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(54) **DNA PLASMIDS WITH IMPROVED EXPRESSION**

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(57) **ABSTRACT**

The present invention relates to the production and use of covalently closed circular (ccc) recombinant plasmids, and more particulary to vector modifications that improve express of said DNA molecules in the target organism.

Specification includes a Sequence Listing.

FIGURE 1

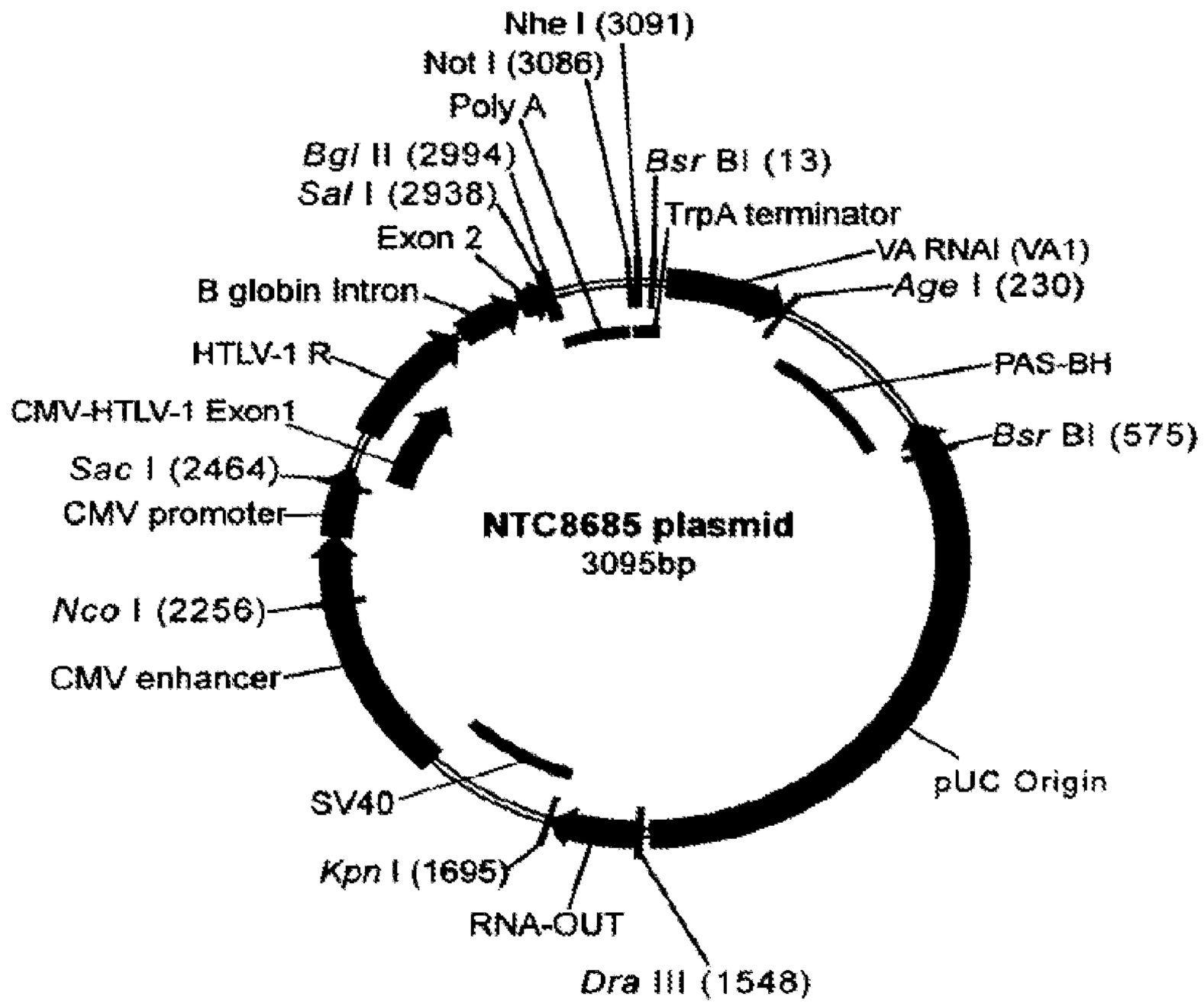

Nhe I (3091)
Not I (3086)
Poly A
Bgl II (2994)
Sal I (2938)
Exon 2
B globin intron
HTLV-1 R
CMV-HTLV-1 Exon1
Sac I (2464)
CMV promoter
Nco I (2256)
CMV enhancer
SV40
Kpn I (1695)
RNA-OUT
Dra III (1548)
Bsr BI (13)
TrpA terminator
VA RNAI (VA1)
Age I (230)
PAS-BH
Bsr BI (575)
pUC Origin
NTC8685 plasmid
3095bp

FIGURE 2

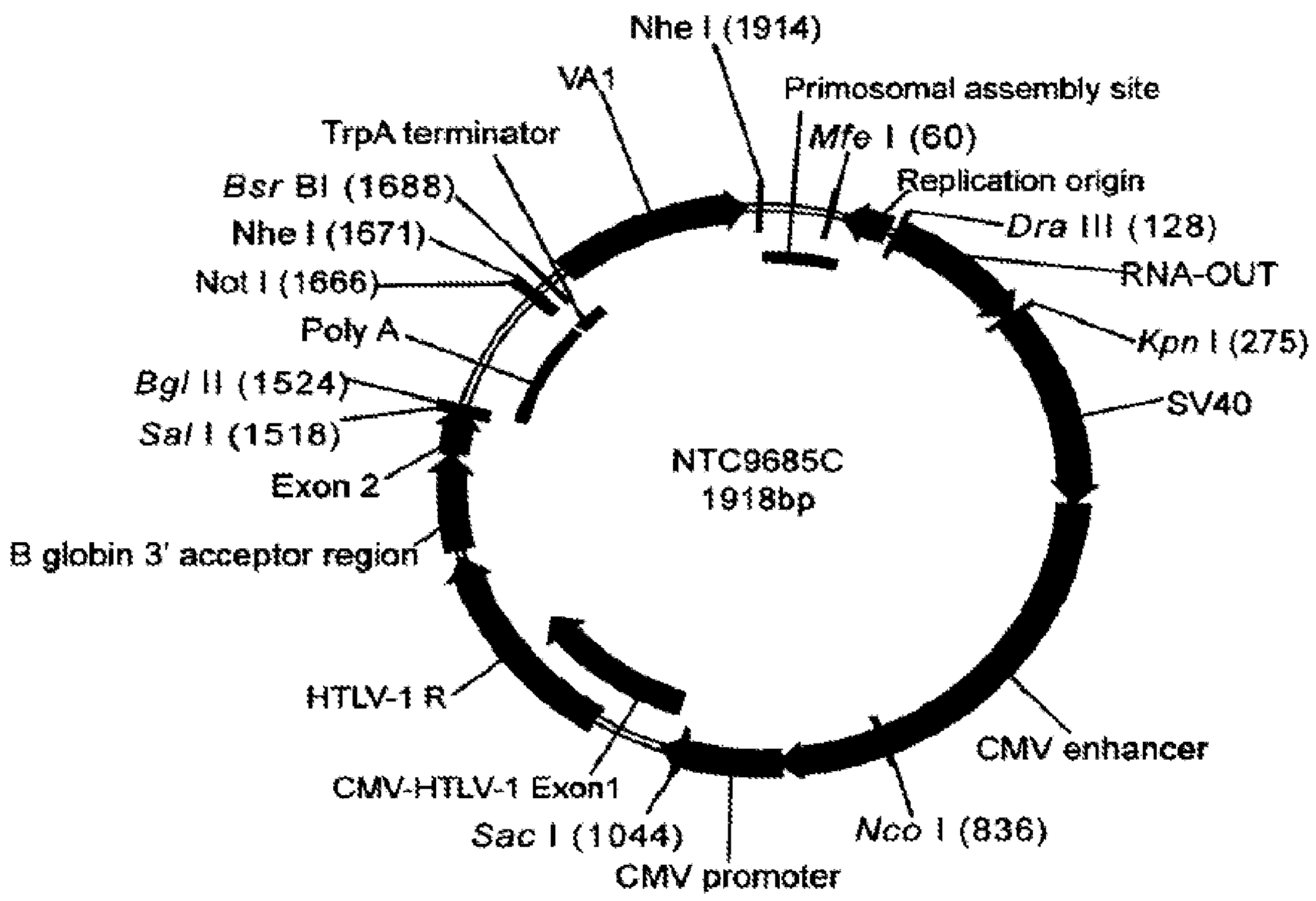

Nhe I (1914)
VA1
Primosomal assembly site
TrpA terminator
Mfe I (60)
Bsr BI (1688)
Replication origin
Nhe I (1671)
Dra III (128)
Not I (1666)
RNA-OUT
Poly A
Kpn I (275)
Bgl II (1524)
SV40
Sal I (1518)
NTC9685C
1918bp
Exon 2
B globin 3' acceptor region
HTLV-1 R
CMV enhancer
CMV-HTLV-1 Exon1
Sac I (1044)
Nco I (836)
CMV promoter

FIGURE 3

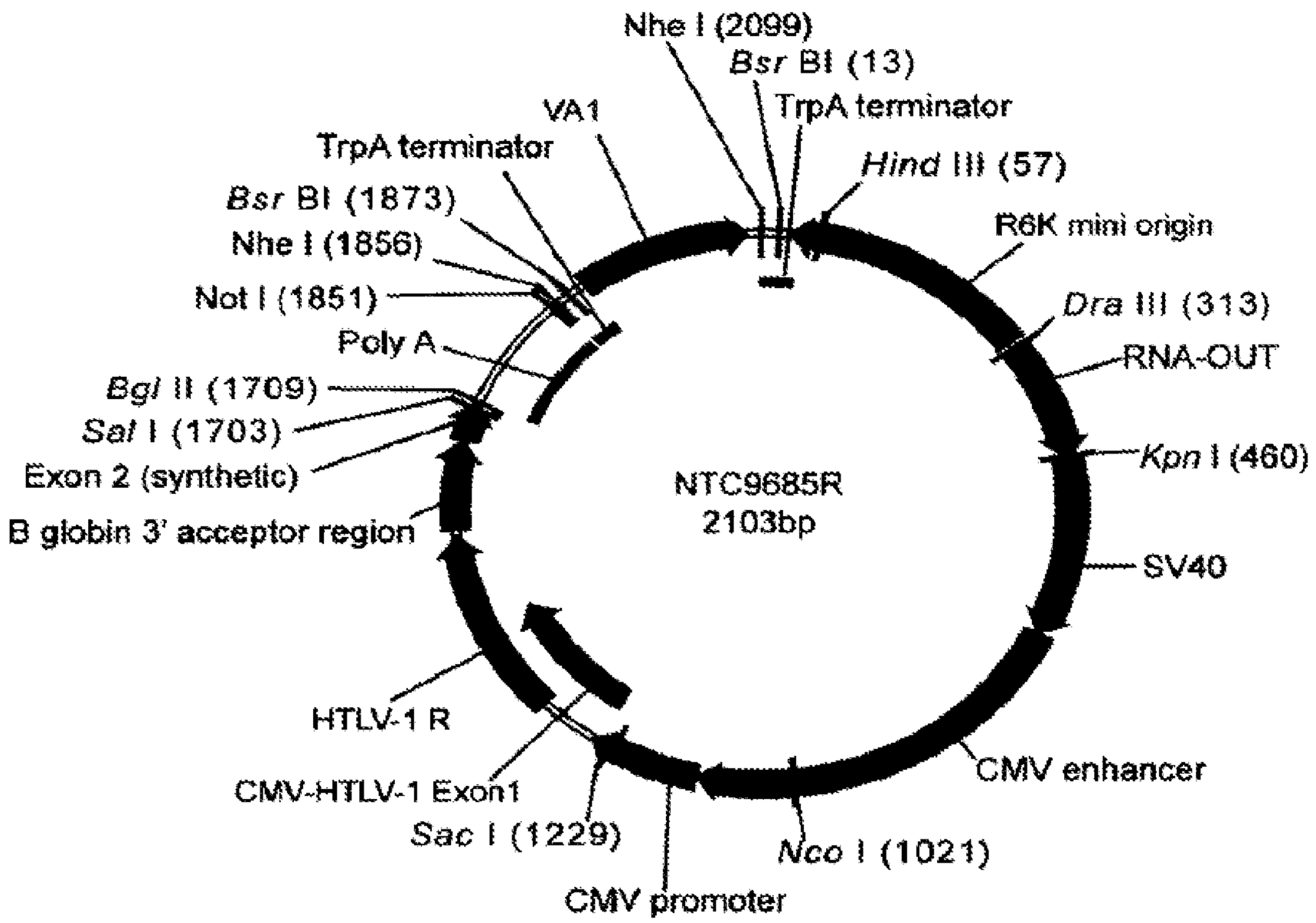

Nhe I (2099)
Bsr BI (13)
TrpA terminator
Hind III (57)
R6K mini origin
Dra III (313)
RNA-OUT
Kpn I (460)
SV40
CMV enhancer
Nco I (1021)
CMV promoter
Sac I (1229)
CMV-HTLV-1 Exon1
HTLV-1 R
B globin 3' acceptor region
Exon 2 (synthetic)
Sal I (1703)
Bgl II (1709)
Poly A
Not I (1851)
Nhe I (1856)
Bsr BI (1873)
TrpA terminator
VA1
NTC9685R
2103bp

FIGURE 4

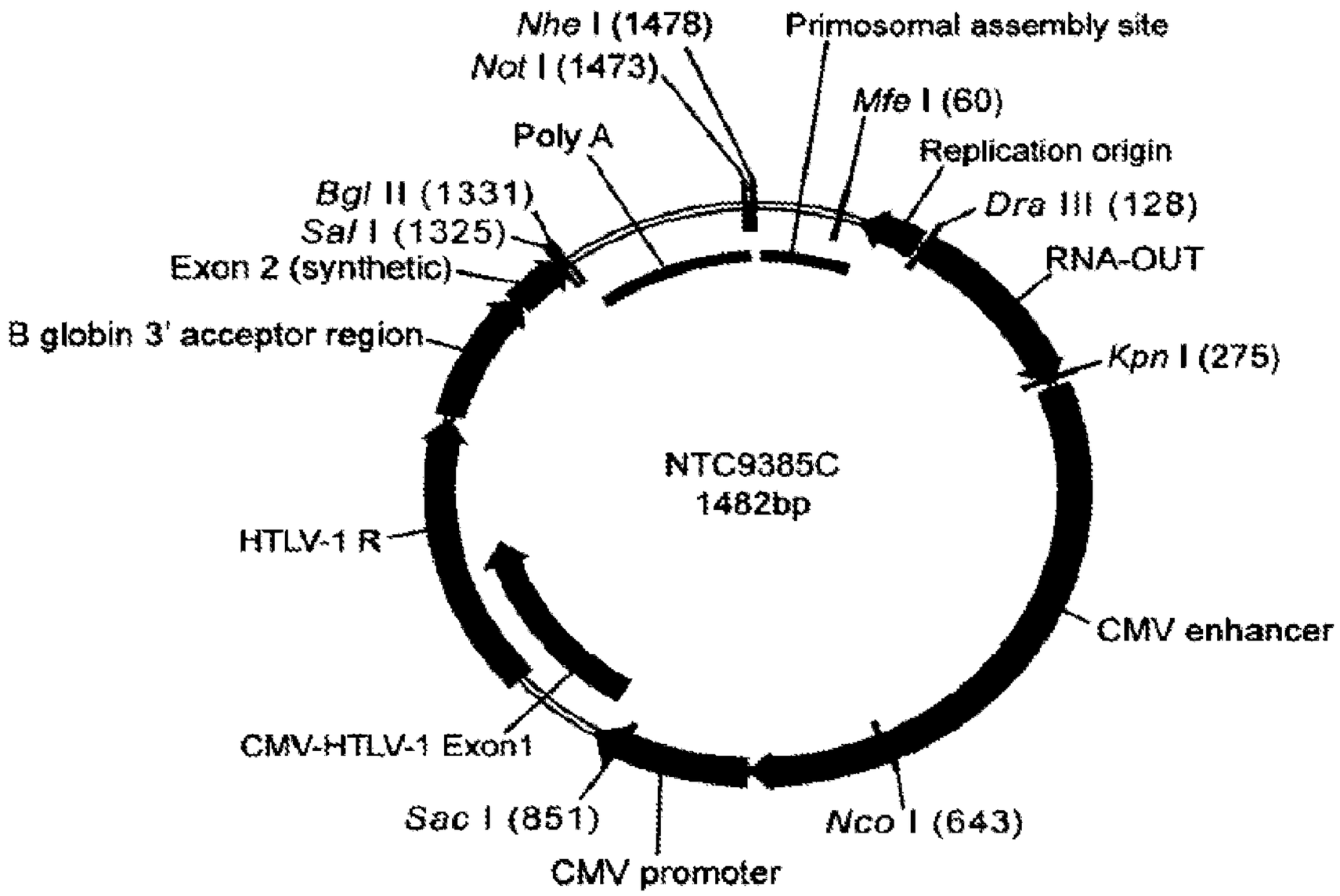

Nhe I (1478)
Not I (1473)
Primosomal assembly site
Mfe I (60)
Poly A
Replication origin
Bgl II (1331)
Sal I (1325)
Dra III (128)
Exon 2 (synthetic)
RNA-OUT
B globin 3' acceptor region
Kpn I (275)
NTC9385C
1482bp
HTLV-1 R
CMV enhancer
CMV-HTLV-1 Exon1
Sac I (851)
Nco I (643)
CMV promoter

FIGURE 5

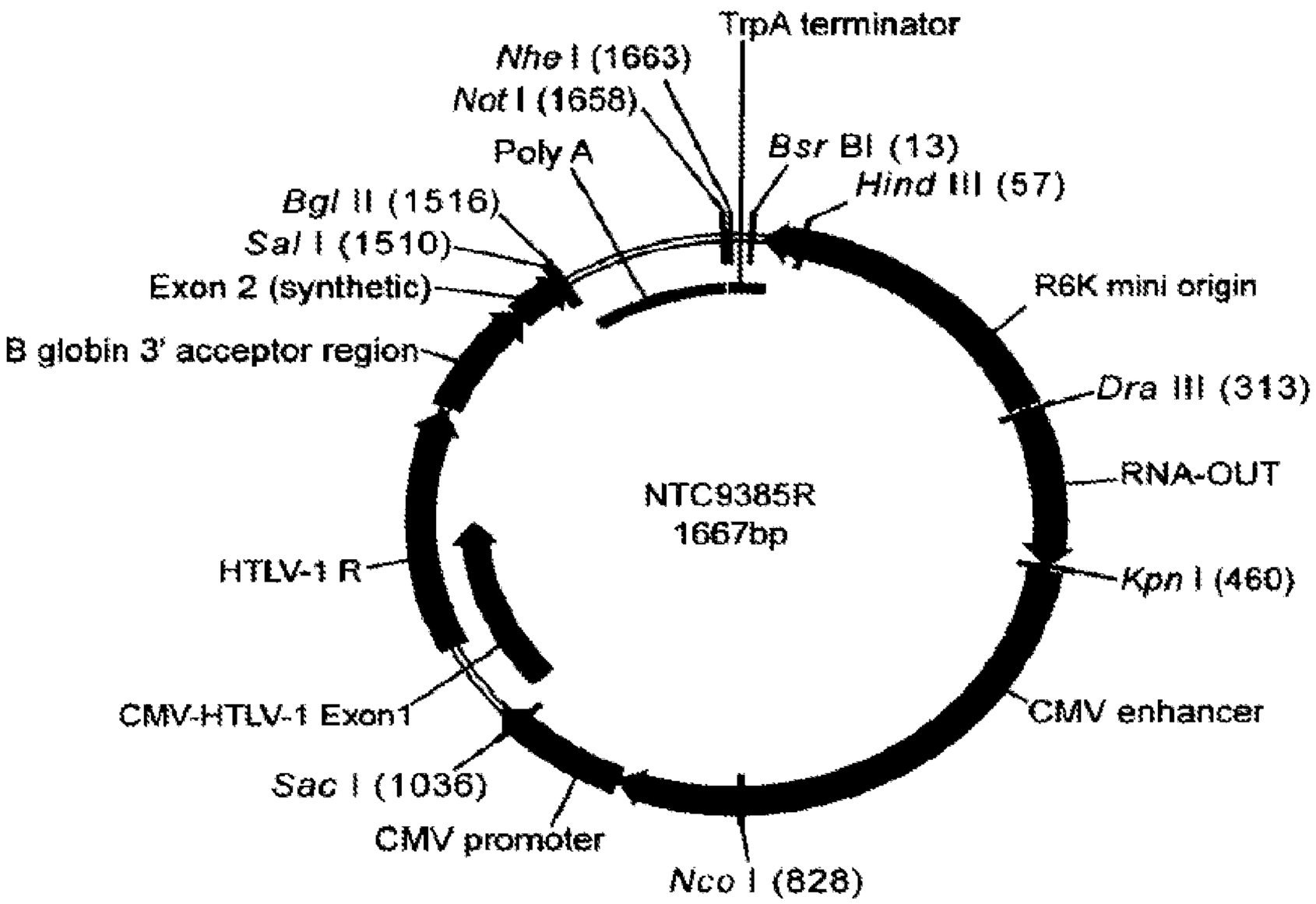

TrpA terminator
Nhe I (1663)
Not I (1658)
Poly A
Bsr BI (13)
Hind III (57)
Bgl II (1516)
Sal I (1510)
Exon 2 (synthetic)
B globin 3' acceptor region
R6K mini origin
Dra III (313)
RNA-OUT
NTC9385R
1667bp
HTLV-1 R
Kpn I (460)
CMV-HTLV-1 Exon1
CMV enhancer
Sac I (1036)
CMV promoter
Nco I (828)

FIGURE 6

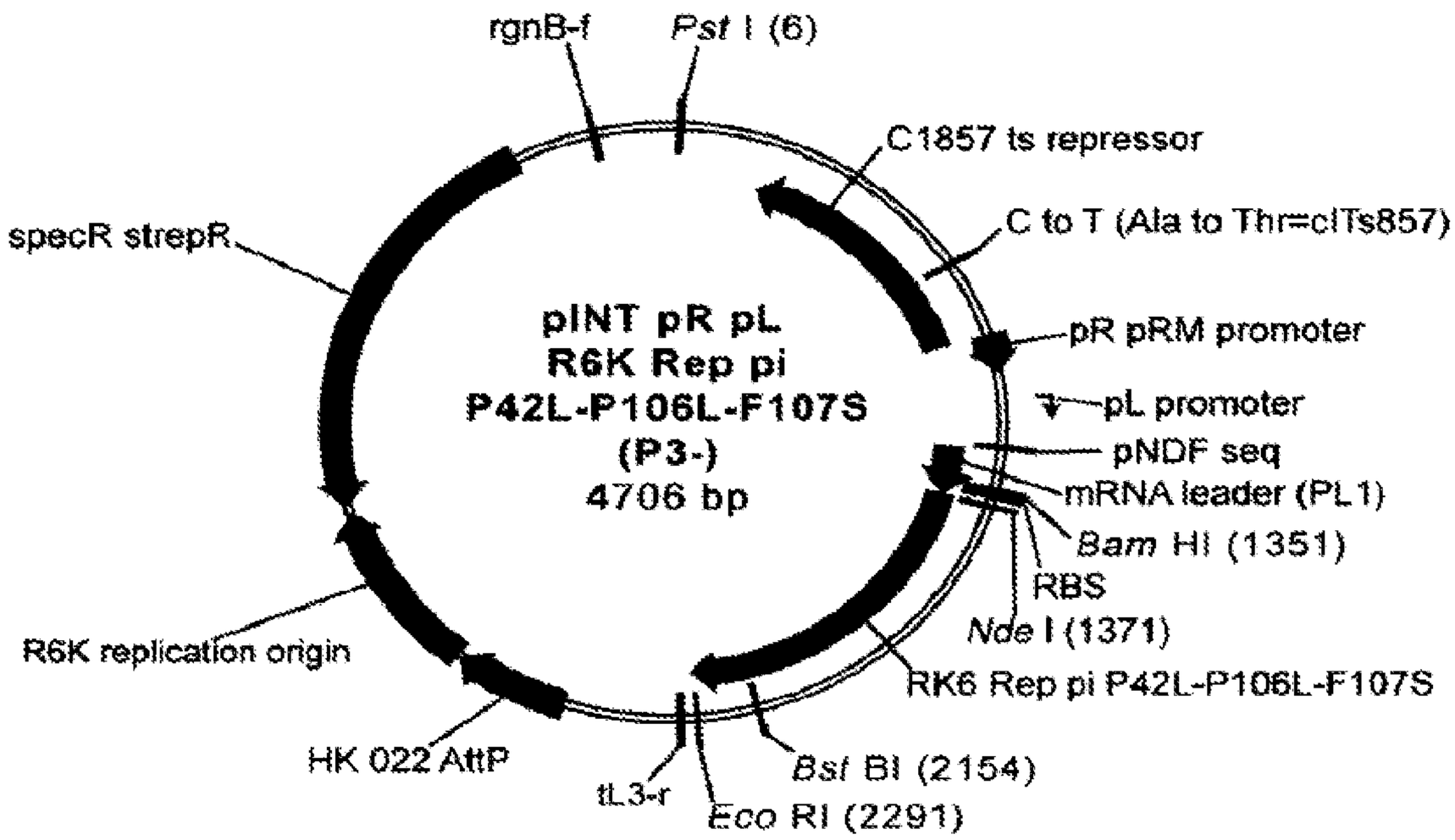
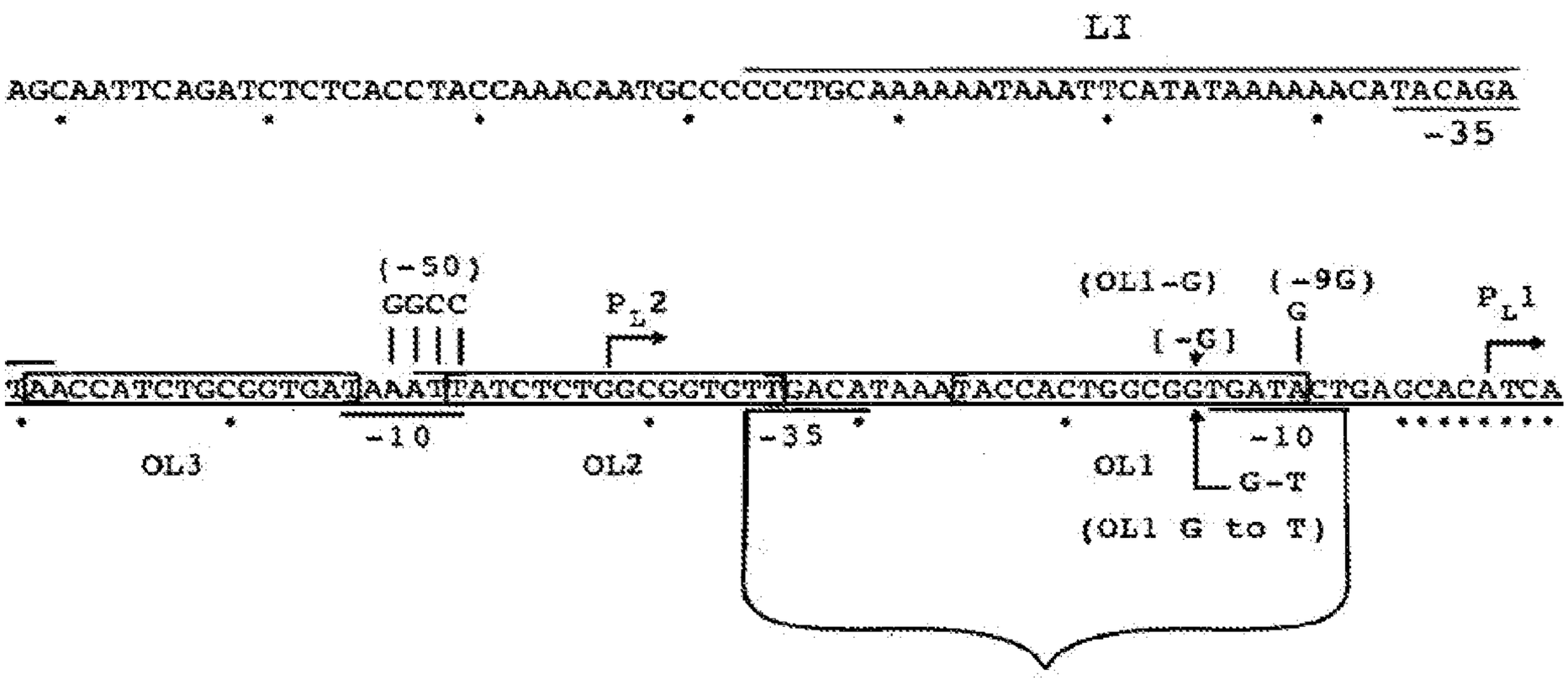

rgnB-f
Pst I (6)
C1857 ts repressor
C to T (Ala to Thr=cITs857)
pR pRM promoter
pL promoter
pNDF seq
mRNA leader (PL1)
Bam HI (1351)
RBS
Nde I (1371)
RK6 Rep pi P42L-P106L-F107S
Bsl BI (2154)
Eco RI (2291)
tL3-r
HK 022 AttP
R6K replication origin
specR strepR
pINT pR pL
R6K Rep pi
P42L-P106L-F107S
(P3-)
4706 bp

FIGURE 7



SEQ ID # 10 - WILD TYPE
11 - OL1-G
12 - OLI-G to T

FIGURE 8

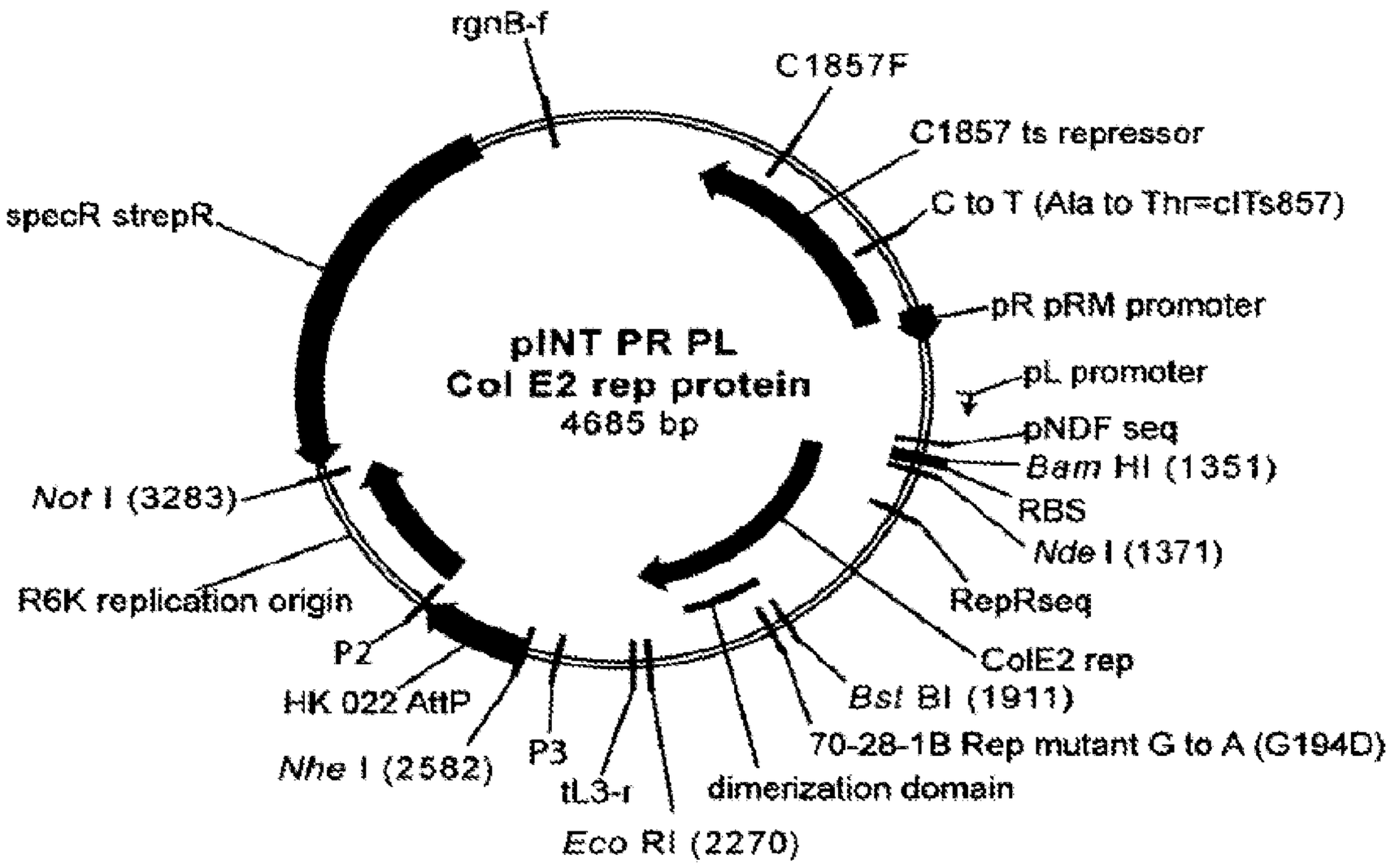

rgnB-f
C1857F
C1857 ts repressor
C to T (Ala to Thr=cITs857)
specR strepR
pR pRM promoter
pINT PR PL
Col E2 rep protein
4685 bp
pL promoter
pNDF seq
Bam HI (1351)
RBS
Nde I (1371)
Not I (3283)
RepRseq
R6K replication origin
ColE2 rep
P2
Bsl BI (1911)
HK 022 AttP
70-28-1B Rep mutant G to A (G194D)
Nhe I (2582)
P3
tL3-I
dimerization domain
Eco RI (2270)

FIGURE 9

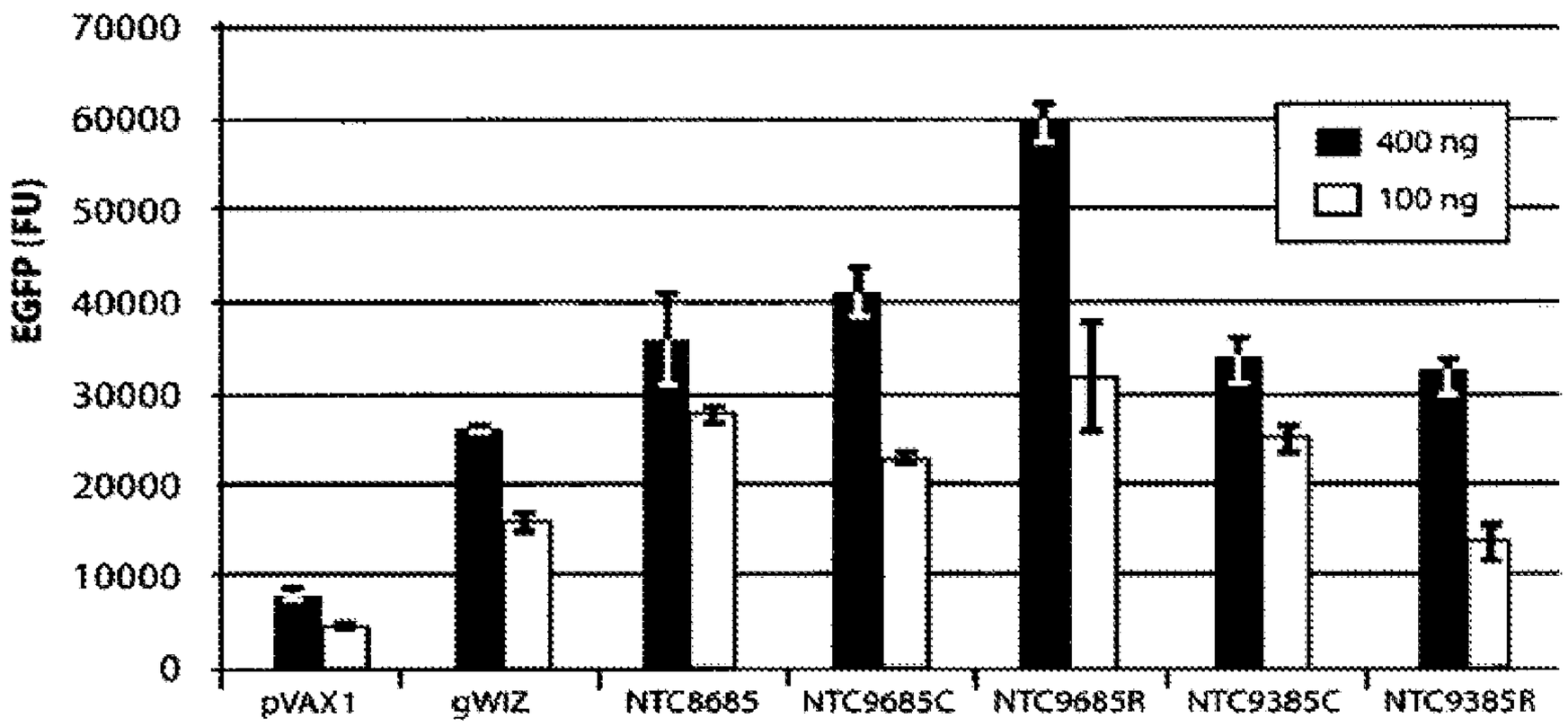

70000
60000
50000
40000
30000
20000
10000
0
EGFP (FU)
400 ng
100 ng
pVAX1
gWIZ
NTC8685
NTC9685C
NTC9685R
NTC9385C
NTC9385R

FIGURE 10

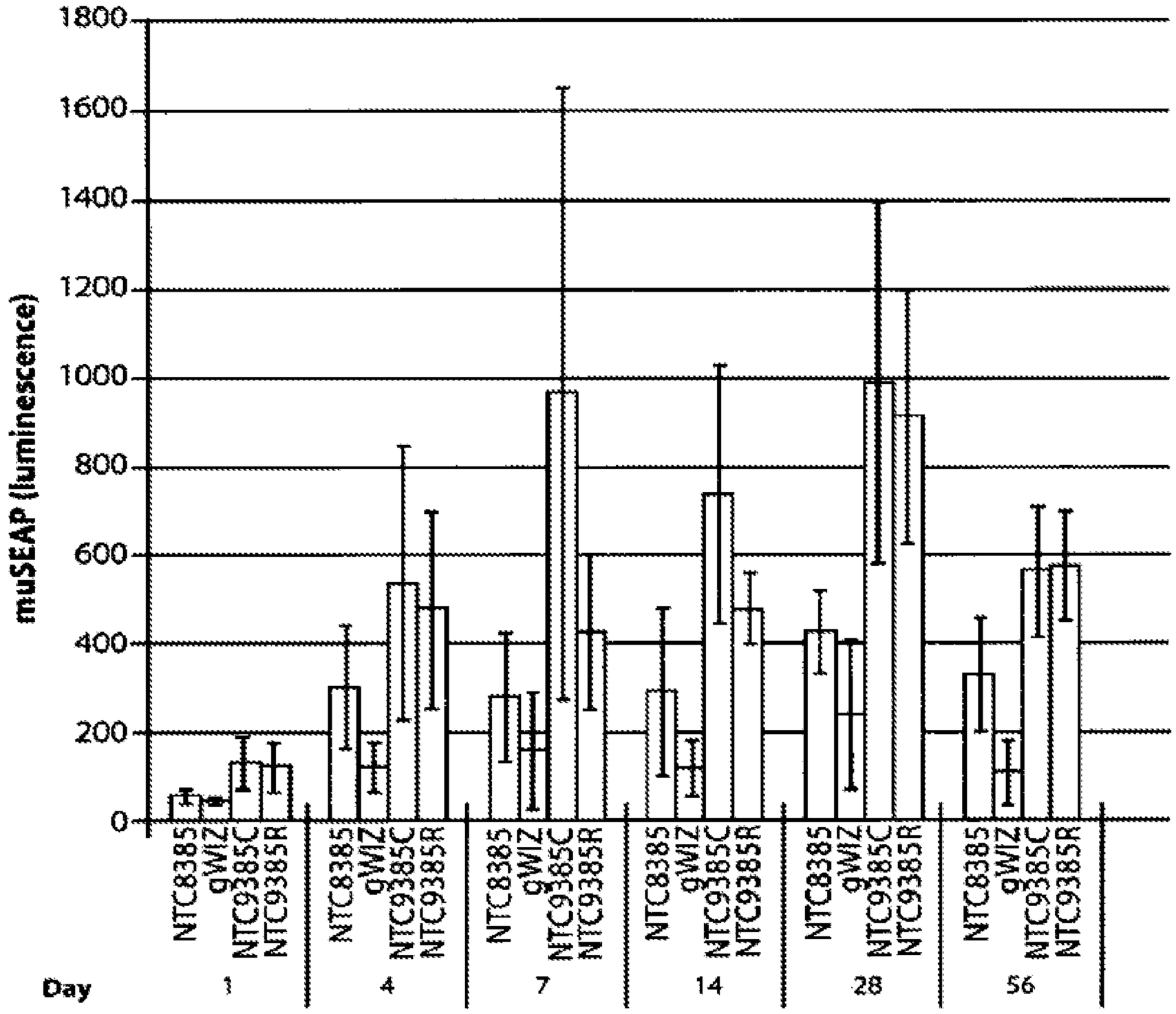

muSEAP (luminescence)
1800
1600
1400
1200
1000
800
600
400
200
0
NTC8385
gWiz
NTC9385C
NTC9385R
Day
1
4
7
14
28
56

FIGURE 11

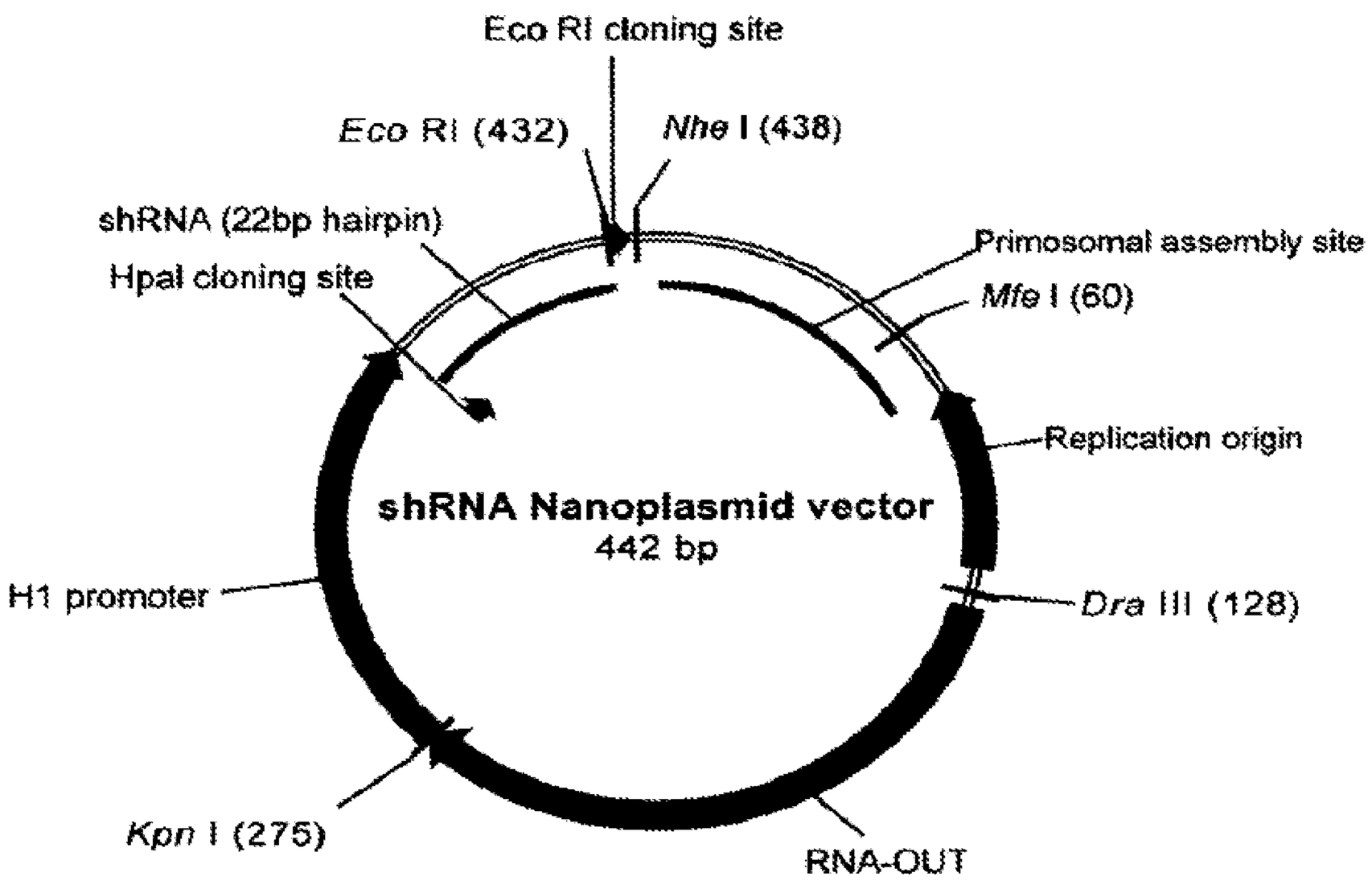
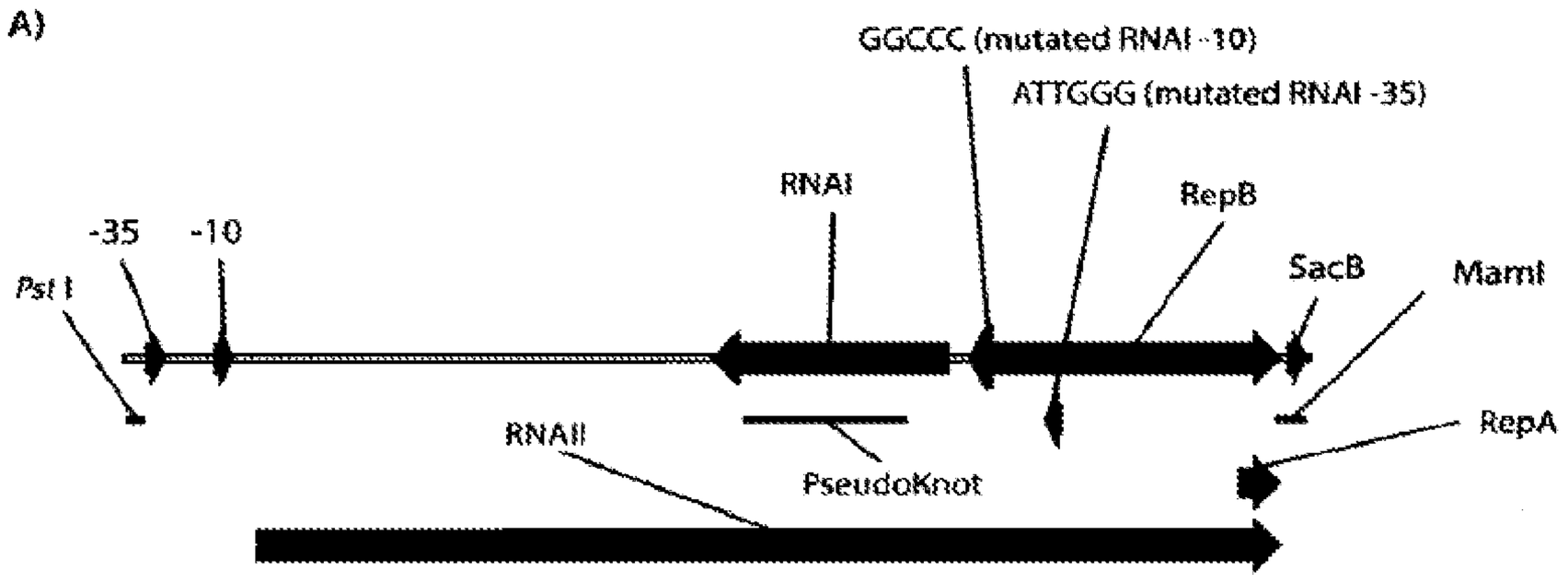

Eco RI cloning site
Eco RI (432)
Nhe I (438)
shRNA (22bp hairpin)
HpaI cloning site
Primosomal assembly site
Mfe I (60)
Replication origin
shRNA Nanoplasmid vector
442 bp
H1 promoter
Dra III (128)
Kpn I (275)
RNA-OUT

FIGURE 12


A)
GGCCC (mutated RNAI -10)
ATTGGG (mutated RNAI -35)
RNAI
RepB
-35
-10
Psi I
SacB
MamI
RepA
RNAII
PseudoKnot
IncB RNAII-SacB synthetic gene
362 bp
B)
Dra III
-35
-10
RNAI
Kpn I
IncB RNAI
124 bp

DNA PLASMIDS WITH IMPROVED EXPRESSION

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation of U.S Pat. Application No. 17/112,918, filed Dec. 4, 2020, which is a reissue of U.S. Pat. Application No. 15/375,215, filed Dec. 12, 2016, now U.S. Pat. 10,144,935, issued Dec. 4, 2018, which is a division of U.S. Pat. Application No. 14/422,865, filed Feb. 20, 2015, now U.S. Pat. 9,550,998, issued Jan. 24, 2017, which is a 371 U.S. National Phase Application of International Application Number PCT/US2013/000068, filed Mar. 14, 2013 which claims the benefit of U.S. Provisional Application No. 61/743,219, filed Aug. 29, 2012 entitled "DNA Plasmids With Improved Expression". The entire disclosures of each of the above-identified applications are incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was supported in part with government support under Grant No. R44GM080768, awarded by the National Institutes of Health. The government has certain rights in this invention.

INCORPORATION-BY- REFERENCE OF A SEQUENCE LISTING

[0003] The sequence listing contained in the file "85535-372741_Sequence_Listing.xml", created on Dec. 14, 2022, file size 85,575 bytes, is incorporated by reference in its entirety herein.

FIELD OF THE INVENTION

[0004] The present invention relates to a family of eukaryotic expression plasmids useful for gene therapy, obtaining improved genetic immunization, natural interferon production, and more particularly, for improving the expression of plasmid encoded antigens, therapeutic proteins and RNAs.

[0005] The present invention also relates to the production of covalently closed circular (ccc) recombinant DNA molecules such as plasmids, cosmids, bacterial artificial chromosomes (BACs), bacteriophages, viral vectors and hybrids thereof, and more particularly to strain modifications that improve production yield of said DNA molecules in fermentation culture.

[0006] Such recombinant DNA molecules are useful in biotechnology, transgenic organisms, gene therapy, therapeutic vaccination, agriculture and DNA vaccines.

BACKGROUND OF THE INVENTION

[0007] *E. coli* plasmids have long been an important source of recombinant DNA molecules used by researchers and by industry. Today, plasmid DNA is becoming increasingly important as the next generation of biotechnology products (e.g., gene medicines and DNA vaccines) make their way into clinical trials, and eventually into the pharmaceutical marketplace. Plasmid DNA vaccines may find application as preventive vaccines for viral, bacterial, or parasitic diseases; immunizing agents for the preparation of hyper immune globulin products; therapeutic vaccines for infectious diseases; or as cancer vaccines. Plasmids are also utilized in gene therapy or gene replacement applications, wherein the desired gene product is expressed from the plasmid after administration to the patient.

[0008] Therapeutic plasmids often contain a pMB1, ColE1 or pBR322 derived replication origin. Common high copy number derivatives have mutations affecting copy number regulation, such as ROP (Repressor of primer gene) deletion, with a second site mutation that increases copy number (e.g. pMB1 pUC G to A point mutation, or ColE1 pMM1). Higher temperature (42° C.) can be employed to induce selective plasmid amplification with pUC and pMM1 replication origins.

[0009] U.S. Pat. No. 7,943,377 (Carnes, A E and Williams, J A, 2011) disclose methods for fed-batch fermentation, in which plasmid-containing *E. coli* cells were grown at a reduced temperature during part of the fed-batch phase, during which growth rate was restricted, followed by a temperature upshift and continued growth at elevated temperature in order to accumulate plasmid; the temperature shift at restricted growth rate improved yield and purity of plasmid. Other fermentation processes for plasmid production are described in Carnes A. E. 2005 *BioProcess Intl*; 3:36-44, which is incorporated herein by reference in its entirety.

[0010] The art teaches that one of the limitations of application of plasmid therapies and plasmid vaccines is regulatory agency (e.g. Food and Drug Administration, EMEC) safety concerns regarding 1) plasmid transfer and replication in endogenous bacterial flora, or 2) plasmid encoded selection marker expression in human cells, or endogenous bacterial flora. Additionally, regulatory agency guidances recommend removal of all non essential sequences in a vector. Plasmids containing a pMB1, ColE1 or pBR322 derived replication origin can replicate promiscuously in *E. coli* hosts. This presents a safety concern that a plasmid therapeutic gene or antigen will be transferred and replicated to a patient's endogenous flora. Ideally, a therapeutic or vaccine plasmid would be replication incompetent in endogenous *E. coli* strains. This requires replacement of the pMB1, ColE1 or pBR322 derived replication origin with a conditional replication origin that requires a specialized cell line for propagation. As well, regulatory agencies such as the EMEA and FDA are concerned with utilization of antibiotic resistance or alternative protein markers in gene therapy and gene vaccine vectors, due to concerns that the gene (antibiotic resistance marker or protein marker) may be expressed in a patients cells. Ideally, plasmid therapies and plasmid vaccines would be 1) replication incompetent in endogenous *E. coli* strains, 2) would not encode a protein based selection marker and 3) be minimalized to eliminate all non essential sequences.

[0011] The art further teaches that one of the limitations of application of plasmid therapies and vaccines is that antigen expression is generally very low. Vector modifications that improve antigen expression (e.g. codon optimization of the gene, inclusion of an intron, use of the strong constitutive CMV or CAGG promoters versus weaker or cell line specific promoter) are highly correlative with improved in vivo expression and, where applicable, immune responses (reviewed in Manoj S, Babiuk LA, van Drunen Little-van den Hurk S. 2004 *Crit Rev Clin Lab Sci* 41: 1-39). A hybrid CMV promoter (CMV/R), which increased antigen expression, also improved cellular immune responses to HIV DNA vaccines in mice and nonhuman primates (Barouch D H,

Yang Z Y, Kong W P, Korioth-Schmitz B, Sumida S M, Truitt D M, Kishko M G, Arthur J C, Miura A, Mascola J R, Letvin N L, Nabel G J. 2005 *J Virol.* 79: 8828-8834). A plasmid containing the woodchuck hepatitis virus posttranscriptional regulatory element (a 600 bp element that increases stability and extranuclear transport of RNA resulting in enhanced levels of mRNA for translation) enhanced antigen expression and protective immunity to influenza hemagglutinin (HA) in mice (Garg S, Oran A E, Hon H, Jacob J. 2004 *J Immunol.* 173: 550-558). These studies teach that improvement in expression beyond that of current CMV based vectors may generally improve immunogenicity and, in the case of gene therapeutics, efficacy.

SUMMARY OF THE INVENTION

[0012] The present invention relates to a family of minimalized eukaryotic expression plasmids that are replication incompetent in endogenous flora and have dramatically improved in vivo expression. These vectors are useful for gene therapy, genetic immunization and or interferon therapy.

[0013] The present invention also relates generally to methods of increasing production yield of covalently closed super-coiled plasmid DNA.

[0014] Improved vectors that utilize novel replication origins that unexpectedly improve antigen expression are disclosed.

[0015] One object of the invention is to provide improved expression plasmid vectors. Yet another object of the invention is to provide methods for improving plasmid copy number.

[0016] According to one object of the invention, a method of increasing expression from an expression plasmid vector comprises modifying the plasmid DNA to replace the pMB1, ColE1 or pBR322 derived replication origin and selectable marker with an alternative replication origin selected from the group consisting of an minimal pUC origin, a R6K gamma replication origin, a ColE2-P9 replication origin, and a ColE2-P9 related replication origin and an RNA selectable marker; transforming the modified plasmid DNA into a bacterial cell line rendered competent for transformation; and isolating the resultant transformed bacterial cells. The resultant plasmid surprisingly has higher in vivo expression levels than the parent pMB1, ColE1 or pBR322 derived replication origin expression plasmid vector.

[0017] According to one object of the invention, a composition for construction of a eukaryotic expression vector comprises an R6K origin with at least 90% sequence identity to the sequence set forth as SEQ ID NO: 1, and a RNA selectable marker, wherein said R6K origin is operably linked to said RNA selectable marker and a eukaryotic region. According to another object of the invention, said R6K origin-RNA selectable marker improves said vector expression in vivo compared to a corresponding vector containing a pMB1, ColE1 or pBR322 derived replication origin. According to still another object of the invention, said vector has at least 95% sequence identity to a sequence selected from the group consisting of: SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 41.

[0018] According to one object of the invention, a composition for construction of a eukaryotic expression vector comprises a ColE2-P9 origin with at least 90% sequence identity to the sequence set forth as SEQ ID NO: 4, 5, 6, or 7, and a a RNA selectable marker, wherein said ColE2-P9 origin—a RNA selectable marker is operably linked to a eukaryotic region. According to another object of the invention, said ColE2-P9 origin-RNA selectable marker improves said vector expression in vivo compared to a corresponding vector containing a pMB1, ColE1 or pBR322 derived replication origin. According to still another object of the invention, said vector has at least 95% sequence identity to a sequence selected from the group consisting of: SEQ ID NO: 8, SEQ ID NO: 9. According to still another object of the invention, a primosomal assembly site (ssiA) is optionally incorporated into the vector adjacent to the ColE2-P9 origin.

[0019] According to another object of the invention, production cell lines are disclosed that improve plasmid yield in shake flask and or fermentation culture with said R6K gamma replication origin, ColE2-P9 replication origin, or ColE2-P9 related replication origin plasmid vectors of the current invention.

[0020] According to another object of the invention, production cell lines providing heat inducible induction of R6K gamma replication origin, ColE2-P9 replication origin, or ColE2-P9 related replication origin plasmid vectors for DNA production are disclosed. These cell lines contain one or more copies of the corresponding R6K gamma replication origin, ColE2-P9 replication origin, or ColE2-P9 related replication protein integrated into the genome and expressed from the group consisting of: the heat inducible P_L promoter (SEQ ID NO: 10), the heat inducible P_L promoter incorporating the OL1-G deletion (SEQ ID NO: 11), the heat inducible P_L promoter incorporating the OL1-G to T substitution (SEQ ID NO: 12).

[0021] According to another object of the invention, mutant R6K replication proteins that improve heat inducible induction of R6K gamma replication origin vectors are disclosed. These cell lines contain one or more copies of the mutant R6K gamma replication origin replication protein integrated into the genome and expressed from the group consisting of: the heat inducible P_L promoter (SEQ ID NO: 10), the heat inducible P_L promoter incorporating the OL1-G deletion (SEQ ID NO: 11), the heat inducible P_L promoter incorporating the OL1-G to T substitution (SEQ ID NO: 12). The mutant R6K gamma replication origin replication protein are selected from the group consisting of: P42L-P113S (SEQ ID NO: 13), P42L-P106L-F107S (SEQ ID NO: 14).

[0022] According to another object of the invention, a mutant ColE2-P9 replication protein that improve heat inducible induction of ColE2-P9 replication origin vectors is disclosed. These cell lines contain one or more copies of the mutant ColE2-P9 replication origin replication protein integrated into the genome and expressed from the group consisting of: the heat inducible P_L promoter (SEQ ID NO: 10), the heat inducible P_L promoter incorporating the OL1-G deletion (SEQ ID NO: 11), the heat inducible P_L promoter incorporating the OL1-G to T substitution (SEQ ID NO: 12). The mutant ColE2-P9 replication origin replication protein is ColE2-P9 Rep mut G194D (SEQ ID NO: 16).

[0023] Further objects and advantages of the invention will become apparent from a consideration of the drawings and ensuing description.

BRIEF DESCRIPTION OF THE FIGURES

[0024] FIG. **1** depicts the NTC8685 pUC origin expression vector;
[0025] FIG. **2** depicts the NTC9685C ColE2 origin expression vector;
[0026] FIG. **3** depicts the NTC9685R R6K origin expression vector;
[0027] FIG. **4** depicts the NTC9385C ColE2 origin expression vector;
[0028] FIG. **5** depicts the NTC9385R R6K origin expression vector;
[0029] FIG. **6** depicts the pINT pR pL R6K Rep pi P42L-P106L- F107S (P3-) integration vector;
[0030] FIG. **7** depicts SEQ ID NO: 44, which includes the P_L promoter with OL1 mutations OL1-G and OL1-G to T;
[0031] FIG. **8** depicts the pINT pR pL ColE2 Rep protein integration vector;
[0032] FIG. **9** shows Nanoplasmid expression in vitro after lipofectamine transfection of HEK293 cell line;
[0033] FIG. **10** shows Nanoplasmid expression in vivo after intramuscular injection with EP;
[0034] FIG. **11** depicts a ColE2 origin Nanoplasmid shRNA expression vector; and
[0035] FIG. **12** depicts an IncB RNAI based RNA selectable marker.
[0036] Table 1: P_L promoter with OL1 mutations OL1-G and OL1-G to T improve plasmid yields in HyperGRO fermentation
[0037] Table 2: NTC9385R-EGFP LB media shake flask production yields in R6K production strains
[0038] Table 3: NTC9385C-Luc plasmid performance in different processes and production cell lines
[0039] Table 4: ColE2 Origin EGFP vector production in NTC701131 ColE2 production cell line
[0040] Table 5: NTC9382C, NTC9385C, NTC9382R, NTC9385R, NTC9682C, NTC9685C, NTC9682R, and NTC9685R vectors
[0041] Table 6: gWIZ and NTC9385C Nanoplasmid expression compared to NTC8685
[0042] Table 7: SR vector expression in vitro and in vivo
[0043] Table 8: RNA Pol III Nanoplasmid vector expression
[0044] Table 9: High level expression is obtained with pMB1 RNAI or RNA-OUT antisense RNA vectors
[0045] SEQ ID NO: 1: R6K gamma Origin
[0046] SEQ ID NO: 2: NTC9385R vector backbone
[0047] SEQ ID NO: 3: NTC9685R vector backbone
[0048] SEQ ID NO: 4: ColE2 Origin (+7)
[0049] SEQ ID NO: 5: ColE2 Origin (+7, CpG free)
[0050] SEQ ID NO: 6: ColE2 Origin (Min)
[0051] SEQ ID NO: 7: ColE2 Origin (+16)
[0052] SEQ ID NO: 8: NTC9385C vector backbone
[0053] SEQ ID NO: 9: NTC9685C vector backbone
[0054] SEQ ID NO: 10: P_L Promoter (-35 to -10)
[0055] SEQ ID NO: 11: P_L Promoter OL1-G (-35 to -10)
[0056] SEQ ID NO: 12: P_L Promoter OL1-G to T(-35 to -10)
[0057] SEQ ID NO: 13: R6K Rep protein P42L-P113S
[0058] SEQ ID NO: 14: R6K Rep protein P42L-P106L-F107S
[0059] SEQ ID NO: 15: ColE2 Rep protein (wild type)
[0060] SEQ ID NO: 16: ColE2 Rep protein mut (G194D)
[0061] SEQ ID NO: 17: pINT pR pL R6K Rep piP42L-P106L- F107S (P3-)
[0062] SEQ ID NO: 18: pINT pR pL ColE2 Rep protein mut (G194D)
[0063] SEQ ID NO: 19: NTC9385R and NTC9685R Bacterial region. [NheI site-trpA terminator-R6K Origin-RNA-OUT- KpnI site]
[0064] SEQ ID NO: 20: NTC9385C and NTC9685C Bacterial region. [NheI site-ssiA-ColE2 Origin (+7)-RNA-OUT-KpnI site]
[0065] SEQ ID NO: 21: NTC9385C and NTC9685C CpG free ssiA [from plasmid R6K]
[0066] SEQ ID NO: 22: CpG free R6K origin
[0067] SEQ ID NO: 23: RNA-OUT selectable marker from NTC9385C, NTC9685C, NTC9385R, and NTC9685R
[0068] SEQ ID NO: 24: RNA-OUT Sense strand RNA from NTC9385C, NTC9685C, NTC9385R, NTC9685R, and NTC9385Ra
[0069] SEQ ID NO: 25: TPA secretion sequence
[0070] SEQ ID NO: 26: PCR primer 15061101
[0071] SEQ ID NO: 27: PCR primer 15061102
[0072] SEQ ID NO: 28: ColE2 core replication origin
[0073] SEQ ID NO: 29: +7(CpG free)-ssiA ColE2 origin
[0074] SEQ ID NO: 30: HTLV-IR-Rabbit β globin hybrid intron
[0075] SEQ ID NO: 31: pMB1 RNAI antisense repressor RNA (origin antisense partner of RNAII)
[0076] SEQ ID NO: 32: pMB1 RNAI selectable Marker, RNAI RNA (Sense strand)
[0077] SEQ ID NO: 33: IncB RNAI antisense repressor RNA (IncB plasmid origin RNAII antisense partner)
[0078] SEQ ID NO: 34: IncB RNAI selectable Marker. DraIII-KpnI restriction fragment
[0079] SEQ ID NO: 35: IncB RNAII-SacB. PstI-MamI restriction fragment
[0080] SEQ ID NO: 36: CpG free RNA-OUT selection marker—flanked by KpnI and Bg1II-EcoRI sites
[0081] SEQ ID NO: 37: CpG free R6K gamma—RNA-OUT bacterial region (CpG free R6K origin-CpG free RNA-OUT selection marker)—flanked by EcoRI-SphI and Bglll-EcoRI sites
[0082] SEQ ID NO: 38: CpG free ColE2 bacterial region (CpG free ssiA-CpG free ColE2 origin-CpG free RNA-OUT selection marker)-flanked by EcoRI-SphI and BglII-EcoRI sites
[0083] SEQ ID NO: 39: NTC9385Ra-02 vector backbone
[0084] SEQ ID NO: 40: NTC9385Ra-01 vector backbone
[0085] SEQ ID NO: 41: NTC9385R-BE vector backbone
[0086] SEQ ID NO: 42: P_{min} minimal pUC replication origin
[0087] SEQ ID NO: 43: pUC (0.85) Bacterial region [NheI site—trpA terminator-P_{min} pUC replication origin (minimal)- RNA-OUT-KpnI site]
[0088] SEQ ID NO: 44: artificial sequence including a P_L promoter

DEFINITION OF TERMS

[0089] A_{405}: Absorbance at 405 nanometers
[0090] AF: Antibiotic-free
[0091] APC: Antigen Processing Cell, for example, langerhans cells, plasmacytoid or conventional dendritic cells
[0092] Approximately: As used herein, the term "approximately" or "about," as applied to one or more values of interest, refers to a value that is the same or similar to a stated reference value
[0093] BAC: Bacterial artificial chromosome

[0094] Bacterial region: Region of a plasmid vector required for propagation and selection in the bacterial host
[0095] BE: Boundary element: Eukaryotic sequence that that blocks the interaction between enhancers and promoters. Also referred to as insulator element. An example is the AT-rich unique region upstream of the CMV enhancer Spe1 site that can function as an insulator/boundary element (Angulo A, Kerry D, Huang H, Borst E M, Razinsky A, Wu J et al. 2000 *J Virol* 74: 2826-2839)
[0096] bp: basepairs
[0097] ccc: Covalently Closed Circular
[0098] cI: Lambda repressor
[0099] cITs857: Lambda repressor further incorporating a C to T (Ala to Thr) mutation that confers temperature sensitivity. cITs857 is a functional repressor at 28-30° C., but is mostly inactive at 37-42° C. Also called cI857
[0100] Cm^R: Chloramphenicol resistance
[0101] cmv: Cytomegalovirus
[0102] CMV promoter boundary element: AT-rich region of the human cytomegalovirus (CMV) genome between the UL127 open reading frame and the major immediate-early (MIE) enhancer. Also referred to as unique region (Angulo et al. Supra, 2000)
[0103] ColE2-P9 replication origin: a region which is specifically recognized by the plasmid-specified Rep protein to initiate DNA replication. Includes but not limited to ColE2-P9 replication origin sequences disclosed in SEQ ID NO: 4: ColE2 Origin (+7), SEQ ID NO: 5: ColE2 Origin (+7, CpG free), SEQ ID NO: 6: ColE2 Origin (Min) and SEQ ID NO: 7: ColE2 Origin (+16) and replication functional mutations as disclosed in Yagura et al 2006, *J Bacteriol* 188:999-1010 included herein by reference
[0104] ColE2 related replication origin: The ColE2-P9 origin is highly conserved across the ColE2-related plasmid family. Fifteen ColE2 related plasmid members including ColE3 are compared in Eiiraga et al 1994, *J Bacteriol*. 176:7233 and 53 ColE2 related plasmid members including ColE3 are compared in Yagura et al Supra, 2006. These sequences are included herein by reference
[0105] ColE2-P9 plasmid: a circular duplex DNA molecule of about 7 kb that is maintained at about 10 to 15 copies per host chromosome. The plasmid encodes an initiator protein (Rep protein), which is the only plasmid-specified trans-acting factor essential for ColE2-P9 plasmid replication ColE2-P9 replication origin RNA-OUT bacterial region: Contains a ColE2-P9 replication origin for propagation and the RNA-OUT selection marker. Optionally includes a PAS, for example, the R6K plasmid CpG free ssiA primosomal assembly site (SEQ ID NO: 21) or alternative ØX174 type or ABC type primosomal assembly sites, such as those disclosed in Nomura et al 1991 Gene 108:15
[0106] ColE2 plasmid: NTC9385C and NTC9685C vectors disclosed herein, as well as modifications and alternative vectors containing a ColE2-P9 replication origin delivery methods: Methods to deliver gene vectors [e.g. poly(lactide-co-glycolide) (PLGA), ISCOMs, liposomes, niosomes, virosomes, chitosan, and other biodegradable polymers, electroporation, piezoelectric permeabilization, sonoporation, ultrasound, corona plasma, plasma facilitated delivery, tissue tolerable plasma, laser microporation, shock wave energy, magnetic fields, contactless magneto-permeabilization, gene gun, microneedles, naked DNA injection, hydrodynamic delivery, high pressure tail vein injection, needle free biojector, liposomes, microparticles, microspheres, nanoparticles, virosomes, bacterial ghosts, bacteria, attenuated bacteria, etc] as known in the art and included herein by reference
[0107] DNA replicon: A genetic element that can replicate under its own control; examples include plasmids, cosmids, bacterial artificial chromosomes (BACs), bacteriophages, viral vectors and hybrids thereof
[0108] *E. coli*: *Escherichia coli*, a gram negative bacteria
[0109] EGFP: Enhanced green fluorescent protein
[0110] EP: Electroporation
[0111] Eukaryotic expression vector: A vector for expression of mRNA, protein antigens, protein therapeutics, shRNA, RNA or microRNA genes in a target organism
[0112] Eukaryotic region: The region of a plasmid that encodes eukaryotic sequences and/or sequences required for plasmid function in the target organism. This includes the region of a plasmid vector required for expression of one or more transgenes in the target organism including RNA Pol II enhancers, promoters, transgenes and polyA sequences. A eukaryotic region may express protein or RNA genes using one or more RNA Pol II promoters, or express RNA genes using one or more RNA Pol III promoters or encode both RNA Pol II and RNA Pol III expressed genes. Additional functional eukaryotic region sequences include RNA Pol I or RNA Pol III promoters, RNA Pol I or RNA Pol III expressed transgenes or RNAs, transcriptional terminators, S/MARs, boundary elements, etc
[0113] FU: Fluorescence units
[0114] g: Gram, kg for kilogram
[0115] Hr(s): Hour(s)
[0116] HTLV-I R: HTLV-I R 5′ untranslated region (UTR). Sequences and compositions were disclosed in Williams, J A 2008 World Patent Application W02008153733 and included herein by reference
[0117] IM: Intramuscular
[0118] immune response: Antigen reactive cellular (e.g. antigen reactive T cells) or antibody (e.g. antigen reactive IgG) responses
[0119] IncB RNAI: plasmid pMU720 origin encoded RNAI (SEQ ID NO: 33) that represses RNA II regulated targets (Wilson IW, Siemering K R, Praszkier J, Pittard A J. 1997. J Bacteriol 179:742)
[0120] kan: Kanamycin
[0121] kanR: Kanamycin Resistance gene
[0122] Kd: Kilodalton
[0123] kozak sequence: Optimized sequence of consensus DNA sequence gccRccATG (R=G or A) immediately upstream of an ATG start codon that ensures efficient tranlation initiation. A Sail site (GTCGAC) immediately upstream of the ATG start codon (GTCGACATG) is an effective Kozak sequence Minicircle: Covalently closed circular plasmid derivatives in which the bacterial region has been removed from the parent plasmid by in vivo or in vitro intramolecular (cis-) site specific recombination or in vitro restriction digestion/ligation
[0124] mSEAP: Murine secreted alkaline phosphatase Nanoplasmid vector: Vector combining an RNA selection marker with a R6K or ColE2 related replication origin. For example, NTC9385C, NTC9685C, NTC9385R, NTC9685R, NTC9385R-BE, NTC9385Ra-01 and NTC9385Ra-02 vectors described herein and modifications thereof
[0125] NTC7382 promoter: A chimeric promoter comprising the CMV enhancer-CMV promoter-HTLV R-syn-

thetic rabbit β globin 3′ intron acceptor-exon 2-SRF protein binding site-kozak sequence, with or without an upstream SV40 enhancer. The creation and application of this chimeric promoter is disclosed in Williams J A Supra, 2008 and included herein by reference

[0126] NTC8385: NTC8385 and NTC8685 plasmids are antibiotic-free vectors that contain a short RNA (RNA-OUT) selection marker in place of the antibiotic resistance marker (kanR) The creation and application of these RNA-OUT based antibiotic-free vectors are disclosed in Williams, J A Supra, 2008 and included herein by reference

[0127] NTC8685: NTC8685 (FIG. **1**) is an antibiotic-free vector that contains a short RNA (RNA-OUT) selection marker in place of the antibiotic resistance marker (kanR) The creation and application of NTC8685 is disclosed in Williams, J A 2010 U.S. Pat. Application 20100184158 and included herein by reference

[0128] OL1: Lambda repressor binding site in the P_L promoter (FIG. **7**). Repressor binding to OL1 is altered by mutations in OL1, such as OL1-G (FIG. **7**; this is a single base deletion that also reduces the distance between the P_L promoter -35 and -10 boxes from optimal 17 bp to 16 bp) and OL1-G to T (FIG. **7**; this is a G to T substitution that maintains the distance between the P_L promoter -35 and -10 boxes at the optimal 17 bp; this is the V2 mutation described by Bailone A and Galibert F, 1980. *Nucleic Acids Research* 8:2147)

[0129] OD_{600}: optical density at 600 nm

[0130] PAS: Primosomal assembly site. Priming of DNA synthesis on a single stranded DNA ssi site. ØX174 type PAS: DNA hairpin sequence that binds priA, which, in turn, recruits the remaining proteins to form the preprimosome [priB, dnaT, recruits dnaB (delivered by dnaC)], which then also recruits primase (dnaG), which then, finally, makes a short RNA substrate for DNA polymerase I. ABC type PAS: DNA hairpin binds dnaA, recruits dnaB (delivered by dnaC) which then also recruits primase (dnaG), which then, finally, makes a short RNA substrate for DNA polymerase I. See Masai et al, 1990 *J Biol Chem* 265:15134. For example, the R6K plasmid CpG free ssiA primosomal assembly site (SEQ ID NO: 21) or alternative ØX174 type or ABC type primosomal assembly sites, such as those disclosed in Nomura et al Supra, 1991

[0131] PAS-BH: Primosomal assembly site on the heavy (leading) strand

[0132] PAS-BH region: pBR322 origin region between ROP and PAS-BL (approximately pBR322 2067-2351)

[0133] PAS-BL: Primosomal assembly site on the light (lagging) strand

[0134] PBS: Phosphate buffered Saline

[0135] PCR: Polymerase Chain Reaction

[0136] pDNA: Plasmid DNA

[0137] pINT pR pL vector: The pINT pR pL integration expression vector is disclosed in Luke et al 2011 *Mol Biotechnol* 47:43 and included herein by reference. The target gene to be expressed is cloned downstream of the pL1 promoter (FIG. **7**). The vector encodes the temperature inducible cI857 repressor, allowing heat inducible target gene expression. P_L promoter: Lambda promoter left (FIG. **7**). P_L is a strong promoter that is repressed by the cI repressor binding to OL1, OL2 and OL3 repressor binding sites. The temperature sensitive cI857 repressor allows control of gene expression by heat induction since at 30° C. the cI857 repressor is functional and it represses gene expression, but at 37-42° C. the repressor is inactivated so expression of the gene ensues

[0138] Plasmid: An extra chromosomal DNA molecule separate from the chromosomal DNA which is capable of replicating independently from the chromosomal DNA

[0139] pMB1 RNAI: pMB1 plasmid origin encoded RNAI that represses RNAII regulated targets (SEQ ID NO: 31; SEQ ID NO: 32) that represses RNAII regulated targets such as those described in Grabherr R, Pfaffenzeller I. 2006 U.S. Pat. Application US20060063232 and Cranenburgh R M. 2009; U.S. Pat. No. 7,611,883

[0140] P_{min}: Minimal 678 bp pUC replication origin SEQ ID NO: 42 and functional variants with base substitutions and/or base deletions. Vectors described herein incorporating P_{min} include NTC8385-Min and NTC8885MP-U6

[0141] Pol: Polymerase

[0142] polyA: Polyadenylation signal or site. Polyadenylation is the addition of a poly(A) tail to an RNA molecule. The poly-adenylation signal is the sequence motif recognized by the RNA cleavage complex. Most human polyadenylation sites contain an AAUAAA motif and conserved sequences 5′ and 3′ to it. Commonly utilized polyA sites are derived from the rabbit β globin (NTC8685; FIG. **1**), bovine growth hormone (gWIZ; pVAXl), SV40 early, or 5V40 late polyA signals pUC replication origin: pBR322-derived replication origin, with G to A transition that increases copy number at elevated temperature and deletion of the ROP negative regulator

[0143] pUC plasmid: Plasmid containing the pUC origin

[0144] R6K plasmid: NTC9385R, NTC9685R, NTC9385Ra-O1 and RNA9385Ra-O2 vectors disclosed herein, as well as modifications, and alternative R6K vectors known in the art including but not limited to pCOR vectors (Gencell), pCpG- free vectors (Invivogen), and CpG free University of Oxford vectors including pGM169

[0145] R6K replication origin: a region which is specifically recognized by the plasmid-specified Rep protein to initiate DNA replication. Includes but not limited to R6K replication origin sequence disclosed as SEQ ID NO: 1: R6K Origin, and CpG free versions (SEQ ID NO: 22) as disclosed in Drocourt et al U.S. Pat. No. 7,244,609 and incorporated herein by reference

[0146] R6K replication origin-RNA-OUT bacterial region: Contains a R6K replication origin for propagation and the RNA-OUT selection marker

[0147] Rep: Replication

[0148] Rep protein dependent plasmid: A plasmid in which replication is dependent on a replication (Rep) protein provided in Trans. For example, R6K replication origin, ColE2-P9 replication origin and ColE2 related replication origin plasmids in which the Rep protein is expressed from the host strain genome. Numerous additional Rep protein dependent plasmids are known in the art, many of which are summarized in del Solar et al 1998 *Microbiol. Mol. Biol. Rev* 62:434-464 which is included herein by reference

[0149] RNA-IN: Insertion sequence 10 (IS10) encoded RNA-IN, an RNA complementary and antisense to RNA-OUT. When RNA-IN is cloned in the untranslated leader of a mRNA, annealing of RNA-IN to RNA-OUT reduces translation of the gene encoded downstream of RNA-IN

[0150] RNA-IN regulated selection marker: A genomically expressed RNA-IN regulated selectable marker. In the presence of plasmid borne RNA-OUT, expression of a protein encoded downstream of RNA-IN is repressed. An

RNA-IN regulated selection marker is configured such that RNA-IN regulates either 1) a protein that is lethal or toxic to said cell per se or by generating a toxic substance (e.g. SacB), or 2) a repressor protein that is lethal or toxic to said bacterial cell by repressing the transcription of a gene that is essential for growth of said cell (e.g. murA essential gene regulated by RNA-IN tetR repressor gene). For example, genomically expressed RNA-IN-SacB cell lines for RNA-OUT plasmid propagation are disclosed in Williams, J A Supra, 2008 (SEQ ID NO: 23) and included herein by reference. Alternative selection markers described in the art may be substituted for SacB

[0151] RNA-OUT: Insertion sequence 10 (IS 10) encoded RNA- OUT (SEQ ID NO: 24), an antisense RNA that hybridizes to, and reduces translation of, the transposon gene expressed downstream of RNA-IN. The sequence of the core RNA- OUT sequence (SEQ ID NO: 24) and complementary RNA- IN SacB genomically expressed RNA-IN-SacB cell lines can be modified to incorporate alternative functional RNA-IN/RNA-OUT binding pairs such as those disclosed in Mutalik et al. 2012 *Nat Chem Biol* 8:447, including, but not limited to, the RNA-OUT A08/RNA-IN S49 pair, the RNA-OUT A08/RNA-IN S08 pair, and CpG free modifications of RNA-OUT A08 that modify the CG in the RNA-OUT 5′ TT CGCT sequence to a non-CpG sequence

[0152] RNA-OUT Selectable marker: An RNA-OUT selectable marker DNA fragment including *E. coli* transcription promoter and terminator sequences flanking an RNA-OUT RNA. An RNA-OUT selectable marker, utilizing the RNA-OUT promoter and terminator sequences, that is flanked by DraIII and KpnI restriction enzyme sites, and designer genomically expressed RNA-IN-SacB cell lines for RNA-OUT plasmid propagation, are disclosed in Williams, J A Supra, 2008 (SEQ ID NO: 23) and included herein by reference. The RNA-OUT promoter and terminator sequences flanking the RNA-OUT RNA may be replaced with heterologous promoter and terminator sequences. For example, the RNA-OUT promoter may be substituted with a CpG free promoter known in the art, for example the I-EC2K promoter or the P⅝ ⅝ or P⅝ 6/6 promoters disclosed in Williams, J A Supra, 2008 and included herein by reference

[0153] RNA selectable marker: also RNA selection marker. An RNA selectable marker is a plasmid borne expressed non translated RNA that regulates a chromosomally expressed target gene to afford selection. This may be a plasmid borne nonsense suppressing tRNA that regulates a nonsense suppressible selectable chromosomal target as described by Crouzet J and Soubrier F 2005 U.S. Pat. No. 6,977,174 included herein by reference. This may also be a plasmid borne antisense repressor RNA, a non limiting list included herein by reference includes RNA-OUT that represses RNA-IN regulated targets, pMB1 plasmid origin encoded RNAI that represses RNAII regulated targets (SEQ ID NO: 31; SEQ ID NO: 32; Grabherr and, Pfaffenzeller Supra, 2006; Cranenbuigh R M. Supra, 2009), IncB plasmid pMU720 origin encoded RNAI that represses RNA II regulated targets (SEQ ID NO: 33; SEQ ID NO: 34; Wilson et al Supra, 1997), ParB locus Sok of plasmid R1 that represses Hok regulated targets, Flm locus FlmB of F plasmid that represses flmA regulated targets (Morsey M A, 1999 U.S. Pat. No. 5,922,583). An RNA selectable marker may be another natural antisense repressor RNAs known in the art such as those described in Wagner E G H, Altuvia S, Romby P. 2002. *Adv Genet* 46:361 and Franch T, and Gerdes K. 2000. *Current Opin Microbiol* 3:159. An RNA selectable marker may also be an engineered repressor RNAs such as synthetic small RNAs expressed SgrS, MicC or MicF scaffolds as described in Na D, Yoo S M, Chung H, Park H, Park J H, Lee S Y. 2013. *Nat Biotechnol* 31:170

[0154] ROP: Repressor of primer

[0155] sacB: Structural gene encoding *Bacillus subtilis levansucrase*. Expression of sacB in gram negative bacteria is toxic in the presence of sucrose

[0156] SEAP: Secreted alkaline phosphatase

[0157] shRNA: Short hairpin RNA

[0158] SR: Spacer region. As used herein, spacer region is the region linking the 5′ and 3′ ends of the eukaryotic region sequences. The eukaryotic region 5′ and 3′ ends are typically separated by the replication origin and selection marker. In simple single RNA Pol II transcription vectors this will be between the RNA Pol II promoter region (5′ to a promoter, enhancer, boundary element, S/MAR) and the RNA Pol II polyA region (3′ to a polyA sequence, eukaryotic transcriptional terminator sequence, boundary element, S/MAR). For example, in NTC9385R (FIG. **5**) the spacer region is region between NheI site at 1663 and KpnI site at 460. In dual RNA Pol II transcription vectors, the eukaryotic sequences separated by the spacer will depend on the orientation of the two transcription elements. For example, with divergent or convergent RNA Pol II transcription units, the spacer region may separate two polyA sequences or two enhancers respectively. In RNA Pol II, RNA Pol III dual expression vectors, the spacer region may separate an RNA Pol II enhancer and a RNA Pol III promoter. The spacer region may encode bacterial or eukaryotic selectable markers, bacterial transcription terminators, eukaryotic transcription terminators, boundary elements, S/MARs, RNA Pol I or RNA Pol III expressed sequences or other functionalities

[0159] ssi: Single stranded initiation sequences

[0160] SV40 enhancer: Region containing the 72 bp and optionally the 21 bp repeats

[0161] target antigen: Immunogenic protein or peptide epitope, or combination of proteins and epitopes, against which an immune response can be mounted. Target antigens may by derived from a pathogen for infectious disease applications, or derived from a host organism for applications such as cancer, allergy, or autoimmune diseases. Target antigens are well defined in the art. Some examples are disclosed in Williams, Supra, 2008 and are included herein by reference TE buffer: A solution containing approximately 10 mM Tris pH 8 and 1 mM EDTA

[0162] Transcription terminator: Bacterial: A DNA sequence that marks the end of a gene or operon for transcription. This may be an intrinsic transcription terminator or a Rho-dependent transcriptional terminator. For an intrinsic terminator, such as the trpA terminator, a hairpin structure forms within the transcript that disrupts the mRNA-DNA-RNA polymerase ternary complex. Alternatively, Rho-dependent transcriptional terminators require Rho factor, an RNA helicase protein complex, to disrupt the nascent mRNA-DNA-RNA polymerase ternary complex. Eukaryotic: PolyA sites are not ‘terminators’, instead internal cleavage at PolyA sites leaves an uncapped 5′ end on the 3′UTR RNA for nuclease digestion. Nuclease catches up to RNA Pol II and causes termination. Termination can be promoted within a short region of the poly A site by introduction of

RNA Pol II pause sites (eukaryotic transcription terminator). Pausing of RNA Pol II allows the nuclease introduced into the 3′ UTR mRNA after PolyA cleavage to catch up to RNA Pol II at the pause site. A nonlimiting list of eukaryotic transcription terminators know in the art include the C2x4 terminator (Ashfield R, Patel A J, Bossone S A, Brown H, Campbell R D, Marcu K B, Proudfoot N J. 1994. *EMBO J* 13:5656) and the gastrin terminator (Sato K, Ito R, Baek K H, Agarwal K, 1986. *Mol. Cell. Biol.* 6:1032). Terminator element may stabilize mRNA by enhancing proper 3′-end processing of mRNA (Kim D, Kim J D, Baek K, Yoon Y, Yoon J. 2003. *Biotechnol Prog* 19:1620)

[0163] Transgene: Target antigen or protein or RNA gene that is cloned into a vector

[0164] ts: Temperature sensitive

[0165] μg: microgram

[0166] μl: microliter

[0167] UTR: Untranslated region of a mRNA (5′ or 3′ to the coding region)

[0168] VARNA: Adenoviral virus associated RNA, including VAR-NAI (VAI or VA1) and or VARNAII (VAII or VA2) from any Adenovirus serotype, for example, serotype 2, serotype 5 or hybrids thereof

[0169] VARNAI: Adenoviral virus associated RNAI, also referred to as VAI, or VAI, from any Adenovirus serotype, for example, serotype 2, serotype 5 or hybrids thereof

[0170] Vector: A gene delivery vehicle, including viral (e.g. alphavirus, poxvirus, lentivirus, retrovirus, adenovirus, adenovirus related virus, etc) and nonviral (e.g. plasmid, midge, transcriptionally active PCR fragment, minicircles, bacteriophage, etc) vectors. These are well known in the art and are included herein by reference

[0171] Vector backbone: Eukaryotic region and bacterial region of a vector, without the transgene or target antigen coding region

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0172] The invention relates generally to plasmid DNA compositions and methods to improve plasmid expression and plasmid production. The invention can be practiced to improve expression of vectors such as eukaryotic expression plasmids useful for gene therapy, genetic immunization and or interferon therapy. The invention can be practiced to improve the copy number of vectors such as eukaryotic expression plasmids useful for gene therapy, genetic immunization and or interferon therapy. It is to be understood that all references cited herein are incorporated by reference in their entirety.

[0173] According to one preferred embodiment, the present invention provides for method of increasing in vivo expression of transgene from covalently closed super-coiled plasmid DNA, which comprises modifying the plasmid DNA to replace the pMB1, ColE1 or pBR322 derived replication origin and selectable marker with a replication origin selected from the group consisting of an P_{min} minimal pUC replication origin, ColE2-P9 replication origin, ColE2 related replication origin, and R6K replication origin and a RNA selectable marker; transforming the modified plasmid DNA into a Rep protein producing bacterial cell line rendered competent for transformation; and isolating the resultant transformed bacterial cells. The modified plasmid produced from these cells has increased transgene expression in the target organism.

[0174] In one preferred embodiment, the spacer region encoded pMB1, ColE1 or pBR322 derived replication origin is replaced with a CpG free ColE2 origin. In another preferred embodiment, a primosome assembly site is incorporated into a ColE2 plasmid DNA backbone to improve plasmid copy number. In yet another preferred embodiment, the pMB1, ColE1 or pBR322 derived replication origin is replaced with a CpG free R6K origin.

[0175] The methods of plasmid modification of the present invention have been surprisingly found to improve plasmid expression in the target organism. Increased expression vectors may find application to improve the magnitude of DNA vaccination mediated antigen reactive B or T cell responses for preventative or therapeutic vaccination, increase RNA and or protein transgene levels to improve gene replacement therapy or gene knockdown therapy, increase plasmid based expression levels of DNA vector expressed therapeutic antibodies that neutralize infectious diseases such as influenza, EHV, malaria, hepatitis C virus, tuberculosis, etc.

[0176] Plasmid encoded transgene expression in the target organ-ism is preferably increased by employing specific constructs or compositions incorporated in a vector. According to one preferred embodiment, the present invention provides a composition for construction of a vector, comprising a ColE2 origin with at least 90% sequence identity to the sequences set forth as SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, and a RNA selectable marker and a eukaryotic region, wherein the ColE2 origin is operably linked to the RNA selectable marker and eukaryotic region. It has been surprisingly found that this ColE2 origin-RNA selectable marker improves plasmid encoded transgene expression in the target organism. According to another preferred embodiment, the resultant vector of the invention has at least 95% sequence identity to a sequence selected from the group consisting of: SEQ ID NO: 8, SEQ ID NO: 9.

[0177] According to another preferred embodiment, the present invention provides a composition for construction of a vector, comprising an R6K origin with at least 90% sequence identity to the sequences set forth as SEQ ID NO: 1, SEQ ID NO: 22, and a RNA selectable marker and a eukaryotic region, wherein the R6K origin is operably linked to the RNA selectable marker and eukaryotic region. It has been surprisingly found that this R6K origin-RNA selectable marker improves plasmid encoded transgene expression in the target organism. According to another preferred embodiment, the resultant vector of the invention has at least 95% sequence identity to a sequence selected from the group consisting of: SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41.

[0178] As used herein, the term "sequence identity" refers to the degree of identity between any given query sequence, e.g., SEQ ID NO: 2, and a subject sequence. A subject sequence may, for example, have at least 90 percent, at least 95 percent, or at least 99 percent sequence identity to a given query sequence. To determine percent sequence identity, a query sequence (e.g., a nucleic acid sequence) is aligned to one or more subject sequences using any suitable sequence alignment program that is well known in the art, for instance, the computer program ClustalW (version 1.83, default parameters), which allows alignments of nucleic

acid sequences to be carried out across their entire length (global alignment). Chema et al., 2003 *Nucleic Acids Res.*, 31:3497-500. In a preferred method, the sequence alignment program (e.g., ClustalW) calculates the best match between a query and one or more subject sequences, and aligns them so that identities, similarities, and differences can be determined Gaps of one or more nucleotides can be inserted into a query sequence, a subject sequence, or both, to maximize sequence alignments. For fast pair-wise alignments of nucleic acid sequences, suitable default parameters can be selected that are appropriate for the particular alignment program. The output is a sequence alignment that reflects the relationship between sequences. To further determine percent identity of a subject nucleic acid sequence to a query sequence, the sequences are aligned using the alignment program, the number of identical matches in the alignment is divided by the length of the query sequence, and the result is multiplied by 100. It is noted that the percent identity value can be rounded to the nearest tenth. For example, 78.11, 78.12, 78.13, and 78.14 are rounded down to 78.1, while 78.15, 78.16, 78.17, 78.18, and 78.19 are rounded up to 78.2.

[0179] According to another preferred embodiment, the present invention provides methods and compositions for production of a Rep protein dependent plasmid vector. Production cell lines providing improved heat inducible P_L promoter expression of a Rep protein integrated into the genome and expressed from the heat inducible P_L promoter incorporating the OL1-G deletion (SEQ ID NO: 11), or the heat inducible P_L promoter incorporating the OL1-G to T substitution (SEQ ID NO: 12). It has been surprisingly found that these promoter modifications improves Rep protein dependent plasmid vector copy number in shake flask and fermentation cultures.

[0180] Turning now to the drawings, FIG. **1**. shows an annotated map of the antibiotic free NTC8685 pUC origin expression vector with the locations of the pUC origin, PAS-BH primosomal assembly site, SV40 enhancer and other key elements indicated. The replication origin (PAS-BH and pUC origin) is from the AgeI (230) site to the DraIII (1548) site (1318 bp total). The antibiotic free RNA-OUT selection marker is between the DraIII (1548) and KpnI (1695) sites (147 bp total). The spacer region encoded bacterial region (replication and selection) of this vector is 1465 bp.

[0181] FIG. **2** shows an annotated map of the antibiotic-free NTC9685C ColE2 origin expression vector with the locations of the primosomal assembly site, ColE2 Replication origin (Replication origin) and other key elements indicated. The spacer region encoded bacterial region (replication and selection) of this vector is 281 bp [NheIsite-ssiA-ColE2 Origin (+7)-RNA-OUT-KpnI site] (SEQ ID NO: 20).

[0182] FIG. **3** shows an annotated map of the antibiotic-free NTC9685R R6K origin expression vector with the locations of the primosomal assembly site, R6K Replication origin (R6K mini-origin) and other key elements indicated. The spacer region encoded bacterial region (replication and selection) of this vector is 466 bp [NheIsite-trpA terminator-R6K Origin-RNA-OUT-KpnI site] (SEQ ID NO: 19).

[0183] FIG. **4** shows an annotated map of the antibiotic-free NTC9385C ColE2 origin expression vector with the locations of the primosomal assembly site, ColE2 Replication origin (Replication origin) and other key elements indicated. The spacer region encoded bacterial region (replication and selection) of this vector is 281 bp [NheIsite-ssiA-ColE2 Origin (+7)-RNA-OUT-KpnI site] (SEQ ID NO: 20). This vector differs from NTC9685C in that the VA1 RNA and SV40 enhancer are not present.

[0184] FIG. **5** shows an annotated map of the antibiotic-free NTC9385R R6K origin expression vector with the locations of the primosomal assembly site, R6K Replication origin (R6K mini-origin) and other key elements indicated. The spacer region encoded bacterial region (replication and selection) of this vector is 466 bp [NheIsite-trpA terminator-R6K Origin-RNA-OUT-KpnI site] (SEQ ID NO: 19). This vector differs from NTC9685R in that the VA1 RNA and SV40 enhancer are not present.

[0185] FIG. **6** shows an annotated map of the pINT pR pL R6K Rep pi P42L-P106L-F107S (P3-) integration vector; key features such as the c1857 is repressor, P_L promoter, R6K Rep protein, HK022 phage attachment site for site specific integration into the *E. coli* genome, R6K replication origin and spectinomycin/streptomycin resistance marker (SpecR StrepR) are shown.

[0186] FIG. **7** show an annotated sequence of the P_L promoter with locations of the P_L promoter OL1, OL2 and OL3 repressor binding sites, -10 and -35 promoter elements for P_L1 and P_L2 promoters. The OL1 mutations OL1-G and OL1-G to T alterations are shown.

[0187] FIG. **8** shows an annotated map of the pINT pR pL ColE2 Rep protein integration vector; key features such as the c1857 is repressor, P_L promoter, ColE2 Rep protein, HK022 phage attachment site for site specific integration into the *E. coli* genome, R6K replication origin and spectinomycin/ streptomycin resistance marker (SpecR StrepR) are shown.

[0188] FIG. **9** shows Nanoplasmid expression in vitro after lipofectamine transfection of HEK293 cell line of various EGFP transgene encoding vectors.

[0189] FIG. **10** shows Nanoplasmid expression in vivo after intramuscular injection with EP of various muSEAP transgene encoding vectors.

[0190] FIG. **11** shows a ColE2 origin Nanoplasmid shRNA expression vector. In this vector, a 22 bp shRNA is expressed from the RNA Polymerase III H1 promoter, with a TTTTTT terminator. The bacterial region is the NTC9385C and NTC9685C Bacterial region (SEQ ID NO: 20).

[0191] FIG. **12** shows an IncB RNAI based RNA selection marker. A) Genomically expressed target of IncB RNAI RNA selection marker (SEQ ID NO: 35). Plasmid expressed RNAI binding to the pseudoknot in the complementary genomically expressed RNAII target prevents translation of the downstream SacB gene, conferring sucrose resistance. The RNAI -10 and -35 promoter elements are mutated to prevent RNAI expression. B) Structure of plasmid expressed IncB RNAI RNA selection marker (SEQ ID NO: 34) encoding the IncB RNAI antisense repressor (SEQ ID NO: 33).

[0192] The invention also relates to compositions and methods for producing high expression level plasmids. The present invention provides sequences that, when introduced into a vector backbone, increase plasmid expression.

[0193] The surprising observation that a ColE2 replication origin-RNA selection marker or R6K replication origin-RNA selection marker can be utilized as a plasmid expression enhancer is disclosed.

[0194] As described herein, plasmid expression is increased by replacement of the pMB1, ColE1 or pBR322

derived origin-selection marker bacterial region with an R6K origin-RNA selection marker in the plasmid backbone. In yet another preferred embodiment, the R6K origin is CpG free. In yet another preferred embodiment, the R6K origin is included with an RNA-OUT selection marker. In yet another preferred embodiment, the R6K origin is included with an pMB 1 RNAI selection marker. In yet another preferred embodiment, the R6K origin is included with an IncB RNAI selection marker.

[0195] In yet another preferred embodiment, plasmid expression is increased by replacement of the pMB1, ColE1 orpBR322 derived origin-selection marker bacterial region with a ColE2 origin-RNA selection marker in the plasmid backbone. In yet another preferred embodiment, the ColE2 origin is CpG free. In yet another preferred embodiment, the ColE2 origin is included with an RNA-OUT selection marker. In yet another preferred embodiment, the ColE2 origin is included with an pMB 1 RNAI selection marker. In yet another preferred embodiment, the ColE2 origin is included with an IncB RNAI selection marker. In yet another preferred embodiment, the ColE2 origin is included with a primosome assembly site.

[0196] In yet another preferred embodiment, plasmid expression is increased by replacement of the pMB1, ColE1 or pBR322 derived origin-selection marker with a P_{min} minimal pUC, ColE2 or a R6K origin in the plasmid backbone spacer region and an RNA selection marker in an intron. In yet another preferred embodiment, the R6K or ColE2 origin is CpG free. In yet another preferred embodiment, the RNA selection marker is the RNA-OUT selection marker. In yet another preferred embodiment, the RNA selection marker is the pMB1 RNAI selection marker. In yet another preferred embodiment, the RNA selection marker is the IncB RNAI selection marker.

EXAMPLES

[0197] The methods of the invention are further illustrated by the following examples. These are provided by way of illustration and are not intended in any way to limit the scope of the invention.

Example 1: Heat Inducible R6K Replication Origin Plasmid Production

Fermentation

[0198] Fermentations were performed using proprietary fed-batch media (NTC3019, HyperGRO media) in New Brunswick BioFlo 110 bioreactors as described (Carnes and Williams, Supra, 2011). The seed cultures were started from glycerol stocks or colonies and streaked onto LB medium agar plates containing 6% sucrose. The plates were grown at 30-32° C.; cells were resuspended in media, and used to provide approximately 0.1% inoculums for the fermentations that contained 0.5% sucrose to select for RNA-OUT plasmids.

[0199] Antibiotic-free RNA-OUT plasmid fermentations were performed in *E. coli* strain XLlBlue [recAl endAl gyrA96 thi-1 hsdR17 supE44 relAl lac [F′ proAB lacIqZΔM15TnlO (Tef] (Stratagene, La Jolla, Calif.)] or GT115 [F—mcrA Δ(mrr-hsdRMS-mcrBC) φ801 acZΔM15 ΔlacX74 recAl rspL (StrA) endAl Δdcm uidA(ΔMluI)::pir-116 ΔsbcC-sbcD (Invivogen, San Diego)] strains containing chromosomally integrated pCAH63-CAT RNA-IN-SacB (P⅚ 6/6) at the phage lambda integration site as disclosed in Williams, J A Supra, 2008. SacB (*Bacillus subtilis levansucrase*) is a counterselectable marker which is lethal to *E. coli* cells in the presence of sucrose. Translation of SacB from the RNA-IN-SacB transcript is inhibited by plasmid encoded RNA-OUT. This facilitates plasmid selection in the presence of sucrose, by inhibition of SacB mediated lethality.

Analytical Methods

[0200] Culture samples were taken at key points and at regular intervals during all fermentations. Samples were analyzed immediately for biomass (OD_{600}) and for plasmid yield. Plasmid yield was determined by quantification of plasmid obtained from Qiagen Spin Miniprep Kit preparations as described (Carnes and Williams, Supra, 2011). Briefly, cells were alkaline lysed, clarified, plasmid was column purified, and eluted prior to quantification. Agarose gel electrophoresis analysis (AGE) was performed on 0.8-1% Tris/acetate/ EDTA (TAE) gels as described in Carnes and Williams J A, Supra, 2011.

R6K Background

[0201] The R6K gamma plasmid replication origin requires a single plasmid replication protein it that binds as a monomer to multiple repeated 'iteron' sites (seven core repeats containing TGAGNG consensus) and as a dimer to repressive sites [TGAGNG (dimer repress) as well as to iterons with reduced affinity]. Various host factors are used including IHF, DnaA, and primosomal assembly proteins DnaB, DnaC, DnaG (Abhyankar et al 2003 *J Biol Chem* 278: 45476-45484). The R6K core origin contains binding sites for DnaA and IHF that affect plasmid replication (π, IHF and DnaA interact to initiate replication).

[0202] Different versions of the R6K gamma replication origin have been utilized in various eukaryotic expression vectors, for example pCOR vectors (Soubrier et al 1999, *Gene Therapy* 6:1482) and a CpG free version in pCpGfree vectors (Invivogen, San Diego Calif.), and pGM169 (University of Oxford). Incorporation of the R6K replication origin does not improve expression levels compared to an optimized pUC origin vector (Soubrier et al Supra, 1999). However, use of a conditional replication origin such as R6K gamma that requires a specialized cell line for propagation adds a safety margin since the vector will not replicate if transferred to a patients endogenous flora.

[0203] A highly minimalized R6K gamma derived replication origin that contains core sequences required for replication (including the DnaA box and stb 1-3 sites; Wu et al, 1995. *J Bacteriol.* 177: 6338-6345), but with the upstream π dimer repressor binding sites and downstream π promoter deleted (by removing one copy of the iterons, as with pCpG; see map below) was designed (SEQ ID NO: 1) and NTC9685R and NTC9385R expression vectors incorporating it constructed (see Example 3).

[0204] Typical R6K production strains incorporate the π protein derivative PIR116 that contains a P106L substitution that increases copy number (by reducing π dimerization; π monomers activate while π dimers repress). Fermentation results with pCOR (Soubrier et al., Supra, 1999) and pCpG plasmids (Hebel H L, Cai Y, Davies L A, Hyde S C, Pringle I A, Gill D R. 2008. *Mol Ther* 16: SI 10) were low, around 100 mg/L in PIR116 cell lines.

[0205] As expected, fermentation yields of the R6K expression vector NTC9685R-EGFP in R6K plasmid production cell line NTC641642 (GT115-SacB; GT115 modified for RNA-OUT AF vector selection by insertion of pCAH63-CAT RNA-IN-SacB (P⅚ 6/6) into the genome. The GT115 genome encoded endogenous π gene P3 promoter constitutively expresses R6K replication protein π containing the pir-116 mutation; Metcalf et al, 1994; Gene 138; 1-7) were low (Table 1). Mutagenesis of the pir-116 replication protein and selection for increased copy number has been used to make new production strains. For example, the TEX2pir42 strain contains a combination of P106L and P42L. The P42L mutation interferes with DNA looping replication repression. The TEX2pir42 cell line improved copy number and fermentation yields with pCOR plasmids with reported yields of 205 mg/L (Soubrier F. 2004. World Patent Application WO2004033664). Methods to improve R6K origin yields are needed.

[0206] Other combinations of π copy number mutants have been shown to improve copy number. This includes 'P42L and P113S' and 'P42L, P106L and F107S' (Abhyankar et al 2004. *J Biol Chem* 279:6711-6719).

[0207] Two cell lines using the endogenous π gene P3 promoter to express π mutants 'P42L and P113S' (SEQ ID NO: 13) (NTC640722 cell line) and 'P42L, P106L and F107S' (SEQ ID NO: 14) were constructed and tested for copy number improvement with NTC9685R-EGFP. Two additional cell lines using the P_L promoter in addition to the endogenous π gene P3 promoter to express π mutants 'P42L and P113S' (NTC641981 cell line) and 'P42L, P106L and F107S' (NTC641053 cell line) were made and tested for copy number improvement with NTC9685R-EGFP. R6K production cell lines were made in XL1-Blue SacB (XL1-Blue attλ:P⅚ 6/6-RNAIN-SacB, CmR).

[0208] These cell lines were constructed as follows. The R6K replication proteins π were cloned into the pINT pR pL integration vectors as described in Luke et al Supra, 2011 40 and included herein by reference. Constructed R6K Rep protein vectors were integrated into the genome at the HK022 phage attachment site as described in Luke et al, Supra, 2011. Briefly the pINT pINT pR pL R6K Rep vectors were amplified by PCR to delete the R6K replication origin, 45 ligated to form a circle, and integrated into the HK022 attachment site using the pAH69 helper plasmid as described.

[0209] The results (Table 1) demonstrated that constitutive expression of 'P42L and P113S' or 'P42L, P106L and F107S' resulted in much higher levels of NTC9685R-EGFP than the NTC641642 encoded P106L Rep protein. However, constitutive expression from P3 resulted in low overall biomass and plasmid multimerization with P42L, P106L and F107S (RF306, RF314), due to high plasmid levels and resultant metabolic burden during the growth phase

TABLE 1

2.6 kb NTC9685R-EGFP R6K Nanoplasmid fermentation yields in R6K rep cell lines

Ferm #	Cell line	Rep Gene	Promoter	Growth phase temp (C.)	Growth spec yield[a]	Induced phase temp (C.)	Induced spec yield[a]	Final OD_{600}	Final plasmid yield (mg/L)	Plasmid multimerization
RF310	NTC641642	P106L	Const P3	37	1.1	NA, 37	1.2	43	53	
RF323	NTC641642	P106L	Const P3	37	ND	NA, 37	0.9	54	49	Monomer
RF305	NTC640722	P42L-P1138	Const P3	30	3.4	37	3.6	96	345	Monomer
RF321	NTC641981	P43L-P1138	p8, PL & P3	32	4.4-5.0	43	3.8	93	259	Monomer[b]
RF346	NTC641053	P42L-P186L-P1078	P8, PL & P3	30	3.1	37	6.6	86	567	Monomer
RF314	NTC641083	P42L-P106L-P1075	pR PL & P3	32	2.6-4.7	42	3.1	79	558	Monomer
RF351	NTC661135	P42L-P166L-F1078	p8, PL only	32	0.4	42	1.49	88	128	Monomer
RF326	NTC661138-MLT	P42L-P106L-F107S	pR PL OL1 G	32	0.64	42	6.7	81	548	Monomer
RF358	NTC711055	P42L-P106L-F1078	p8, PL OL1 G	32	1	42	5.9	118	690	Monomer
RF339	NTC711231	P42L-P106L-P1038	p8 PL OL1 G to 7	30	1.8	42	8.8	82	695	Monomer

NTC640321 - NTC5402-P42L-P10L-F1078
NTCM0722 - NTC8402-P42L-P1338
NTC541083 - NTC9332-pK pL P42L-P302S
NTC641642 -Gt15-S32B (c1845) pl136-P106L
NTC641981 - NTC6402 p8 PL P42L-P1338
NTC64135 - NTC54208-p8 pL P42L-P106L-P302S(P3-)
NTC661135-ML1 - NTC64208-p8 pL (pL-3) P42L-P105L-P1078 (P3-)
NTC661134 - NTC6423-pK PL P42L-P3138 (P3-)
NTC513848 - NTC54168-p8 pL (OL1-G) P42L-P108L-P1078 (P3-)
NTC311231 - NTC84208-p8 pL (OL1-G to 7) P42L-P108L-P107S (P3-)
ND - Not discontinued
NA - Not applicable
[a]Specific Yield - ms plasmid LODGO
[b]Some smx ground
NTC6402 - XL181x,SwB
NTC54208 - XL1Ble SwB, dwx

[0210] Heat inducible versions were then made by deletion of the P3 promoter to determine if P_L promoter mediated replication protein induction in a temperature shift improved R6K plasmid production yields and quality by reduction of plasmid copy number and metabolic burden during the reduced temperature growth phase. A strain encoding a deletion of the P3 promoter expressing P42L, P106L and F107S (NTC661135, incorporating a single copy of the pINT pR pL R6K Rep pi P42L-P106L-F107S (P3-) integration vector; FIG. **6**, SEQ ID NO: 17) constructed as described above dramatically reduced copy number during the reduced temperature growth phase with copy number induction after temperature upshift (Table 1; RF351). However, the yield (128 mg/L) was overall lower than with the P3 promoter (567, 558 mg/L).

[0211] However, excellent results were obtained after fermentation with a second NTC661135 cell line (RF326) in which plasmid copy number was increased 10 fold by temperature shifting, resulting in excellent final plasmid yields of 545 mg/L. PCR amplification and sequencing of the P42L, P106L and F107S expression cassette from the RF326 cell line (NTC661135-MUT) and the RF351 cell line (NTC661135) demonstrated that NTC661135-MUT contained a mutation in the OL1 lambda repressor binding site in the P_L promoter (FIG. **7**; OL1-G this is a single base deletion that also reduces the distance between the P_L1 promoter -35 and -10 boxes from optimal 17 bp to 16 bp).

[0212] This mutation was introduced into the pINT pR pL R6K Rep pi P42L-P106L-F107S (P3-) integration vector by PCR mutagenesis and a sequence verified clone incorporating the OL1-G mutation integrated into the genome (NTC711055) as described above. Fermentation evaluation of this cell line with the NTC9685R-EGFP plasmid (Table 1; RF358) dem-onstrated similar dramatic 6 fold heat inducible plasmid copy number induction, resulting in excellent final plasmid yields of 690 mg/L.

[0213] Repressor binding to OL1 is altered by mutations in OL1, such as OL1-G (FIG. **7**; SEQ IDNO:11) and V2 (OL1-G to T; FIG. **7**; SEQ ID NO: 12; this is a G to T substitution that maintains the distance between the P_L promoter -35 and -10 boxes at the optimal 17 bp; this is the V2 mutation described by Bailone and Galibert, Supra, 1980).

[0214] The OL1-G to T (V2) mutation was introduced into the pINT pR pL R6K Rep pi P42L-P106L-F107S (P3-) integration vector by PCR mutagenesis and a sequence verified clone incorporating the OL1-G mutation integrated into the genome as described above to create NTC711231. Fermentation evaluation of this cell line with the NTC9685R-EGFP plasmid (Table 1; RF359) demonstrated, similar to OL1G, a dramatic 5 fold heat inducible plasmid copy number induction, resulting in excellent final plasmid yields of 695 mg/L.

[0215] Cell lines incorporating the pINT pR pL R6K Rep pi P42L-P106L-F107S (P3-) integration vector containing either the wildtype P_L promoter (NTC661135, SEQ ID NO: 10), the OL1-G mutation (NTC711055, SEQ ID NO: 11) or the OL1-G to T mutation (NTC711231, SEQ ID NO: 12) were transformed with the NTC9385R-EGFP plasmid and yields in shake flask determined (Table 2). The results demonstrated the OL1-G and OL1-G to T mutations dramatically improve temperature inducible R6K plasmid yields in shake flask culture. Improved yield with two different R6K plasmids (NTC9385R, Table 2; NTC9685R, Table 1) in either LB shake flask media or HyperGRO fermentation media demonstrates improved temperature inducible R6K plasmid is generic, and is not plasmid or growth media specific. Thus the invention can be utilized with a plurality of R6K origin vectors, in various plasmid growth media described in the art and various temperature induction profiles.

[0216] Likewise the pINT pR pL R6K Rep plasmids can be integrated into alternative *E. coli* strains to create production hosts. Any strain that is acceptable for plasmid production, such as JM108, BL21, DH5, DH1, DH5a, GT115, GT116, DH10B, EC 100, can be converted to a high yield temperature inducible R6K plasmid production host by integration of a pINT pR pL R6K Rep plasmid into the genome. The pR pL R6K Rep expression cassette may alternatively be removed from the pINT vector backbone and directly integrated into the chromosome, for example, using Red Gam recombination cloning (for example, using the methods described in Datsenko and Wanner 2000 *Proc Natl Acad Sci USA* 97:6640-6645). The pR pL R6K Rep expression cassette may alternatively be transferred to a different vector backbone, such as integration vectors that target different phage attachment sites, for example, those described by Haldimann and Warmer 2001, *J Bacteriol* 183:6384-6393.

TABLE 2

NTC9385R-EGFP LB media shake flask production yields in R6K production strains

Cell Line	Rep Gene	Rep Gene Promoter	30° C. Spec yield[a]	32° C. Spec yield[a]	37° C. Spec yield[a]
NTC661135	P42L-P106L-F107S	P_R P_L	1.2	2.1	0.6
NTC711055	P42L-P106L-F107S	P_R P_L (OLI-G)	0.5	1.3	9.1
NTC711231	P42L-P106L-F107S	P_R P_L (OL1-G to T)	1.3	7.0	9.3

[a]Specific yield = mg plasmid/L/OD_{600}

[0217] These results are surprising since the art teaches that P_L promoter mutations in the OL1 binding site such as V2 (OL1-G to T) are constitutively active due to an inability of the lambda repressor to stop expression from the P_L promoter (Bailone and Galibert, Supra, 1980). While not limliting the application of the invention, it is possible that the lambda repressor is able to repress the P_L promoter through binding to the OL2 and OL3 sites (FIG. **7**) when the P_L promoter is integrated in the genome; the lambda repressor may not be able to bind multiple copies of the mutated P_L promoter as in a phage infection.

[0218] The application of two independent OL1 mutations (OL1-G and OL1-G to T) to create cell lines for high yield R6K plasmid production demonstrates the general utility of P_L promoters incorporating OL1 mutations to improve heat inducible chromosomal expression of a target protein. Any

OL1 mutation is contemplated for use in the current invention. New OL1 mutations can be defined by standard methods known in the art, for example error prone mutagenesis of the OL1 region, with subsequent selection of beneficial OL1 mutations by screening for heat inducible taiget protein production. The taiget protein can be a Rep protein as described herein, or a fluorescent marker, or any target protein or RNA. Thus application of P_L promoters incorporating OL1 mutations is contemplated generally as a platform for improved heat inducible chromosomal expression of any recombinant protein or RNA. This can be applied to improve heat inducible chromosomal expression of any recombinant protein or RNA using either shake flask (Table 2) or fermentation (Table 1) culture.

[0219] These cell lines may also be used to produce alternative R6K plasmids, such as CpGfree vectors, pCOR vectors, pGM169, etc. P_L promoter vectors with the OL1 mutations may be used to improve expression of alternative taiget proteins or mRNAs from the genome.

[0220] These cell lines may also be used to produce alternative Rep protein dependent plasmids, such as ColE2-P9 replication origin plasmids (Examples 2 and 3), ColE2 related replication origin plasmids, etc. Numerous additional Rep protein dependent plasmids known in the art may also be produced using the cell lines of the invention. Many Rep protein dependent plasmids are described in del Solar et al Supra, 1998 which is included herein by reference.

[0221] Heat inducible target protein production may be further improved, by further mutating OL1 -G and OL1-G to T or an alternative OL1 mutation to incorporate a mutation in the P_L-10 GATACT sequence to make it more closely match the consensus TATAAT (-35 is already consensus TTGACA (FIG. **7**).

[0222] Alternative temperature sensitive (ts) lambda repressors (cI) may be substituted for the cITs857 mutation utilized in the pINT vectors. Multiple alternative ts lambda repressors have been defined (for example, see Lieb M. 1979 *J Virol* 32:162 incorporated herein by reference) or new ts lambda repressors may be isolated by screening for temperature sensitive cI function.

[0223] Alternative integration methods rather than the described pINT pR pL integration vectors may be utilized such as integration of the pR pL expression cassette into the genome at defined sites using Red Gam recombination cloning (for example, using the methods described in Datsenko and Wanner Supra, 2000).

Example 2: ColE2-P9 Replication Origin Plasmid Production

[0224] Similar to plasmid R6K, the ColE2 replication origin is separate from the replication protein, so the ColE2 replication origin theoretically may be utilized to construct Rep protein dependent plasmids. Here application of the ColE2 replication origin, using ColE2-P9 as an example, to produce ColE2 Rep protein dependent plasmids is demonstrated (Example 3).

ColE2 Background

[0225] The ColE2 replication origin (for example, ColE2-P9) is highly conserved across the ColE2-related plasmid family (15 members are compared in Hiraga et al Supra, 1994, and 53 ColE2 related plasmid members including ColE3 are compared in Yagura et al Supra, 2006, both references are included herein by reference). Plasmids containing this origin are normally 10 copies/cell (low copy #). For application in gene therapy or DNA vaccination vectors, the copy number of ColE2 replication origin vectors needs to be improved dramatically.

[0226] Expression of the ColE2-P9 replication (Rep) protein is regulated by antisense RNA (RNAI). Copy number mutations have been identified that interfere with this regulation and raise the copy number to 40/cell (Takechi et al 1994 *Mol Gen Genet* 244:49-56). pINT pR pL ColE2 Rep Protein Cell Line NTC641711:

[0227] The ColE2 Rep protein (SEQ ID NO: 15) was expressed using the heat inducible pINT pR pL vector as described in Example 1. The ColE2 RNAI region was removed and replaced with an optimal kozak-ATG region. This modification deletes the RNAI -10 promoter box. The Rep internal RNAI -35 box (Yasueda et al 1994 *Mol Gen Genet* 244: 41-48) was mutagenized (from (opposite strand) TTGAAG to CTGAAG) to lower the consensus. A high copy mutation in the Rep coding region (C139T; Nagase et al 2008 Plasmid 59:36-44) was also incorporated.

[0228] These changes do not alter the Rep protein amino acid sequence (SEQ ID NO: 15).

[0229] The ColE2 Rep gene was PCR amplified from CGSC Strain #8203 with following primers 15061101 :

(SEQ ID NO : 26) ggaacgggatccagaaggagatatacatatgag tgccgtacttcagcgcttcaggga

15061102 :

(SEQ ID NO : 27) ggaacggaattcttatcattttgcgagatctgg atcacat

[0230] The 920 bp PCR product was digested with BamHI/ EcoRI and cloned into BamHI/EcoRI digested pINT pR pL BamHI/EcoRI (3766, polylinker). Recombinant clones were selected by restriction digestion and sequence verified. The map of the resultant pINT pR pL CoE2 Rep integration vector is shown in FIG. **8**. The integration plasmid was integrated into NTC54208 (XL1BluesacB [dcm-]) to create cell line NTC641711 as described in Example 1.

[0231] A kanR ColE2-P9 replication origin fluorescent reporter plasmid (pDNAVACCUltra5-C2-P5%,4/6-T7RBS EGFP) was constructed to select for copy number improving mutations. The 1067 bp pUC replication origin was removed from the kanR pDNAVACCUltra5-P5%,4/6-T7RBS EGFP vector (the pDNAVACCUltra5-EGFP vector disclosed in Williams J A, 2006 World patent application WO06078979, modified to express the EGFP reporter in *E. coli* utilizing the weak constitutive P5%,4/6 promoter disclosed in Lissemore J L, Jankowski J T, Thomas C B, Mascotti D P, deFlaseth P L. 2000. *Biotechniques* 28: 82-89 and included herein by reference) by NheI-DraIII digestion, and replaced with a 132 bp ColE2-P9 replication origin (+7-ssiA; see below). Recombinant clones were recovered in cell line NTC641711 and the ColE2 origin confirmed by restriction digestion and sequence verification. This demonstrates that the ColE2-P9 Rep protein cell line NTC641711

can be used to select and propagate ColE2 replication origin containing plasmids. ColE2 Rep Protein Mutagenesis, Selection of Copy Number Increasing Mutants

Background

[0232] The ColE2 Rep protein binds as a monomer to the ColE2 replication origin. However, Rep protein exists mostly as a dimer in solution; Rep dimerization will limit the amount of active monomeric Rep which is hypothesized will maintain ColE2 plasmid at a low copy number (Flan M, Aoki K, Yagura M, Itoh T. 2007. *Biochem Biophys Res Commun* 353:306). Copy number autoregulation by Rep protein dimerization is a common copy number control mechanism. Significantly, R6K Rep protein mutations such as P106L (PIR116) utilized in Example 1 that interfere with dimer formation dramatically increase copy number (Abhyankar et al Supra, 2004). It was hypothesized that ColE2 plasmid copy number can also be increased with a dimerization deficient Rep mutation.

Mutagenesis

[0233] ColE2 Rep protein functional domains have been mapped and a region responsible for dimerization defined (FIG. **8**). The dimerization region was mutagenized using the Gen-eMorph II Random Mutagenesis Kit (Stratagene) as described (Lanza A M, Alper El S. 2011. Methods in Molecular Biology, Vol. 765, Strain Engineering: Methods and Protocols, Ed. J. A. Williams, Flumana Press Inc., Totowa, N.J. pp 253-274). The Rep gene was error prone PCR amplified from the pINT pR pL ColE2 Rep vector with the kit enzyme. The mutagenized dimerization domain (359 bp BstBl/EcoRI fragment; FIG. **8**) was cloned back into the pINT pR pL ColE2 Rep vector replacing the non mutagenized 359 bp BstBl/EcoRI fragment. An integrated pINT pR pL ColE2 Rep library was then made by mass genome integration without purification of the mutagenized plasmid pool into NTC54208 containing the pAF169 integration plasmid. The integrated Rep library was transformed with the kanR pDNAVACCUltra5-C2-P⅚,4/6-T7RBS EGFP fluorescent ColE2 reporter plasmid and transformants plated on LB+kanamycin agar plates and grown at 37° C. This EGFP reporter plasmid allows 1) visual selection of plasmid copy number improvement using a Dark Reader for agar plate illumination; and 2) quantitative copy number evaluation (fluorescence is linear with copy number) in liquid culture using a fluorometer (BioTek FLx800 microplate fluorescence reader).

[0234] Two colonies were isolated from 30,000 screened cells with significantly higher colony fluorescence. Both cell lines were verified to have improved pDNAVACCUltra5-C2-P5/ 6,4/6-T7RBS EGFP plasmid copy number in liquid culture demonstrating increased fluorescence corresponds to increased copy number.

[0235] The lambda repressor-P_L-ColE2 Rep regions from genomic DNA from these two cell lines were amplified by PCR and sequenced to determine the basis for improvement. One colony had a mutation in the lambda repressor which presumably reduces the activity of the repressor leading to Rep protein overexpression. This demonstrates that alternations to the vector backbone that increase P_L promoter activity improve ColE2 plasmid copy number. Thus ColE2 copy number, like R6K plasmids, will be improved by making a cell line with the ColE2 Rep protein (or Rep protein copy number improving mutations) expressed from pINT pR pL vectors incorporating the lambda repressor binding site OL1 mutations (OL1-G and OL1-G to T) identified in Example 1.

[0236] The second colony had a mutation in the Rep protein (G194D; SEQ ID NO: 16). This mutation was introduced back into the pINT pR pL ColE2 Rep vector to create the pINT pR pL ColE2 Rep protein mutant (G194D) (SEQ ID NO: 18). The integration plasmid was integrated into NTC54208 (XLlBlue-sacB [dcm-]) to create cell line NTC701131 as described in Example 1. ColE2 plasmid production yields were improved in the ColE2 Rep protein mutant cell line NTC701131, compared to the parental ColE2 Rep protein cell line NTC641711 in both shake flask and fermentation culture (Table 3). This demonstrates that the ColE2 Rep protein, like the R6K Rep protein, can be mutagenized to create copy number improving variants.

[0237] Combining the ColE2 Rep protein G194D mutant with pINT pR pL vector incorporating the lambda repressor binding site OL1 OL1 -G to T mutation identified in Example 1 further increased copy number (cell line NTC710351=NTC54208-pR pL (OL1-G to T) ColE2 rep G194D) and fermentation production yields (Example 3).

TABLE 3

NTC938SC-Luc plasmid performance in different processes and production cell lines[a]

ColB2 Plasimid	LB shake flask (37 C.)			Plasmid + Shake flask (37 C.) [c]			HyperGRO fermentation		
production cell line	OD_{600}	mg/L	Spec yield[b]	OD_{600}	mg/L	Spec yield[b]	OD_{600}	mg/L	Spec yield[b]
NTC641711	3.4	1.4	0.4	13.0	12.3	0.93	148	61	0.4
NTC701131	3.4	3.1	0.9	16.6	17.9	1.1	113	110	1.0
Rep mutant							140	142	1.0

[a]All plasmid preparations at harvest were high quality monomers.

[b]Specific yield = mg plasmid/L/OD_{600}

[c]Plasmid + media from Thomson Instruments Company

[0238] Additional rounds of mutagenesis of the wild type Rep protein, or mutagenesis of mutant Rep protein such as G194D may be performed to further improve copy number. The entire Rep protein or subfragments can be mutagenized (e.g. BamHl-EcoRI fragment for entire Rep protein; FIG. **8**). The ideal mutant will be similar to the R6K Rep protein mutants 'P42L and P113S' and 'P42L, P106L and F107S' (Example 1) with higher copy number at 37-42° C. (i.e. higher levels of replication inducing monomeric Rep protein are produced from the heat inducible P_R P_L promoters) to facilitate adaptation into NTC's inducible fermentation plasmid production process as in Example 1.

ColE2 Origin Vectors

[0239] The following vectors containing the minimal ColE2-P9 origin (Yagura and Itoh 2006 *Biochem Biophys Res Commun* 345:872-877) and various origin region modifications were constructed.

+7-ssiA

[0240] This combines the ColE2 origin (+7) (SEQ ID NO: 4) with ssiAfrom plasmid R6K (SEQ ID NO: 21). Thus ssiA vectors contain, in addition to the ColE2-P9 origin, a downstream primosome assembly site. Like most plasmid origins, the ColE2 origin contains a primosomal assembly site about 100 bp downstream of the origin (Nomura et al Supra, 1991). This site primes lagging strand DNA replication (Masai et al 1990 *J Biol Chem*265:15124-15133) which may improve plasmid copy number or plasmid quality. The ColE2 PAS (ssiA) is similar to PAS-BF1 (ColE1 ssiA=-PAS-BL Marians et al 1982 *J Biol Chem* 257:5656-5662) and both sites (and PAS-BH) are CpG rich ØX174 type PAS. A CpG free PAS (ssiAfrom R6K; Nomura et al Supra, 1991; SEQ ID NO: 21) that acts as a dnaA, dnaB dnaC (ABC) primosome on a dnaA box hairpin sequence (Masai et al 1990 *J Biol Chem* 265:15134-15144) was selected for inclusion in the +7-ssiA vectors. Alternative ABC or ØX174 type PAS sequences are functionally equivalent to ssiA from R6K, and may be substituted for ssiA in these ColE2 replication origin vectors.

[0241] +7-ssiA vectors were constructed by replacing the pUC origin NheI-DraIII region (FIG. **1**) with a NheI-DraIII compatible synthetic ssiA-+7 ColE2 origin restriction fragment (FIG. **2**, FIG. **4**). Plasmids were transformed into ColE2 plasmid production NTC641711. The correct ColE2 vectors were identified by restriction digestion and sequence verified.

+7 (No ssiA)

[0242] This deletes the ssiA sequence from +7-ssiA while retaining the ColE2 origin (+7) (SEQ ID NO: 4). The ssiA sequence was removed by NheI-MfeI digestion, the sites blunted with Klenow and the vector religated to delete the 64 bp ssiA region. Plasmids were transformed into ColE2 plasmid production host NTC641711. The correct ColE2 vector was identified by restriction digestion and sequence verified.

+7 CpG-ssiA

[0243] This combines the ColE2 replication origin (+7 CpG) (SEQ ID NO: 5) with ssiA from plasmid R6K (SEQ ID NO: 21). The single CpG in the ColE2 replication origin (Table 4) was removed from the vector by site directed mutagenesis. Plasmids were transformed into ColE2 plasmid production host NTC641711. The correct ColE2 vector was identified by restriction digestion and sequence verified.
+16-ssiA:

[0244] This combines the ColE2 replication origin (+16) (SEQ 30 ID NO:7) with ssiA from plasmid R6K (SEQ ID NO: 21). A 16 bp region of homology downstream of the ColE2-P9 replication origin is conserved with the ColE3 replication origin. This 16 bp region was added to the vector by site directed mutagenesis. Plasmids were transformed into ColE2 plasmid production host NTC641711. The correct ColE2 vector was identified by restriction digestion and sequence verified.

Min-ssiA

[0245] This combines the ColE2 Min replication origin (SEQ ID NO:6) with ssiA from plasmid R6K (SEQ ID NO: 21). This is the minimal 32 bp ColE2 sequence sufficient for replication defined by Yasueda et al 1989 *Mol Gen Genet* 215:209) (SEQ ID NO: 28), extended by an additional 6 bp (Table 4). This vector was created by site directed mutagenesis of the +7-ssiA clone. Plasmids were transformed into ColE2 plasmid production host NTC641711. The correct ColE2 vector was identified by restriction digestion and sequence verified.

[0246] The series of plasmids were transformed into ColE2 plasmid production host NTC701131 (Rep mutant). The resultant cell lines were then used determine plasmid copy number and quality (Table 4). Two different backbones were evaluated with the +7-ssiA and +16-ssiA ColE2 replication origins to determine the effect of plasmid sequence alterations.

[0247] The results demonstrate that the four replication origin variants containing the ssiA sequence [+7-ssiA; +16-ssiA; +7 (CpG free) ssiA; Min-ssiA] are functional in NTC701131, replicating to a similar copy number (0.73-1 x). All plasmids were high quality monomer. This demonstrates that any of these minimal ColE2 origin variants can function as a plasmid replication origin to produce high quality plasmid.

[0248] Yagura et al Supra, 2006 have demonstrated that the Min ColE2 Replication origin (SEQ ID NO: 28, which is reverse complement of residues 7-38, in FIG. **1** of Yagura et al Supra, 2006) can be further deleted without eliminating replication function. Yagura et al, Supra, 2006, demonstrated that the core sequence is residues 8-35, with residues 5-36 are required for full activity. The +7 ColE2 Replication origin (SEQ ID NO: 4; which is the reverse complement of residues 0-44 in FIG. **1** of Yagura et al Supra, 2006) could therefore be reduced to span residues 8-35 or 5-36 of FIG. **1** of Yagura et al Supra, 2006. Such vectors should replicate similarly to the disclosed vectors. As well, a number of base changes can be made within the core ColE2 origin 8-34 region that do not affect ColE2 replication (see changes to residues that retain function in Table 2 in Yagura et al Supra, 2006).

[0249] A surprising observation that is contrary to the teachings of Yagura et al Supra, 2006 is that the +7(CpG free)-ssiA ColE2 origin is fully functional. This origin contains a change of a G to C in residue 36 (FIG. **1** Yagura et al Supra, 2006). This change is predicted to reduce origin activity (Relative transformation frequency 5 fold reduced with 36 G-C to C-G; Table 2 in Yagura et al Supra, 2006). This may be due to the different context in the +7(CpG free)-ssiA ColE2 origin, or the longer origin fragment (0-44). Regardless, the 121 bp +7(CpG free)-ssiA ColE2 origin (SEQ ID NO 29) or +7(CpG free) ColE2 origin (SEQ ID NO 5) are smaller CpG free replication origin alternatives to the 260 bp CpG free R6K replication origins (SEQ ID NO: 22). CpG free ColE2 origins may be utilized to construct CpG free plasmid vectors, or to retrofit the replication origin in existing vectors with a CpG free alternative replication origin. Combinations of a CpG free ColE2 or R6K replication origin with a CpG free RNA-OUT selection mar-

ker may be utilized to construct antibiotic free CpG free plasmid vectors, or to retrofit the selection marker-replication origin region in existing vectors with an antibiotic free-CpG free alternative selection marker-replication origin.

[0250] The ssiA sequence was not necessary for plasmid replication, although removal of ssiA in +7 (no ssiA) reduced copy number to 55% of +7 (ssiA). Thus inclusion of a primosomal assembly site is beneficial to ColE2 plasmid copy number.**[text missing or illegible when filed]**

Example 3: NTC9382C, NTC9385C, NTC9382R, NTC9385R, NTC9682C, NTC9685C, NTC9682R, and NTC9685R Vectors

[0251] A series of AF eukaryotic expression vectors incorporating these novel ColE2-P9 derived vector origins were made. To replace the pUC origin, the +7 (ssiA) ColE2 origin from Example 2 was selected as well as the R6K origin (SEQ ID NO: 1) from Example 1. The features of these vectors (NTC9382C, NTC9385C, NTC9382R, NTC9385R, NTC9682C, NTC9685C, NTC9682R, and NTC9685R) are summarized in Table 5.

[0252] NTC9682C, NTC9685C (FIG. **2**), NTC9682R, NTC9685R (FIG. **3**) are antibiotic-free RNA-OUT ColE2 origin (C) or R6K origin (R) versions of the pUC origin NTC8682, NTC8685 (FIG. **1**) equivalents disclosed in Williams J A, Supra, 2010. These vectors contain the SV40 enhancer upstream of the CMV enhancer, and Adenoviral serotype 5 VA RNAI regulatory RNA (VARNAI).

[0253] NTC9382C, NTC9385C (FIG. **4**), NTC9382R, NTC9385R (FIG. **5**) are versions without the SV40 enhancer or VARNAI sequences.

[0254] NTC9682C, NTC9682R, NTC9382C, and NTC9382R all express the secreted transgene product as TPA fusion proteins while NTC9685C, NTC9685R, NTC9385C, and NTC9385R all express the native transgene product from a vector encoded ATG start codon.

[0255] The remainder of the vector sequences is identical between the different vectors, with the exception that the two R vectors NTC9682R and NTC9382R (FIG. **3**, FIG. **5**) contain the trpA bacterial terminator, which is absent in the two C vectors NTC9682C and NTC9382C (FIG. **2**, FIG. **4**).

[0256] An R6K gamma origin vector was constructed by swapping in the R6K gamma origin (SEQ ID NO: 1) in a NotI-DraIII R6K origin synthetic gene for the corresponding NotI-DraIII pUC origin region in NTC8685. The NTC9682R, NTC9685R NTC9382R, NTC9385R vectors were made by standard restriction digestion mediated fragment swaps. The ColE2 origin vectors were constructed in a similar fashion, by swapping in the +7 ssiA ColE2 origin in a Nhel-Dralll synthetic gene for the corresponding Nhel-DraIII pUC origin region. The NTC9682C, NTC9685C, NTC9382C, NTC9385C vectors were made by standard restriction digestion mediated fragment swaps. The 466 bp Bacterial region [NheI site-trpA terminator-R6K Origin-RNA-OUT-KpnI site] for NTC9385R and NTC9685R is shown in SEQ ID NO: 19. The 281 bp Bacterial region [NheI site-ssiA-ColE2 Origin (+7)-RNA-OUT-KpnI site] for NTC9385C and NTC9685C is shown in SEQ ID NO: 20.

[0257] High fermentation yields in HyperGRO media are obtained with these vectors. For example 695 mg/mL with NTC9685R-EGFP in R6K production cell line NTC711231 (Table 1) and 672 mg/L with NTC9385C-EGFP in ColE2 production cell line NTC710351.

[0258] These are just a few possible nonlimiting vector configurations. Many alternative vector configurations incorporating the novel R6K or ColE2 origin vector modifications may also be made, including but not limited to vectors with alternative selection markers, alternative promoters, alternative terminators, and different orientations of the various vector-encoded elements or alternative R6K or ColE2 origins as described in Examples 1 and 2.

TABLE 5

NTC9382C, NTC9385C, NTC9382R, NTC9385R, NTC9682C, NTC9685C, NTC9682R, and NTC9685R vectors

Vector	Origin	VA RNAI presence	SV40 enhancer	Transgene targeting
NTC9382C	ColE2-P9	No	No	Secretion (TPA)
NTC9382R	R6K	No	No	Secretion (TPA)
NTC9682C	ColE2-P9	Yes	Yes	Secretion (TPA)
NTC9682R	R6K	Yes	Yes	Secretion (TPA)
NTC9385C	ColE2-P9	No	No	Native
(SEQ ID NO: 8) NTC9385R	R6K	No	No	Native
(SEQ ID NO: 2) NTC9685C	ColE2-P9	Yes	Yes	Native
(SEQ ID NO: 9) NTC9685R	R6K	Yes	Yes	Native
(SEQ ID NO: 3)				

An example strategy for cloning into these vectors is outlined below.

```
GTCGACATG--------Gene of interest----Stop codon
  ⍰

------AGATCT
      ⍰

⍰ indicates text missing or illegible when filed
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[0259] For the NTC9385C, NTC9685C, NTC9385R, and NTC9685R vectors, the ATG start codon (double underlined) is immediately preceded by a unique SaII site. The SaII site is an effective Kozak sequence for translational initiation. In NTC9382C, NTC9682C, NTC9382R, and NTC9682R, the SaII site is downstream in frame with the optimized TPA secretion sequence (SEQ ID NO: 25). The TEA ATG start codon is double underlined and the SaII site single underlined.

```
SEQ ID NO: 25: TPA secretion sequence
atggatgcaatgaagagagggctctgctgtgtgctgctgctgtgtggag
cagtctt cgtttcgcccagcggtaccggatccgtcgac
```

[0260] For precise cloning, genes are copied by PCR amplification from clones, cDNA, or genomic DNA using primers with Sail (5′ end) and BgIII (3′ end) sites. Alternatively, genes are synthesized chemically to be compatible with the unique SaII/BgIII cloning sites in these vectors.

[0261] For NTC9385C, NTC9685C, NTC9385R, and NTC9685R, the start codon ATG may immediately follow the SaII site (GTCGACATG) since the SaII site is a high function Kozak sequence. For all vectors one or two stop codons (preferably TAA or TGA) may be included after the open reading frame, prior to the BgIII site. A PCR product or synthetic gene designed for NTC9385C, NTC9685C, NTC9385R, and NTC9685R is compatible with, and can also be cloned into, the NTC9382C, NTC9682C, NTC9382R, and NTC9682R vectors.

[0262] EGFP and muSEAP transgene versions NTC9385C, NTC9685C, NTC9385R, and NTC9685R were constructed by standard restriction fragment swaps. The muSEAP gene is secreted using its endogenous secretion signal, while EGFP is cell associated. Expression levels in vitro were determined using EGFP, while expression levels in vivo were determined using muSEAP Expression levels were compared to the NTC8685 parent vector, the gWIZ vector, and a minicircle comparator.

[0263] Adherent HEK293 (human embryonic kidney), A549 (human lung carcinoma), cell lines were obtained from the American Type Culture Collection (Manassas, Va., USA). Cell lines were propagated in Dulbecco's modified Eagle's medium/F12 containing 10% fetal bovine serum and split (0.25% trypsin-EDTA) using Invitrogen (Carlsbad, Calif., USA) reagents and conventional methodologies. For trans-fections, cells were plated on 24-well tissue culture dishes, plasmids were transfected into cell lines using Lipo-fectamine 2000 following the manufacturer's instructions (Invitrogen).

[0264] Total cellular lysates for EGFP determination were prepared by resuspending cells in cell lysis buffer (BD Biosciences Pharmingen, San Diego, Calif., USA), lysing cells by incubating for 30 min at 37° C., followed by a freeze-thaw cycle at -80° C. Lysed cells were clarified by centrifugation and the supernatants assayed for EGFP by FLX800 microplate fluorescence reader (Bio-Tek, Winooski, Vt., USA). The results are summarized in FIG. **9** and Table 6.

[0265] Groups of five mice were injected with plasmid DNA in an IACUC-approved study. Five micrograms of muSEAP plasmid in 25 or 50 μL of phosphate-buffered saline (PBS) was injected intramuscularly (IM) into a tibialis cranialis muscles of female BALB/c mice or ND4 Swiss Webster mice (6 to 8 weeks old) followed by Ichor TriGrid electroporation. SEAP levels in serum were determined using the Phospha-light SEAP Reporter Gene Assay System from Applied Biosystems (Foster City, Calif.) according to the manufacturer's instructions. The results are summarized in FIG. **10** and Table 6.

[0266] The NTC9385C, NTC9685C, NTC9385R, and NTC9685R vectors had similar expression to the parent NTC8685 vector in vitro, and higher expression than the gWIZ comparator (FIG. **9**). Thus substitution of the R6K or ColE2 replication origin for the pUC origin was not detrimental for eukaryotic cell expression. However, surprisingly, in vivo expression was dramatically improved compared to NTC8685 or gWIZ with the ColE2 and R6K origin vectors (FIG. **10**). For example the NTC9385C vector was unexpectedly improved 1.5 to 3.8x that of NTC8385 (Table 6) or NTC8685 (not shown) after IM delivery with EP

TABLE 6

gWIZ and NTC9385C Nanoplasmid expression compared to NTC8685

Plasmid	% NTC8685 expression in vitro[a]	% NTC8685 expression T =7 day BALB/c[b]	% NTC8685 expression T =7 days ND4[b]	% NTC8685 expression T =28 days BALB/c[b]	% NTC8685 expression T =28 days ND4[b]
gWIZ	58	59	57	21	57
NTC8385	NA	NA	101	NA	101
NTC9385C	92	377	349	150	233
Minicircle[c]	NA	89	NA	40	NA

[a] 100 μg/well BGFP transgene vectors transfected with lipofectamine into HEK293 cells

[b] murine SEAP (muSEAP) transgene vectors in 8-10 week old BALB/c ND4 Swiss Webster female mice. 5 μg dose with EP intramuscular into one anterior muscle followded by Labor TriGrid electroporation. 25 μL dose for ND4 mice, 50 μL dose gor BALB/c.

[c] Minicircle equivalent to NTC9385C or NTC9385R, with Nbel-KpaI region containing the replication origin and RNA-OUT selection marker (bacteral region) removed from NTC8385-muSEAP by SpeI/Nbel digestion, gel purification of the enkaryotic region in vitro ligation and superceiling with DNA gyrase. The SpeI site is the same site used to truncate the CMV promoter to make NTC8685 and the NTC9385C muSEAP vector so the minicircle cukaryotic region is the same as NTC9385C-muSEAP, the difference being the C2 and RNA-OUT region including the KpaI site is deleted in the minicircle. NA = Not assayed

[0267] This improved in vivo expression was not specific to the CMV promoter. Versions of NTC8685-muSEAP and NTC9385C-muSEAP were constructed in which the murine creatine kinase (MCK) promoter (3 copies of the MCK Enhancer upstream of the MCK promoter and 50 bp of the MCK exon 1 leader sequence; Wang B, Li J, Fu F H, Chen C, Zhu X, Zhou L, Jiang X, Xiao X. 2008. *Gene Ther* 15:1489) was substituted for the CMV promoter. The swaps replaced the entire CMV enhancer CMV promoter-exon 1 leader (NTC8685: from a Xbal site immediately after the SV40 enhancer to a SacII site in the CMV derived exon 1 leader sequence FIG. **1**; NTC9385C: from the KpnI site to a SacII site in the CMV derived exon 1 leader sequence FIG. **4**) with the MCK enhancer, MCK promoter-exon 1 leader retaining the HTLV-I R portion of exon 1. Purified plasmid DNA from the resultant vectors, NTC8685-MCK-muSEAP (4847 bp) and NTC9385C-MCK-muSEAP (3203 bp), was injected IM into one anterior tibialis muscle of 8-10 week old BALB/c female mice (5 mice/group), 5 μg dose in 50 μL, followed by Ichor TriGrid electroporation as described in Table 6. SEAP levels in serum was determined on day 28 (T=28) post delivery. The NTC9385C-MCK-muSEAP vector (98.4±55.8) had 4.5x higher average expression than NTC8685-muSEAP (22.0±10.9). All 5 NTC9385C-MCK-muSEAP injected

mice had higher muSEAP levels than any of the NTC8685-muSEAP mice. This demonstrates that improved in vivo expression with the Nanoplasmid vectors of the invention is not specific to the CMV promoter.

[0268] While the basis for expression improvement is unknown, it is not simply due to the size difference between the parent pUC origin vectors and the modified R6K origin-RNA selection marker or ColE2 origin-RNA selection marker vectors of the invention, since expression was not improved with a minicircle comparator vector that contains no bacterial region (Table 6). This demonstrates improved in vivo expression with the R6K origin-RNA selection marker or ColE2 origin-RNA selection marker vectors is not the result of simple elimination of a threshold amount of bacterial region sequences.

[0269] Reduction of the vector spacer region size as described herein by replacement of the spacer region replication origin and selection marker with the R6K, ColE2 origin-RNA selection marker vectors of the invention will also increase the duration of in vivo expression since expression duration is improved with plasmid vectors in which the bacterial region is removed (minicircle) or replaced with a spacer region of up to at least 500 bp (Lu J, Zhang F, Xu S, Fire A Z, Kay M A. 2012. *Mol Ther.* 20:2111-9). Thus the replicative minicircle vectors of the invention also have additional utility for applications requiring extended duration expression, such as: liver gene therapy using hydrodynamic delivery with transgenes such as a-1 antitrypsin (AAT) for AAT deficiency, Coagulation Factor VIII for Hemophilia A Therapy or Coagulation Factor IX for Hemophilia B Therapy etc: lung gene therapy with transgenes such as Cystic fibrosis transmembrane conductance regulator 30 (CFTR) for cystic fibrosis etc; muscle gene therapy with transgenes such as the GNE gene for Hereditary inclusion body myopathies (HIBM), or dystrophin or dystrophin minigenes for duchenne muscular dystrophy (DMD).

Example 4: Spacer Region and Intron Modified Nanoplasmid Vectors

[0270] NTC8685 (SR=1465 bp) has much lower expression than NTC9385R (SR=466 bp) and NTC9385C (SR=281 bp). A minimal pUC origin vector was constructed with an 866 bp spacer region (NTC8385-Min; contains Pml,, minimal pUC origin-RNA-OUT). These vectors were tested for expression in vitro (lipofectamine 2000 delivery) and in vivo after intradermal electroporation delivery. As with Intramuscular injection (Example 3), the results (Table 7) demonstrated ColE2 and R6K origin vector dramatically improved in vivo expression after intradermal delivery compared to NTC8685. For example the NTC9385C vector was unexpectedly improved 2.7 to 3.1x compared to NTC8685 while the NTC9385R vector was unexpectedly improved 5.3 to 6.3x that of NTC8685 (Table 7). The 866 bp minimal pUC origin vector also improved expression to 1.4-1.9x that of NTC8685. This demonstrates improved in vivo expression with the NTC9385C and NTC9385R vectors is not limited to muscle tissue, and is observed also after intradermal delivery. Inclusion of the C2x4 eukaryotic transcription terminator in the NTC9385C vector further improved in vivo expression to 2.9 to 4.1x compared to NTC8685. This demonstrates improved in vivo expression with Nanoplasmid vectors may be obtained with alternative/additional sequences flanking the bacterial region.

[0271] A NTC9385R derivative was made in which the RNA-OUT antibiotic free marker was transferred to the intron (NTC9385Ra-02 SEQ ID NO: 39; RNA-OUT SEQ ID NO:23) inserted into the unique HpaI site in the intron (SEQ ID NO: 30). This vector encodes the R6K replication origin in the spacer region (SR=306 bp). To determine splicing accuracy NTC9385Ra-02-EGFP was transfected into the A549 cell line and cytoplasmic RNA isolated. The RNAwas reverse transcribed using an EGFP specific primer, and PCR amplified using Exon 1 and Exon 2 specific primers. The resultant PCR product (a single band) was determined by 5 sequencing to be the correct spliced exonl -exon2 fragment. This demonstrated that intronic RNA-OUT is accurately removed by splicing and does not interfere with splicing accuracy. NTC9385Ra-02-EGFP also demonstrated improved in vivo expression compared to NTC8685 (Table to 7: 1.6-3.5x). This demonstrates that Nanoplasmid vectors with improved expression of the current invention may encode the RNA selection marker in the intron rather than the spacer region.

[0272] The improved expression level after intradermal delivery demonstrates the application of Nanoplasmid vectors of the invention for cutaneous gene therapy applications, for example, for wound healing, bums, diabetic foot ulcer, or critical limb ischemia therapies using growth factors such as hypoxia inducible factor, hypoxia inducible factor 1α, keratinocyte growth factor, vascular endothelial growth factor (VEGF), fibroblast growth factor-1 (FGF-1, or acidic FGF), FGF-2 (also known as basic FGF), FGF-4, placental growth factor (P1GF), angiotensin-1 (Ang-1), hepatic growth factor (HGF), Developmentally Regulated Endothelial Locus 25 (Del-1), stromal cell derived factor-1 (SDF-1), etc.

TABLE 7

SR vector expression in vitro and in vivo

muSEAP Vector[b]	SR[a]	SR (bp) Intron[a]	A549 (A_{405})[d]	HEK-293 (A_{405})[d]	ID + EP[e] (pg/mL) T = 4	ID + EP[?] (pg/mL) T = 7	ID + EP[e] (pg/mL) T = 14
NTC8685	T-Val-BH-A[?]→	1465 HR- β	0.240 = 0.029 (1.0x)	3.002 ± 0.188 (1.0x)	1.9 ± 1.2 (1.0x)	6.7 ± 4.1 (1.0x)	5.0 ± 3.9 (1.0x)
NTC8385-Min[?]	A[?]→	866 HR- β	0.495 ± 0.027 (1.0x)	2.713 ± 0.177 (1.0x)	3.7 ± 2.7 (1.0x)	12.4 ± 8.1 (1.0x)	7.1 ± 5.2 (1.0x)
NTC9385R (SEQ ID NO: 2)	T←R-A[?]→	466 HR- β	0.604 ± 0.04 (1.0x)	3.036 ± 0.169 (1.0x)	13.0 ± 7.4 (1.0 x)	35.5 ± 31.1 (1.0 x)	29.9.± 23.4 (1.0 x)
NTC9385C (SEQ ID NO: 8)	←C-A[?]→	281 HR-β	0.267 ± 0.053 (1.1x)	2.720 ± 0.238 (0.9x)	5.8 ± 3.0 (3.1 x)	20.8 ± 9.6 (3.1 x)	13.5 ± 9.8 (2.7x)

TABLE 7-continued

SR vector expression in vitro and in vivo							
muSEAP Vector[b]	SR[a]	SR (bp) Intron[a]	A549 (A_{405})[d]	HEK-293 (A_{405})[d]	ID + EP[e] (pg/mL) T = 4	ID + EP? (pg/mL) T = 7	ID + EP[e] (pg/mL) T = 14
NTC9385C (2×4	←C-A?→	281 HR- β	0.214 ± 0.017 (0.89x)	2.472 ± 0.197 (0.82x)	5.6 ± 2.3 (2.9 x)	27.7 ± 20.3 (4.1 x)	16.0 ± 14.3 (3.2x)
NTC9385R a-O2 (SEQ ID NO: 39)	T←R	306 HR-←AF- β	0.524 ± 0.071 (2.2x)	3.065 ± 0.220 (1.0x)	3.6 ± 2.8 (1.9x)	23.4 ± 16.5 (3.5 x)	7.8 ± 8.0 (1.6 x)
a?							
b?							
c?							
d?							
e?							

? indicates text missing or illegible when filed

Example 5: RNA Pol III Nanoplasmid Vectors

[0273] An example Nanoplasmid vector for RNA Pol III directed expression of RNA is shown in FIG. **11**. This vector contains the human H1 RNA Pol III promoter, but an alternative promoter such as the murine U6 promoter can be substituted. This example vector expresses a 22 bp shRNA target RNA, but alternative RNAs may be expressed, including shorter or longer shRNAs, microRNAs, aptamer RNAs, hairpin RNAs, etc. This example vector is very small, with a monomer size of 442 bp. Small size is advantageous, since vectors <1.2 kb are highly resistant to shear forces used with gene therapy delivery formulation (Catanese et al 2012. *Gene Ther* 19:94-100).

[0274] RNA Pol III Nanoplasmid vectors were made by standard restriction digestion mediated fragment swaps to combine either U6 or HI RNA Pol III promoter-target RNA-TTTTTT terminator (Eukaryotic region) with either the 466 bp Bacterial region [NheI site-trpA terminator-R6K Origin-RNA-OUT-KpnI site; SEQ ID NO: 19] for NTC9385R-U6 and NTC9385RE-U6 vectors (Table 8) or the 281 bp Bacterial region [NheI site-ssiA-ColE2 Origin (+7)-RNA-OUT-KpnI site; SEQ ID NO: 20] for NTC9385C-U6 and NTC9385CE-U6 vectors (Table 8). Versions were modified to express from the U6 promoter eRNA18, a single stranded RNA the expression of which can be quantified by Reverse transcriptase dependent RT-PCR. Vector performance (U6 promoter mediated eRNA18 RNA expression) was determined in total RNA extracted from HEK293 at either 25 or 48 hrs after lipofectamine 2000 mediated transfection as described (Luke J, Simon G G, Soderholm J, Errett J S, August J T, Gale M Jr, Flodgson C P, Williams J A. 2011. *J Virol.* 85:1370). These results (Table 9) demonstrate Nanoplasmid RNA Pol III vectors direct dramatically improved RNA expression relative to a plasmid RNA Pol III vector (NTC7485-U6-eRNA18) comparator.

[0275] Random 22 bp shRNA (KP2F11) versions of NTC9385CE-U6 (903 bp NTC9385CE-U6-KP2F11 shRNA propagated in ColE2 rep cell line NTC710351) and NTC9385R-U6 (855 bp NTC9385R-U6-KP2F11 shRNA propagated in R6K rep cell line NTC711231) were fermented in FlyperGRO media as described in Example 1 except fermentation and cultures for inoculations were grown at 37° C. throughout. Final yields were 149 mg/L (NTC9385CE-U6-KP2F11) and 216 mg/L (NTC9385R-U6- KP2F11 shRNA). This demonstrates that Nanoplasmid vectors for RNA Pol III expression (and RNA Pol II; Example 3) have superior manufacturing simplicity and yield com-pared to shRNA expressing minicircle vectors (Zhao et al 2011. *Gene Ther* 18:220-224). For example, optimal manu-facture of minicircle vectors yields only 5 mg of minicircle per liter culture (Kay M A, He C Y, Chen Z Y. 2010. Nat 5 *Biotechnol* 28:1287-1289).

TABLE 8

RNA Pol III Nanoplasmid vector expression						
			Transfection 1: RNA isolated 48 hr post transfection		Transfection 2: RNA isolated 25 hr post transfection	
Vector	Pol II Enhancer	Size (bp)	HEK pg RNA/100 ng mRNA[a]	HEK Std[b]	HEK pg RNA/100 ng mRNA[a]	HEK Std[b]
NTC9385R-EGFP (negative control)	None	NA	0.0 ± 0.0	0%		
NTC8885MP-U6-cRNA18	SV40	1578[c]	62.2 ± 5.9 (1.3x)	69%		
NTC9385RE-U6-eRNA18	SV40	1178	119.1 ± 13.9 (2.5x)	98%		
NTC9385R-U6-eRNA18	None	945[d]	123.7 = 8.0 (2.6x)	82%		
NTC9385CE-U6-eRNA18	SV40	993	119.0 ± 13.9 (2.5x)	83%		
NTC9385C-U6-eRNA18	None	760[e]	131.1 ± 10.5 (2.7x)	70%	57.3 ± 2.6 (5.0x)	127%
NTC7485-U6-eRNA18	SV40	2978	48.0 ± 1.3 (1x control)	100% (100%	11.5 ± 1.5 (1x)	100% (control)

TABLE 8-continued

RNA Pol III Nanoplasmid vector expression						
			Transfection 1: RNA isolated 48 hr post transfection		Transfection 2: RNA isolated 25 hr post transfection	
Vector	Pol II Enhancer	Size (bp)	HEK pg RNA/ 100 ng mRNA[a]	HEK Std[b]	HEK pg RNA/100 ng mRNA[a]	HEK Std[b]
				control)		

[a]pg eRNA18 target/100 ng total RNA isolated post-transfection.

[b]Standarized mU6 expression compared to NTC7485-U6 shRNA eRNA18 vector (C) = test vector average pg RNA × test vector size (bp)/2978 × 100%

[c]Pmin minimal pUC origin (SEQ ID NO: 42) and RNA-OUT (bacterial region = SEQ ID NO: 43) with SV40) is 1035 bp

[d]R6K origin and RNA-OUT (bacterial region = SEQ ID NO: 19). HI promoter version (with shRNA) is 635 bp

[e]C2 origin and RNA-OUT (bacteria region = SEQ ID NO: 20). HI promoter version (with shRNA) is 442 bp (FIG. **11**)

Example 6: Alternative RNA Selection Marker Nanoplasmid Vectors

[0276] Expression of Nanoplasmid vectors encoding RNA-OUT in the intron (both orientations of RNA-OUT SEQ ID NO: 23 inserted into the unique HpaI site in the intron SEQ ID NO: 30; NTC9385Ra-01 dual and NTC9385Ra-02 dual) demonstrated robust expression with RNA-OUT in either orientation in the intron (Table 9). Consistent with this, similarly high levels of expression are obtained with NTC9385Ra-01 (SEQ ID NO: 40) and NTC9385Ra-02 (SEQ ID NO: 39) which have opposite orientations of intronic RNA-OUT marker and the R6K origin in the spacer region. Nanoplasmid variants with the pMB1 antisense RNA RNAI (SEQ ID NO: 31) with promoter and terminator region (RNAI selectable marker: SEQ ID NO: 32 flanked by DraIII-KpnI restriction sites for cloning as described previously for RNA-OUT) substituted for RNA-OUT were constructed and tested for expression to determine if alternative selection markers may be utilized in place of RNA-OUT. The results (Table 9) demonstrate alternative RNA selection markers may be substituted for RNA-OUT. Substitution of 60 RNAI for RNA-OUT in the vector backbone (NTC9385Ra- RNAI-O1) or in the intron in either orientation (NTC9385R-RNAI-O1 and NTC9385R-RNAI-O2) did not reduce expression relative to the corresponding RNA-OUT construct. To determine splicing accuracy, NTC9385R-RNAI-O1-EGFP and NTC9385R-RNAI-O2-EGFP were transfected into the A549 cell line and cytoplasmic RNA isolated from transfected HEK293 and A549 cells using the protein and RNA isolation system (PARIS kit, Ambion, Austin Tex.) and quantified by A_{260}. Samples were DNase treated (DNA-free DNase; Ambion, Austin Tex.) prior to reverse transcription using the Agpath-ID One step RT-PCR kit (Ambion, Austin Tex.) with a EGFP transgene specific complementary strand primer. Intron splicing was determined by PCR amplification of the reverse transcribed cytoplasmic RNA with the exon 1 and exon 2 specific primers. The resultant PCR product (a single band in each case) was determined by sequencing to be the correct spliced exon1-exon2 fragment. This demonstrated that, like intronic RNA-OUT, intronic RNAI in either orientation is accurately removed by splicing and does not interfere with splicing accuracy. This demonstrates that alternative RNA based selection markers could be substituted for RNA-OUT in the spacer region or the intron and that pMB 1 RNAI is a preferred RNA based selection marker.

[0277] The RNAI transcription unit (SEQ ID NO: 32) may be substituted for the RNA-OUT selection marker (SEQ ID NO: 23) in any of the constructs described in Examples 1-6. Alternatively, the 108 bp RNAI antisense repressor RNA (SEQ ID NO: 31) may be substituted for the 70 bp RNA-OUT antisense repressor RNA (SEQ ID NO: 24) retaining the flanking RNA-OUT transcription control sequences in any of the constructs described in Examples 1-6. RNAI regulated vectors may be grown in RNAII-SacB regulated cell lines further expressing, as required, R6K, ColE2-P9, or ColE2 related rep protein. RNAII-SacB regulated cell lines may be made replacing the RNA-IN sequence in pCAH63-CAT RNA-IN-SacB (P⅝ 6/6) with a RNAII taiget sequence as described in Williams, J A Supra, 2008 included herein by reference. Alternatively, RNAI regulated vectors may be grown in any of the RNAII regulated chromosomal selection marker cell lines disclosed in Grabherr and, Pfaffenzeller Supra, 2006; Cranenburgh Supra, 2009. These cell lines would be modified for expression, as required, of R6K, ColE2-P9, or ColE2 related rep protein.

[0278] Another preferred RNA based selection marker, IncB plasmid RNAI (SEQ ID NO: 33; SEQ ID NO: 34), is shown in FIG. **12**. A cell line for antibiotic free sucrose selection of IncB RNAI expressing plasmid vectors is created by modi-fication of the genomically expressed RNA-IN-SacB cell lines for RNA-OUT plasmid propagation disclosed in Williams, J A Supra, 2008 by replacement of the 68 bp RNA-IN regulator in a PstI-MamI restriction fragment with a 362 bp PstI-MamI IncB RNAII regulator (SEQ ID NO: 35). Alternatively, RNA-OUT may be substituted with one of the many RNA based selection markers know in the art.

TABLE 9

High level expression is explained with pMBI RNAI or RNA-OUT universe RNA vectors				
Vector (EGFP)	Spacer region[a]	SR (bp) Intron[a]	A549 FU[b] (T = 48 hr mean + SD)	HEK293 FU[b] (T = 48 hr mean + SD)
NTC8685	T-VAI-BH-P-AF-SV40	1465 HR- β[a]	8546 = 1163 (1.0x)	62068 = 1760 (1.0x)
NTC8385 (0.85 kb)[d]	T-P_{min}-AF-BB	866 HR- β[c]	9364 ± 966 (1.10x)	31482 ± 1822 (0.51x)

TABLE 9-continued

High level expression is explained with pMBI RNAI or RNA-OUT universe RNA vectors				
Vector (EGFP)	Spacer region[a]	SR (bp) Intron[a]	A549 FU[b] (T = 48 hr mean + SD)	HEK293 FU[b] (T = 48 hr mean + SD)
NTC9385C (SEQ ID NO: 8)	←C - AF→	281 HR- β[c]	8860 ± 382 (1.04x)	3.3356 ±1489 (0.54x)
NTC9385R (SEQ ID NO: 2)	←R - AF→	466 HR- β[a]	16237 ± 2520 (1.90x)	55919 ± 6371 (0.90x)
NTC9385R-n-O2 (SEQ ID NO: 39)	←R	306 HR ← AF - β	14510 ± 835 (1.70x)	49526 ± 2179 (0.50x)
NTC9385R-a-O1 dual	←R -AF→	466 HR-AF→ β	13929 ± 129 (1.63x)	56552 ± 2714 (0.91x)
NTC9385R-a-O2 dual	←R -AF→	466 HR-←AF- β	12543 ± 245 (1.47x)	54379 ± 1244 (0.89x)
NTC9385R-a-RNAI-O1	←RRNAI→	488 HR-AF→ β	15773 ± 238 (1.85x)	55468 ± 6619 (0.89x)
NTC9385R-RNAI-O1	←R-AF→	466 HR← RNAI - β	14296 ± 287 (1.67x)	60630 ± 2176 (0.98x)
NTC9385R-RNAI-O2	←R -AF →	466 HR - RNAI → β	12271 ± 466 (1.44x)	6069 ± 6482 (0.98x)

[a] rPa term = TMS-M =HR: B globin I′ accptor side δ: RNA-OUT sucrose reaction maker = AF: pHC region RNAI variance RNA =RNAI (. p) /(origin = R: CoE2 origin = C; BH = PAS-BH VP = upstrream pUC plasmid derived DNA

[b] EGFP plasmid DNA transfected with Lipofectmine 2000. Fluorescence nids (FU) reported Mese FU standardized m NTC8685

[c] HR p irdron is 225 bp

[d] Pmin minimal pUC origin (SEQ ID NO: 42) abd RNA-OUT (bacterial region = SEQ ID NO: 43)

[0279] Thus, the reader will see that the improved expression vectors of the invention provide for a rational approach to improve plasmid expression.

[0280] While the above description contains many examples, so these should not be construed as limitations on the scope of the invention, but rather should be viewed as an exempli-fication of preferred embodiments thereof. Many other variations are possible. For example, the RNA-OUT selectable marker may be substituted with an alternative RNA-OUT sequence variant that functionally binds RNA-IN to repress expression, for example, a CpG free RNA-OUT (SEQ ID NO: 36). A CpG free R6K-RNA-OUT bacterial region (SEQ ID NO: 37) or CpG free ColE2-RNA-OUT bacterial region (SEQ ID NO: 38) may be utilized. Likewise, the RNA-OUT promoter and/or terminator could be substituted with an alternative promoter and/or terminator. Likewise, the ColE2-P9 or R6K replication origin may be substituted with a ColE2 related replication origin, and propagated in a strain expressing the ColE2 related replication origin replication protein. Likewise, the ColE2-P9 or R6K origin may be substituted with an origin from one of the numerous additional Rep protein dependent plasmids that are know in the art, for example the Rep protein dependent plasmids described in del Solar et al Supra, 1998 which is included herein by reference. Likewise, the vectors may encode a diversity of transgenes different from the examples provided herein, for example, antigen genes for a variety of pathogens, or therapeutic genes such as hypoxia inducible factor, keratinocyte growth factor, factor IX, factor VIII, etc, or RNA genes such as microRNAs or shRNA. to Likewise, the eukaryotic region may express RNA from a RNA Pol III promoter as described herein. The orientation of the various vector-encoded elements may be changed relative to each other. The vectors may optionally contain additional functionalities, such as nuclear localizing sequences, and/or immunostimulatory RNA elements as disclosed in Williams, Supra, 2008. The vectors may include a boundary element between the bacterial region and the eukaryotic region, for example, the CMV promoter boundary element upstream of the CMV enhancer (or heterologous promoter enhancer) may be included in the vector design (e.g. NTC9385R-BE; SEQ ID NO: 41). The vectors may include a eukaryotic transcriptional terminator between the bacterial region and the eukaryotic region, for example, the 4xC2 terminator or the gastrin terminator. Likewise, the vectors may utilize a diversity of RNA Pol II promoters different from the CMV promoter examples provided herein, for example, constitutive promoters such as the elongation factor 1 (EF1) promoter, the chicken ß-actin promoter, the ß-actin promoter from other species, the elongation factor-1a (EF1a) promoter, the phosphoglycerokinase (PGK) promoter, the Rous sarcoma virus (RSV) promoter, the human serum albumin (SA) promoter, the α-1 antitrypsin (AAT) promoter, the thyroxine binding globulin (TBG) promoter, the cytochrome P450 2E1 (CYP2E1) promoter, etc. The vectors may also utilize combination promoters such as the chicken ß-actin/CMV enhancer (CAG) promoter, the human or murine CMV-derived enhancer elements combined with the elongation factor 1α (EF1α) promoters, CpG free versions of the human or murine CMV-derived enhancer elements combined with the elongation factor 1a (EF1α) promoters, the albumin promoter combined with an α-feto- protein MERII enhancer, etc, or the diversity of tissue specific or inducible promoters know in the art such as the muscle specific promoters muscle creatine kinase (MCK), and C5-12 described herein or the liver-specific promoter apolipoprotein A-I (ApoAI).

[0281] Accordingly, the scope of the invention should be determined not by the embodiments illustrated, but by the appended claims.

SEQUENCE LISTING

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ttcttccaac tgatctgcgc gcgaggccaa gcgatcttct tcttgtccaa gataagcctg  3420
tctagcttca agtatgacgg gctgatactg ggccggcagg cgctccattg cccagtcggc  3480
agcgacatcc ttcggcgcga ttttgccggt tactgcgctg taccaaatgc gggacaacgt  3540
aagcactaca tttcgctcat cgccagccca gtcgggcggc gagttccata gcgttaaggt  3600
ttcatttagc gcctcaaata gatcctgttc aggaaccgga tcaaagagtt cctccgccgc  3660
tggacctacc aaggcaacgc tatgttctct tgcttttgtc agcaagatag ccagatcaat  3720
gtcgatcgtg gctggctcga agatacctgc aagaatgtca ttgcgctgcc attctccaaa  3780
ttgcagttcg cgcttagctg gataacgcca cggaatgatg tcgtcgtgca caacaatggt  3840
gacttctaca gcgcggagaa tctcgctctc tccaggggaa gccgaagttt ccaaaaggtc  3900
gttgatcaaa gctcgccgcg ttgtttcatc aagccttacg gtcaccgtaa ccagcaaatc  3960
aatatcactg tgtggcttca ggccgccatc cactgcggag ccgtacaaat gtacggccag  4020
caacgtcggt tcgagatggc gctcgatgac gccaactacc tctgatagtt gagtcgatac  4080
ttcggcgatc accgcttccc tcatgatgtt taactttgtt ttagggcgac tgccctgctg  4140
cgtaacatcg ttgctgctcc ataacatcaa acatcgaccc acggcgtaac gcgcttgctg  4200
cttggatgcc cgaggcatag actgtacccc aaaaaaacag tcataacaag ccatgaaaac  4260
cgccactgcg ccgttaccac cgctgcgttc ggtcaaggtt ctggaccagt tgcgtgagcg  4320
catacgctac ttgcattaca gcttacgaac cgaacaggct tatgtccact gggttcgtgc  4380
cttcatccgt atcgatggcc cccgatggta gtgtggggtc tccccatgcg agagtaggga  4440
actgccaggc atcaaataaa acgaaaggct cagtcgaaag actgggcctt tcgttttatc  4500
tgttgtttgt cggtgaacgc tctcctgagt aggacaaatc cgccgggagc ggatttgaac  4560
gttgcgaagc aacggcccgg agggtggcgg gcaggacgcc cgccataaac tgccaggcat  4620
caaattaagc agaaggccat cctgacggat ggccttttg cgtggccagt gccaagcttg  4680
catgc                                                             4685
```

```
SEQ ID NO: 19          moltype = DNA  length = 466
FEATURE                Location/Qualifiers
source                 1..466
                       mol_type = other DNA
                       organism = synthetic construct
misc_feature           1..466
                       note = NTC9385R and NTC9685R Bacterial region [NheI
                        site-trpA terminator-R6K Origin-RNA-OUT-KpnI site]
SEQ ID NO: 19
gctagcccgc ctaatgagcg ggctttttt tggcttgttg tccacaaccg ttaaacctta  60
aaagctttaa aagccttata tattcttttt tttcttataa aacttaaaac cttagaggct  120
atttaagttg ctgatttata ttaattttat tgttcaaaca tgagagctta gtacgtgaaa  180
catgagagct tagtacgtta gccatgagag cttagtacgt tagccatgag ggtttagttc  240
gttaaacatg agagcttagt acgttaaaca tgagagctta gtacgtacta tcaacaggtt  300
gaactgctga tccacgttgt ggtagaattg gtaaagagag tcgtgtaaaa tatcgagttc  360
gcacatcttg ttgtctgatt attgattttt ggcgaaacca tttgatcata tgacaagatg  420
tgtatctacc ttaacttaat gattttgata aaaatcatta ggtacc                 466

SEQ ID NO: 20          moltype = DNA  length = 281
FEATURE                Location/Qualifiers
source                 1..281
                       mol_type = other DNA
                       organism = synthetic construct
misc_feature           1..281
                       note = NTC9385C and NTC9685C Bacterial region [NheI
```

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site-ssiA-ColE2 Origin (+7)-RNA-OUT-KpnI site]

SEQ ID NO: 20

gctagctaca atggctcatg tggaaaaacc attggcagaa aaacacctgc caacagtttt 60
accacaattg ccacttaacc cacaaaaggg cgctgttatc tgataaggct tatctggtct 120
cattttgcac gttgtggtag aattggtaaa gagagtcgtg taaaatatcg agttcgcaca 180
tcttgttgtc tgattattga tttttggcga aaccatttga tcatatgaca agatgtgtat 240
ctaccttaac ttaatgattt tgataaaaat cattaggtac c 281

SEQ ID NO: 21 moltype = DNA length = 76
FEATURE Location/Qualifiers
source 1..76
mol_type = other DNA
organism = synthetic construct
misc_feature 1..76
note = NTC9385C and NTC9685C CpG free ssiA [from plasmid R6K]

SEQ ID NO: 21

tacaatggct catgtggaaa aaccattggc agaaaaacac ctgccaacag ttttaccaca 60
attgccactt aaccca 76

SEQ ID NO: 22 moltype = DNA length = 260
FEATURE Location/Qualifiers
source 1..260
mol_type = other DNA
organism = synthetic construct
misc_feature 1..260
note = CpG free R6K origin

SEQ ID NO: 22

aaaccttaaa acctttaaaa gccttatata ttctttttt tcttataaaa cttaaaacct 60
tagaggctat ttaagttgct gatttatatt aattttattg ttcaaacatg agagcttagt 120
acatgaaaca tgagagctta gtacattagc catgagagct tagtacatta gccatgaggg 180
tttagttcat taaacatgag agcttagtac attaaacatg agagcttagt acatactatc 240
aacaggttga actgctgatc 260

SEQ ID NO: 23 moltype = DNA length = 137
FEATURE Location/Qualifiers
source 1..137
mol_type = other DNA
organism = synthetic construct
misc_feature 1..137
note = RNA-OUT Selectable Marker from NTC9385C, NTC9685C, NTC9385R, NTC9685R

SEQ ID NO: 23

agaattggta aagagagtcg tgtaaaatat cgagttcgca catcttgttg tctgattatt 60
gatttttggc gaaaccattt gatcatatga caagatgtgt atctacctta acttaatgat 120
tttgataaaa atcatta 137

SEQ ID NO: 24 moltype = DNA length = 70
FEATURE Location/Qualifiers
source 1..70
mol_type = other DNA
organism = synthetic construct
misc_feature 1..70
note = RNA-OUT Sense strand RNA from NTC9385C, NTC9685C, NTC9385R, NTC9685R, NTC9385Ra

SEQ ID NO: 24

ttcgcacatc ttgttgtctg attattgatt tttggcgaaa ccatttgatc atatgacaag 60
atgtgtatct 70

SEQ ID NO: 25 moltype = DNA length = 87
FEATURE Location/Qualifiers
source 1..87
mol_type = other DNA
organism = synthetic construct
misc_feature 1..87
note = TPA secretion sequence

SEQ ID NO: 25

atggatgcaa tgaagagagg gctctgctgt gtgctgctgc tgtgtggagc agtcttcgtt 60
tcgcccagcg gtaccggatc cgtcgac 87

-continued

SEQ ID NO: 26 moltype = DNA length = 57
FEATURE Location/Qualifiers
source 1..57
mol_type = other DNA
organism = synthetic construct
misc_feature 1..57
note = PCR primer 15061101
SEQ ID NO: 26
ggaacgggat ccagaaggag atatacatat gagtgccgta cttcagcgct tcaggga 57

SEQ ID NO: 27 moltype = DNA length = 40
FEATURE Location/Qualifiers
source 1..40
mol_type = other DNA
organism = synthetic construct
misc_feature 1..40
note = PCR primer 15061102
SEQ ID NO: 27
ggaacggaat tcttatcatt ttgcgagatc tggatcacat 40

SEQ ID NO: 28 moltype = DNA length = 32
FEATURE Location/Qualifiers
source 1..32
mol_type = other DNA
organism = synthetic construct
misc_feature 1..32
note = ColE2 core replication origin
SEQ ID NO: 28
ggcgctgtta tctgataagg cttatctggt ct 32

SEQ ID NO: 29 moltype = DNA length = 121
FEATURE Location/Qualifiers
source 1..121
mol_type = other DNA
organism = synthetic construct
misc_feature 1..121
note = +7(CpG free)-ssiA ColE2 origin
SEQ ID NO: 29
tacaatggct catgtggaaa aaccattggc agaaaaacac ctgccaacag ttttaccaca 60
attgccactt aacccacaaa agggggctgt tatctgataa ggcttatctg gtctcatttt 120
g 121

SEQ ID NO: 30 moltype = DNA length = 228
FEATURE Location/Qualifiers
source 1..228
mol_type = other DNA
organism = synthetic construct
misc_feature 1..228
note = HTLV- IR-Rabbit Beta globin hybrid intron
SEQ ID NO: 30
aggtaagttt aaagctcagg tcgagaccgg gcctttgtcc ggcgctccct tggagcctac 60
ctagactcag ccggctctcc acgctttgcc tgaccctgct tgctcaactc tagttctctc 120
gttaacttaa tgagacagat agaaactggt cttgtagaaa cagagtagtc gcctgctttt 180
ctgccaggtg ctgacttctc tcccctgggc ttttttcttt ttctcagg 228

SEQ ID NO: 31 moltype = DNA length = 108
FEATURE Location/Qualifiers
source 1..108
mol_type = other DNA
organism = synthetic construct
misc_feature 1..108
note = pMB1 RNAI antisense repressor RNA (origin antisense
partner of RNAII)
SEQ ID NO: 31
acagtatttg gtatctgcgc tctgctgaag ccagttacct tcggaaaaag agttggtagc 60
tcttgatccg gcaaacaaac caccgctggt agcggtggtt tttttgtt 108

SEQ ID NO: 32 moltype = DNA length = 156
FEATURE Location/Qualifiers
source 1..156

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                     mol_type = other DNA
                     organism = synthetic construct
misc_feature         1..156
                     note = pMB1 RNAI selectable Marker, RNAI RNA (Sense strand)
SEQ ID NO: 32
ttgaagtggt ggcctaacta cggctacact agaagaacag tatttggtat ctgcgctctg  60
ctgaagccag ttaccttcgg aaaaagagtt ggtagctctt gatccggcaa acaaaccacc  120
gctggtagcg gtggtttttt tgtttgcaag cagcag                           156

SEQ ID NO: 33        moltype = DNA  length = 73
FEATURE              Location/Qualifiers
source               1..73
                     mol_type = other DNA
                     organism = synthetic construct
misc_feature         1..73
                     note = IncB RNAI antisense repressor RNA (IncB plasmid
                      origin RNAII antisense partner)
SEQ ID NO: 33
gtattctgtg aggcccccat tatttttctg cgttccgcca agttcgagga aaaatagtgg  60
gggttttcct tta                                                    73

SEQ ID NO: 34        moltype = DNA  length = 124
FEATURE              Location/Qualifiers
source               1..124
                     mol_type = other DNA
                     organism = synthetic construct
misc_feature         1..124
                     note = IncB RNAI selectable Marker. DraIII-KpnI restriction
                      fragment.
SEQ ID NO: 34
cacgttgtgt tgaatctctg gtacggtttc atatatactt atcccgtatt ctgtgaggcc  60
cccattattt ttctgcgttc cgccaagttc gaggaaaaat agtgggggtt ttcctttagg  120
tacc                                                              124

SEQ ID NO: 35        moltype = DNA  length = 362
FEATURE              Location/Qualifiers
source               1..362
                     mol_type = other DNA
                     organism = synthetic construct
misc_feature         1..362
                     note = IncB RNAII-SacB. PstI-MamI restriction fragment
SEQ ID NO: 35
ctgcagttca aagcggtgga aaaggggtat attgcggatc gttattcagt ggcttttggg  60
atcctcgcgg tccggaaagc cagaaaacgg cagaatgcgc cataaggcat tcaggacgta  120
tggcagaaac gacggcagtt tgccggtgcc ggaaggctga aaaaagtttc agaagaccat  180
aaaggaaaac ccccactatt tttcctcgaa cttggcggaa cgcagaaaaa taatgggggc  240
ctcacagaat acgggatagg gcccatgaaa ccgtaccaga gattgggccc tgtgcagtgt  300
ataaatacac ggcacaatcg ctccgccata agcgacagct tgtggcaggt ctgatgaaca  360
tc                                                                362

SEQ ID NO: 36        moltype = DNA  length = 159
FEATURE              Location/Qualifiers
source               1..159
                     mol_type = other DNA
                     organism = synthetic construct
misc_feature         1..159
                     note = CpG free RNA-OUT selection marker - flanked by KpnI
                      and BglII- EcoRI sites
SEQ ID NO: 36
ggtacctggt agaattggta aagagagttg tgtaaaatat tgagttagca catcttgttg  60
tctgattatt gatttttggg gaaaccattt gatcatatga caagatgtgt atctaccttа  120
acttaatgat tttgataaaa atcattaaga tctgaattc                        159

SEQ ID NO: 37        moltype = DNA  length = 432
FEATURE              Location/Qualifiers
source               1..432
                     mol_type = other DNA
                     organism = synthetic construct
misc_feature         1..432
                     note = CpG free R6K gamma - RNA-OUT bacterial region (CpG
```

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free R6K origin-CpG free RNA-OUT selection marker)-flanked
by EcoRI-SphI and BglII-EcoRI sites

SEQ ID NO: 37
gaattcagca tgcaaacctt aaaaccttta aaagccttat atattctttt ttttcttata 60
aaacttaaaa ccttagaggc tatttaagtt gctgatttat attaatttta ttgttcaaac 120
atgagagctt agtacatgaa acatgagagc ttagtacatt agccatgaga gcttagtaca 180
ttagccatga gggtttagtt cattaaacat gagagcttag tacattaaac atgagagctt 240
agtacatact atcaacaggt tgaactgctg atcggtacct ggtagaattg gtaaagagag 300
ttgtgtaaaa tattgagtta gcacatcttg ttgtctgatt attgattttt ggggaaacca 360
tttgatcata tgacaagatg tgtatctacc ttaacttaat gattttgata aaaatcatta 420
agatctgaat tc 432

SEQ ID NO: 38 moltype = DNA length = 292
FEATURE Location/Qualifiers
source 1..292
mol_type = other DNA
organism = synthetic construct
misc_feature 1..292
note = CpG free ColE2 bacterial region (CpG free ssiA-CpG
free ColE2 origin-CpG free RNA-OUT selection marker)- -
flanked by EcoRI-SphI and BglII-EcoRI sites
SEQ ID NO: 38
gaattcagca tgctacaatg gctcatgtgg aaaaaccatt ggcagaaaaa cacctgccaa 60
cagttttacc acaattgcca cttaacccac aaaaggggac tgttatctga taaggcttat 120
ctggtctcat tttggtacct ggtagaattg gtaaagagag ttgtgtaaaa tattgagtta 180
gcacatcttg ttgtctgatt attgattttt ggggaaacca tttgatcata tgacaagatg 240
tgtatctacc ttaacttaat gattttgata aaaatcatta agatctgaat tc 292

SEQ ID NO: 39 moltype = DNA length = 1660
FEATURE Location/Qualifiers
source 1..1660
mol_type = other DNA
organism = synthetic construct
misc_feature 1..1660
note = NTC9385Ra-02 vector backbone
SEQ ID NO: 39
ccgcctaatg agcgggcttt tttttggctt gttgtccaca accgttaaac cttaaaagct 60
ttaaaagcct tatatattct tttttttctt ataaaactta aaaccttaga ggctatttaa 120
gttgctgatt tatattaatt ttattgttca aacatgagag cttagtacgt gaaacatgag 180
agcttagtac gttagccatg agagcttagt acgttagcca tgagggttta gttcgttaaa 240
catgagagct tagtacgtta aacatgagag cttagtacgt actatcaaca ggttgaactg 300
ctgatccacc ccggctctag ttattaatag taatcaatta cggggtcatt agttcatagc 360
ccatatatgg agttccgcgt tacataactt acggtaaatg gcccgcctgg ctgaccgccc 420
aacgacccccc gcccattgac gtcaataatg acgtatgttc ccatagtaac gccaataggg 480
actttccatt gacgtcaatg ggtggagtat ttacggtaaa ctgcccactt ggcagtacat 540
caagtgtatc atatgccaag tacgcccccct attgacgtca atgacggtaa atggcccgcc 600
tggcattatg cccagtacat gaccttatgg gactttccta cttggcagta catctacgta 660
ttagtcatcg ctattaccat ggtgatgcgg ttttggcagt acatcaatgg gcgtggatag 720
cggtttgact cacggggatt tccaagtctc caccccattg acgtcaatgg gagtttgttt 780
tggcaccaaa atcaacggga ctttccaaaa tgtcgtaaca actccgcccc attgacgcaa 840
atgggcggta ggcgtgtacg gtgggaggtc tatataagca gagctcgttt agtgaaccgt 900
cagatcgcct ggagacgcca tccacgctgt tttgacctcc atagaagaca ccgggaccga 960
tccagcctcc gcggctcgca tctctccttc acgcgcccgc cgccctacct gaggccgcca 1020
tccacgccgg ttgagtcgcg ttctgccgcc tcccgcctgt ggtgcctcct gaactgcgtc 1080
cgccgtctag gtaagtttaa agctcaggtc gagaccgggc ctttgtccgg cgctcccttg 1140
gagcctacct agactcagcc ggctctccac gctttgcctg accctgcttg ctcaactcta 1200
gttctctcgt tctaatgatt tttatcaaaa tcattaagtt aaggtagata cacatcttgt 1260
catatgatca aatggtttcg ccaaaaatca ataatcagac aacaagatgt gcgaactcga 1320
tattttacac gactctcttt accaattcta ccacaactta atgagacaga tagaaactgg 1380
tcttgtagaa acagagtagt cgcctgcttt tctgccaggt gctgacttct ctcccctggg 1440
ctttttttctt tttctcaggt tgaaaagaag aagacgaaga agacgaagaa gacaaaccgt 1500
cgtcgacaga tctttttccc tctgccaaaa attatgggga catcatgaag ccccttgagc 1560
atctgacttc tggctaataa aggaaattta ttttcattgc aatagtgtgt tggaattttt 1620
tgtgtctctc actcggaagg acataagggc ggccgctagc 1660

SEQ ID NO: 40 moltype = DNA length = 1660
FEATURE Location/Qualifiers
source 1..1660
mol_type = other DNA
organism = synthetic construct

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misc_feature 1..1660
note = NTC9385Ra-01 vector backbone

SEQ ID NO: 40

```
ccgcctaatg agcgggcttt tttttggctt gttgtccaca accgttaaac cttaaaagct  60
ttaaaagcct tatatattct ttttttttct ataaaactta aaaccttaga ggctatttaa  120
gttgctgatt tatattaatt ttattgttca aacatgagag cttagtacgt gaaacatgag  180
agcttagtac gttagccatg agagcttagt acgttagcca tgagggttta gttcgttaaa  240
catgagagct tagtacgtta aacatgagag cttagtacgt actatcaaca ggttgaactg  300
ctgatccacc ccggctctag ttattaatag taatcaatta cggggtcatt agttcatagc  360
ccatatatgg agttccgcgt tacataactt acggtaaatg gcccgcctgg ctgaccgccc  420
aacgaccccc gcccattgac gtcaataatg acgtatgttc ccatagtaac gccaataggg  480
actttccatt gacgtcaatg ggtggagtat ttacggtaaa ctgcccactt ggcagtacat  540
caagtgtatc atatgccaag tacgcccccc attgacgtca atgacggtaa atggcccgcc  600
tggcattatg cccagtacat gaccttatgg gactttccta cttggcagta catctacgta  660
ttagtcatcg ctattaccat ggtgatgcgg ttttggcagt acatcaatgg gcgtggatag  720
cggtttgact cacggggatt tccaagtctc caccccattg acgtcaatgg gagtttgttt  780
tggcaccaaa atcaacggga ctttccaaaa tgtcgtaaca actccgcccc attgacgcaa  840
atgggcggta ggcgtgtacg gtgggaggtc tatataagca gagctcgttt agtgaaccgt  900
cagatcgcct ggagacgcca tccacgctgt tttgacctcc atagaagaca ccgggaccga  960
tccagcctcc gcggctcgca tctctccttc acgcgcccgc cgccctacct gaggccgcca  1020
tccacgccgg ttgagtcgcg ttctgccgcc tcccgcctgt ggtgcctcct gaactgcgtc  1080
cgccgtctag gtaagtttaa agctcaggtc gagaccgggc ctttgtccgg cgctcccttg  1140
gagcctacct agactcagcc ggctctccac gctttgcctg accctgcttg ctcaactcta  1200
gttctctcgt tgtggtagaa ttggtaaaga gagtcgtgta aaatatcgag ttcgcacatc  1260
ttgttgtctg attattgatt tttggcgaaa ccatttgatc atatgacaag atgtgtatct  1320
accttaactt aatgatttttg ataaaaatca ttagaactta atgagacaga tagaaactgg  1380
tcttgtagaa acagagtagt cgcctgcttt tctgccaggt gctgacttct ctcccctggg  1440
ctttttttctt tttctcaggt tgaaaagaag aagacgaaga agacgaagaa gacaaaccgt  1500
cgtcgacaga tctttttccc tctgccaaaa attatgggga catcatgaag ccccttgagc  1560
atctgacttc tggctaataa aggaaattta ttttcattgc aatagtgtgt tggaattttt  1620
tgtgtctctc actcggaagg acataagggc ggccgctagc                        1660
```

SEQ ID NO: 41 moltype = DNA length = 1763
FEATURE Location/Qualifiers
source 1..1763
mol_type = other DNA
organism = synthetic construct
misc_feature 1..1763
note = NTC9385R-BE vector backbone

SEQ ID NO: 41

```
ccgcctaatg agcgggcttt tttttggctt gttgtccaca accgttaaac cttaaaagct  60
ttaaaagcct tatatattct ttttttttct ataaaactta aaaccttaga ggctatttaa  120
gttgctgatt tatattaatt ttattgttca aacatgagag cttagtacgt gaaacatgag  180
agcttagtac gttagccatg agagcttagt acgttagcca tgagggttta gttcgttaaa  240
catgagagct tagtacgtta aacatgagag cttagtacgt actatcaaca ggttgaactg  300
ctgatccacg ttgtggtaga attggtaaag agagtcgtgt aaaatatcga gttcgcacat  360
cttgttgtct gattattgat ttttggcgaa accatttgat catatgacaa gatgtgtatc  420
taccttaact taatgatttt gataaaaatc attaggtacc ccggctctag atggccattg  480
catacgttgt atccatatca taatatgtac atttatattg gctcatgtcc aacattaccg  540
ccatgttgac attgattatt gactagttat taatagtaat caattacggg gtcattagtt  600
catagcccat atatggagtt ccgcgttaca taacttacgg taaatggccc gcctggctga  660
ccgcccaacg acccccgccc attgacgtca ataatgacgt atgttcccat agtaacgcca  720
atagggactt tccattgacg tcaatgggtg gagtatttac ggtaaactgc ccacttggca  780
gtacatcaag tgtatcatat gccaagtacg ccccctattg acgtcaatga cggtaaatgg  840
cccgcctggc attatgccca gtacatgacc ttatgggact ttcctacttg gcagtacatc  900
tacgtattag tcatcgctat taccatggtg atgcggtttt ggcagtacat caatgggcgt  960
ggatagcggt ttgactcacg gggatttcca agtctccacc ccattgacgt caatgggagt  1020
ttgttttggc accaaaatca acgggacttt ccaaaatgtc gtaacaactc cgccccattg  1080
acgcaaatgg gcggtaggcg tgtacggtgg gaggtctata taagcagagc tcgtttagtg  1140
aaccgtcaga tcgcctggag acgccatcca cgctgttttg acctccatag aagacaccgg  1200
gaccgatcca gcctccgcgg ctcgcatctc tccttcacgc gcccgccgcc ctacctgagg  1260
ccgccatcca cgccggttga gtcgcgttct gccgcctccc gcctgtggtg cctcctgaac  1320
tgcgtccgcc gtctaggtaa gtttaaagct caggtcgaga ccgggccttt gtccggcgct  1380
cccttggagc ctacctagac tcagccggct ctccacgctt tgcctgaccc tgcttgctca  1440
actctagttc tctcgttaac ttaatgagac agatagaaac tggtcttgta gaaacagagt  1500
agtcgcctgc ttttctgcca ggtgctgact tctctccccc gggctttttt ctttttctca  1560
ggttgaaaag aagaagacga agaagacgaa gaagacaaac cgtcgtcgac agatcttttt  1620
ccctctgcca aaaattatgg ggacatcatg aagccccttg agcatctgac ttctggctaa  1680
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taaaggaaat ttattttcat tgcaatagtg tgttggaatt ttttgtgtct ctcactcgga  1740
aggacataag ggcggccgct agc                                          1763

SEQ ID NO: 42          moltype = DNA  length = 678
FEATURE                Location/Qualifiers
source                 1..678
                       mol_type = other DNA
                       organism = synthetic construct
misc_feature           1..678
                       note = Pmin minimal pUC replication origin
SEQ ID NO: 42
cgcgttgctg gcgtttttca taggctccgc ccccctgacg agcatcacaa aaatcgacgc  60
tcaagtcaga ggtggcgaaa cccgacagga ctataaagat accaggcgtt tccccctgga  120
agctccctcg tgcgctctcc tgttccgacc ctgccgctta ccggatacct gtccgccttt  180
ctcccttcgg gaagcgtggc gctttctcat agctcacgct gtaggtatct cagttcggtg  240
taggtcgttc gctccaagct gggctgtgtg cacgaacccc ccgttcagcc cgaccgctgc  300
gccttatccg gtaactatcg tcttgagtcc aacccggtaa gacacgactt atcgccactg  360
gcagcagcca ctggtaacag gattagcaga gcgaggtatg taggcggtgc tacagagttc  420
ttgaagtggt ggcctaacta cggctacact agaagaacag tatttggtat ctgcgctctg  480
ctgaagccag ttaccttcgg aaaaagagtt ggtagctctt gatccggcaa acaaaccacc  540
gctggtagcg gtggtttttt tgtttgcaag cagcagatta cgcgcagaaa aaaaggatct  600
caagaagatc ctttgatctt ttctacgggg tctgacgctc agtggaacga aaactcacgt  660
taagggattt tggtcatg                                                678

SEQ ID NO: 43          moltype = DNA  length = 866
FEATURE                Location/Qualifiers
source                 1..866
                       mol_type = other DNA
                       organism = synthetic construct
misc_feature           1..866
                       note = pUC (0.85) Bacterial region [NheI site- trpA
                        terminator-Pmin pUC replication origin
                        (minimal)-RNA-OUT-KpnI site]
SEQ ID NO: 43
gctagcccgc ctaatgagcg ggctttttt tcttaggcct cgcgttgctg gcgtttttca  60
taggctccgc cccctgacg agcatcacaa aaatcgacgc tcaagtcaga ggtggcgaaa  120
cccgacagga ctataaagat accaggcgtt tccccctgga agctccctcg tgcgctctcc  180
tgttccgacc ctgccgctta ccggatacct gtccgccttt ctcccttcgg gaagcgtggc  240
gctttctcat agctcacgct gtaggtatct cagttcggtg taggtcgttc gctccaagct  300
gggctgtgtg cacgaacccc ccgttcagcc cgaccgctgc gccttatccg gtaactatcg  360
tcttgagtcc aacccggtaa gacacgactt atcgccactg gcagcagcca ctggtaacag  420
gattagcaga gcgaggtatg taggcggtgc tacagagttc ttgaagtggt ggcctaacta  480
cggctacact agaagaacag tatttggtat ctgcgctctg ctgaagccag ttaccttcgg  540
aaaaagagtt ggtagctctt gatccggcaa acaaaccacc gctggtagcg gtggtttttt  600
tgtttgcaag cagcagatta cgcgcagaaa aaaaggatct caagaagatc ctttgatctt  660
ttctacgggg tctgacgctc agtggaacga aaactcacgt taagggattt tggtcatggt  720
ggtagaattg gtaaagagag tcgtgtaaaa tatcgagttc gcacatcttg ttgtctgatt  780
attgattttt ggcgaaacca tttgatcata tgacaagatg tgtatctacc ttaacttaat  840
gattttgata aaaatcatta ggtacc                                       866

SEQ ID NO: 44          moltype = DNA  length = 146
FEATURE                Location/Qualifiers
source                 1..146
                       mol_type = other DNA
                       organism = synthetic construct
misc_feature           1..146
                       note = PL promoter with locations   of the PL promoter OL1,
                        OL2 and OL3 repressor binding sites, -10 and -35 promoter
                        elements for Pll and PL2 promoters
SEQ ID NO: 44
agcaattcag atctctcacc taccaaacaa tgccccccctg caaaaaataa attcatataa  60
aaaacataca gataaccatc tgcggtgata aattatctct ggcggtgttg acataaatac  120
cactggcggt gatactgagc acatca                                       146
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Claimed is:

1. An engineered bacterial cell comprising a Rep protein-dependent plasmid vector, a Rep protein gene that expresses a Rep protein, and a P_L promoter that controls the expression of the Rep protein gene, and wherein the P_L promoter comprises an OL1 mutation, wherein the OL1 mutation comprises either a single base substitution or a single base deletion that decreases or prevents repressor biding to OL1, and wherein

the Rep protein-dependent plasmid vector comprises an R6K replication origin.

2. The engineered bacterial cell of claim **1**, wherein the P_L promoter comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:10 and SEQ ID NO: 44.

3. The engineered bacterial cell of claim **1**, wherein said Rep protein comprises an amino acid sequence of SEQ ID NO: 13, wherein the amino acid sequence comprises two mutations at amino acids 106 and 107 of SEQ ID NO:13.

4. The engineered bacterial cell of claim **1**, wherein the sequences of the P_L promoter comprising said OL1 mutation is selected from the group consisting of SEQ ID NO: 11 and SEQ ID NO: 12.

5. The engineered bacterial cell of claim **1**, wherein the R6K replication origin comprises a nucleic acid sequence that possesses at least 90% sequence identity to SEQ ID NO: 1 or SEQ ID NO: 22.

6. The engineered bacterial cell of claim **1**, wherein the Rep protein-dependent plasmid vector further comprises an RNA-OUT selectable marker.

7. The engineered bacterial cell of claim **6**, wherein the RNA-OUT selectable marker comprises a nucleic acid sequence with at least 90% sequence identity to SEQ ID NO: 23 or SEQ ID NO: 36.

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