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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 July 2017 (20.07.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/123242 A1

(51) International Patent Classification:
C12N 15/64 (2006.01) *A61K 48/00* (2006.01)
C12N 15/63 (2006.01)

(21) International Application Number:
PCT/US2016/013588

(22) International Filing Date:
15 January 2016 (15.01.2016)

(25) Filing Language: English

(26) Publication Language: English

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

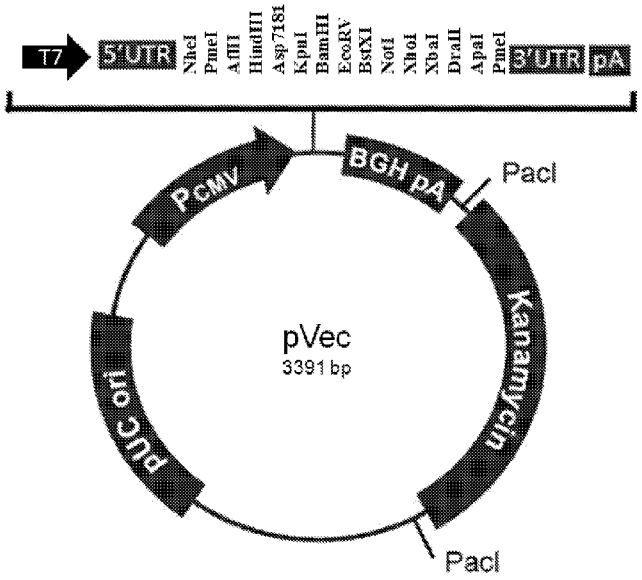
(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*
— *with amended claims (Art. 19(1))*
— *with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description (Rules 13bis.4(d)(i) and 48.2(a)(viii))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) **Title**: A UNIVERSAL NUCLEIC ACID DRUG VECTOR ENHANCING MRNA STABILITY AND TRANSLATABILITY

Fig. 1



(57) **Abstract**: In order to improve mRNA stability and translatabihty in cells, the present invention provides pVec that is obtained through in turn constructing pcDNA3.1 -5'UTR-MCS-3'UTR- pA, pcDNA3.1 -5'UTR-MCS (no SpeI, BamHI/EcoRI)-3'UTR-pA, pVecO-5'UTR-MCS (no SpeI, BamHI/EcoRI)-3'UTR-pA, achieving pVecI -5'UTR-MCS (no SpeI, BamHI/EcoRI)- 3'UTR-pA, referred to as pVec. It contains CMV enhancer/promoter, T7 promoter, 5'UTR, MCS, 3'UTR, poly A (120A)-TTATT, BGH poly (A) signal, kanamycin resistance gene and pUC origin, etc., and is mainly characterized in being able to enhance the stability and translatabihty of the in vitro transcribed mRNA in cells. pVec can be both a DNA vaccine or drug vector and an mRNA vaccine or drug vector. The present invention also provides the construction of pVec-GM-CSF, pVec-hlL-12 and pVAX1 -hlL-12, which are as application examples of evaluating the benefits of pVec.

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A UNIVERSAL NUCLEIC ACID DRUG VECTOR ENHANCING MRNA STABILITY AND TRANSLATABILITY

DESCRIPTION

BACKGROUND OF THE INVENTION

The present invention in the field of biotechnology relates to a nucleic acid drug. In particular, the present invention relates to a universal nucleic acid drug vector enhancing mRNA stability and translatability.

The nucleic acid drug applies DNA and RNA as a vaccine or a therapeutic drug of prevention and treatment of diseases for clinical use.

For many years, RNA has been considered to be unstable and susceptible to degradation. Thus most research in nucleic acid drugs, in particular nucleic acid vaccines, is based on DNA vaccines. A DNA vaccine is a plasmid DNA containing a foreign antigen gene sequence. It is delivered into the host body and enters the nucleus through the cellular and nuclear membranes. In the nucleus, the delivered foreign antigen gene DNA is transcribed into mRNA, which is then transported to the cytoplasm and translated into protein. The expressed protein can induce an immune response against the antigen, achieving the purpose of prevention and treatment of diseases such as cancer and viral diseases. Conventional DNA vaccine vectors include pcDNA3.1 and pVAX1. Among them, pcDNA3.1 contains an ampicillin resistance gene, which means the vector is banned by US Food and Drug Administration (FDA) from human clinical use. Since a DNA vaccine does not easily pass through the cellular and nuclear membranes, only a few DNA molecules can enter the nucleus, making it difficult to stimulate the body and further elicit a strong immune response. Therefore, no DNA vaccine has yet been approved for human clinical use. Currently, the employed electroporation method greatly improves the immune effect of DNA vaccines, but there are still concerns regarding whether the plasmid DNA can be integrated into the host cell's genome.

In recent years, improvements in plasmid vectors have increased the stability of the in vitro transcribed mRNA, turning our attention to RNA drugs, especially to mRNA vaccines. Based on pGEM4Z/A64 vector, pGEM4Z/GFP/A64 and pGEM4Z/OVA/A64 are constructed and made into templates for producing the in vitro transcribed mRNAs, which are inoculated via the intranasal route to induce anti-tumor immunity (Ref 1). Using pcDNA3.1-

64A and pSP73-Sph/A64, several vectors containing tumor-associated antigens (TAA), glucocorticord-induced TNFR-related protein monoclonal antibody (GITR mAb) and cytotoxic T-lymphocyte-associated protein-4 mAb (CTLA-4 mAb), are respectively constructed and used for producing the corresponding in vitro transcribed mRNAs, which are electroporated into dendritic cells (DC). Subsequently the DC-mRNA vaccines are used for enhancing anti-tumor immunity (Ref 2). pSpjC-βglacZβga$_n$ and pT7TSβggfpβga$_n$ are constructed, characterized in that both sides of LacZ and GFP genes are respectively 5'-untranslated region (UTR) and 3'UTR from xenopus laevis β-globin (Ref 3). The plasmid vectors containing TAA such as mucin1 (MUC1), carcinoembryonic antigen (CEA), human epidermal growth factor receptor 2 (Her-2/neu), telomerase, survivin and melanoma-associated antigen 1 (MAGE-1) are respectively constructed utilizing pSP64-Poly (A)-EGFP-2 provided by V.F.I. Van Tendeloo and taken as the templates for producing the in vitro transcribed mRNAs, which are used for anti-tumor immunity (Ref 4). Also 5'Top UTR is artificially synthesized and applied for increasing mRNA stability (Pat 1). In 2015 several plasmids containing multiple mutant major histocompatibility complex (MHC) class II epitope sequences are respectively constructed using pST1-Sp-MITD-2hBgUTR-A120 and used for producing the in vitro transcribed mRNAs, which are inoculated into the body for generating personalized anti-cancer immunity (Ref 5).

Among the above mentioned vectors, pGEM4Z/A64, pcDNA3.1-64A, pSP73-Sph/A64 and pSP64-Poly (A)-EGFP-2 don't have 5'UTR and 3'UTR, and contain only a short polyadenylation (poly A) tail (64A) so that the mRNA in vitro transcribed utilizing the above vectors is susceptible to degradation. Although containing 5'UTR and 3'UTR, pSpjC-βglacZβga$_n$ and pT7TSβggfpβga$_n$ contain 3'UTR with only a xenopus laevis β-globin so that their effect of stabilizing the in vitro transcribed mRNA is not ideal. pST1-Sp-MITD-2hBgUTR-A120 contains 3'UTR (with two β-globin) and poly A (120A), but it does not contain the TTATT sequence as a terminator after poly A (120A) and its 5'UTR is not ideal. Therefore, there is still room for improvement. Other reported mRNA cancer vaccine vectors that are not mentioned here are mostly made with minor improvements on the above plasmids.

Currently almost all the bacterial antibiotic resistance genes of plasmid vectors for generating the in vitro transcribed mRNA vaccines are ampicillin resistance genes. Before the in vitro transcribed mRNA can be deemed effective for human clinical use, it is

necessary to check whether the ampicillin resistance gene remains in the final product. In addition, according to the provisions of the FDA, the plasmid vectors containing the ampicillin resistance gene cannot be used as DNA vaccines for human clinical use.

Patent citations

Cited patent	Filing date	Publication date	Applicant	Title
1. US 20150050302 A1	Mar 27, 2013	Feb 19, 2015	Andreas Thess	Artificial nucleic acid molecules comprising a 5'top utr.

REFERENCES

1.Phua KK, Staats HF, Leong KW, Nair SK. Intranasal mRNA nanoparticle vaccination induces prophylactic and therapeutic anti-tumor immunity. Sci Rep. 2014; 4:5128.

2. Pruitt SK, Boczkowski D, de Rosa N, et al. Enhancement of anti-tumor immunity through local modulation of CTLA-4 and GITR by dendritic cells. Eur J Immunol. 2011; 41(12): 3553-63.

3. Hoerr I, Obst R, Rammensee HG, Jung G. In vivo application of RNA leads to induction of specific cytotoxic T lymphocytes and antibodies. Eur J Immunol. 2000; 30 (1): 1-7.

4. Rittig SM, Haentschel M, Weimer KJ, et al. Intradermal vaccinations with RNA coding for TAA generate CD8+ and CD4+ immune responses and induce clinical benefit in vaccinated patients. Mol Ther. 2011; 19 (5): 990-9.

5. Kreiter S, Vormehr M, van de Roemer N, et al. Mutant MHC class II epitopes drive therapeutic immune responses to cancer. Nature 2015; 520 (7549): 692-6.

SUMMARY OF THE INVENTION

The object of the present invention is to provide a universal nucleic acid drug vector enhancing mRNA stability and translatability.

To achieve the object of the present invention, the technical program used is as follows.

First of all, conventional pcDNA3.1 is taken as the vector backbone and inserted with the fragment containing restriction endonuclease AgeI, ClaI, SacII and SpeI sites obtained by polymerase chain reaction (PCR) method after ApaI and PmeI sites of multiple cloning sites (MCS) of the vector. Then the fragment containing poly A (120A) and TTATT (termination sequence) is inserted between SacII and SpeI sites of the above vector; the fragment containing the first human β-globin 3'UTR is inserted between ClaI and SacII sites; the fragment containing the second human β-globin 3'UTR is inserted between AgeI and ClaI sites; the fragment containing artificially designed and synthesized DNA as 5'UTR is inserted before NheI site. Through the above steps, pcDNA3.1-5'UTR-MCS-3'UTR-pA is constructed.

To delete SpeI site in the MCS, pcDNA3.1-5'UTR-MCS-3'UTR-pA is digested with BamHI and EcoRI, blunted and then self-ligated by head to tail connection, obtaining pcDNA3.1-5'UTR-MCS (no SpeI, BamHI/EcoRI)-3'UTR-pA.

To replace the ampicillin resistance gene of the vector with a kanamycin resistance gene, the fragment containing MluI-MCS-BbsI region of pcDNA3.1-5'UTR-MCS (no SpeI, BamHI/EcoRI)-3'UTR-pA is obtained by digesting pcDNA3.1-5'UTR-MCS (no SpeI, BamHI/EcoRI)-3'UTR-pA with MluI and BbsI, and then subcloned between MluI and BbsI sites of pVAX1, obtaining pVec0-5'UTR-MCS (no SpeI, BamHI/EcoRI)-3'UTR-pA.

To conveniently replace the kanamycin resistance gene of the vector with other non-bacterial antibiotic resistance gene in the future, the fragment containing BbsI-PacI-KanR-PacI-BspHI region obtained by PCR is subcloned into BbsI and BspHI (second BspHI) sites of pVec0-5'UTR-MCS (no SpeI, BamHI/EcoRI)-3'UTR-pA, obtaining pVec1-5'UTR-MCS (no SpeI, BamHI/EcoRI)-3'UTR-pA (with BbsI-PacI-KanR-PacI-BspHI), referred to as pVec.

In order to evaluate the benefits of pVec, the present invention also provides the constructed pVec-GM-CSF, which shows that pVec can be a DNA vaccine or drug vector as well as an mRNA vaccine or drug vector. In addition, the present invention also provides the constructed pVec-hIL-12 and pVAX1-hIL-12, which demonstrate that the stability of the in vitro transcribed mRNA generated by taking pVec-hIL-12 as a template is firm and the amount of the corresponding hIL-12 expression is high.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig.1 shows pVec vector map

The nucleotide length of pVec: 3391 bp

CMV enhancer: bases 36-415

CMV promoter: bases 416-619

T7 promoter: bases 664-682

5'UTR: bases 702-785

Multiple cloning sites: bases 786-878

3'UTR: bases 885-1149

Poly A: bases 1156-1275

BGH poly (A) signal: bases 1304-1528

Kanamycin resistance gene: bases 1709-2503

pUC origin: bases 2738-3326

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides pVec, which is constructed using conventional molecular biotechnologies through the following steps.

Taking conventional pcDNA3.1 as a template, the fragment containing restriction endonuclease AgeI, ClaI, SacII and SpeI sites (SEQ ID NO: 1) is obtained via polymerase chain reaction (PCR) using the forward primer (SEQ ID NO: 2) and the reverse primer (SEQ ID NO: 3), subcloned after ApaI and PmeI sites of the MCS of pcDNA3.1 and transformed into top10 chemically competent E.coli cells or DH5 alpha competent cells, obtaining pcDNA3.1-MCS-ApaI-PmeI-AgeI-ClaI-SacII-SpeI.

To insert poly A (120A) tail-TTATT sequence, several synthesized oligonucleotides including polyAF1 (SEQ ID NO: 4), polyAF2 (SEQ ID NO: 5), polyAF3 (SEQ ID NO: 6), polyAR1 (SEQ ID NO: 7) and polyAR2 (SEQ ID NO: 8) are phosphorylated with T4 polynucleotide kinase (New England Biolabs, Cat #: M0201S), denatured at 94°C for 10 minutes, annealed at room temperature for 30 minutes and ligated with T4 DNA ligase, and then subcloned into dephosphorylated SacII and SpeI sites of pcDNA3.1-MCS-ApaI-PmeI-AgeI-ClaI-SacII-SpeI, obtaining pcDNA3.1-MCS-ApaI-PmeI-AgeI-ClaI-SacII-poly A (120A)-TTATT-SpeI. The nucleotide sequence of the inserted poly A (120A)-TTATT sequence is as set forth in SEQ ID NO: 9.

To insert 3'UTR (from human β-globin), the synthesized oligonucleotides including 3'UTRClaIF1 (SEQ ID NO: 10), 3'UTRClaIF2 (SEQ ID NO: 11), 3'UTRSacIIR1 (SEQ ID NO: 12) and 3'UTRSacIIR2 (SEQ ID NO: 13) are phosphorylated, denatured, annealed and ligated with T4 DNA ligase, then subcloned into dephosphorylated ClaI and SacII sites of pcDNA3.1-MCS-ApaI-PmeI-AgeI-ClaI-SacII-poly A (120 A)-TTATT-SpeI, obtaining pcDNA3.1-MCS-ApaI-PmeI-AgeI-ClaI-3'UTR (β-globin)-SacII-poly A (120 A)-TTATT-SpeI.

To insert another 3'UTR (β-globin), the synthesized oligonucleotides including 3'UTRAgeIF1 (SEQ ID NO: 14), 3'UTRAgeIF2 (SEQ ID NO: 15), 3'UTRClaIR1 (SEQ ID NO: 16) and 3'UTRClaIR2 (SEQ ID NO: 17) are phosphorylated, denatured, annealed and ligated with T4 DNA ligase, and then subcloned into dephosphorylated AgeI and ClaI sites of pcDNA3.1-MCS-ApaI-PmeI-AgeI-ClaI-3'UTR (β-globin)-SacII-poly A (120 A)-TTATT-SpeI, obtaining pcDNA3.1-MCS-ApaI-PmeI-AgeI-3'UTR (β-globin)-ClaI-3'UTR (β-globin)-SacII-poly A (120 A)-TTATT-SpeI. The nucleotide sequence of 3'UTR is as set forth in SEQ ID NO: 18.

To insert 5'UTR, the oligonucleotides including 5'UTRF1 (SEQ ID NO: 19), 5'UTRF2 (SEQ ID NO: 20), 5'UTRR1 (SEQ ID NO: 21) and 5'UTRR2 (SEQ ID NO: 22) designed and synthesized by referencing eukaryotic 18s rRNA sequence are phosphorylated, denatured, annealed and ligated with T4 DNA ligase, and then subcloned into dephosphorylated NheI and AflII sites of pcDNA3.1-MCS-ApaI-PmeI-AgeI-3'UTR (β-globin)-ClaI-3'UTR (β-globin)-SacII-poly A (120 A)-TTATT-SpeI, resulting in 5'UTR inserted before NheI and obtaining pcDNA3.1-5'UTR-MCS-3'UTR-pA. The nucleotide sequence of 5'UTR is as set forth in SEQ ID NO: 23.

To delete SpeI site between BamHI and EcoRI sites of the MCS, pcDNA3.1-5'UTR-MCS-3'UTR-pA is digested with BamHI and EcoRI, blunted and then self-ligated by head to tail connection, obtaining pcDNA3.1-5'UTR-MCS (no SpeI, BamHI/EcoRI)-3'UTR-pA.

To replace the ampicillin resistance gene of the vector with a kanamycin resistance gene, the fragment containing MluI-MCS-BbsI region of pcDNA3.1-5'UTR-MCS (no SpeI, BamHI/EcoRI)-3'UTR-pA is obtained by digesting pcDNA3.1-5'UTR-MCS (no SpeI, BamHI/EcoRI)-3'UTR-pA with MluI and BbsI, and then subcloned between MluI and BbsI sites of pVAX1, obtaining pVec0-5'UTR-MCS (no SpeI, BamHI/EcoRI)-3'UTR-pA.

To conveniently replace the kanamycin resistance gene of the vector with other non-bacterial antibiotic resistance genes in the future, the fragment containing BbsI-PacI-KanR-PacI-BspHI region is obtained via PCR by taking pVec0-5'UTR-MCS (no SpeI, BamHI/EcoRI)-3'UTR-pA as a template and using the forward primer (SEQ ID NO: 24) and the reverse primer (SEQ ID NO: 25), subsequently subcloned into BbsI and BspHI (second BspHI) sites of pVec0-5'UTR-MCS (no SpeI, BamHI/EcoRI)-3'UTR-pA, achieving pVec1-5'UTR-MCS (no SpeI, BamHI/EcoRI)-3'UTR-pA (with BbsI-PacI-KanR-PacI-BspHI), referred to as pVec.

pVec complete nucleotide sequence is as SEQ ID NO: 26.

The present invention provides pVec that contains CMV enhancer/promoter, T7 promoter, 5'UTR, MCS, 3'UTR, poly A (120A)-TTATT, BGH poly (A) signal, kanamycin resistance gene and pUC origin, etc. pVec has the following characteristics: 1) the vector is relatively small (3,391 bp), which can accommodate large exogenous gene sequences; 2) after T7 promoter of the vector, there are 5'UTR, MCS, 3'UTR, poly (120A)-TTATT, etc., which can enhance the stability and translatability of the in vitro transcribed mRNA; 3) restriction endonuclease SpeI site of the MCS is deleted and another SpeI site is inserted after poly A (120A)-TTATT sequence of the vector so that it is easy to generate the linearized plasmid DNA with SpeI digestion, further produce the in vitro transcribed mRNA and RNA vaccines or therapeutic drugs; 4) the vector contains pUC origin, CMV enhancer/promoter, MCS, BGH poly (A) signal and kanamycin resistance gene, which can be used as DNA vaccines or therapeutic drugs for human clinical use; 5) when used as a DNA vaccine or drug, the components comprising 5'UTR, MCS, 3'UTR and poly A (120A)-TTATT etc. of the vector can effectively enhance the stability and translatability of the mRNA transcribed from the

vector in cells than that of other conventional DNA vaccine vectors such as pcDNA3.1 and pVAX1; 6) on both sides of the kanamycin resistance gene of the vector, the restriction endonuclease PacI sites are respectively inserted so that the kanamycin resistance gene of the vector can be easily replaced with other non-antibiotic resistance genes, further generating the DNA vaccine with the non-antibiotic selection gene.

Example 1: Construction and expression of pVec-GM-CSF

Taking pCMV-SPORT6-GM-CSF [purchased from Open Biosystems, human granulocyte macrophage colony stimulating factor (GM-CSF), GenBank accession number: BC108724.1] as a template, the product obtained by PCR amplification using the forward primer designed and synthesized according to Kozak sequence principle (SEQ ID NO: 27) and the reverse primer (SEQ ID NO: 28) is subcloned into HindIII and XhoI sites of pVec, which is transformed into E.coli cells (e.g., top10 chemically competent E. coli cells or DH5 alpha competent cells), obtaining pVec-GM-CSF.

pVec-GM-CSF is purified with Qiaprep spin miniprep kit (Qiagen, Cat #: 27106), digested with restriction endonuclease SpeI, obtaining the linearized plasmid DNA. A small amount of the above SpeI cut plasmid DNA is used for detecting whether pVec-GM-CSF is completely linearized by 1% agarose gel electrophoresis. The mixture of 100 μl SpeI cut plasmid DNA reaction solution with about 500 μl Buffer PB is transferred into a spin column, centrifuging for 30 seconds and discarding the effluent (flow-through). Then 750 μl Buffer PE is added into the above spin column, centrifuging for 30 seconds and draining the effluent, again centrifuging for 1 minute. The spin column is put into a clean micro-centrifuge tube, adding 30 μl H_2O to the spin column, standing for 1 minute and centrifuging for 1 minute. The concentration of the purified linearized pVec-GM-CSF is checked, further adjusting the concentration to 0.5 to 1 μg/μl.

The in vitro transcribed mRNA from pVec-GM-CSF is generated using HiScribe™ T7 High Yield RNA Synthesis Kit (New England Biolabs, Cat #: E2040S) and 3'-0-Me-m^7G(5')ppp(5')G RNA Cap Structure Analog (ARCA, New England Biolabs, Cat #: S1411S) through the following steps.

In detail, the following reagents are added into a 1.5 ml micro-centrifuge tube at room temperature.

Nuclease-free water	x µl	
10 X reaction buffer	2 µl	
ATP (100 mM)	2 µl	10 mM final
UTP (100 mM)	2 µl	10 mM final
CTP (100 mM)	2 µl	10 mM final
GTP (20 mM)	2 µl	2 mM final
ARCA (40 mM)	4 µl	8 mM final
Template DNA (linearized)	x µl	1 µg
T7 RNA polymerase mix	2 µl	
Total reaction volume	20 µl	

After mixing well and pulse-spinning, the above reaction tube is incubated at 37 °C for 2 hours. To remove the template DNA, 70 µl nuclease-free H_2O, 10 µl of 10X DNase I buffer and 2 µl DNase I (New England Biolabs, Cat #: M0303S) are added into the above reaction tube, incubating at 37 °C for 15 minutes.

Using RNeasy mini kit (Qiagen, Cat #: 74104), the in vitro transcribed mRNA from pVec-GM-CSF is purified by the following steps.

About 20 to 30 µl of the above in vitro transcribed mRNA is taken and transferred into a micro-centrifuge tube (nuclease-free), 350 µl Buffer RLT containing 1% β-mercaptoethanol (β-ME) is added into the above tube. After thoroughly mixing with pipette, adding an equal volume of 70% ethanol and mixing again, the above mixture is transferred into a spin column for centrifuging and draining the effluent (flow-through). 700 µl Buffer RW1 is added into the above spin column, draining the effluent after centrifugation. 500 µl Buffer RPE is added into the above spin column, centrifuging, draining the effluent and repeating twice. After centrifuging for 1 minute, the spin column is transferred into a clean micro-centrifuge tube (nuclease-free) and 30 µl nuclease-free H_2O is added into the spin column, standing

for 1 minute and then centrifuging. The resulting product is the purified in vitro transcribed mRNA from pVec-GM-CSF. The concentration of the above mRNA is checked using nanodrop spectrophotometer and then its quality is detected by 1 % formaldehyde agarose gel electrophoresis.

pVec-GM-CSF DNA (5 μg) and the in vitro transcribed mRNA from pVec-GM-CSF (5 μg) are respectively electroporated into $1X10^6$ cells (e.g., mouse B16F10 cells or D5LacZ cells, etc.) in a 0.2 cm cuvette at the condition of 350 V and 500 μs. The above cells electroporated with the DNA or mRNA are cultured in a cell culture medium at 5% CO_2, 37°C for 36 hours and the supernatants are respectively collected.

Using human GM-CSF enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, Cat #: 88-8337-22), human GM-CSF expression in the supernatant is detected by the following steps.

The ELISA plate is coated with 100 μl capture antibody diluted with 1X coating buffer at the ratio of 1:250 for each well, sealed and incubated at 4°C overnight.

After discarding the coating solution, rinsing with wash buffer [1X phosphate-buffered saline (PBS) containing 0.05% Tween-20] 3 times, at least 1 minute each time, and patting dry, the above plate is added with 200 μl of 1X ELISA/ELISPOT Diluent for each well and incubated at room temperature for 1 hour.

According to the previous method, the above plate is washed and then added with 100 μl of 1X ELISA / ELISPOT Diluent diluted standard human GM-CSF or 100 μl of the collected supernatant for each well, sealed and incubated at room temperature for 2 hours.

The plate is washed according to the previous method 3 to 5 times, then added with 100 μl of 1X ELISA/ELISPOT Diluent diluted detection antibody for each well, sealed and incubated at room temperature for 1 hour.

The plate is washed according to the above method 3 to 5 times, then added with 100 μl of 1X ELISA/ELISPOT Diluent diluted Avidin-HRP for each well, sealed and incubated at room temperature for 30 minutes.

The plate is washed according to the above method 5 to 7 times, and added with 100 μl of 1X TMB solution for each well, and incubated at room temperature for 15 minutes.

Then 50 µl of 2 M H_2SO_4 stop solution is added into each well of the above plate. The concentration of human GM-CSF expressed in the cell supernatant is determined by measuring O.D value at 450 nm using micro-plate reader.

The results show that both the cells electroporated with pVec-GM-CSF DNA and the cells with the in vitro transcribed mRNA from pVec-GM-CSF can express human GM-CSF. In addition, experiments demonstrate that the in vitro transcribed mRNA from pVec-GM-CSF can remain stable at room temperature more than three weeks.

Example 2: Construction of pVec-hIL-12 and the comparison with pVAX1-hIL-12

Human interleukin-12 (hIL-12) gene is obtained by digesting pORF-hIL-12 G2 (InvivoGen) with SalI and NheI, and subcloned into XhoI and XbaI sites of pVec, obtaining pVec-hIL-12. Also, hIL-12 gene is subcloned into XhoI and XbaI sites of pVAX1 (invitrogen), obtaining pVAX1-hIL-12.

Using the above mentioned method, pVec-hIL-12 and pVAX1-hIL-12 are respectively purified with Qiaprep spin miniprep kit (Qiagen, Cat #: 27106) and linearized by SpeI digestion, obtaining the corresponding linearized plasmid DNAs. The concentration of the resultant linearized pVec-hIL-12 and pVAX1-hIL-12 is checked, then adjusting their concentration to 0.5 to 1 µg/µl.

The in vitro transcribed mRNAs respectively from pVec-hIL-12 and pVAX1-hIL-12 are generated by the previous indicated method. The obtained mRNAs are respectively purified using RNeasy mini kit (Qiagen, Cat #: 74104). The concentration of the mRNAs is checked using nanodrop spectrophotometer and their quality is detected by 1% formaldehyde agarose gel electrophoresis.

pVec-hIL-12 DNA, the in vitro transcribed mRNA from pVec-hIL-12, pVAX1-hIL-12 DNA and the in vitro transcribed mRNA from pVAX1-hIL-12 (5 µg/ each) are respectively electroporated into 1X 10^6 cells (such as mouse B16F10 cells or D5LacZ cells, etc.) in a 0.2 cm cuvette at the condition of 350 V and 500 µs. The above electroporated cells are cultured in a cell growth medium at 5% CO_2, 37°C for 36 hours, the supernatants of the above cells are respectively collected.

The collected supernatants are respectively used for detecting human IL-12 expression using human IL-12 ELISA kit (eBioscience, Cat #: 88-7126-88) by the previous mentioned protocol.

The ELISA plate is coated with 100 µl capture antibody diluted with 1X coating buffer at the ratio of 1:250 for each well, sealed and incubated at 4°C overnight.

After discarding the coating solution containing capture antibody, rinsing with wash buffer (1X PBS containing 0.05% Tween-20) 3 times, at least 1 minute each time, and patting dry, the above plate is added with 200 µl of 1X ELISA/ELISPOT Diluent for each well and incubated at room temperature for 1 hour.

According to the previous mentioned method, the above plate is washed and then added with 100 µl of 1X ELISA / ELISPOT Diluent diluted standard human IL-12 or 100 µl of the collected supernatant for each well, sealed and incubated at room temperature for 2 hours.

The plate is washed according to the previous method 3 to 5 times, then added with 100 µl of 1X ELISA/ELISPOT Diluent diluted detection antibody for each well, sealed and incubated at room temperature for 1 hour.

The plate is washed according to the above method 3 to 5 times, then added with 100 µl of 1X ELISA/ELISPOT Diluent diluted Avidin-HRP for each well, sealed and incubated at room temperature for 30 minutes.

The plate is washed according to the above method 5 to 7 times, added with 100 µl of 1X TMB solution for each well, and incubated at room temperature for 15 minutes.

Then 50 µl of 2 M H_2SO_4 stop solution is added into each well of the above plate. Further, the concentration of human IL-12 expressed in the cell supernatant is determined by measuring O.D value at 450 nm using micro-plate reader.

The experiments show that the cells electroporated with pVec-hIL-12 DNA, the in vitro transcribed mRNA from pVec-hIL-12, pVAX1-hIL-12 DNA and the in vitro transcribed mRNA from pVAX1-hIL-12 can express hIL-12 respectively. Furthermore, pVec-hIL-12 as a template is used for generating the in vitro transcribed mRNA, which has better stability. The amount of hIL-12 expressed by the in vitro transcribed mRNA from pVec-hIL-12 is also higher than that of the in vitro transcribed mRNA from pVAX1-hIL-12.

CLAIMS

1 pVec is a universal nucleic acid drug vector, which contains CMV enhancer/promoter, T7 promoter, 5'-untranslated region (5'UTR), multiple cloning sites (MCS), 3'UTR, poly A (120A)-TTATT, BGH poly (A) signal, kanamycin resistance gene and pUC origin, etc.

2 pVec of claim 1 is obtained through in turn constructing pcDNA3.1-5'UTR-MCS-3'UTR-pA, pcDNA3.1-5'UTR-MCS (no SpeI, BamHI/EcoRI)-3'UTR-pA, pVec0-5'UTR-MCS (no SpeI, BamHI/EcoRI)-3'UTR-pA, achieving pVec1-5'UTR-MCS (no SpeI, BamHI/EcoRI)-3'UTR-pA, referred to as pVec.

3 pVec of claim 1, wherein said complete nucleotide sequence is as set forth in SEQ ID NO: 26.

4 pVec of claim 1, wherein said the vector contains 3391 bp of the complete nucleotide sequence, which is relatively small so that pVec can accommodate large exogenous gene sequences.

5 pVec of claim 1, wherein after T7 promoter, there are 5'UTR, MCS, 3'UTR, poly A (120A)-TTATT, etc., which can enhance the in vitro transcribed mRNA stability and translatability in cells.

6 pVec of claim 1, wherein said restriction endonuclease SpeI site of the MCS is deleted and another SpeI site is inserted after poly A (120A)-TTATT sequence of the vector so that it is easy to generate the linearized plasmid DNA with SpeI digestion, produce the in vitro transcribed mRNA, and further prepare RNA vaccines or therapeutic drugs.

7 pVec of claim 1, wherein said 5'UTR nucleotide sequence of the vector is as set forth in SEQ ID NO: 23.

8 pVec of claim 1, wherein said 3'UTR nucleotide sequence of the vector is as set forth in SEQ ID NO: 18.

9 pVec of claim 1, wherein said poly A (120A)-TTATT nucleotide sequence of the vector is as set forth in SEQ ID NO: 9.

10 pVec of claim 1, wherein said vector contains pUC origin, CMV enhancer/promoter, MCS, BGH poly (A) signal and kanamycin resistance gene, which can be used as a DNA vaccine or therapeutic drug vector.

11 pVec of claim 1, wherein said vector contains 5'UTR, 3'UTR, poly A (120A)-TTATT, etc., when used as a DNA vaccine or drug vector, can enhance the stability of mRNA transcribed from the vector and its translatability in cells.

12 pVec of claim 1, wherein said two restriction endonuclease PacI sites are respectively inserted on both sides of the kanamycin resistance gene of pVec so that it is easy to replace the kanamycin resistance gene of the vector with other non-antibiotic selection genes, which can be the DNA vaccine with a non-antibiotic selection gene.

13 To evaluate the beneficial effect of pVec of claim 1, pVec-GM-CSF is constructed.

14 Forward and reverse primers used for PCR amplifying human GM-CSF and constructing pVec-GM-CSF of claim 13 are respectively as set forth in SEQ ID NOS: 27-28.

15 According to claim 13, both pVec-GM-CSF DNA and the corresponding in vitro transcribed mRNA can express human GM-CSF in cells, demonstrating that pVec can be a DNA vaccine or drug vector as well as an mRNA vaccine or drug vector.

16 To further evaluate the beneficial effect of pVec of claim 1, pVec-hIL-12 and pVAX1-hIL-12 are respectively constructed.

17 According to claim 16, the stability of the in vitro transcribed mRNA from pVec-hIL-12 is firm.

18 According to claim 16, the amount of hIL-12 expressed by the in vitro transcribed mRNA from pVec-hIL-12 is relatively high.

AMENDED CLAIMS
received by the International Bureau on 6 May 2016 (06.05.2016)

1. pVec, the complete nucleotide sequence is as set forth in SEQ ID NO: 26.

2. A nucleotide sequence is at least 90% identical to SEQ ID NO: 26.

3. pVec of claim 1 having the size of 3,391 bp is relatively small so that pVec can accommodate large exogenous gene sequences.

4. pVec of claim 1, wherein the 3'UTR nucleotide sequence is as set forth in SEQ ID NO: 18.

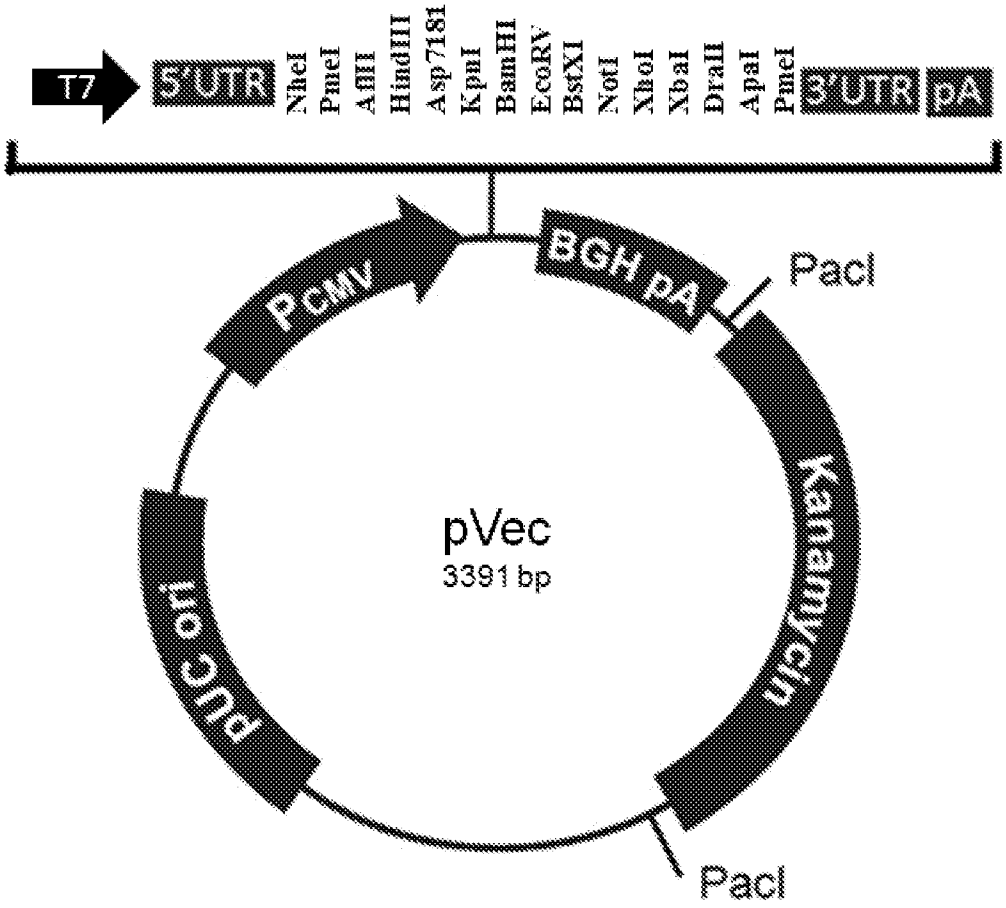
5. A nucleotide sequence is at least 96% identical to SEQ ID NO: 18.

6. Forward and reverse primers used for PCR amplifying human GM-CSF and constructing pVec-GM-CSF are respectively as set forth in SEQ ID NOS: 27-28.

7. A nucleotide sequence is at least 85% identical to SEQ ID NO: 27.

8. A nucleotide sequence is at least 95% identical to SEQ ID NO: 28.

Fig. 1


T7
5'UTR
NheI
PmeI
AflII
HindIII
Asp718I
KpnI
BamHI
EcoRV
BstXI
NotI
XhoI
XbaI
DraII
ApaI
PmeI
3'UTR
pA
PCMV
BGH pA
PacI
Kanamycin
pUC ori
pVec
3391 bp
PacI

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/13588

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 15/64, C12N 15/63, A61K 48/00 (2016.01)
CPC - C12N 15/64, C12N 15/63, A61K 48/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C12N 15/64, C12N 15/63, A61K 48/00 (2016.01)
CPC - C12N 15/64, C12N 15/63, A61K 48/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched (keyword limited; terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, Google Scholar
Search terms: vector, plasmid, pVec, CMV, T7, enhancer, promoter, 5' untranslated region, 5' UTR, 5'-UTR, 3' untranslated region, 3' UTR, 3'-UTR, multiple cloning sites, MCS, polyadenylation, poly A, poly (A), poly-A, poly-(A), BGH, TTATT, RNA, mRNA, stability, transla

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y --- A	US 2013/0274129 A1 (GENEART AG et al.) 17 October 2013 (17.10.2013) para [0011], [0022], [0039], [0060], [0067], [0133], [0136], [0137], [0364], [0557], [0574]	1-2, 5-7, 9-13, 15-18 --- 3-4, 8, 14
Y --- A	US 2007/0083334 A1 (MINTZ et al.) 12 April 2007 (12.04.2007) para [0296]; SEQ ID NO: 496365	1-2, 5-7, 9-13, 15-18 --- 3-4, 8, 14
Y --- A	US 2013/0078275 A1 (TAO) 28 March 2013 (28.03.2013) para [0062], [0067], [0091], [0099]	1-2, 5-7, 9-13, 15-18 --- 3-4, 8, 14
Y	US 2010/0266546 A1 (RAMACHANDRA et al.) 21 October 2010 (21.10.2010) para [0165], [0166]	6, 12
Y	US 2014/0243399 A1 (MODERNA THERAPEUTICS, INC.) 28 August 2014 (28.08.2014) para [0009]; SEQ ID NO: 2	7
Y	US 2002/0102546 A1 (NOLAN et al.) 01 August 2002 (01.08.2002) para [0139], [0212]	16-18

☒ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 April 2016	22 APR 2016
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/13588

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☒ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

      ☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/13588

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CN1690207 A (FIRST HOSPITAL OF JILIN UNIVER) 02 November 2011 (02.11.2011) claim 5, see machine translation	3-4
A	US 2010/0048679 A1 (GARREN et al.) 25 February 2010 (25.02.2010) SEQ ID NO: 3	3-4
A	US 2014/0227237 A1 (The Trustees Of The University Of Pennsylvania) 14 August 2014 (14.08.2014) SEQ ID NO: 26	8
A	US 2003/0104625 A1 (Cheng et al.) 05 June 2003 (05.06.2003) SEQ ID NO: 19	14
A	CN 101270367 A (UNIV SOOCHOW) 24 September 2008 (24.09.2008) sequence of Example 3, see machine translation	14