGGGT GGGGCAGGAC AGCAAGGGGG
3901 AGGATTGGGA AGACAATAGC AGGCATGCTG GGGATGCGGT GGGCTCTATG
3951 GGTACCCAGG TGCTGAAGAA TTGACCCGGT TCCTCCTGGG CCAGAAAGAA
4001 GCAGGCACAT CCCCTTCTCT GTGACACACC CTGTCCACGC CCCTGGTTCT
4051 TAGTTCCAGC CCCACTCATA GGACACTCAT AGCTCAGGAG GGCTCCGCCT
4101 TCAATCCCAC CCGCTAAAGT ACTTGGAGCG GTCTCTCCCT CCCTCATCAG
4151 CCCACCAAAC CAAACCTAGC CTCCAAGAGT GGGAAGAAAT TAAAGCAAGA
4201 TAGGCTATTA AGTGCAGAGG GAGAGAAAAT GCCTCCAACA TGTGAGGAAG
4251 TAATGAGAGA AATCATAGAA TTTTAAGGCC ATGATTTAAG GCCATCATGG
4301 CCTTAATCTT CCGCTTCCTC GCTCACTGAC TCGCTGCGCT CGGTCGTTCG

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4351 GCTGCGGCGA GCGGTATCAG CTCACTCAAA GGCGGTAATA CGGTTATCCA
4401 CAGAATCAGG GGATAACGCA GGAAAGAACA TGTGAGCAAA AGGCCAGCAA
4451 AAGGCCAGGA ACCGTAAAAA GGCCGCGTTG CTGGCGTTTT TCCATAGGCT
4501 CCGCCCCCCT GACGAGCATC ACAAAAATCG AGCTAGC
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[SEQ ID NO: 5]

HIV-1 gp145ΔCF1 - ORF:

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   1 ATGCGCGTGC GCGGCATCCA GACCAGCTGG CAGAACCTGT GGCGCTGGGG
  51 CACCATGATC CTGGGCATGC TGGTGATCTA CAGCGCCGCC GAGAACCTGT
 101 GGGTGGCCGT GTACTACGGC GTGCCCGTGT GGAAGGACGC CGAGACCACC
 151 CTGTTCTGCG CCAGCGACGC CAAGGCCTAC GACACCGAGG TGCACAACGT
 201 GTGGGAGACC CACGCCTGCG TGCCCACCGA CCCCAACCCC CAGGAGATCC
 251 ACCTGGAGAA CGTGACCGAG GACTTCAACA TGTGGCGCAA CAACATGGTG
 301 GAGCAGATGC ACACCGACAT CATCAGCCTG TGGGACCAGA GCCTGAAGCC
 351 CTGCGTGAAG CTGACCCCCC TGTGCGTGAC CCTGGACTGC AACGCCACCG
 401 CCAGCAACGT GACCAACGAG ATGCGCAACT GTTGCTTCAA CATCACCACC
 451 GAGCTGAAGG ACAAGAAGCA GCAGGTGTAC AGCCTGTTCT ACAAGCTGGA
 501 CGTGGTGCAG ATCAACGAGA AGAACGAGAC CGACAAGTAC CGCCTGATCA
 551 ACTGCAACAC CAGCGCCATC ACCCAGGCCT GCCCCAAGGT GAGCTTCGAG
 601 CCCATCCCCA TCCACTACTG CGCCCCCGCC GGCTTCGCCA TCCTGAAGTG
 651 CAAGGACACC GAGTTCAACG GTACCGGCCC CTGCAAGAAC GTGAGCACCG
 701 TGCAGTGCAC CCACGGCATC CGACCGGTGA TCAGCACCCA GCTGCTGCTG
 751 AACGGCAGCC TGGCCGAGGA GGGCATCCAG ATCCGCAGCG AGAACATCAC
 801 CAACAACGCC AAGACCATCA TCGTGCAGCT GGATAAGGCC GTGAAGATCA
 851 ACTGCACCCG CCCCAACAAC AACACCCGCA AGGGCGTGCG CATCGGCCCC
 901 GGCCAGGCCT TCTACGCCAC CGGCGGCATC ATCGGCGACA TCCGCCAGGC
 951 CCACTGCCAC GTGAGCCGCG CCAAGTGGAA CGACACCCTG CGCGGCGTGG
1001 CCAAGAAGCT GCGCGAGCAC TTCAAGAACA AGACCATCAT CTTCGAGAAG
1051 AGCAGCGGCG GCGACATCGA GATCACCACC CACAGCTTCA TCTGCGGCGG
1101 CGAGTTCTTC TACTGCAACA CCAGCGGCCT GTTCAACAGC ACCTGGGAGA
1151 GCAACAGCAC CGAGAGCAAC AACACCACCA GCAACGACAC CATCACCCTG
1201 ACCTGCCGCA TCAAGCAGAT CATCAACATG TGGCAGAAGG TGGGCCAGGC
1251 CATGTACCCC CCCCCCATCC AGGGCGTGAT CCGCTGCGAG AGCAACATCA
1301 CCGGCCTGCT GCTGACCCGC GACGGCGGCA ACAACAGCAC CAACGAGATC
1351 TTCCGCCCCG GCGGCGGCAA CATGCGCGAC AACTGGCGCA GCGAGCTGTA
1401 CAAGTACAAG GTGGTGAAGA TCGAGCCCCT GGGCGTGGCC CCCAGCCGCG
1451 CCAAGCTTAC CGCCCAGGCC CGCCAGCTGC TGAGCGGCAT CGTGCAGCAG
1501 CAGAGCAACC TGCTGCGCGC CATCGAGGCC CAGCAGCACA TGCTGAAGCT
1551 GACCGTGTGG GGCATCAAGC AGCTGCAGGC CCGCGTGCTG GCCGTGGAGC
1601 GCTACCTGAA GGACCAGCAG CTCGAGATCT GGGACAACAT GACCTGGCTG
1651 CAGTGGGACA AGGAGATCAG CAACTACACC CAGATCATCT ACAACCTGAT
1701 CGAGGAGAGC CAGAACCAGC AGGAGAAGAA CGAGCAGGAC CTGCTGGCCC
1751 TGGACAAGTG GGCCAGCCTG TGGAACTGGT TCGACATCAG CCGCTGGCTG
1801 TGGTACATCA AGATCTTCAT CATGATCGTG GGCGGCCTGA TCGGCCTGCG
1851 CATCGTGTTC GCCGTGCTGA GCGTGATCTG A
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[SEQ ID NO: 6]

HBVs(S)-DU - expression cassette:

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   1 GCTAGCGATG TACGGGCCAG ATATACGCGT TGACATTGAT TATTGACTAG
  51 TTATTAATAG TAATCAATTA CGGGGTCATT AGTTCATAGC CCATATATGG
 101 AGTTCCGCGT TACATAACTT ACGGTAAATG GCCCGCCTGG CTGACCGCCC
 151 AACGACCCCC GCCCATTGAC GTCAATAATG ACGTATGTTC CCATAGTAAC
 201 GCCAATAGGG ACTTTCCATT GACGTCAATG GGTGGACTAT TTACGGTAAA
 251 CTGCCCACTT GGCAGTACAT CAAGTGTATC ATATGCCAAG TACGCCCCCT
 301 ATTGACGTCA ATGACGGTAA ATGGCCCGCC TGGCATTATG CCCAGTACAT
 351 GACCTTATGG GACTTTCCTA CTTGGCAGTA CATCTACGTA TTAGTCATCG
 401 CTATTACCAT GGTGATGCGG TTTTGGCAGT ACATCAATGG GCGTGGATAG
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 451 CGGTTTGACT CACGGGGATT TCCAAGTCTC CACCCCATTG ACGTCAATGG
 501 GAGTTTGTTT TGGCACCAAA ATCAACGGGA CTTTCCAAAA TGTCGTAACA
 551 ACTCCGCCCC ATTGACGCAA ATGGGCGGTA GGCGTGTACG GTGGGAGGTC
 601 TATATAAGCA GAGCTCTCTG GCTAACTAGA GAACCCACTG CTTAACTGGC
 651 TTATCGAAAT TAATACGACT CACTATAGGG AGACCCAAGC TTGGTACCGG
 701 GCCCCCCCTC GAGGATTGGG GACCCTGCGC TGAACATGGA GAACATCACA
 751 TCAGGATTCC TAGGACCCCT TCTCGTGTTA CAGGCGGGGT TTTTCTTGTT
 801 GACAAGAATC CTCACAATAC CGCAGAGTCT AGACTCGTGG TGGACTTCTC
 851 TCAATTTTCT AGGGGGAACT ACCGTGTGTC TTGGCCAAAA TTCGCAGTCC
 901 CCAACCTCCA ATCACTCACC AACCTCTTGT CCTCCAACTT GTCCTGGTTA
 951 TCGCTGGATG TGTCTGCGGC GTTTTATCAT CTTCCTCTTC ATCCTGCTGC
1001 TATGCCTCAT CTTCTTGTTG GTTCTTCTGG ACTATCAAGG TATGTTGCCC
1051 GTTTGTCCTC TAATTCCAGG ATCCTCAACA ACCAGCACGG GACCATGCCG
1101 GACCTGCATG ACTACTGCTC AAGGAACCTC TATGTATCCC TCCTGTTGCT
1151 GTACCAAACC TTCGGACGGA AATTGCACCT GTATTCCCAT CCCATCATCC
1201 TGGGCTTTCG GAAAATTCCT ATGGGAGTGG GCCTCAGCCC GTTTCTCCTG
1251 GCTCAGTTTA CTAGTGCCAT TTGTTCAGTG GTTCGTAGGG CTTTCCCCCA
1301 CTGTTTGGCT TTCAGTTATA TGGATGATGT GGTATTGGGG GCCAAGTCTG
1351 TACAGCATCT TGAGTCCCTT TTTACCGCTG TTACCAATTT TCTTTTGTCT
1401 TTGGGTATAC ATTTAAACCC TAACAAAACA AAGAGATGGG GTTACTCTCT
1451 AAATTTTATG GGTTATGTCA TTGGATGTTA TGGGTCCTTG CCACAAGAAC
1501 ACATCATACA AAAAATCAAA GAATGTTTTA GAAAACTTCC TATTAACAGG
1551 CCTATTGATT GGAAAGTATG TCAACGAATT GTGGGTCTTT TGGGTTTTGC
1601 TGCCCCTTTT ACACAATGTG GTTATCCTGC GTTGATGCCT TTGTATGCAT
1651 GTATTCAATC TAAGCAGGCT TTCACTTTCT CGCCAACTTA CAAGGCCTTT
1701 CTGTGTAAAC AATACCTGAA CCTTTACCCC GTTGCCCGGC AACGGCCAGG
1751 TCTGTGCCAA GTGTTTGCTG ACGCAACCCC CACTGGCTGG GGCTTGGTCA
1801 TGGGCCATCA GCGCATGCGT GGAACCTTTT CGGCTCCTCT GCCGATCCAT
1851 ACTGCGGAAC TCCTAGCCGC TTGTTTTGCT CGCAGCAGGT CTGGAGCAAA
1901 CATTATCGGG ACTGATAACT CTGTTGTCCT ATCCCGCAAA TATACATCGT
1951 TTCCATGGCT GCTAGGCTGT GCTGCCAACT GGATCCTGCG CGGGACGTCC
2001 TTTGTTTACG TCCCGTCGGC GCTGAATCCT GCGGACGACC CTTCTCGGGG
2051 TCGCTTGGGA CTCTCTCGTC CCCTTCTCCG TCTGCCGTTC CGACCGACCA
2101 CGGGGCGCAC CTCTCTTTAC GCGGACTCCC CGTCTGTGCC TTCTCATCTG
2151 CCGGACCGTG TGCACTTCGC TTCACCTCTG CACGTCGCAT GGAGACCACC
2201 GTGAACGCCC ACCAAATATT GCCCAAGGTC TTACATAAGA GGACTCTTGG
2251 ACTCTCAGCA ATGTCAACGA CCGACCTTGA GGCATACTTC AAAGACTGTT
2301 TGTTTAAAGA CTGGGAGGAG TTGGGGGAGG AGATTAGGTT AAAGGTCTTT
2351 GTACTAGGAG GCTGTAGGCA TAAATTGGTC TGCGCACCAG CACCATGCAA
2401 CTTTTTCACC TCTGCCTAAT CATCTCTTGT TCATGTCCTA CTGTTCAAGC
2451 CTCCAAGCTG TGCCTTGGGT GGCTTTGGGG CATGGACATC GACCCTTATA
2501 AAGAATTTGG AGCTACTGTG GAGTTACTCT CGTTTTTGCC TTCTGACTTC
2551 TTTCCTTCAG TACGAGATCT GGCCAGGATC CACTAGTTCT AGAGCGGCCG
2601 CCACCGCGGT GGAGCTCCAG CTTTTGTTCC CTTTAGTGAG GGTTAATTGC
2651 GCG
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[SEQ ID NO: 7]

HBVs(S) - ORF:

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   1 ATGGAGAACA TCACATCAGG ATTCCTAGGA CCCCTTCTCG TGTTACAGGC
  51 GGGGTTTTTC TTGTTGACAA GAATCCTCAC AATACCGCAG AGTCTAGACT
 101 CGTGGTGGAC TTCTCTCAAT TTTCTAGGGG GAACTACCGT GTGTCTTGGC
 151 CAAAATTCGC AGTCCCCAAC CTCCAATCAC TCACCAACCT CTTGTCCTCC
 201 AACTTGTCCT GGTTATCGCT GGATGTGTCT GCGGCGTTTT ATCATCTTCC
 251 TCTTCATCCT GCTGCTATGC CTCATCTTCT TGTTGGTTCT TCTGGACTAT
 301 CAAGGTATGT TGCCCGTTTG TCCTCTAATT CCAGGATCCT CAACAACCAG
 351 CACGGGACCA TGCCGGACCT GCATGACTAC TGCTCAAGGA ACCTCTATGT
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401 ATCCCTCCTG TTGCTGTACC AAACCTTCGG ACGGAAATTG CACCTGTATT
451 CCCATCCCAT CATCCTGGGC TTTCGGAAAA TTCCTATGGG AGTGGGCCTC
501 AGCCCGTTTC TCCTGGCTCA GTTTACTAGT GCCATTTGTT CAGTGGTTCG
551 TAGGGCTTTC CCCCACTGTT TGGCTTTCAG TTATATGGAT GATGTGGTAT
601 TGGGGGCCAA GTCTGTACAG CATCTTGAGT CCCTTTTTAC CGCTGTTACC
651 AATTTTCTTT TGTCTTTGGG TATACATTTA A
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[SEQ ID NO: 8]

HA-DU - H1N1 expression cassette:

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   1 GCGGCCGCAC GCGTGGAGCT AGTTATTAAT AGTAATCAAT TACGGGGTCA
  51 TTAGTTCATA GCCCATATAT GGAGTTCCGC GTTACATAAC TTACGGTAAA
 101 TGGCCCGCCT GGCTGACCGC CCAACGACCC CCGCCCATTG ACGTCAATAA
 151 TGACGTATGT TCCCATAGTA ACGTCAATAG GGACTTTCCA TTGACGTCAA
 201 TGGGTGGAGT ATTTACGGTA AACTGCCCAC TTGGCAGTAC ATCAAGTGTA
 251 TCATATGCCA AGTACGCCCC CTATTGACGT CAATGACGGT AAATGGCCCG
 301 CCTGGCATTA TGCCCAGTAC ATGACCTTAT GGGACTTTCC TACTTGGCAG
 351 TACATCTACG TATTAGTCAT CGCTATTACC ATGGTGATGC GGTTTTGGCA
 401 GTACATCAAT GGGCGTGGAT AGCGGTTTGA CTCACGGGGA TTTCCAAGTC
 451 TCCACCCCAT TGACGTCAAT GGGAGTTTGT TTTGCACCAA AATCAACGGG
 501 ACTTTCCAAA ATGTCGTAAC AACTCCGCCC CATTGACGCA AATGGGCGGT
 551 AGGCGTGTAC GGTGGGAGGT CTATATAAGC AGAGCTCGTT TAGTGAACCG
 601 TCAGATCGCC TGGAGACGCC ATCCACGCTG TTTTGACCTC CATAGAAGAC
 651 ACCGGGACCG ATCCAGCCTC CGCGGATTCG AATCCCGGCC GGGAACGGTG
 701 CATTGGAACG CGGATTCCCC GTGCCAAGAG TGACGTAAGT ACCGCCTATA
 751 GAGTCTATAG GCCCACAAAA AATGCTTTCT TCTTTTAATA TACTTTTTTG
 801 TTTATCTTAT TTCTAATACT TTCCCTAATC TCTTTCTTTC AGGGCAATAA
 851 TGATACAATG TATCATGCCT CTTTGCACCA TTCTAAAGAA TAACAGTGAT
 901 AATTTCTGGG TTAAGGCAAT AGCAATATTT CTGCATATAA ATATTTCTGC
 951 ATATAAATTG TAACTGATGT AAGAGGTTTC ATATTGCTAA TAGCAGCTAC
1001 AATCCAGCTA CCATTCTGCT TTTATTTTAT GGTTGGGATA AGGCTGGATT
1051 ATTCTGAGTC CAAGCTAGGC CCTTTTGCTA ATCATGTTCA TACCTCTTAT
1101 CTTCCTCCCA CAGCTCCTGG GCAACGTGCT GGTCTGTGTG CTGGCCCATC
1151 ACTTTGGCAA AGAATTGGGA TTCGAACATC GATTGAATTC CCCGGGGATC
1201 CCCCGGGCTG CAGGAATTCG ATAGCTTAAT GAAGGCAAAC CTACTGGTCC
1251 TGTTATGTGC ACTTGCAGCT GCAGATGCAG ACACAATATG TATAGGCTAC
1301 CATGCGAACA ATTCAACCGA CACTGTTGAC ACAGCACTCG AGAAGAATGT
1351 GACAGTGACA CACTCTGTTA ACCTGCTCGA AGACAGCCAC AACGGAAAAC
1401 TATGTAGATT AAAAGGAATA GCCCCACTAC AATTGGGGAA ATGTAACATC
1451 GCCGGATGGC TCTTGGGAAA CCCAGAATGC GACCCACTGC TTCCAGTGAG
1501 ATCATGGTCC TACATTGTAG AAACACCAAA CTCTGAGAAT GGAATATGTT
1551 ATCCAGGAGA TTTCATCGAC TATGAGGAGC TGAGGGAGCA ATTGAGCTCA
1601 GTGTCATCAT TCGAAAGATT CGCAATATTT CCCAAAGAAA GCTCATGGCC
1651 CAACCACAAC ACAAACAAAG GAGTAACGGC AGCATGCTCC CATGCGGGGA
1701 AAAGCAGTTT TTACAGAAAT TTGCTATGGC TGACGGAGAA GGAGGGCTCA
1751 TACCCAAAGC TGAAAAATTC TTATGTGAAC AAGAAAGGGA AAGAAGTCCT
1801 TGTACTGTGG GGTATTCATC ACCCGTCTAA CAGTAAGGAT CAACAGAATC
1851 TCTATCAGAA TGAAAATGCT TATGTCTCTG TAGTGACTTC AAATTATAAC
1901 AGGAGATTTA CCCCGGAAAT AGCAGAAAGA CCCAAAGTAA GAGATCAAGC
1951 TGGGAGGATG AACTATTACT GGACCTTGCT AAAACCCGGA GACACAATAA
2001 TATTTGAGGC AAATGGAAAT CTAATAGCAC CAATGTATGC TTTCGCACTG
2051 AGTAGAGGCT TTGGGTCCGG CATCATCACC TCAAACGCAT CAATGCATGA
2101 GTGTAACACG AAGTGTCAAA CACCCCTGGG AGCTATAAAC AGCAGTCTCC
2151 CTTTCCAGAA TATACACCCA GTCACAATAG GAGAGTGCCC AAAATACGTC
2201 AGGAGTGCCA AATTGAGGAT GGTTACAGGA CTAAGGAACA TTCCGTCCAT
2251 TCAATCCAGA GGTCTATTTG GAGCCATTGC CGGTTTTATT GAAGGGGGAT
2301 GGACTGGAAT GATAGATGGA TGGTATGGTT ATCATCATCA GAATGAACAG
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2351 GGATCAGGCT ATGCAGCGGA TCAAAAAAGC ACACAAAATG CCATTAACGG
2401 GATTACAAAC AAGGTGAACT CTGTTATCGA GAAAATGAAC ATTCAATTCA
2451 CAGCTGTGGG TAAAGAATTC AACAAATTAG AAAAAAGGAT GGAAAATTTA
2501 AATAAAAAAG TTGATGATGG ATTTCTGGAC ATTTGGACAT ATAATGCTGA
2551 ATTGTTAGTT CTACTGGAAA ATGAAAGGAC TCTGGATTTC CATGACTCAA
2601 ATGTGAAGAA TCTGTATGAG AAAGTAAAAA GCCAATTAAA GAATAATGCC
2651 AAAGAAATCG GAAATGGATG TTTTGAGTTC TACCACAAGT GTGACAATGA
2701 ATGCATGGAA AGTGTAAGAA ATGGGACTTA TGCTTATCCC AAATATTCAG
2751 AAGAGTCAAA GTTGAACAGG GAAAAGGTAG ATGGAGTGAA ATTGGAATCA
2801 ATGGGGATCT ATCAGATTCT GGCGATCTAC TCAACTGTCG CCAGTTCACT
2851 GGTGCTTTTG GTCTCCCTGG GGGCAATCAG TTTCTGGATG TGTTCTAATG
2901 GATCTTTGCA GTGCAGAATA TGCATCTGAG CCTAGATCAA GCTTGCCTCG
2951 AGCAGCGCTG CTCGAGAGAT CTACGGGTGG CATCCCTGTG ACCCCTCCCC
3001 AGTGCCTCTC CTGGCCCTGG AAGTTGCCAC TCCAGTGCCC ACCAGCCTTG
3051 TCCTAATAAA ATTAAGTTGC ATCATTTTGT CTGACTAGGT GTCCTTCTAT
3101 AATATTATGG GGTGGAGGGG GGTGGTATGG AGCAAGGGGC AAGTTGGGAA
3151 GACAACCTGT AGGGCCTGCG GGGTCTATTG GGAACCAAGC TGGAGTGCAG
3201 TGGCACAATC TTGGCTCACT GCAATCTCCG CCTCCTGGGT TCAAGCGATT
3251 CTCCTGCCTC AGCCTCCCGA GTTGTTGGGA TTCCAGGCAT GCATGACCAG
3301 GCTCAGCTAA TTTTTGTTTT TTTGGTAGAG ACGGGGTTTC ACCATATTGG
3351 CCAGGCTGGT CTCCAACTCC TAATCTCAGG TGATCTACCC ACCTTGGCCT
3401 CCCAAATTGC TGGGATTACA GGCGTGAACC ACTGCTCCCT TCCCTGTCCT
3451 TCTGATTTTG TAGGTAACCA CGTGCGGACC GA

[SEQ ID NO: 9]

HA (H1N1) - ORF:

1 ATGAAGGCAA ACCTACTGGT CCTGTTATGT GCACTTGCAG CTGCAGATGC
51 AGACACAATA TGTATAGGCT ACCATGCGAA CAATTCAACC GACACTGTTG
101 ACACAGCACT CGAGAAGAAT GTGACAGTGA CACACTCTGT TAACCTGCTC
151 GAAGACAGCC ACAACGGAAA ACTATGTAGA TTAAAAGGAA TAGCCCCACT
201 ACAATTGGGG AAATGTAACA TCGCCGGATG GCTCTTGGGA AACCCAGAAT
251 GCGACCCACT GCTTCCAGTG AGATCATGGT CCTACATTGT AGAAACACCA
301 AACTCTGAGA ATGGAATATG TTATCCAGGA GATTTCATCG ACTATGAGGA
351 GCTGAGGGAG CAATTGAGCT CAGTGTCATC ATTCGAAAGA TTCGCAATAT
401 TTCCCAAAGA AAGCTCATGG CCCAACCACA ACACAAACAA AGGAGTAACG
451 GCAGCATGCT CCCATGCGGG GAAAAGCAGT TTTTACAGAA ATTTGCTATG
501 GCTGACGGAG AAGGAGGGCT CATACCCAAA GCTGAAAAAT TCTTATGTGA
551 ACAAGAAAGG GAAAGAAGTC CTTGTACTGT GGGGTATTCA TCACCCGTCT
601 AACAGTAAGG ATCAACAGAA TCTCTATCAG AATGAAAATG CTTATGTCTC
651 TGTAGTGACT TCAAATTATA ACAGGAGATT TACCCCGGAA ATAGCAGAAA
701 GACCCAAAGT AAGAGATCAA GCTGGGAGGA TGAACTATTA CTGGACCTTG
751 CTAAAACCCG GAGACACAAT AATATTTGAG GCAAATGGAA ATCTAATAGC
801 ACCAATGTAT GCTTTCGCAC TGAGTAGAGG CTTTGGGTCC GGCATCATCA
851 CCTCAAACGC ATCAATGCAT GAGTGTAACA CGAAGTGTCA AACACCCCTG
901 GGAGCTATAA ACAGCAGTCT CCCTTTCCAG AATATACACC CAGTCACAAT
951 AGGAGAGTGC CCAAAATACG TCAGGAGTGC CAAATTGAGG ATGGTTACAG
1001 GACTAAGGAA CATTCCGTCC ATTCAATCCA GAGGTCTATT TGGAGCCATT
1051 GCCGGTTTTA TTGAAGGGGG ATGGACTGGA ATGATAGATG GATGGTATGG
1101 TTATCATCAT CAGAATGAAC AGGGATCAGG CTATGCAGCG GATCAAAAAA
1151 GCACACAAAA TGCCATTAAC GGGATTACAA ACAAGGTGAA CTCTGTTATC
1201 GAGAAAATGA ACATTCAATT CACAGCTGTG GGTAAAGAAT TCAACAAATT
1251 AGAAAAAAGG ATGGAAAATT TAAATAAAAA AGTTGATGAT GGATTTCTGG
1301 ACATTTGGAC ATATAATGCT GAATTGTTAG TTCTACTGGA AAATGAAAGG
1351 ACTCTGGATT TCCATGACTC AAATGTGAAG AATCTGTATG AGAAAGTAAA
1401 AAGCCAATTA AAGAATAATG CCAAAGAAAT CGGAAATGGA TGTTTTGAGT
1451 TCTACCACAA GTGTGACAAT GAATGCATGG AAAGTGTAAG AAATGGGACT

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1501 TATGCTTATC CCAAATATTC AGAAGAGTCA AAGTTGAACA GGGAAAAGGT
1551 AGATGGAGTG AAATTGGAAT CAATGGGGAT CTATCAGATT CTGGCGATCT
1601 ACTCAACTGT CGCCAGTTCA CTGGTGCTTT TGGTCTCCCT GGGGGCAATC
1651 AGTTTCTGGA TGTGTTCTAA TGGATCTTTG CAGTGCAGAA TATGCATCTG
1701 A
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[SEQ ID NO: 10]

B5R-DU - Small Pox expression cassette:

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   1 ACGCGTTGAC ATTGATTATT GACTAGTTAT TAATAGTAAT CAATTACGGG
  51 GTCATTAGTT CATAGCCCAT ATATGGAGTT CCGCGTTACA TAACTTACGG
 101 TAAATGGCCC GCCTGGCTGA CCGCCCAACG ACCCCCGCCC ATTGACGTCA
 151 ATAATGACGT ATGTTCCCAT AGTAACGCCA ATAGGGACTT TCCATTGACG
 201 TCAATGGGTG GACTATTTAC GGTAAACTGC CCACTTGGCA GTACATCAAG
 251 TGTATCATAT GCCAAGTACG CCCCCTATTG ACGTCAATGA CGGTAAATGG
 301 CCCGCCTGGC ATTATGCCCA GTACATGACC TTATGGGACT TTCCTACTTG
 351 GCAGTACATC TACGTATTAG TCATCGCTAT TACCATGGTG ATGCGGTTTT
 401 GGCAGTACAT CAATGGGCGT GGATAGCGGT TTGACTCACG GGGATTTCCA
 451 AGTCTCCACC CCATTGACGT CAATGGGAGT TTGTTTTGGC ACCAAAATCA
 501 ACGGGACTTT CCAAAATGTC GTAACAACTC CGCCCCATTG ACGCAAATGG
 551 GCGGTAGGCG TGTACGGTGG GAGGTCTATA TAAGCAGAGC TCTCTGGCTA
 601 ACTAGAGAAC CCACTGCTTA CTGGCTTATC GAAATTAATA CGACTCACTA
 651 TAGGGAGACC CAAGCTGGCT AGCGTTTAAA CTTAAGCTTG CCGCCACCAT
 701 GGATTGGACT TGGATCTTAT TTTTAGTTGC TGCTGCTACT AGAGTTCATT
 751 CTAAAACGAT TTCCGTTGTT ACGTTGTTAT GCGTACTACC TGCTGTTGTT
 801 TATTCAACAT GTACTGTACC CACTATGAAT AACGCTAAAT TAACGTCTAC
 851 CGAAACATCG TTTAATGATA AACAGAAAGT TACGTTTACA TGTGATCAGG
 901 GATATCATTC TTCGGATCCA AATGCTGTCT GCGAAACAGA TAAATGGAAA
 951 TACGAAAATC CATGCAAAAA AATGTGCACA GTTTCTGATT ACATCTCTGA
1001 ATTATATAAT AAACCGCTAT ACGAAGTGAA TTCCACCATG ACACTAAGTT
1051 GCAACGGCGA AACAAAATAT TTTCGTTGCG AAGAAAAAAA TGGAAATACT
1101 TCTTGGAATG ATACTGTTAC GTGTCCTAAT GCGGAATGTC AACCTCTTCA
1151 ATTAGAACAC GGATCGTGTC AACCAGTTAA AGAAAAATAC TCATTTGGGG
1201 AATATATGAC TATCAACTGT GATGTTGGAT ATGAGGTTAT TGGTGCTTCG
1251 TACATAAGTT GTACAGCTAA TTCTTGGAAT GTTATTCCAT CATGTCAACA
1301 AAAATGTGAT ATGCCGTCTC TATCTAATGG ATTAATTTCC GGATCTACAT
1351 TTTCTATCGG TGGCGTTATA CATCTTAGTT GTAAAAGTGG TTTTACACTA
1401 ACGGGGTCTC CATCATCCAC ATGTATCGAC GGTAAATGGA ATCCCGTACT
1451 CCCAATATGT GTACGAACTA ACGAAGAATT TGATCCAGTG GATGATGGTC
1501 CCGACGATGA GACAGATTTG AGCAAACTCT CGAAAGACGT TGTACAATAT
1551 GAACAAGAAA TAGAATCGTT AGAAGCAACT TATCATATAA TCATAGTGGC
1601 GTTAACAATT ATGGGCGTCA TATTTTTAAT CTCCGTTATA GTATTAGTTT
1651 GTTCCTGTGA CAAAAATAAT GACCAATATA AGTTCCATAA ATTGCTACCG
1701 TACCCATACG ATGTTCCAGA TTACGCTTAA GTCTAGAGGG CCCGTTTAAA
1751 CCCGCTGATC AGCCTCGACT GTGCCTTCTA GTTGCCAGCC ATCTGTTGTT
1801 TGCCCCTCCC CCGTGCCTTC CTTGACCCTG GAAGGTGCCA CTCCCACTGT
1851 CCTTTCCTAA TAAAATGAGG AAATTGCATC GCATTGTCTG AGTAGGTGTC
1901 ATTCTATTCT GGGGGGTGGG GTGGGGCAGG ACAGCAAGGG GGAGGATTGG
1951 GAAGACAATA GCAGGCATGC TGGGGATGCG GTGGGCTCTA TGGCTTCTAC
2001 TGGGCGGTTT TATGGACAGC AAGCGAACCG GAATTGCC
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[SEQ ID NO: 11]

B5R-DU - Small Pox ORF:

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   1 ATGGATTGGA CTTGGATCTT ATTTTTAGTT GCTGCTGCTA CTAGAGTTCA
  51 TTCTAAAACG ATTTCCGTTG TTACGTTGTT ATGCGTACTA CCTGCTGTTG
 101 TTTATTCAAC ATGTACTGTA CCCACTATGA ATAACGCTAA ATTAACGTCT
 151 ACCGAAACAT CGTTTAATGA TAAACAGAAA GTTACGTTTA CATGTGATCA
 201 GGGATATCAT TCTTCGGATC CAAATGCTGT CTGCGAAACA GATAAATGGA
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 251 AATACGAAAA TCCATGCAAA AAAATGTGCA CAGTTTCTGA TTACATCTCT
 301 GAATTATATA ATAAACCGCT ATACGAAGTG AATTCCACCA TGACACTAAG
 351 TTGCAACGGC GAAACAAAAT ATTTTCGTTG CGAAGAAAAA AATGGAAATA
 401 CTTCTTGGAA TGATACTGTT ACGTGTCCTA ATGCGGAATG TCAACCTCTT
 451 CAATTAGAAC ACGGATCGTG TCAACCAGTT AAAGAAAAAT ACTCATTTGG
 501 GGAATATATG ACTATCAACT GTGATGTTGG ATATGAGGTT ATTGGTGCTT
 551 CGTACATAAG TTGTACAGCT AATTCTTGGA ATGTTATTCC ATCATGTCAA
 601 CAAAAATGTG ATATGCCGTC TCTATCTAAT GGATTAATTT CCGGATCTAC
 651 ATTTTCTATC GGTGGCGTTA TACATCTTAG TTGTAAAAGT GGTTTTACAC
 701 TAACGGGGTC TCCATCATCC ACATGTATCG ACGGTAAATG GAATCCCGTA
 751 CTCCCAATAT GTGTACGAAC TAACGAAGAA TTTGATCCAG TGGATGATGG
 801 TCCCGACGAT GAGACAGATT TGAGCAAACT CTCGAAAGAC GTTGTACAAT
 851 ATGAACAAGA AATAGAATCG TTAGAAGCAA CTTATCATAT AATCATAGTG
 901 GCGTTAACAA TTATGGGCGT CATATTTTTA ATCTCCGTTA TAGTATTAGT
 951 TTGTTCCTGT GACAAAAATA ATGACCAATA TAAGTTCCAT AAATTGCTAC
1001 CGTACCCATA CGATGTTCCA GATTACGCTT AA
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[SEQ ID NO: 12]