NTC9 series Nanoplasmid™ Expression Vectors

Instruction Manual

Catalog Numbers

NTC-NPV-9385R-MCS
NTC-NPV-9385R-EGFP
NTC-NPV-9385R-LAMP
NTC-NPV-9382R-TPA
NTC-NPV-9384R
NTC-NPV-9385R-Luc
NTC-NPV-9385R-SEAP
NTC-NPV-9385R-Bgal
NTC-NPV-9385R-DsRed

Version 2
April 2018

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General Information

Contents: 20 µg each of Nanoplasmid™ vector shipped in 1x TE buffer.

Storage: Nanoplasmids™ should be stored at -20˚C

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NTC9 Series Nanoplasmid™ Vector Family

Overview

Nature Technology Corporation's (NTC's) NTC9 series antibiotic free selection Nanoplasmid™ vectors have dramatically higher *in vivo* expression than previous generation antibiotic free selection NTC8 series vectors, which were themselves dramatically improved compared to kanamycin selectable vectors such as gWIZ and pVAX1, and minicircle vectors. Nanoplasmid™ vectors include NTC8 vector generation advantages, with the additional advantage of a reduced bacterial backbone size that dramatically improves *in vivo* and *in vitro* expression level and duration (Williams, 2014).

Introduction

NTC9 series Nanoplasmids™ are modified antibiotic-free Nanoplasmid™ versions of the corresponding NTC8 series vectors.

These NTC9 series Nanoplasmids™ were specifically designed as safe, minimalized, antibiotic-free selection vectors for the expression of recombinant proteins in mammalian cells. This may be for protein production, gene therapy, or induction of neutralizing immune responses by genetic immunization. The vectors combine minimal prokaryotic sequences including an antibiotic-free sucrose selectable marker. The vectors also contain a novel chimeric promoter that directs superior mammalian cell expression (Luke *et al.* 2009).

The vectors are available in transgene-targeting versions. NTC9385R expresses encoded protein without additional targeting sequences. NTC9382R targets encoded protein into the secretory pathway using an optimized tissue plasminogen activator (TPA) signal peptide. NTC9385R-LAMP-Vax™ targets encoded protein to the endosome using an optimized human lysosomal-associated membrane protein 1 (Lamp1) targeting tag. NTC9384R-Ub A76 targets proteins to the proteasome by fusion C-terminal to a destabilizing UbiquitinA76 tag.

The vectors were designed to be responsive to Food and Drug Administration (FDA) regulatory guidances regarding DNA Vaccine vector composition (FDA 1996, FDA 2007; reviewed in Williams *et al*. 2009a). All sequences that were not essential for *Escherichia coli* plasmid replication or mammalian cell expression of the target gene were eliminated. Synthetic, optimized, non-human eukaryotic mRNA leader and polyA sequences were utilized in the vector design to limit DNA sequence homology with the human genome to reduce the possibility of chromosomal integration.

Target gene expression is driven from an optimized chimeric promoter-intron (CMV-HTLV-I R synthetic intron). The chimeric CMV promoter achieves significantly higher expression levels than traditional human cytomegalovirus (CMV) promoter based vectors (Luke *et al.* 2009). The vectors encode a consensus Kozak translation initiation sequence and ATG start codon.

Antibiotic-free Selection

Antibiotic-resistance markers, typically kanamycin resistance (KanR), allow selective retention of plasmid DNA during bacterial fermentation and are the most commonly utilized selectable markers.

To ensure safety, however, regulatory agencies recommend elimination of antibiotic-resistance markers from therapeutic and vaccine plasmid DNA vectors. The presence of an antibiotic resistance gene in the plasmid backbone is considered undesirable by regulatory agencies, due to the potential transfer of antibiotic resistance to endogenous microbial flora and the potential activation and transcription of the genes from mammalian promoters after cellular incorporation into the genome (Reviewed in Williams *et al.* 2009a, Williams 2013).

The NTC9 series antibiotic-free DNA vaccines (*e.g*. NTC9385R-EGFP; NTC9385R-MCS; NTC9382R) incorporate and express a 150 bp RNA-OUT antisense RNA. RNA-OUT that represses expression of a chromosomal counter-selectable marker (*Sac*B) (**Fig. 1**; Luke *et al*., 2009). *Sac*B encodes a levansucrase, which is toxic in the presence of sucrose. Plasmid selection is achieved in sucrose-containing media.

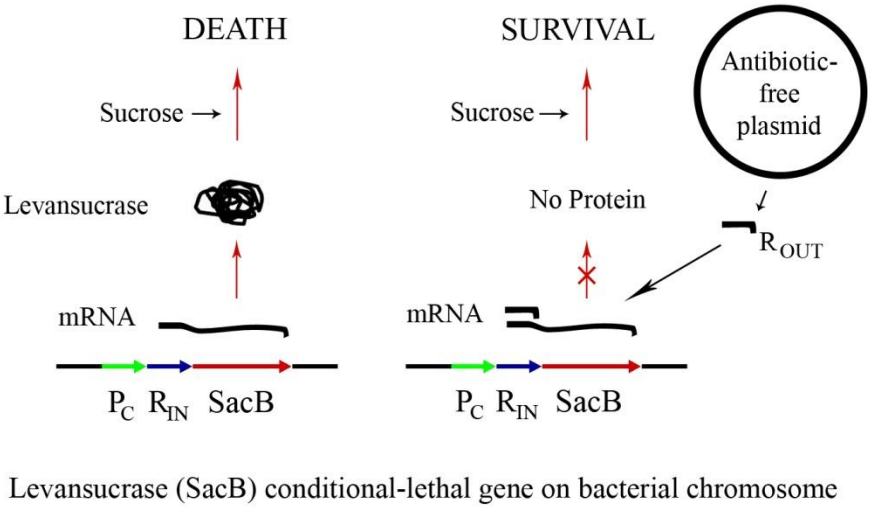


Figure 1: Antibiotic-free selection using NTC att$_\lambda$::P$_{5/6\ 6/6}$-RNA-IN- SacB Antibiotic-free Sucrose Selection Cell Lines

Heat Inducible Replication

NTC9 series sucrose selectable Nanoplasmid™ vectors combine antibiotic-free selection with highly productive heat inducible R6K origin replication (**Fig. 2**). HyperGRO™ fermentation DNA yields up to 2.4 g/L have been obtained with NTC9 series Nanoplasmid™ vectors.

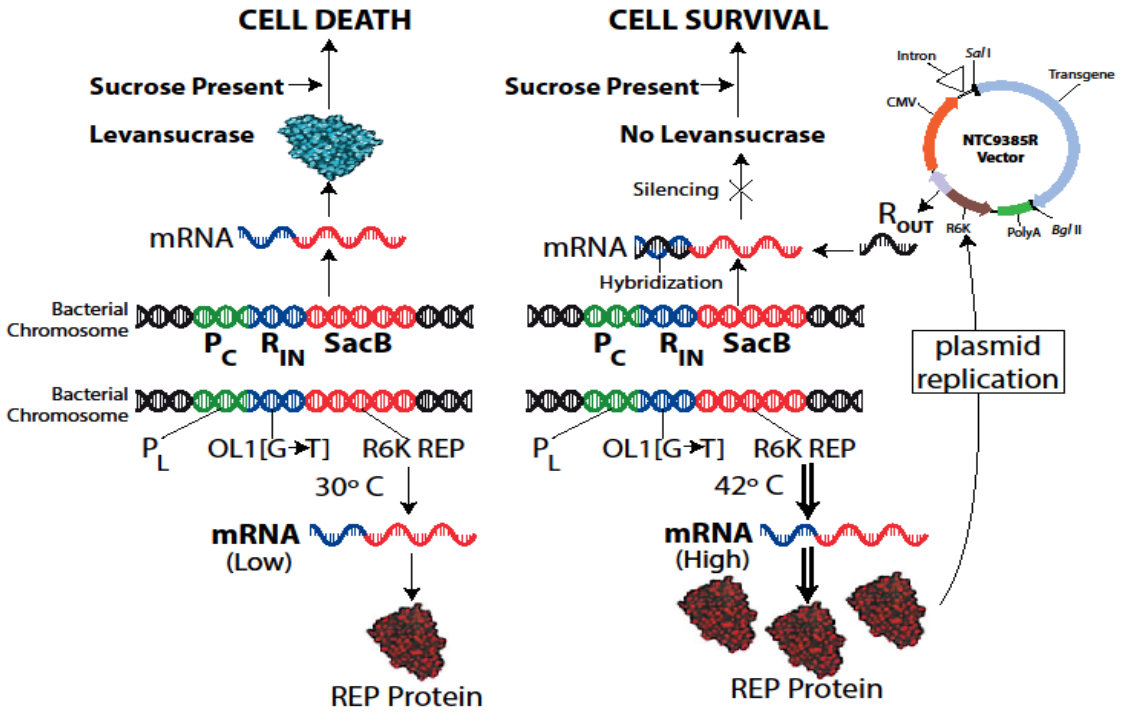


Figure 2: Nanoplasmid™ Antibiotic-Free RNA-OUT Marker selection and heat inducible R6K origin propagation in engineered cell lines NTC711772, NTC821601, NTC711231, NTC1050811 and NTC940211

Chimeric Promoter

The chimeric promoter is composed of:

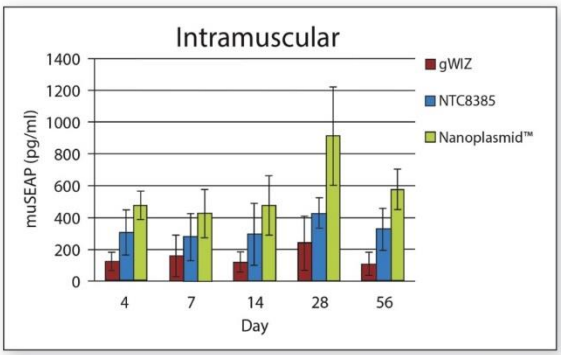
1) CMV promoter and start of exon 1;
2) A HTLV-I R sequence which contains the 5' splice acceptor site;
3) A synthetic 3' acceptor site based on the rabbit β globin intron;
4) Exon 2 splicing enhancer comprised of serine-arginine rich (SR) protein binding site (3 copies of GAAGAAGAC) to improve RNA export (Lavigueur *et al*. 1993);
5) Exon 2 kozak sequence upstream of the start codon for the gene of interest.

Incorporation of the HTLV-I R region downstream of the CMV promoter has been demonstrated to improve expression and cellular immune responses to HIV DNA vaccines in mice and nonhuman primates compared to CMV promoter based vectors (Barouch *et al.* 2005). Expression levels of EGFP from plasmids incorporating the NTC chimeric promoter are also dramatically increased versus CMV promoter containing plasmids (Luke *et al*. 2009).

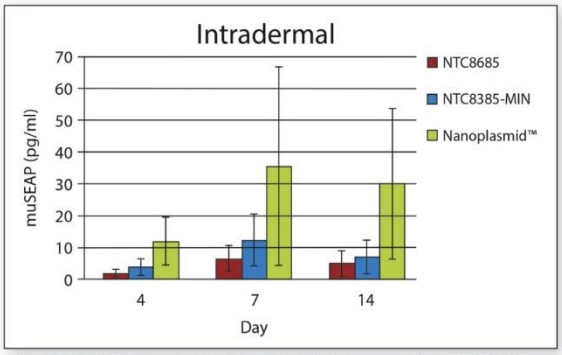
The HTLV-I R region in NTC9 series vectors (incorporated as part of Exon 1 and Intron 1) dramatically increases mRNA translation efficiency, but not overall mRNA levels, after transient transfection (Luke *et al*., 2009).

Nanoplasmid™ Bacterial Backbone Expression Enhancement

The Nanoplasmid™ bacterial backbone comprising the R6K origin and RNA-OUT selection marker is <500 bp. Inclusion of this minimal vector backbone in NTC9 series vectors dramatically increases expression level and duration (**Fig. 3**)



Intramuscular *in vivo* expression of muSEAP, comparing gWIZ, NTC8385 and **Nanoplasmid™** vector.



Intradermal *in vivo* expression of muSEAP, comparing NTC8685, NTC8385-MIN and **Nanoplasmid™** vector.

Figure 3: NTC9385R increases expression compared to gWIZ-EGFP, pVAX1-EGFP, NTC8 series plasmid vectors, and minicircle vectors, *in vivo* after intramuscular or intradermal delivery (Williams, 2015). Improved expression is also observed *in vitro* (Williams, 2015) and in various other tissues. Increased expression has been demonstrated to translate to improved NTC9385R DNA vaccine encoded antigen-mediated B-cell and T-cell adaptive immune responses (Borggren, 2015)

Cloning

For precise cloning into NTC9382-TPA, NTC9384-Ub A76 or NTC9385R-EGFP, genes are copied by PCR amplification from clones or genomic DNA using primers with *Sal*I (5' end) and *Bgl*II (3' end) sites. Alternatively, genes are synthesized chemically to be compatible with the *Sal*I / *Bgl*II cloning sites. Design criteria for gene synthesis have been developed by NTC (Williams *et al.* 2009). Alternatively, NTC9385R contains a multiple cloning site polylinker for cloning existing genes.

In summary, the NTC9 series Nanoplasmid™ vectors offer the following advantages:

- Highest expression level and duration conferred by <500 bp bacterial region (Nanoplasmid™ backbone)
- Robust mammalian cell expression using a chimeric CMV promoter-synthetic intron*
- Small vectors for more efficient transfection
- Antibiotic-free selection in *Escherichia coli* host
- Optional protein targeting tags (secretion, endosome, proteasome)
- Simultaneous cloning into all vectors through use of compatible precision cloning cassettes
- Compliance with regulatory guidance (*i.e*. no antibiotic resistance marker; replication incompetence in standard *E. coli* or native organisms, reduced human genome homology)

****Nanoplasmid™ vectors with alternative promoters (e.g. EF1, CAG) are available***.

NTC9 Series Nanoplasmid™ Vector Features

Feature	Function
Optimized human cytomegalovirus (CMV) immediate-early enhancer promoter	High-level mammalian cell expression *in vitro* and *in vivo*
HTLV-I R	Increase mammalian cell expression
Synthetic Intron	Increase mRNA nuclear export
SR-protein binding sites (3x)	Increase mRNA nuclear export
Precision cloning cassette	Clone target gene into the vector with native, endosome, secreted or proteasomal intracellular trafficking
Rabbit β globin eukaryotic polyadenylation signal- polyA	Effective mRNA transcriptional termination and polyadenylation
trpA prokaryotic terminator	Protection of replication origin from insert initiated transcription
Sucrose selection marker (RNA-OUT)	Plasmid selection in *Escherichia coli* cells[A]
R6K replication origin	High copy number plasmid production in *Escherichia coli* cells[B]
<500 bp bacterial backbone (R6K-RNA-OUT)	Dramatically increased transgene expression level and duration[C]

[A] Selection in engineering host strain DH5α att_{λ}::$P_{5/6\ 6/6}$-RNA-IN- SacB, catR

[B] Heat inducible plasmid replication in engineered cell lines NTC711772, NTC821601, NTC711231, NTC1050811 and NTC940211

[C] Williams, 2014; Williams, 2015

NTC9 Series Nanoplasmid™: Intracellular Targeting

Vector	Protein Destination	Targeting Tag
NTC9382R-TPA	secreted	human tissue plasminogen activator (TPA)
NTC9384R-Ub A76	proteasome	murine Ubiquitin A76
NTC9385R-LAMP-Vax™	endosome	human lysosomal-associated membrane protein 1 (Lamp1)
NTC9385R	native	ATG*****

* Native vectors express the target gene from a vector encoded ATG start codon immediately downstream of an optimized kozak sequence (*Sal*I site-ATG or introduced GCCACC-ATG). Protein targeting (*e.g.* cytoplasmic or nuclear) will be determined by protein-intrinsic factors

Each of these targeting peptides has been demonstrated to efficiently target heterologous proteins to the indicated intracellular destination. The Lamp1 fusion protein (**NTC9385R-LAMP-Vax™**) is utilized to target proteins to the endosome and MHC class II presentation (Wu *et al.* 1995; August *et al.*1997; Weiss *et al.* 2000). The TPA targeting peptide (**NTC9382R-TPA**) has been demonstrated to efficiently target heterologous proteins for secretion (Zhongming *et al.*1999). The destabilizing ubiquitin molecule (UbiquitinA76 versus native UbiquitinG76) in **NTC9384R-Ub A76** enhances entry into proteasomal degradation pathway and MHC class I presentation, and shifts host response towards T_H1 type immunity (Rodriguez *et al.*1998; Delogu *et al.*2000).

Nanoplasmid™ Expression Vector Kits*

Vector	Targeting	Quantity	Catalog Number	Price
NTC9382R-TPA	secreted	20 µg	NTC-NPV-9382R-TPA	$604.00
NTC9384R-Ub A76	Proteasome	20 µg	NTC-NPV-9384R	$$604.00
NTC9385R-LAMP-Vax™	Endosome	20 µg	NTC-NPV-9385R-LAMP	$604.00
NTC9385R-EGFP‡	cytoplasmic EGFP	20 µg	NTC-NPV-9385R-EGFP	$604.00
NTC9385R-MCS	No targeting	20 µg	NTC-NPV-9385R-MCS	$604.00

***** Kit contains Nanoplasmid™ vector and NTC821601 DH5α derived NTC9 series Nanoplasmid™ host strain glycerol stock

‡ The NTC9385R-EGFP Nanoplasmid™ is used as a transfection control for expression in a cell line of interest, and can be used as the NTC9385R backbone for SalI-BglII cloning

RAPID-VACC™ CONTRACT CLONING is an optional service whereby NTC clones investigator-specified genes into the desired expression vectors, providing sequence-validated clones, or purified plasmid DNA ready for animal testing (see *www.natx.com*).

RAPID-VACC™

Seamless cloning gene of interest into any vector	$440.00
1mg endotoxin-free plasmid preparations, each	$369.00

Nanoplasmid™ Reporter Gene Vector Kits*

Vector	Targeting	Quantity	Catalog Number	Price
NTC9385R-Bgal	cytoplasmic β galactosidase	20 µg	NTC-NPV-9385R-BGAL	$604.00
NTC9385R-DsRedExpress	cytoplasmic DsRedExpress	20 µg	NTC-NPV-9385R-DsRed	$604.00
NTC9385R-EGFP	cytoplasmic EGFP	20 µg	NTC-NPV-9385R-EGFP	$604.00
NTC9385R-Luc	cytoplasmic Luciferase	20 µg	NTC-NPV-9385R-Luc	$604.00
NTC9385R-SEAP	Secreted murine SEAP	20 µg	NTC-NPV-9385R-SEAP	$604.00

*****Kit contains Nanoplasmid™ vector and NTC821601 DH5α derived NTC9 series Nanoplasmid™ host strain glycerol stock

NTC offers the following accessory products for use with the NTC9 series Nanoplasmid™ vectors:

Accessory Products

Product	Description	Quantity	Catalog Number	Price
pVAC5'	Forward sequencing primer	500 pmol (10 pmol/µL)	NTC-DVU-SP1	$30.00
pVAC3'	Reverse sequencing primer	500 pmol (10 pmol/µL)	NTC-DVU-SP2	$30.00
RNAOUTF01seq	Nanoplasmid™ backbone primer	500 pmol (10 pmol/µL)	NTC-NPV-SP1	$30.00
RNAOUTR01seq	Nanoplasmid™ backbone primer	500 pmol (10 pmol/µL)	NTC-NPV-SP2	$30.00
CMVF2seq	Nanoplasmid™ backbone primer	500 pmol (10 pmol/µL)	NTC-NPV-SP3	$30.00
CMVF4seq	Nanoplasmid™ backbone primer	500 pmol (10 pmol/µL)	NTC-NPV-SP4	$30.00
NTC711772 DH5α dcm- att$_\lambda$::P$_{5/6\ 6/6}$-RNA-IN- SacB, catR; pL (OL1-G to T) P42L-P106L-F107S (P3-), SpecR StrepR electrocompetent cells	Dcm- host strain for NTC9 series Nanoplasmid™ selection	0.5 mL	NTC-NP-CC1	$285.00
NTC821601 DH5α att$_\lambda$::P$_{5/6\ 6/6}$-RNA-IN- SacB, catR; pL (OL1-G to T) P42L-P106L-F107S (P3-), SpecR StrepR electrocompetent cells	Host strain for NTC9 series Nanoplasmid™ selection	0.5 mL	NTC-NP-CC2	$285.00
NTC711231 XL1Blue dcm- att$_\lambda$::P$_{5/6\ 6/6}$-RNA-IN- SacB, catR; pL (OL1-G to T) P42L-P106L-F107S (P3-), SpecR StrepR electrocompetent cells	Dcm- XL1Blue host strain for NTC9 series Nanoplasmid™ selection	0.5 mL	NTC-NP-CC3	$285.00
NTC940211 DH5α att$_\lambda$::P$_{5/6\ 6/6}$-RNA-IN- SacB, catR; pL (OL1-G to T) P42L-P106**I**-F107S **P113S** (P3-), SpecR StrepR electrocompetent cells	Host strain for NTC9 series Nanoplasmid™ selection	0.5 mL	NTC-NP-CC4	$285.00
NTC1050811 DH5α att$_\lambda$::P$_{5/6\ 6/6}$-RNA-IN- SacB, catR; pL (OL1-G to T) P42L-P106**I**-F107S **P113S** (P3-), SpecR StrepR; pARA-CI857ts, TetR electrocompetent cells	Copy number cutter host strain for NTC9 series Nanoplasmid™ selection[A]	0.5 mL	NTC-NP-CC5	$285.00
Sucrose selection agar	Nanoplasmid™ selection plates	to make 1 L agar[B]	NTC-DVU-MD1	$22
Sucrose selection media	Nanoplasmid™ growth media	to make 1 L media[B]	NTC-DVU-MD2	$22

A Addition of arabinose to 0.02-0.2% induces production of λ repressor CI857ts with strain NTC1050811. This ts repressor reduce R6K rep protein P42L-P106I-F107S P113S production at 30ºC, but not 37°C, to selectively reduce Nanoplasmid™ copy number during 30ºC growth phase.
B Reconstitute with distilled water to 1L and autoclave prior to use.

Cloning Into NTC9 Series Nanoplasmid™ Vectors

Overview: An example strategy for cloning into the NTC9 series Nanoplasmid™ vectors is outlined below.

GTCGACATG-------- Gene of interest----Stop codon ------AGATCT
*Sal*I *Bgl*II

The ATG start codon (double underlined) is immediately preceded by a *Sal*I site. In NTC9385R, the *Sal*I site has been demonstrated to be an effective consensus Kozak sequence for translational initiation. In NTC9382R-TPA, the *Sal*I site is downstream in frame with the optimized TPA secretion sequence. In NTC9384-Ub A76, the *Sal*I site is downstream in frame with the Ubiquitin A76 proteasome targeting sequence.

For precise cloning, genes are copied by PCR amplification from clones or genomic DNA using primers with *Sal*I (5' end) and *Bgl*II (3' end) sites. Alternatively, genes are synthesized chemically to be compatible with the *Sal*I / *Bgl*II cloning sites. Design criteria for gene synthesis are reviewed in Williams *et al.* 2009a.

For NTC9385R, the start codon ATG must immediately follow the *Sal*I site (GTCGACATG) or, optionally, a kozak site (*e.g.* GTCGACGCCACCATG). For NTC9382R-TPA and NTC9384R-Ub A76, the ATG is optional but the same reading frame must be retained. For all three vectors one or two stop codon (preferably TAA or TGA) must be included prior to the *Bgl*II site. A PCR product designed for NTC9385R is compatible with, and can also be cloned into, the NTC9382R-TPA and NTC9384R-Ub A76 vectors.

Protocol: Genes are PCR amplified with primers incorporating a *Sal*I site into the 5' termini and a *Bgl*II site into the 3' termini. Example primers, for cloning a target gene of interest into both vectors are diagramed below.

*Sal*I
GCGCATGTCGACATG-GENE OF INTEREST 5' END
*Bgl*II
GCAGAAAGATCTTTA-GENE OF INTEREST (REVERSE COMPLEMENT) 3' END

Cleavage of the vectors with *Sal*I / *Bgl*II generates sticky ends compatible with the cleaved PCR product. The insert is thus directionally and precisely cloned into the vector. The majority of recovered colonies are recombinant, since the generated sticky ends in the parental vector are not compatible.

If the gene of interest encodes internal *Bgl*II sites, *Bam*HI or *Bcl*I restriction enzymes can be utilized in the PCR primers since they create *Bgl*II compatible sticky ends. Note that both enzymes sites will be lost in the resulting construct.

*Xho*I is not recommended as an alternative to *Sal*I for cloning into NTC9385R, since the ligated hybrid (GTCGAGATG) has not been established as an effective kozak sequence. This is not an issue for cloning downstream of TPA in NTC9382R-TPA or Ub A76 in NTC9384R-Ub A76.

Alternatively, when these restriction sites are present in the gene, the PCR primers may be modified to incorporate *Aar*I (Fermentas, Vilnius Lithuania) type IIS enzyme sites. This enzyme cleaves after +4 and +8 bp downstream of the *Aar*I recognition sequence to create any designed 4 bp 3'-recessed sticky end. *Aar*I primer design to amplify a gene for cloning into NTC9382R-TPA, NTC9384R-Ub A76 and NTC9385R-MCS vectors is shown below.

*Aar*I
5' CTCCAGCACCTGCCTATTCGACATG-GENE OF INTEREST 5' END

5' CGTGAGCACCTGCAACGGATCTTA-GENE OF INTEREST (REVERSE COMPLEMENT) 3' END

Recombinant clones can be identified by restriction digestion. *Sal*I and *Bgl*II release the gene insert when using *Aar*I or *Sal*I / *Bgl*II containing primers. *Bgl*II /*Bam*HI/*Bcl*I ligated cohesive termini in the resultant clone will not cleave with either parent restriction enzyme.

The NTC9385R-LAMP-Vax™ vector uses *Xho*I/ *Eco*RI cloning, rather than *Sal*I and *Bgl*II. The *Xho*I site is downstream in frame with the human LAMP N terminal luminal (N-LAMP) endosomal targeting sequence. The *Eco*RI site is located upstream and in frame with the human LAMP C terminal transmembrane and cytoplasmic domains (C-LAMP). *Xho*I is substituted for *Sal*I and *Eco*RI is substituted for *Bgl*II in the cloning strategies outlined above. However, since the C-LAMP domain is essential for endosomal targeting, ***the transgene cannot contain a stop codon*** (i.e. the reading frame must extend through the C terminus of the transgene in frame with C-LAMP).

The NTC9385R-MCS contains a multiple cloning site polylinker to facilitate restriction enzyme based transfer of cloning existing gene inserts into the NTC9385R backbone using a variety of different restriction enzymes.

The sequence of the gene insert can be determined using the following cloning cassette flanking sequencing primers.

pVAC5': GCTTTTCTGCCAGGTGCTGA
(hybridizes to intron and sequences from 5' end of gene)

pVAC3': GCCAGAAGTCAGATGCTCAA
(hybridizes to polyA region and sequences from 3' end of gene)

Transformation and Bacterial Propagation Overview

NTC RNA-OUT antibiotic-free (AF) DNA vaccines incorporate and express a 150 bp RNA-OUT antisense RNA. RNA-OUT represses expression of a chromosomal counter-selectable marker (*Sac*B) under the control of RNA-IN (att$_{\lambda}$::P$_{5/6\ 6/6}$-RNA-IN- SacB) (**Fig. 1**; Luke *et al*., 2009). *Sac*B encodes a levansucrase, which is toxic in the presence of sucrose. Plasmid selection is achieved in sucrose-containing media.

Instructions for use of NTC att$_{\lambda}$::P$_{5/6\ 6/6}$-RNA-IN- SacB Antibiotic-free sucrose selection cell lines are provided below. ***Detailed, published methodologies for the design and cloning of genes into RNA-OUT encoding, antibiotic-free DNA vaccine vectors using engineered sucrose selection cell lines are available (Luke et al., 2014)***.

NTC Electrocompetent Cells

Electrocompetent cells for use with NTC Antibiotic-Free vectors (catalog items: NTC-NP-CC1 = NTC711772, DH5α dcm- att$_{\lambda}$::P$_{5/6\ 6/6}$-RNA-IN- SacB, catR; pL (OL1-G to T) P42L-P106L-F107S (P3-), SpecR StrepR; NTC-NP-CC2 = NTC821601, DH5α att$_{\lambda}$::P$_{5/6\ 6/6}$-RNA-IN- SacB, catR; pL (OL1-G to T) P42L-P106L-F107S (P3-), SpecR StrepR; NTC-NP-CC3 = NTC711231, XL1Blue dcm- att$_{\lambda}$::P$_{5/6\ 6/6}$-RNA-IN- SacB, catR; pL (OL1-G to T) P42L-P106L-F107S (P3-), SpecR StrepR; NTC-NP-CC4 = NTC940211, DH5α att$_{\lambda}$::P$_{5/6\ 6/6}$-RNA-IN- SacB, catR; pL (OL1-G to T) P42L-P106**I**-F107S **P113S** (P3-), SpecR StrepR; NTC-NP-CC5 = NTC1050811, DH5α att$_{\lambda}$::P$_{5/6\ 6/6}$-RNA-IN- SacB, catR; pL (OL1-G to T) P42L-P106**I**-F107S **P113S** (P3-), SpecR StrepR; pARA-CI857ts, TetR) are available for purchase from NTC, and will work well for both cloning applications and transfer of plasmid from one cell line to another.

See Williams, 2013, and Luke *et al*., 2014 for guidance to select the optimal cell line (DH5α or XL1Blue , dcm+ or dcm-) for RNA-OUT DNA Vaccine or Gene Therapeutic applications.

Competent Cell Preparation

Competent cells may be prepared using the NTC AF cell line glycerol stock provided. We recommend the following procedure to ensure cells are made that will work best with the AF system.

To prepare electrocompetent cells:

1. Streak an LB agar plate or start a small liquid LB culture of the NTC AF cell line using the provided glycerol stock as an inoculum, grow at 28-30°C until cell growth is visible. All NTC AF cell lines are resistant to chloramphenicol at a concentration of 6 µg/mL. LB broth or LB agar plates may be used with or without 6 µg/mL of chloramphenicol, but proper aseptic technique must be used in either case. **Do not use Sucrose Selection medium**.
2. Using the plate or liquid culture, sub-culture the cells to a new 50 mL LB culture and grow at 28-30°C until the OD_{600} of the culture is 0.4-0.6.
3. Chill cells on ice for 10 min.
4. Centrifuge 4000 x *g* at 4°C for 10 minutes.
5. Decant media and resuspend cell pellet with 50 mL 10% ice-cold glycerol.

6. Centrifuge 4000 x *g* at 4°C for 10 minutes.
7. Decant liquid and resuspend cell pellet with 25 mL 10% ice-cold glycerol
8. Centrifuge 4000 x *g* at 4°C for 10 minutes.
9. Carefully decant the glycerol
10. Resuspend the cell pellet to a cell density of 30 OD_{600}
    For example: A 5 mL culture grew to 0.5 OD_{600}; 0.5 OD_{600} × 50 mL = 25 OD_{600}•mL; 25 OD_{600}•mL / 30 OD_{600} = 0.83 mL resuspension volume for a final competent cell OD_{600} of 30.

Chemically competent cells can also be prepared and used for NTC AF vectors, the resuspension OD_{600} should be 1-2. We recommend the Zymo Research Z-comp Kit (see http://www.zymoresearch.com/e-coli/transformation-kits-accessories/z-competent-e-coli-transformation-kit-buffer-set) for chemically competent cell preparation.

RNA-OUT Vector Transformation and Propagation

RNA-OUT represses expression of a sucrose counter-selectable marker (*SacB*) from the host chromosome. NTC RNA-OUT vectors and ligation reactions must therefore be transformed into *SacB* expression strains encoding att_{λ}::$P_{5/6\ 6/6}$-RNA-IN- SacB, catR competent cells.

Transformed cells are plated and propagated on Solid 6% Sucrose Selection Agar and Liquid 6% Sucrose Selection Media (Note: **Plates must not contain NaCl**)

RNA-OUT Vector Transformation Procedure

1. Add RNA-OUT plasmid DNA to a chilled microcentrifuge tube (***see* Notes 1 and 2**)
2. Prechill on ice one 0.1 cm electroporation cuvette for each sample to be electroporated.
3. Thaw NTC electrocompetent cells on wet ice.
4. When cells are thawed, mix cells by tapping gently. Add 25 µl of cells to each chilled microcentrifuge tube containing your DNA to be transfected (***see* Note 2**).
5. Refreeze any unused cells. Although the cells are refreezable, subsequent freeze-thaw cycles will decrease transformation efficiency.
6. Pipette the cell/DNA mixture into a prechilled 0.1 cm cuvette and electroporate. If you are using the BTX®ECM®630 or BioRad GenePulser®II electroporator, we recommend using the following electroporator conditions: 2.0 kV, 200 Ω, 25 µF. (**see Note 3**)
7. To the cells in the cuvette, quickly add 0.5-1 ml of room temperature S.O.C. medium and transfer the solution to a 15 ml snap-cap tube (*e.g.* Falcon™ tube).
8. Shake at 225 rpm (**30°C**) for 1-2 hour (***see* Notes 4, 5**).
9. Spread 50-100 µl on 6% Sucrose Selection agar (or dilute experimental reactions as necessary and spread 50-100 µl on selective plates)
10. Incubate plates overnight at **30°C** (***see* Note 4**).

Notes

1. Transformation efficiencies will be 10- to 100-fold lower for ligation mixtures than for an intact control plasmid. Transform using more pDNA than is typically used with other strains of *E. coli*. We get our best results with electrocompetent cells by transforming at least 5 ng supercoiled pDNA or 30-50 ng for cloning ligation reactions into 25 µL electrocompetent cells.
2. **Salts and buffers severely inhibit electroporation.** Adding too high a volume of DNA decreases transformation efficiency and increases the risk of arcing.
3. If you are using an electroporator other than a BTX®ECM®630 or BioRad GenePulser®II electroporator, you may need to vary the settings to achieve optimal transformation efficiency.
4. The R6K replication protein expressed from the chromosome is expressed from the pL promoter and is thus heat inducible (**Fig. 2**). **Therefore for cloning, transformation, and glycerol stock creation steps, to reduce metabolic burden, you should maintain the plasmid at reduced copy number by growth at 30°C**. For subsequent large scale plasmid production, you can grow at 37°C to induce copy number and improve purification yields.
5. Keep transformed S.O.C. medium outgrowths from step 8 at room temperature overnight. Replate cells from outgrowth if too few or too many colonies are obtained on the plates after overnight incubation at 30ºC.

RNA-OUT Vector Cell Line Propagation

NTC RNA-OUT AF vectors are highly stable once transformed into the att_{λ}::$P_{5/6\ 6/6}$-RNA-IN- SacB cell line. Colonies are inoculated into 6% Sucrose Selection Media. Growth at 30°C will give lower yields but plasmid production can be increased by using 37°C for the incubation temperature when growing larger cultures than those used for the initial selection of clones.

Sucrose Selection Medium

A 50% Sucrose solution should be prepared (wt./vol.) prior to making liquid or solid media. (This should not be autoclaved but rather filter-sterilized with a 0.2 micron filter prior to use)

Solid 6% Sucrose Selection Agar Preparation	Liquid 6% Sucrose Selection Media Preparation
10g tryptone* 5 g yeast extract 15 g agar QS to 880 mL with H_2O Autoclave 20 min 121°C, when cooled to ~50°C, add 120 mL 50% Sucrose (wt./vol.), mix, pour plates.	10g tryptone* 5 g yeast extract QS to 880 mL with H_2O Autoclave 20 min 121°C When cooled to ~50°C, add 120 mL 50% Sucrose (wt./vol.), allow to cool before use.

SOC Medium

1. Add the following to 900ml of distilled H_2O
    - 20g Bacto Tryptone*
    - 5g Bacto Yeast Extract
    - 2ml of 5M NaCl.
    - 2.5ml of 1M KCl.
    - 10ml of 1M $MgCl_2$
    - 10ml of 1M $MgSO_4$
    - 20ml of 1M glucose
2. Adjust to 1L with distilled H_2O
3. Sterilize by autoclaving

*****Soy peptone may be used in place of tryptone when non-animal sourced materials are required.

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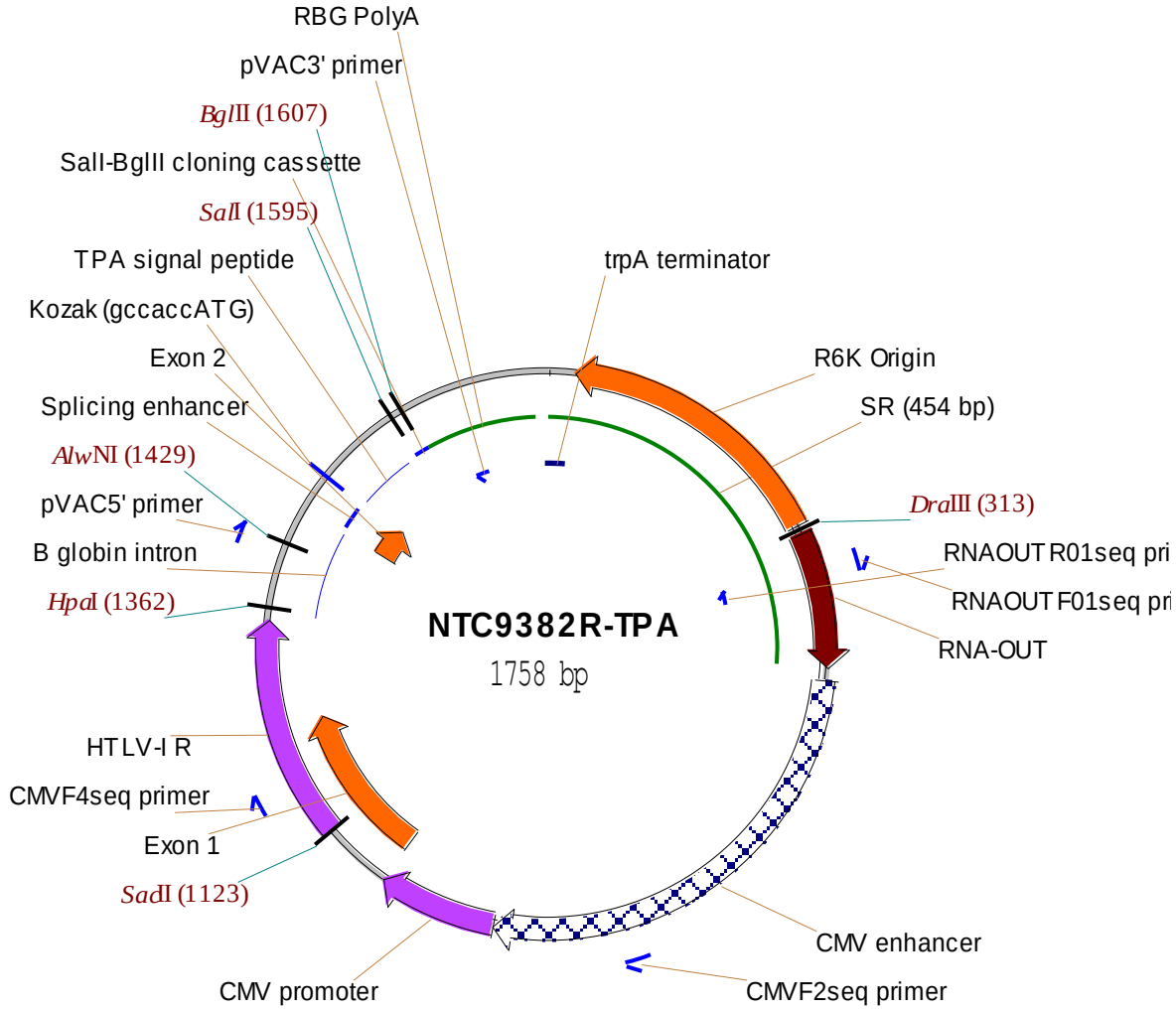

Vector Features

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R6K replication origin: 26-306
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CMV enhancer: 467-933
CMV promoter: 934-1053
Untranslated leader (Exon 1, CMV-HTLV-I R): 1054-1240
HTLV-I R: 1125-1350
Synthetic Rabbit β-globin-based 3' intron: 1359-1465
Intron (HTLV-I R- Rabbit β-globin): 1241-1465
Exon 2 Splicing Enhancer (3x gaagaagac SR binding protein): 1474-1500
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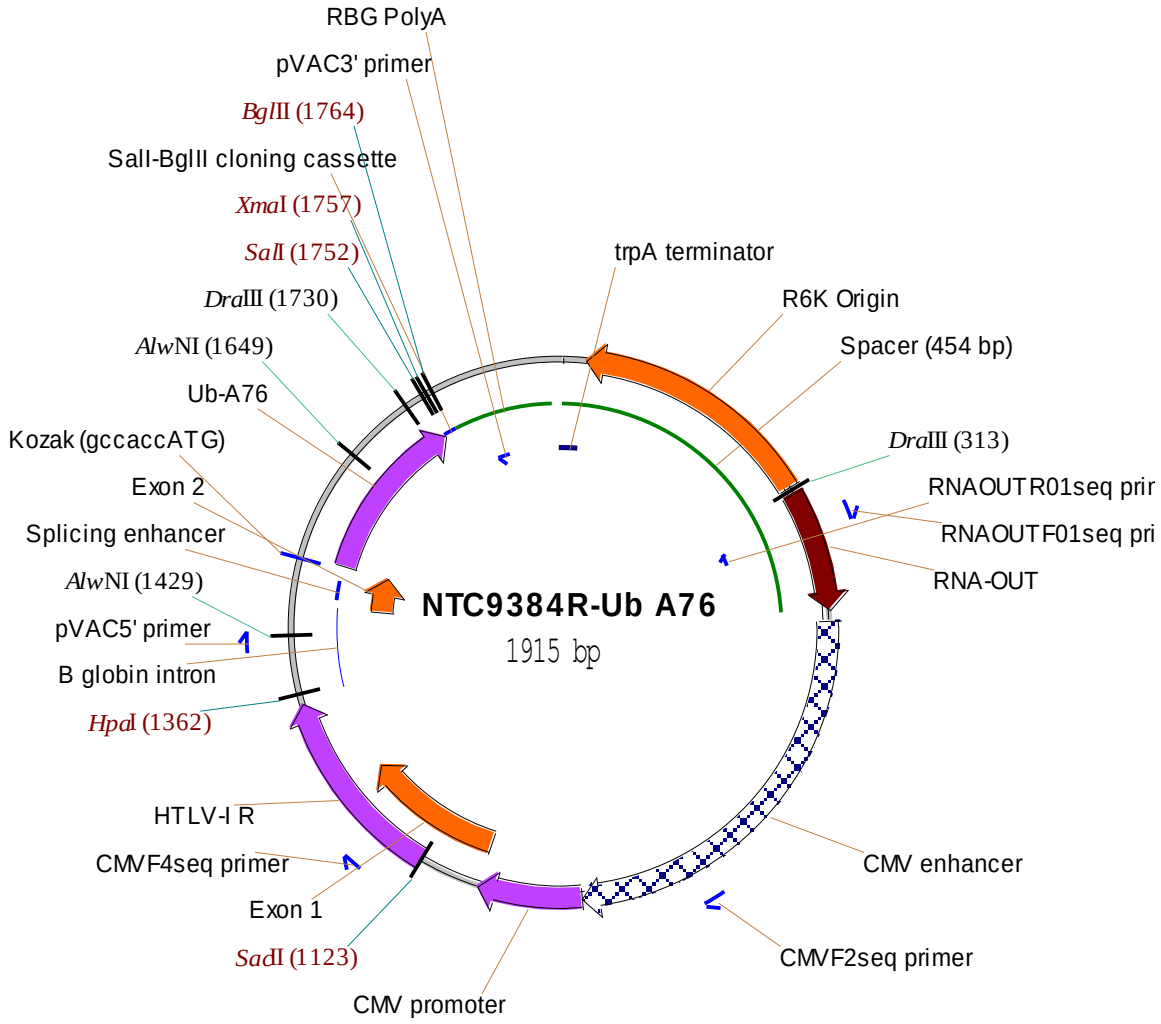

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HTLV-I R: 1125-1350
Synthetic Rabbit β-globin-based 3' intron: 1359-1465
Intron (HTLV-I R- Rabbit β-globin): 1241-1465
Exon 2 Splicing Enhancer (3x gaagaagac SR binding protein): 1474-1500
Exon 2 (SR-protein binding sites-Kozak):1466-1522
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SalI-BglII cloning cassette :1751-1768
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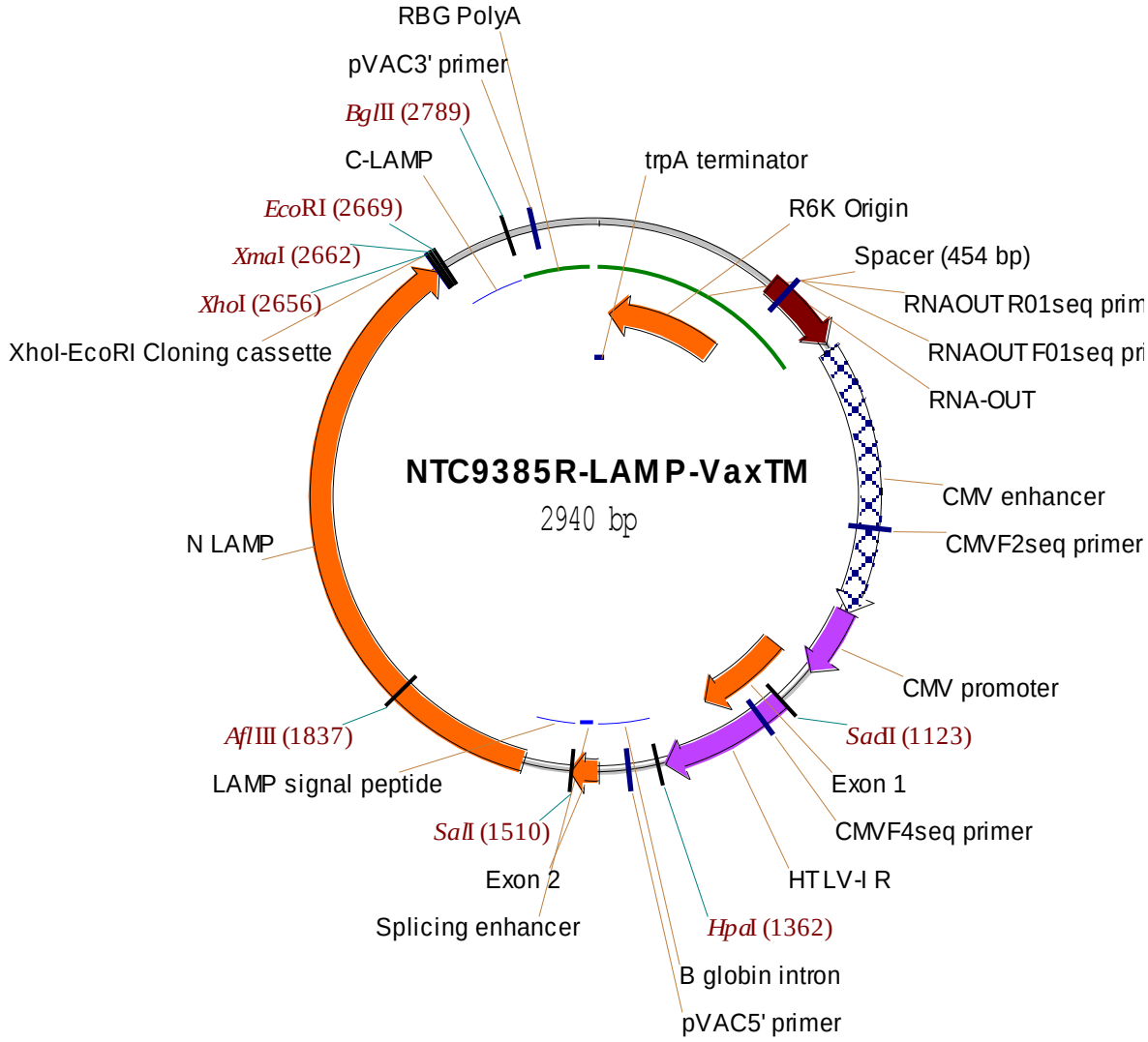

Vector Features

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R6K replication origin: 26-306
Sucrose selection marker (RNA-OUT): 316-454
CMV enhancer: 467-933
CMV promoter: 934-1053
Untranslated leader (Exon 1, CMV-HTLV-I R): 1054-1240
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Synthetic Rabbit β-globin-based 3' intron: 1359-1465
Intron (HTLV-I R- Rabbit β-globin): 1241-1465
Exon 2 Splicing Enhancer (3x gaagaagac SR binding protein): 1474-1500
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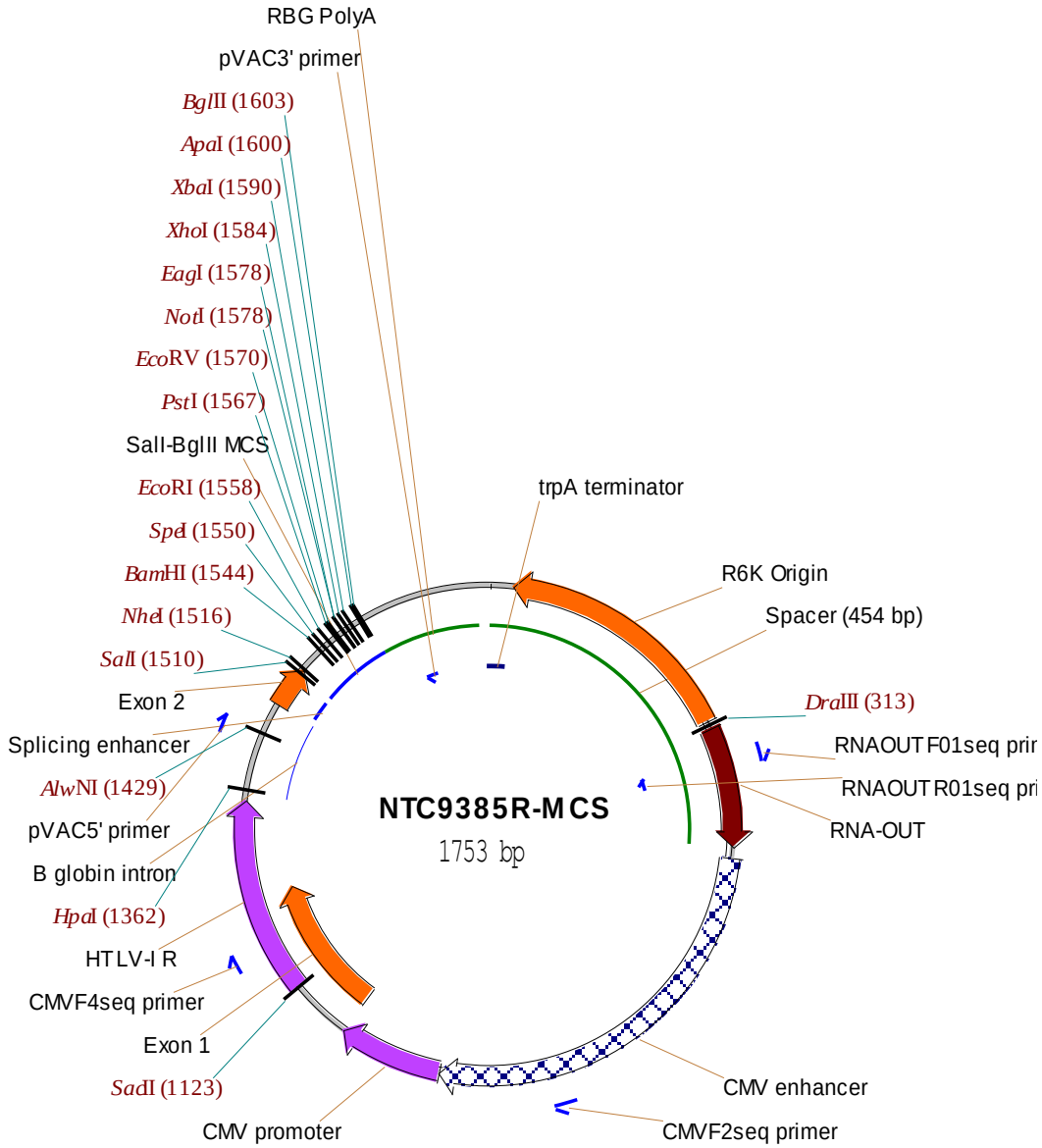

Vector Features

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Sucrose selection marker (RNA-OUT): 316-454
CMV enhancer: 467-933
CMV promoter: 934-1053
Untranslated leader (exon 1, CMV-HTLV-I R): 1054-1240
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Synthetic Rabbit β-globin-based 3' intron: 1359-1465
Intron (HTLV-I R- Rabbit β-globin): 1241-1465
Exon 2 Splicing Enhancer (3x gaagaagac SR binding protein): 1474-1500
Exon 2 (SR-protein binding sites-SalI):1466-1514
Multiple Cloning Site (SalI—BglII): 1509-1607
Rabbit β-globin PolyA: 1608-1742

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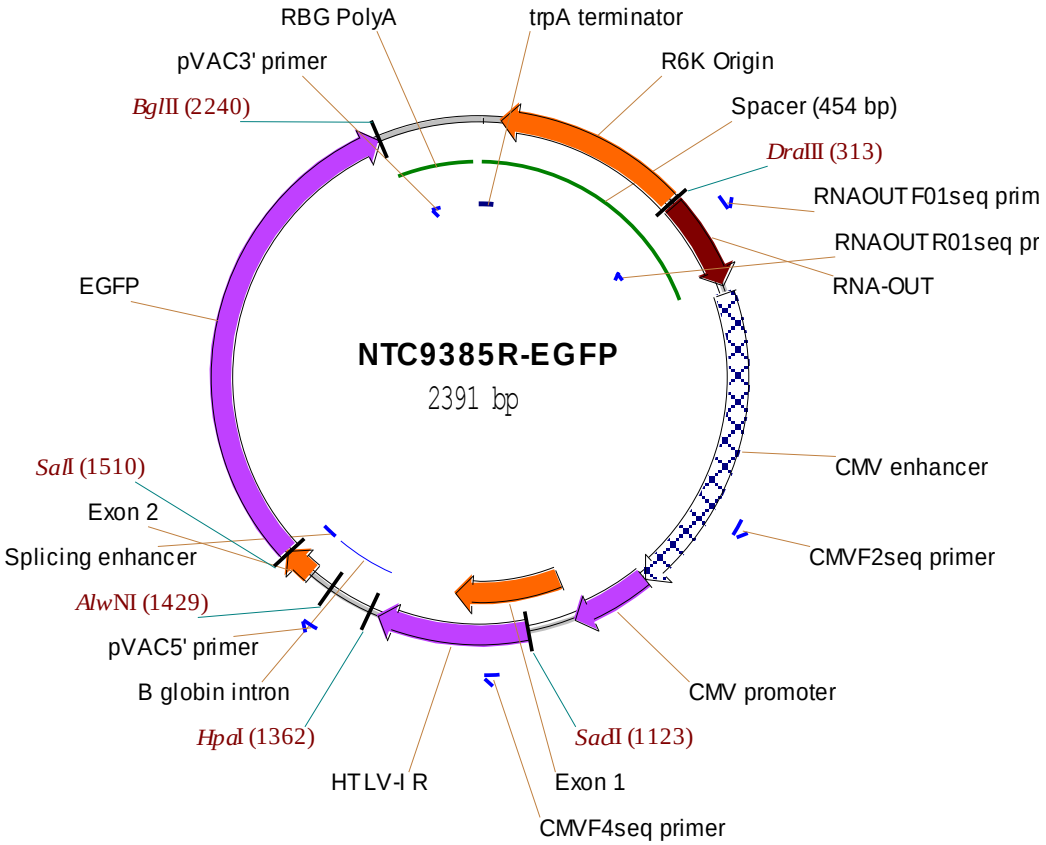

Vector Features

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CMV enhancer: 467-933
CMV promoter: 934-1053
Untranslated leader (exon 1, CMV-HTLV-I R): 1054-1240
HTLV-I R: 1125-1350
Synthetic Rabbit β-globin-based 3' intron: 1359-1465
Intron (HTLV-I R- Rabbit β-globin): 1241-1465
Exon 2 Splicing Enhancer (3x gaagaagac SR binding protein): 1474-1500
Exon 2 (SR-protein binding sites-SalI):1466-1514
EGFP: 1515-2238
Rabbit β-globin PolyA: 2245-2379

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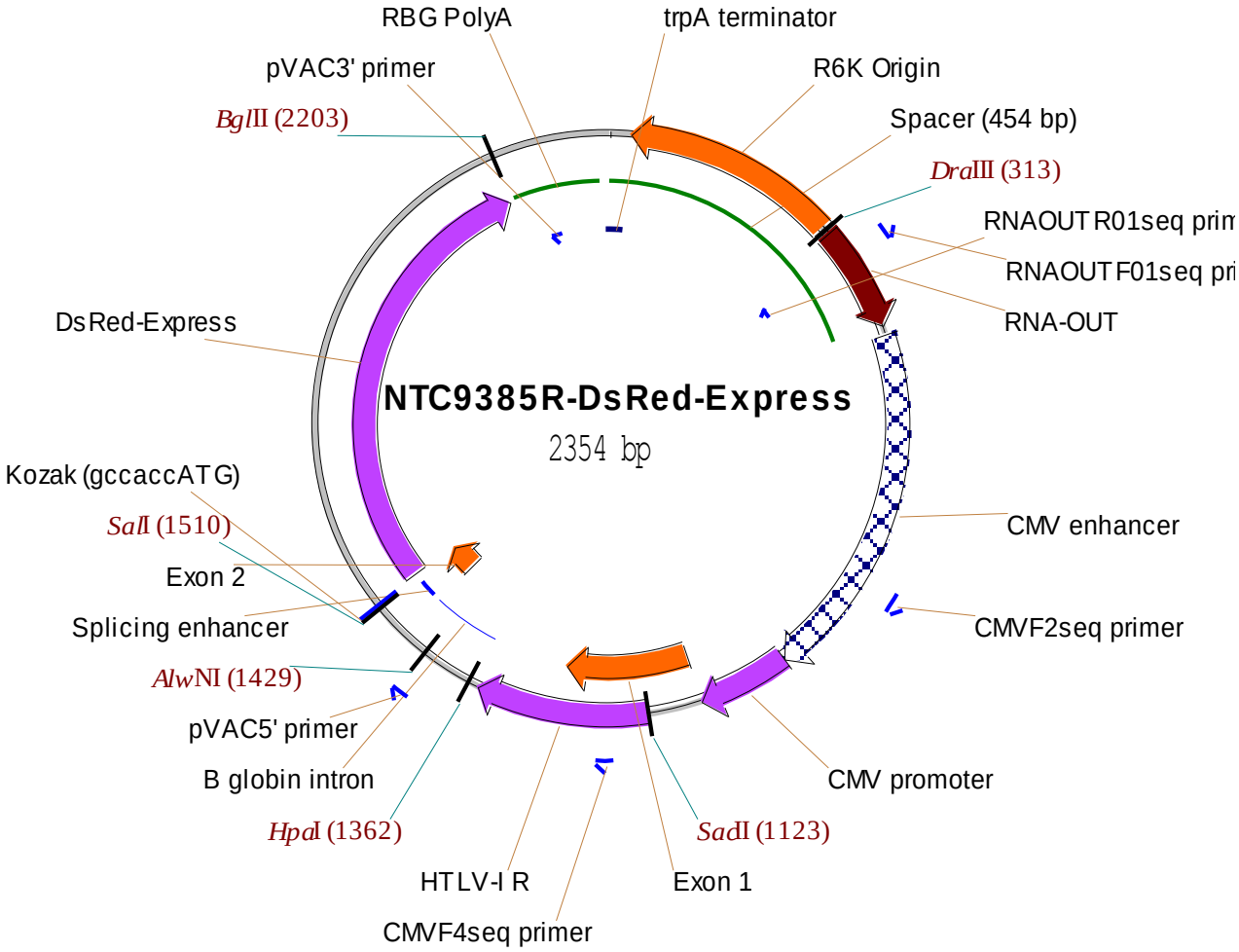

Vector Features

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Sucrose selection marker (RNA-OUT): 316-454
CMV enhancer: 467-933
CMV promoter: 934-1053
Untranslated leader (exon 1, CMV-HTLV-I R): 1054-1240
HTLV-I R: 1125-1350
Synthetic Rabbit β-globin-based 3' intron: 1359-1465
Intron (HTLV-I R- Rabbit β-globin): 1241-1465
Exon 2 Splicing Enhancer (3x gaagaagac SR binding protein): 1474-1500
Exon 2 (SR-protein binding sites-SalI):1466-1514
Kozak (gccaccATG): 1515-1523
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Rabbit β-globin PolyA: 2208-2342

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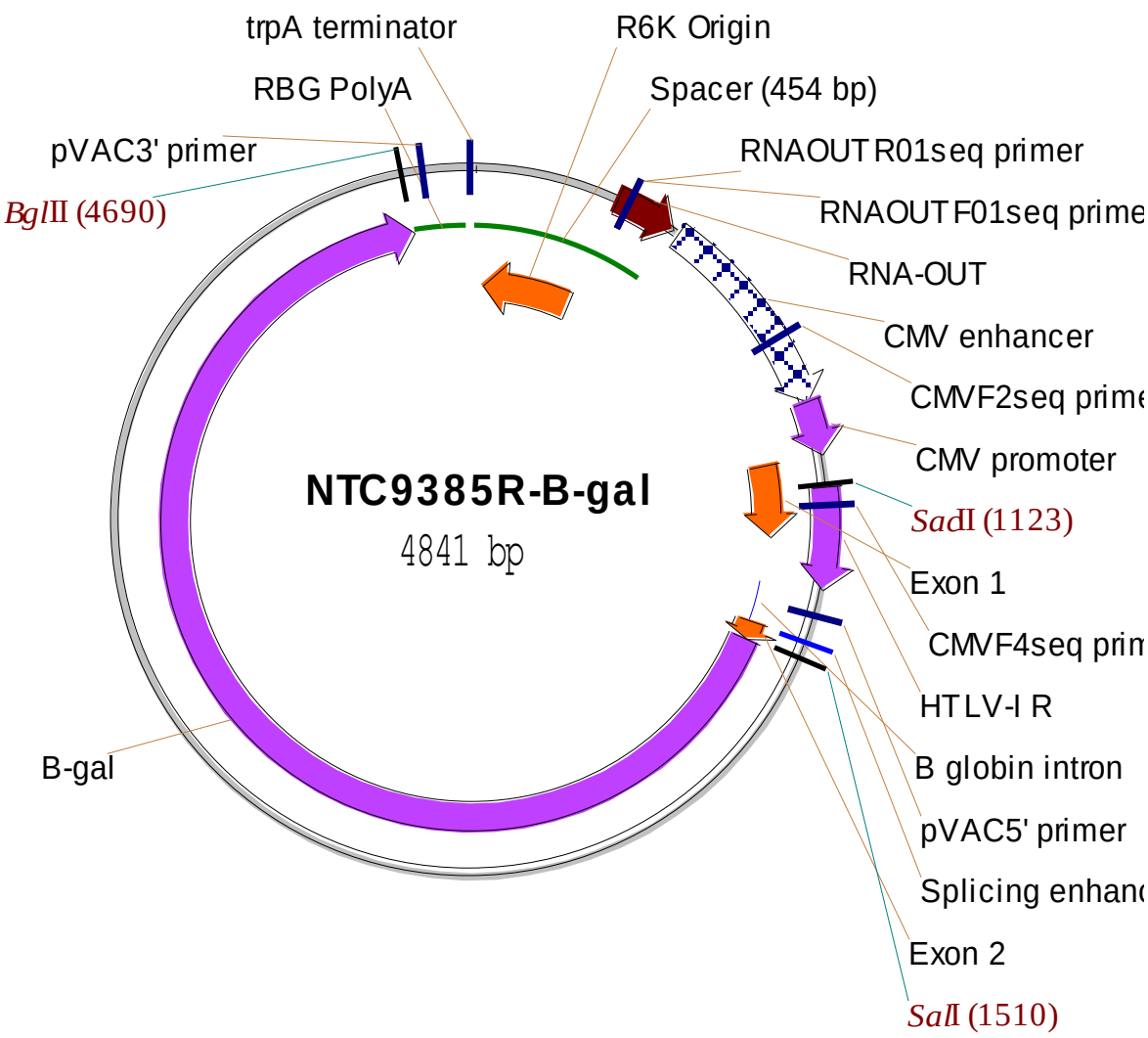

Vector Features
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R6K replication origin: 26-306
Sucrose selection marker (RNA-OUT): 316-454
CMV enhancer: 467-933
CMV promoter: 934-1053
Untranslated leader (exon 1, CMV-HTLV-I R): 1054-1240
HTLV-I R: 1125-1350
Synthetic Rabbit β-globin-based 3' intron: 1359-1465
Intron (HTLV-I R- Rabbit β-globin): 1241-1465
Exon 2 Splicing Enhancer (3x gaagaagac SR binding protein): 1474-1500
Exon 2 (SR-protein binding sites-SalI):1466-1514
N terminal His-tag B-gal: 1515-4688
Rabbit β-globin PolyA: 4695-4829

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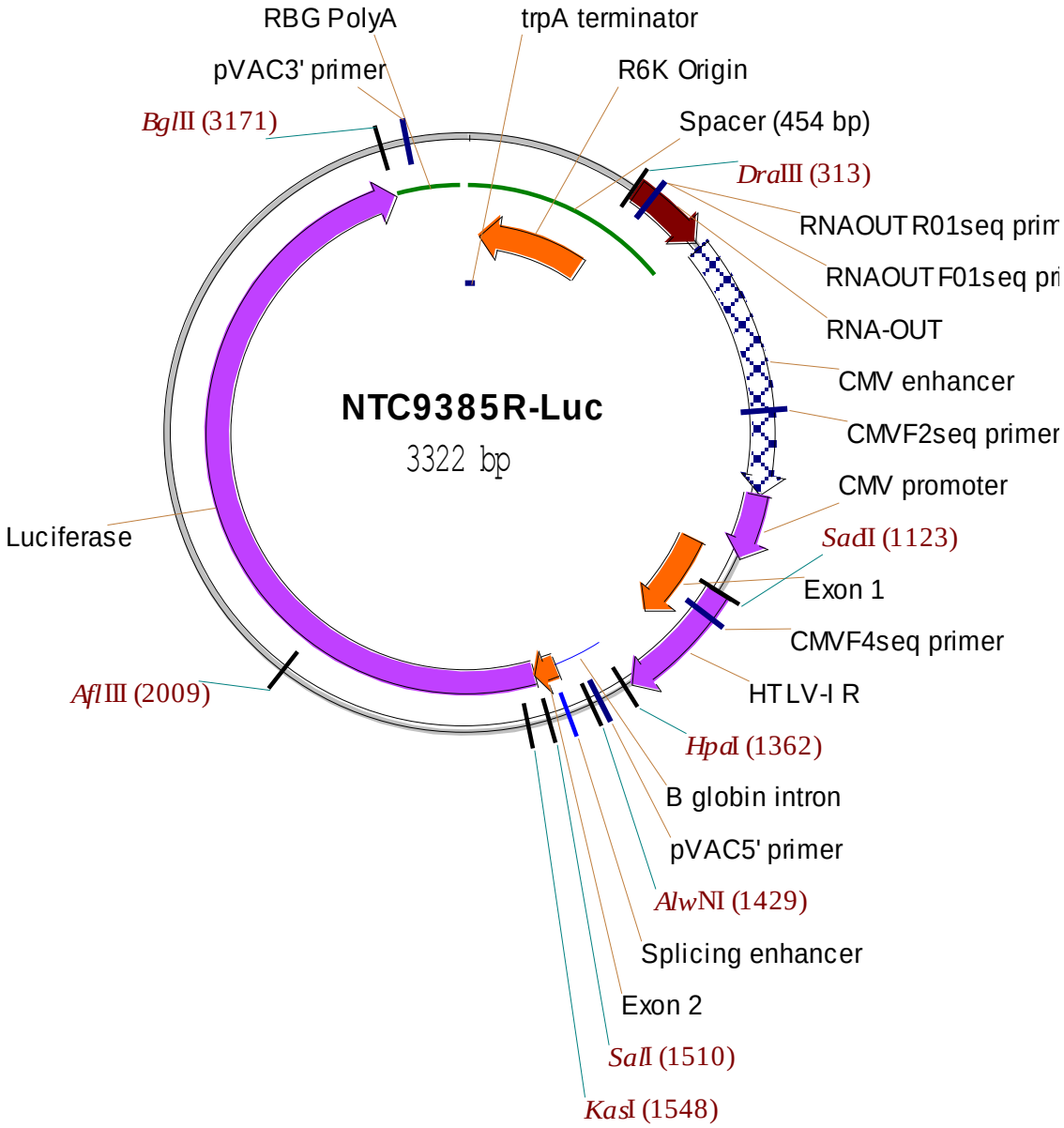

Vector Features

trpA prokaryotic terminator: 3321-26
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Sucrose selection marker (RNA-OUT): 316-454
CMV enhancer: 467-933
CMV promoter: 934-1053
Untranslated leader (exon 1, CMV-HTLV-I R): 1054-1240
HTLV-I R: 1125-1350
Synthetic Rabbit β-globin-based 3' intron: 1359-1465
Intron (HTLV-I R- Rabbit β-globin): 1241-1465
Exon 2 Splicing Enhancer (3x gaagaagac SR binding protein): 1474-1500
Exon 2 (SR-protein binding sites-SalI):1466-1514
Luciferase: 1515-3170
Rabbit β-globin PolyA: 3176-3310

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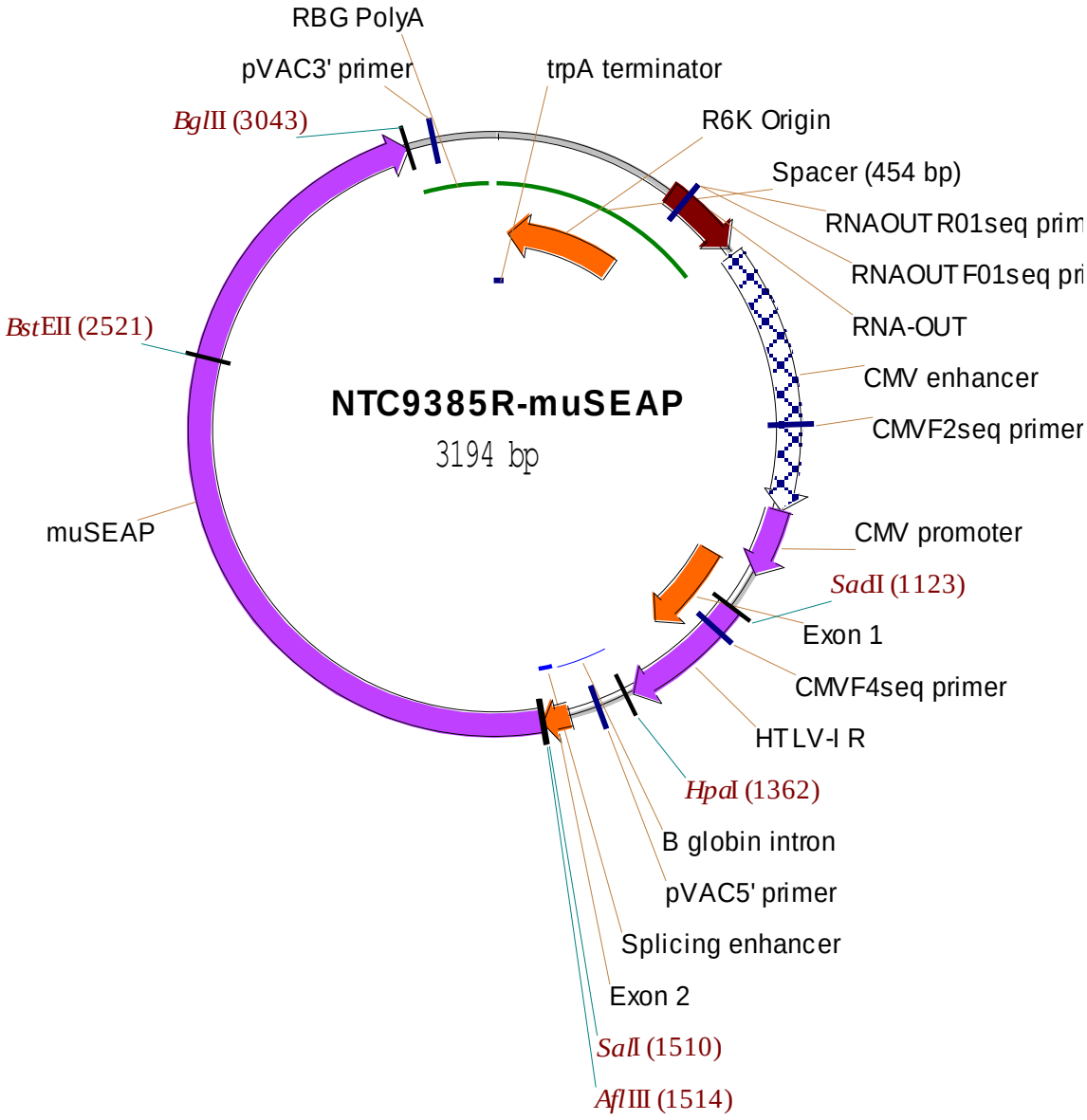

Vector Features

trpA prokaryotic terminator: 3193-26
R6K replication origin: 26-306
Sucrose selection marker (RNA-OUT): 316-454
CMV enhancer: 467-933
CMV promoter: 934-1053
Untranslated leader (exon 1, CMV-HTLV-I R): 1054-1240
HTLV-I R: 1125-1350
Synthetic Rabbit β-globin-based 3' intron: 1359-1465
Intron (HTLV-I R- Rabbit β-globin): 1241-1465
Exon 2 Splicing Enhancer (3x gaagaagac SR binding protein): 1474-1500
Exon 2 (SR-protein binding sites-SalI):1466-1514
Murine SEAP: 1515-3041
Rabbit β-globin PolyA: 3048-3182

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