NTC8482 and NTC8485 Expression Vectors
Instruction Manual

Catalog Numbers
NTC-DV8482
NTC-DV8485-EGFP
NTC- DV8482-LV
NTC- DV8485-LV

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General Information

Contents: 20 ug each of plasmid vector shipped in 1x TE buffer.

Storage: Plasmids should be stored at -20˚C

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NTC8482 and NTC8485 vector family

Introduction

Nature Technology Corporations (NTC's) NTC8482 and NTC8485 plasmids are antibiotic-free versions of the corresponding kanamycin resistant NTC7482 and NTC7485 vectors.

These plasmids 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, 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 two versions. NTC8485 expresses encoded protein without additional sequences. NTC8482 targets encoded protein into the secretory pathway using an optimized tissue plasminogen activator (TPA) signal peptide.

The parent NTC7482 and NTC7485 plasmids were designed to be responsive to Food and Drug Administration (FDA) regulatory guidance's 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 eukaryotic mRNA leader and terminators were utilized in the vector design to limit DNA sequence homology with the human genome to reduce the possibility of chromosomal integration.

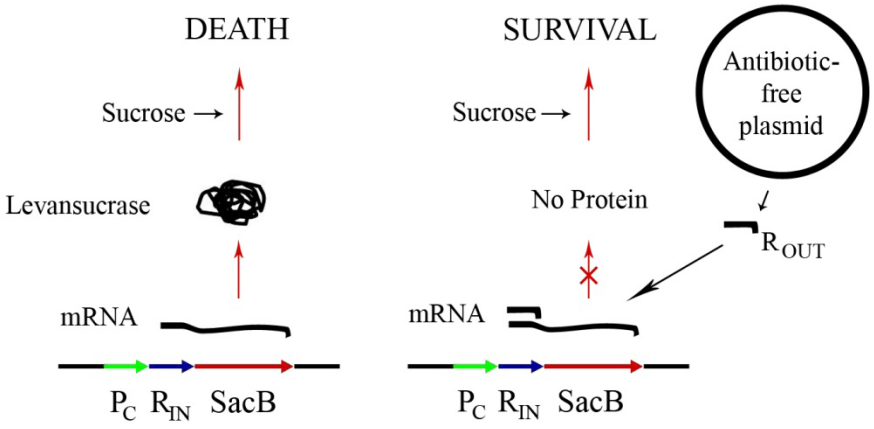
Target gene expression is driven from an optimized chimeric promoter-intron (SV40-CMV-HTLV-1 R synthetic intron). The vectors encode a consensus Kozak translation initiation sequence and ATG start codon. The chimeric CMV promoter achieves significantly higher expression levels than traditional human cytomegalovirus (CMV) promoter based vectors (Luke *et al.* 2009).

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).

NTC has designed an antibiotic-free selection system (*figure below*). Vectors with this selection system incorporate and express a 150 bp RNA-OUT antisense RNA. RNA-OUT represses expression of a counter-selectable marker (*SacB*) from the host chromosome (selection host DH5α att_{λ}::$P_{5/6\ 6/6}$-RNA-IN- SacB, catR). *SacB* encodes a levansucrase, which is toxic in the presence of sucrose. Plasmid selection is achieved in the presence of sucrose.



Levansucrase (SacB) conditional-lethal gene on bacterial chromosome

The kanamycin resistance antibiotic selection marker in parent vectors NTC7482 and NTC7485 were removed and replaced with the sucrose selectable RNA-OUT marker to create NTC8482 and NTC8485, respectively. Plasmid production yields > 1 g/L were verified in fermentation culture (Luke *et al.* 2009).

In summary, the NTC8482 and NTC8485 vectors offer the following advantages

• Highest level expression in a wide range of mammalian cells using an optimized chimeric CMV promoter-synthetic intron
• Antibiotic-free selection in *Escherichia coli* host
• Choice of *Escherichia coli* production strain for higher immunogenicity (dcm+ NTC4862) or higher expression (dcm- NTC48165)
• Superior *Escherichia coli* plasmid production yields using optimized vector backbone
• Optional N-terminal TPA secretion tag (NTC8482) for protein export
• Simultaneous cloning into both vectors through use of compatible precision cloning cassettes
• Small vectors for more efficient transfection
• Compliance with regulatory guidance (*i.e*. Reduced size, elimination of homology to human genomic DNA, elimination of antibiotic resistance marker)

NTC8482 and NTC8485 vector construction

NTC used Gene Self-Assembly (GENSA) technology to create a series of validated modular elements for vector assembly. Each modular element was assigned a position in a circular array, providing a promoter, 5-leader/splice-site, target gene or high throughput cloning site, terminators, and prokaryotic origin/selection/terminator sites. Individual modules were assigned a position and directionality by means of 4bp unique, non-palindromic address tags. Modules were constructed using a novel cloning process that generates unique, non-palindromic address tags. This allowed precise minimal vector design to eliminate all extraneous sequences.

The pDNAVACCultra vectors (Williams *et al.* 2006) were assembled from GENSA modules consisting of products representing:

● Optimized inducible high copy number pMB1-derived pUC prokaryotic replication origin;
● Prokaryotic selectable marker gene (kanamycin);
● Eukaryotic enhancer-promoter (CMV)
● Optimized synthetic eukaryotic untranslated leader-intron-translational initiation sequence (Kozak sequence) cassette
● Gene leader cassette containing the cloning site;
● Optimized synthetic eukaryotic transcriptional terminator.

The kanamycin resistant parent NTC7482 and NTC7485 vectors were constructed by incorporation of designed modifications to the base pDNAVACCultra vectors to improve mammalian cell expression (chimeric promoter) and plasmid production yields (vector backbone modification) in the *Escherichia coli* bacterial host.

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-1 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 (Barouch *et al.* 2005) and improve humoral responses to an influenza pDNAVACCultra based DNA vaccine in mice (J. Williams, unpublished observations) compared to CMV promoter based vectors. Expression levels of EGFP from plasmids incorporating the NTC7382 promoter are dramatically increased versus CMV promoter containing plasmids (Luke *et al*. 2009).

Vector backbone modification: Modifications to the pDNAVACCUltra encoded kanR-pUC replication origin vector backbone to improve production yield included:

1) Extension of the pUC origin to include a primosomal assembly site;
2) Inclusion of the SV40 enhancer adjacent to the kanR gene;

These modifications doubled plasmid fermentation production yields compared to standard kanR-pUC replication origin vectors such as gWiz (Williams *et al.* 2009b).

The kanamycin resistance antibiotic selection marker in NTC7482 and NTC7485 were then removed and replaced with the sucrose selectable RNA-OUT marker to create NTC8482 and NTC8485, respectively.

NTC8482 and NTC8485 vector features

Feature	Function
Optimized human cytomegalovirus (CMV) immediate-early enhancer promoter	High-level mammalian cell expression *in vitro* and *in vivo*
HTLV-1 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 or secreted intracellular trafficking
Synthetic eukaryotic polyadenylation signal-eukaryotic terminator	Effective mRNA transcriptional termination and polyadenylation
trpA prokaryotic terminator	Protection of replication origin from insert initiated transcription
PAS-BH primosomal assembly site	Improved plasmid production yields
pUC replication origin	High copy number plasmid production in *Escherichia coli* cells
Sucrose selection marker (RNA-OUT)	Plasmid selection in *Escherichia coli* cells*
SV40 enhancer	Increased mammalian cell expression Improved plasmid production yields

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

NTC8482 and NTC8485: Intracellular targeting

Vector	Protein Destination	Targeting Tag
NTC8482	secreted	human tissue plasminogen activator (TPA)
NTC8485	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). Protein targeting (*e.g.* cytoplasmic or nuclear) will be determined by protein-intrinsic factors

The TPA targeting peptide has been demonstrated to efficiently target heterologous proteins for secretion (Zhongming *et al.*1999).

Plasmid Expression Vector kit‡

Vector	Targeting	Quantity	Catalog Number	Price
NTC8482	secreted	20 µg	NTC- DV8482	$420.00
NTC8485-EGFP*	cytoplasmic EGFP	20 µg	NTC- DV8485-EGFP	$420.00

* The NTC8485-EGFP control plasmid is used as a transfection control for expression in a cell line of interest, and as the NTC8485 backbone for cloning new genes
‡ Kit contains plasmid and DH5α att_{λ}::$P_{5/6\ 6/6}$-RNA-IN- SacB, catR host strain glycerol stock

Linearized Vector kit‡

Vector	Targeting	Quantity	Catalog Number	Price
NTC8482	secreted	1 µg	NTC- DV8482-LV	$444.00
NTC8485	native	1 µg	NTC- DV8485-LV	$444.00

‡ *Sal*I - *Bgl*II linearized vector sufficient for 20 cloning reactions and DH5α att_{λ}::$P_{5/6\ 6/6}$-RNA-IN- SacB, catR host strain glycerol stock

RAPID-VACC^{tm} 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^{tm}

Seamless cloning gene of interest into any vector	$400.00
1mg endotoxin-free plasmid preparations, each	$335.00

NTC offers the following products for use the NTC8482 and NTC8485 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	$30.00
NTC4862 DH5α att_{λ}::$P_{5/6\ 6/6}$-RNA-IN- SacB, catR electrocompetent cells	Host strain for NTC8482 and NTC8485 selection	0.5 mL	NTC-DVU-CC1	$199.00
NTC48165 DH5α dcm att_{λ}::$P_{5/6\ 6/6}$-RNA-IN- SacB, catR electrocompetent cells	dcm- host strain for NTC8482 and NTC8485 selection	0.5 mL	NTC-DVU-CC2	$199.00
Sucrose selection agar	Plates for NTC8482 and NTC8485 selection	Pouch to make 1 L agar*	NTC-DVU-MD1	$20
Sucrose selection media	Media for NTC8482 and NTC8485 culture	Pouch to make 1 L media*	NTC-DVU-MD2	$20

***** Reconstitute with distilled water to 1L and autoclave prior to use

Cloning into NTC8482 and NTC8485 vectors

Overview: An example strategy for cloning into the NTC8482 and NTC8485 vectors is outlined below.

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

For both vectors, the ATG start codon (double underlined) is immediately preceded by a *Sal*I site. In NTC8485, the *Sal*I site has been demonstrated to be an effective consensus Kozak sequence for translational initiation. In NTC8482, the *Sal*I site is downstream in frame with the optimized TPA secretion 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 NTC8485, the start codon ATG must immediately follow the *Sal*I site (GTCGACATG). For NTC8482, the ATG is optional but the same reading frame must be retained. For both vectors one or two stop codon (preferably TAA or TGA) must be included prior to the *Bgl*II site. A PCR product designed for NTC8485 is compatible with, and can also be cloned into, the NTC8482 vector.

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 NTC8485, 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 NTC8482.

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 NTC8482 and NTC8485 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 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 terminator and sequences from 3' end of gene)

Transformation and bacterial propagation overview

RNA-OUT represses expression of a sucrose counter-selectable marker (*SacB*) from the host chromosome. Ligation reactions must therefore be transformed into *SacB* expression strain DH5α att_{λ}::$P_{5/6\ 6/6}$-RNA-IN- SacB, catR competent cells.

Cells are plated and propagated on 6% sucrose media (Note: **Plates must not contain NaCl**)

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 Media Preparation	Liquid 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.

Transformation Procedure

1. Ligation reactions must be diluted (See note 1) to avoid arcing in the electroporator. Dilute reactions 5-fold with sterile water and add 1ul to a chilled microcentrifuge tube. Prechill on ice one 0.1 cm electroporation cuvette for each sample to be electroporated.
2. Thaw NTC electrocompetent cells on wet ice.
3. When cells are thawed, mix cells by tapping gently. Add 20 µl of cells to each chilled microcentrifuge tube containing your DNA to be transfected.
4. Refreeze any unused cells. Although the cells are refreezable, subsequent freeze-thaw cycles will decrease transformation efficiency.
5. 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 2)
6. 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).
7. Shake at 225 rpm (30°C) for 1 hour. (This step may be omitted if transforming supercoiled plasmid DNA)
8. Spread 50-100 ul on Sucrose selection agar (or dilute experimental reactions as necessary and spread 50-100 µl on selective plates)
9. Incubate plates overnight at 30°C.

Notes

1. Transformation efficiencies will be 10- to 100-fold lower for ligation mixtures than for an intact control plasmid. **Salts and buffers severely inhibit electroporation.** Ligation reactions can be diluted 5-fold with sterile water, and 1 µl added to 20 µl of cells. Adding undiluted ligation mixtures or too high a volume of DNA decreases transformation efficiency and increases the risk of arcing.
2. 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.

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

References

Barouch DH, Yang ZY, Kong WP, Korioth-Schmitz B, Sumida SM, Truitt DM, Kishko MG, Arthur JC, Miura A, Mascola JR, Letvin NL, and Nabel GJ. (2005) A human T-cell leukemia virus type 1 regulatory element enhances the immunogenicity of human immunodeficiency virus type 1 DNA vaccines in mice and nonhuman primates. *J. Virol*. 79: 8828-8834

FDA. (1996) Points to consider on plasmid DNA vaccines for preventive infectious disease indications. US Food and Drug Administration

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Luke J, Carnes AE, Hodgson CP, and Williams JA. (2009) Improved antibiotic-free DNA vaccine vectors utilizing a novel RNA based plasmid selection system. *Vaccine* 27: 6454-6559

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Zhongming L, Howard A, Kelley C, Delogu G, Collins F, and Morris S. (1999) Immunogenicity of DNA vaccines expressing tuberculosis proteins fused to tissue plasminogen activator signal sequences. *Infect Immun*. 67: 4780-4786

Patent and Licensing information

Limited License
Nature Technology Corporation (NTC) grants the end user (purchaser) of the NTC8482 and NTC8485 expression vectors and NTC4862, NTC48165, NTC5402, and NTC54208 sucrose-selection host strains a nontransferable, non-exclusive license to use the plasmids and host strains for non-commercial research purposes only. These vectors are intended for research use only by the purchaser.

The purchaser cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for commercial purposes.

Separate licenses are available from NTC for the express purpose of non-research use or applications of the NTC8482 and NTC8485 vectors and NTC4862, NTC48165, NTC5402, and NTC54208 host strains.

Product Use Limitations
The NTC8482 and NTC8485 vectors and NTC4862, NTC48165, NTC5402, and NTC54208 host strains are sold for research use only. They are not to be used for human diagnostic or included/used in any drug intended for human use.
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Patent Information

The CMV promoter is covered under U.S. Patents 5,168,062 and 5,385,839 and its use is permitted for research purposes only. Any other use of the CMV promoter requires a license from the University of Iowa Research Foundation, 214 Technology Innovation Center, Iowa City, IA 52242.

The polymerase chain reaction (PCR) process is covered by patents owned by Roche and requires a license for use.

NTC makes no representations that the use of the NTC8482 and NTC8485 vectors and/or NTC4862, NTC48165, NTC5402, and NTC54208 host strains will not infringe any patent, copyright, trademark, or other proprietary rights.

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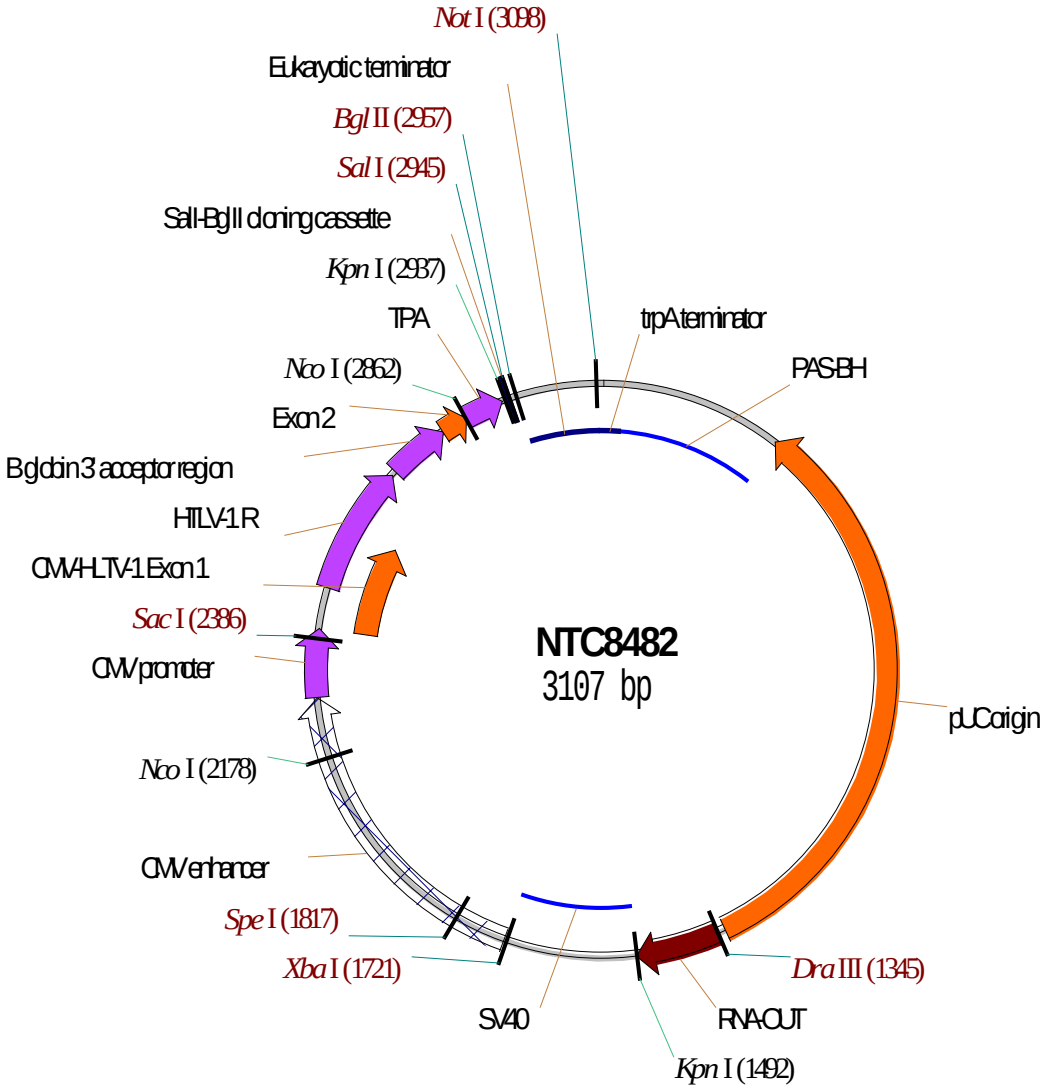
NTC8482



Vector Features

trpA prokaryotic terminator: 3106-28
Primosomal assembly site (PAS-BH) extended origin: 32-316
pUC replication origin: 317-1331
Sucrose selection marker (RNA-OUT): 1348-1492
SV40 enhancer: 1493-1719
CMV enhancer: 1726-2283
CMV promoter: 2284-2403
Untranslated leader (exon 1): 2404-2590
HTLV-1 R: 2475-2700
Synthetic Rabbit β-globin-based 3' intron: 2709-2815
Intron: 2591-2815
Exon 2 (SR-protein binding sites-Kozak):2816-2862
TPA N-terminal targeting tag: 2863-2931
*Sal*I-*Bgl*II cloning cassette: 2944-2961

Eukaryotic terminator: 2962-3096

ccgcctaatgagcgggctttttttttcttaggctgcctcgcgcgtttcggtgatgacggtgaaaacctctgacacatgcagctcccgg
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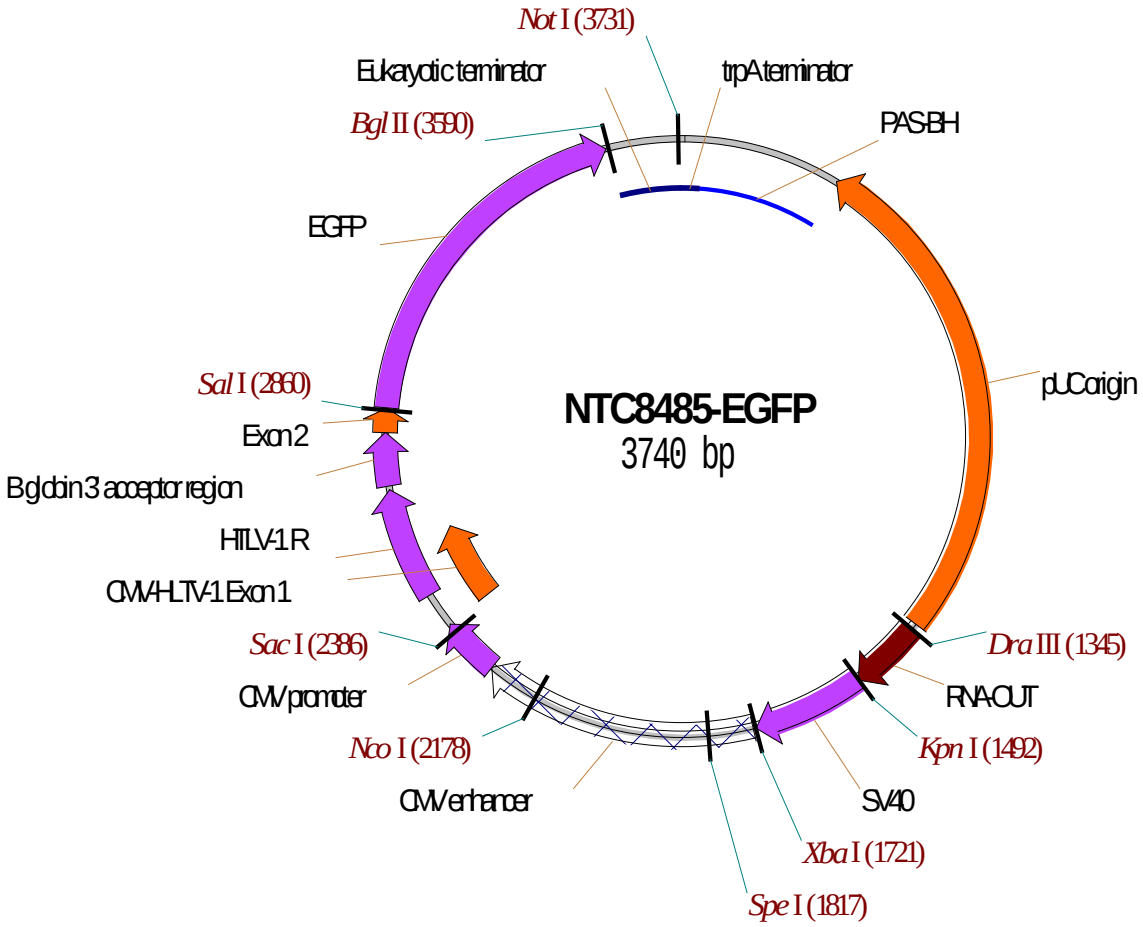
cgctagc

NTC8485-EGFP



Vector Features
trpA prokaryotic terminator: 3739-28
Primosomal assembly site (PAS-BH) extended origin: 32-316
pUC replication origin: 317-1331
Sucrose selection marker (RNA-OUT): 1348-1492
SV40 enhancer: 1493-1719
CMV enhancer: 1726-2283
CMV promoter: 2284-2403
Untranslated leader (exon 1): 2404-2590
HTLV-1 R: 2475-2700
Synthetic Rabbit β-globin-based 3' intron: 2709-2815
Intron: 2591-2815
Exon 2 (SR-protein binding sites-Kozak):2816-2864
EGFP: 2865-3584
*Sal*I-*Bgl*II cloning cassette: 2859-3594
Eukaryotic terminator: 3595-3729

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