NTC8681, NTC8682, NTC8684 & NTC8685

Targeting Expression Vectors
Instruction Manual

Catalog Numbers
NTC-DV8681
NTC-DV8682
NTC-DV8684
NTC-DV8685-EGFP

NTC- DV8681-LV
NTC- DV8682-LV
NTC- DV8684-LV
NTC- DV8685-LV

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General Information

Contents

- 20 µg each of plasmid vector shipped in 1x TE buffer.
- NTC4862 (DH5α att_{λ}::$P_{5/6\ 6/6}$-RNA-IN- SacB, catR) host strain glycerol stock

Storage

- Plasmids should be stored at -20˚C.
- Glycerol stock should be stored at -80˚C

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NTC8681, NTC8682, NTC8684 and NTC8685 vector family

Introduction

Nature Technology Corporations (NTC's) NTC8681, NTC8682, NTC8684 and NTC8685 plasmids are antibiotic-free vectors optimized to combine maximal eukaryotic gene expression with superior bacterial manufacturing yields.

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, 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, 2011).

The vectors are available in four versions. NTC8681 targets encoded protein to the endosome using an optimized human lysosomal-associated membrane protein 1 (Lamp1) targeting tag. NTC8682 targets encoded protein into the secretory pathway using an optimized tissue plasminogen activator (TPA) signal peptide. NTC8684 targets proteins to the proteosome by fusion C-terminal to a destabilizing UbiquitinA76 tag. NTC8685 expresses encoded protein 'native' without targeting sequences.

The vectors 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. The vectors encode a consensus Kozak translation initiation sequence and ATG start codon.

Target gene expression is driven from an optimized chimeric promoter-intron (SV40-CMV-HTLV-1 R synthetic intron). The boundary between the CMV promoter and the SV40 enhancer has been optimized resulting in dramatically improved expression in mammalian cells (Luke *et al*. 2011). This chimeric CMV promoter achieves significantly higher expression levels than traditional human cytomegalovirus (CMV) promoter based vectors (Luke *et al.* 2009, 2011).

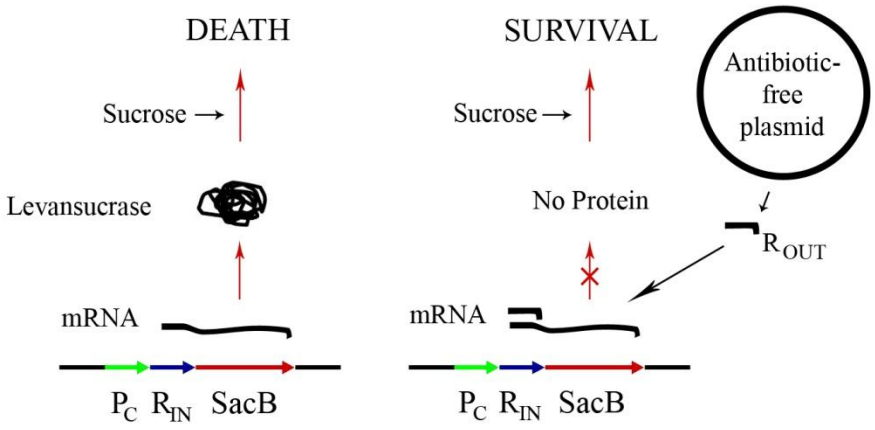
NTC8681, NTC8682, NTC8684 and NTC8685 vectors also incorporate the adenoviral serotype 5 VA RNAI (VA1) transient expression enhancer. VA1 further improves eukaryotic expression without affecting *Escherichia coli* production yields (Carnes *et al*. 2010, 2011).

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*).



Levansucrase (SacB) conditional-lethal gene on bacterial chromosome

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. NTC8685 vector production yields > 1 g/L were verified in *Escherichia coli* fermentation culture (Carnes *et al.* 2010).

In summary, the NTC8681, NTC8682, NTC8684 and NTC8685 vectors offer the following advantages

• Highest level expression in a wide range of mammalian cells using: 1) an optimized chimeric CMV-HTLV-I promoter; 2) Adenoviral VA RNAI transient expression enhancer; and 3) SV40 enhancer (**Fig. 1**)
• Antibiotic-free selection in *Escherichia coli* host
• Choice of *Escherichia coli* production strain for higher immunogenicity (dcm+ NTC4862) or higher expression (dcm- NTC48165; **Fig. 1**) (Carnes *et al*. 2011)
• Superior *Escherichia coli* plasmid production yields using optimized vector backbone
• Optional intracellular antigen targeting
    N-terminal and C terminal LAMP1 tag for endosomal targeting (NTC8681)
    N-terminal TPA signal peptide tag for secretion targeting (NTC8682)
    N-terminal destabilizing Ubiquitin tag for proteosome targeting (NTC8684)
• Simultaneous cloning into NTC8682, NTC8684 and NTC8685 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)

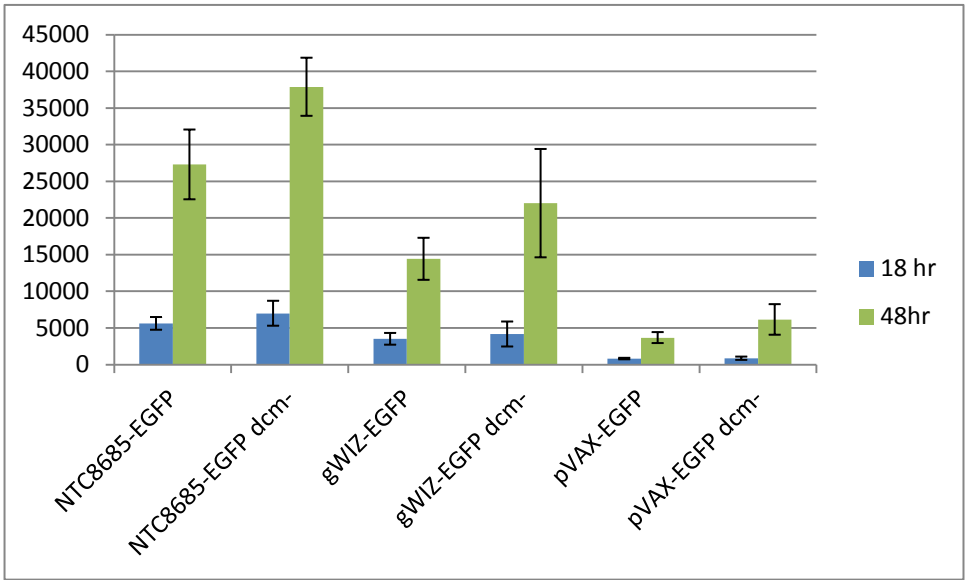


Fig 1. EGFP transgene expression (fluorescence) in human HEK293 cell line after transfection of dcm+ or dcm- NTC8685, gWIZ and pVAX1 CMV promoter plasmid vectors

NTC8681, NTC8682, NTC8684 and NTC8685 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 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 antibiotic free NTC8685 vector was constructed by incorporation of designed modifications to the base pDNAVACCultra vectors to improve mammalian cell expression (chimeric promoter) while eliminating antibiotic selection/improving plasmid production yields in the *Escherichia coli* bacterial host (vector backbone modification).

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 DNA vaccines in mice and rabbits (Luke *et al*. 2009, 2011) compared to CMV promoter based vectors. Expression levels of EGFP from plasmids incorporating the chimeric promoter are dramatically increased versus CMV promoter containing plasmids (Luke *et al*. 2009, 2011).

Vector backbone modification: Modifications to the pUC replication origin vector backbone to improve production yield included:
1) Extension of the pUC origin to include a primosomal assembly site (PAS-BH);
2) Inclusion of the SV40 enhancer adjacent to CMV promoter;

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

The kanR antibiotic selection marker was replaced with the sucrose selectable RNA-OUT marker to create NTC8685. Endosomal (NTC8681) secreted (NTC8682) and proteosomal (NTC8684) targeting derivatives were then constructed.

These vectors also incorporate two modifications that further increase eukaryotic expression. 1) the adenoviral serotype 5 VA RNAI (VA1) transient expression enhancer and; 2) optimization of the boundary between the CMV promoter and the SV40 enhancer. Both modifications enhance eukaryotic expression compared to the parent vectors (Luke *et al.* 2011).

NTC8681, NTC8682, NTC8684 and NTC8685 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 vector defined 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
Adenoviral Serotype 5 VA RNAI (VA1)	Increased mammalian cell expression
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

Intracellular targeting

Vector	Protein destination	Targeting Tag
NTC8681	endosome	human lysosomal-associated membrane protein 1 (Lamp1)
NTC8682	secreted	human tissue plasminogen activator (TPA)
NTC8684	proteasome	murine Ubiquitin A76
NTC8685	native	ATG*****

***** Native vectors express the target gene from a vector encoded ATG start codon immediately downstream of an optimized kozak sequence. 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 (NTC8681) 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 (NTC8682) has been demonstrated to efficiently target heterologous proteins for secretion (Zhongming *et al.*1999). The destabilizing ubiquitin molecule (UbiquitinA76 versus native UbiquitinG76) in NTC8684 enhances entry into proteosomal degradation pathway and MHC class I presentation, and shifts host response towards T_H1 type immunity (Rodriguez *et al.*1998; Delogu *et al.*2000).

Plasmid Expression Vector kit‡

Vector	Targeting	Quantity	Catalog Number	Price
NTC8681	endosome	20 µg	NTC- DV8681	$420.00
NTC8682	secreted	20 µg	NTC- DV8682	$420.00
NTC8684	proteosome	20 µg	NTC- DV8684	$420.00
NTC8685-EGFP*	cytoplasmic EGFP	20 µg	NTC- DV8685-EGFP	$420.00

* The NTC8685-EGFP control plasmid is used as a transfection control for expression in a cell line of interest, and as the NTC8685 backbone for cloning new genes

‡ Kit contains plasmid and NTC4862 (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
NTC8681†	endosome	1 µg	NTC- DV8681-LV	$444.00
NTC8682‡	secreted	1 µg	NTC- DV8682-LV	$444.00
NTC8684‡	proteosome	1 µg	NTC- DV8684-LV	$444.00
NTC8685‡	native	1 µg	NTC- DV8685-LV	$444.00

‡ *Sal*I - *Bgl*II linearized vector sufficient for 20 cloning reactions and NTC4862 (DH5α att_λ::$P_{5/6\ 6/6}$-RNA-IN- SacB, catR) host strain glycerol stock

† *Xho*I - *Eco*RI linearized vector sufficient for 20 cloning reactions and NTC4862 (DH5α att_λ::$P_{5/6\ 6/6}$-RNA-IN- SacB, catR) host strain glycerol stock

NTC offers the following accessory products for use with NTC8681, NTC8682, NTC8684 and NTC8685 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
LAMPFseq	Forward sequencing primer (NTC8681)	500 pmol (10 pmol/µL)	NTC-DVU-SP3	$30.00
NTC4862 DH5α att_{λ}::$P_{5/6\ 6/6}$-RNA-IN- SacB, catR electrocompetent cells	Host strain for NTC8682 and NTC8685 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 NTC8682 and NTC8685 selection	0.5 mL	NTC-DVU-CC2	$199.00
Sucrose selection agar	Plates for NTC8682 and NTC8685 selection	Pouch to make 1 L agar*	NTC-DVU-MD1	$20
Sucrose selection media	Media for NTC8682 and NTC8685 culture	Pouch to make 1 L media*	NTC-DVU-MD2	$20

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

RAPID-VACC^{tm} CONTRACT CLONING is an optional service whereby NTC designs and clones investigator-specified genes into the desired expression vectors, providing sequence-validated clones, or purified plasmid DNA ready for animal testing. NTC also offers a *Codon Optimization Design Service* to design and synthesize (by subcontract at cost) synthetic genes for *RAPID-VACC*^{tm} cloning (see www.natx.com).

RAPID-VACC^{tm} Contract Cloning service
Seamless cloning gene of interest into any vector NTC-RVC1 $400.00
1mg endotoxin-free plasmid preparations, each $335.00

Codon Optimization Design Service NTC-CONS-CODON $400.00†
† No charge with 100 mg endotoxin-free plasmid preparation

Cloning into NTC8682, NTC8684 and NTC8685 vectors

Overview: An example strategy for cloning into the NTC8682, NTC8684 and NTC8685 vectors is outlined below.

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

For all three vectors, the ATG start codon (double underlined) is immediately preceded by a *Sal*I site. In NTC8685, the *Sal*I site has been demonstrated to be an effective consensus Kozak sequence for translational initiation. Inclusion of the ATG is optional for NTC8682 and NTC8684 but the reading frame must be retained. In NTC8682, the *Sal*I site is downstream in frame with the optimized TPA secretion sequence. In NTC8684, the *Sal*I site is downstream in frame with the optimized Ubiquitin destabilization 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 NTC8685, the start codon ATG must immediately follow the *Sal*I site (GTCGACATG). For NTC8682 and NTC8684, 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 NTC8685 is compatible with, and can also be cloned into, the NTC8682 and NTC8684 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 NTC8685, since the ligated hybrid (GTCGAGATG) has not been established as an effective kozak sequence. ATG is optional for cloning downstream of TPA (NTC8682) or ubiquitin (NTC8684).

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 NTC8682, NTC8684 and NTC8685 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)

Cloning into NTC8681 vector

Primers contain *Xho*I (compatible with *Sal*I; the same 5' primer designed for NTC8682, NTC8684 and NTC8685 cloning can be used but both enzymes sites will be lost in the resulting construct) and *Eco*RI sites (rather than *Bgl*II) as outlined below.

CTCGAG-------- Gene of interest--------GAATTC
XhoI *EcoRI*

To facilitate the C terminal extensions needed for endosomal membrane anchoring the NTC8681 *Eco*RI containing 3' primer ***cannot include the TAA stop codon***.

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

LAMPFseq: TGCTCTTCCAGTTCGGGATG
(hybridizes to LAMP1 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 (NTC-DVU-CC1 or NTC-DVU-CC2) 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

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Patent and Licensing information

Limited License
Nature Technology Corporation (NTC) grants the end user (purchaser) of the NTC8681, NTC8682, NTC8684 and NTC8685 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 and host strains 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 NTC8681, NTC8682, NTC8684 and NTC8685 vectors and NTC4862, NTC48165, NTC5402, and NTC54208 host strains.

Product Use Limitations
The NTC8681, NTC8682, NTC8684 and NTC8685 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.

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.

The vectors and cell lines herein are covered by World Patent Application WO2008153733, JA Williams, Vectors and methods for genetic immunization (2008), and by US, European, and Australian patents: US 2010/0303859; EP2333091; and AU 2008262478, respectively.

The LAMP sequence, and related endosomal targeting sequences (e.g. NTC8681, NTC8681-eRNA41H vectors), is covered under U.S. Patents 5,633,234 and 8,318,173 and its use is permitted for research purposes only. Any other use of the LAMP technology requires a license from Immunomic Therapeutics Inc, 15010 Broschart Rd, Suite 250, Rockville, MD 20850.

NTC makes no representations that the use of the NTC8681, NTC8682, NTC8684 and NTC8685 vectors and/or NTC4862, NTC48165, NTC5402, and NTC54208 host strains will not infringe any patent, copyright, trademark, or other proprietary rights.

For more information, please contact: Clague Hodgson
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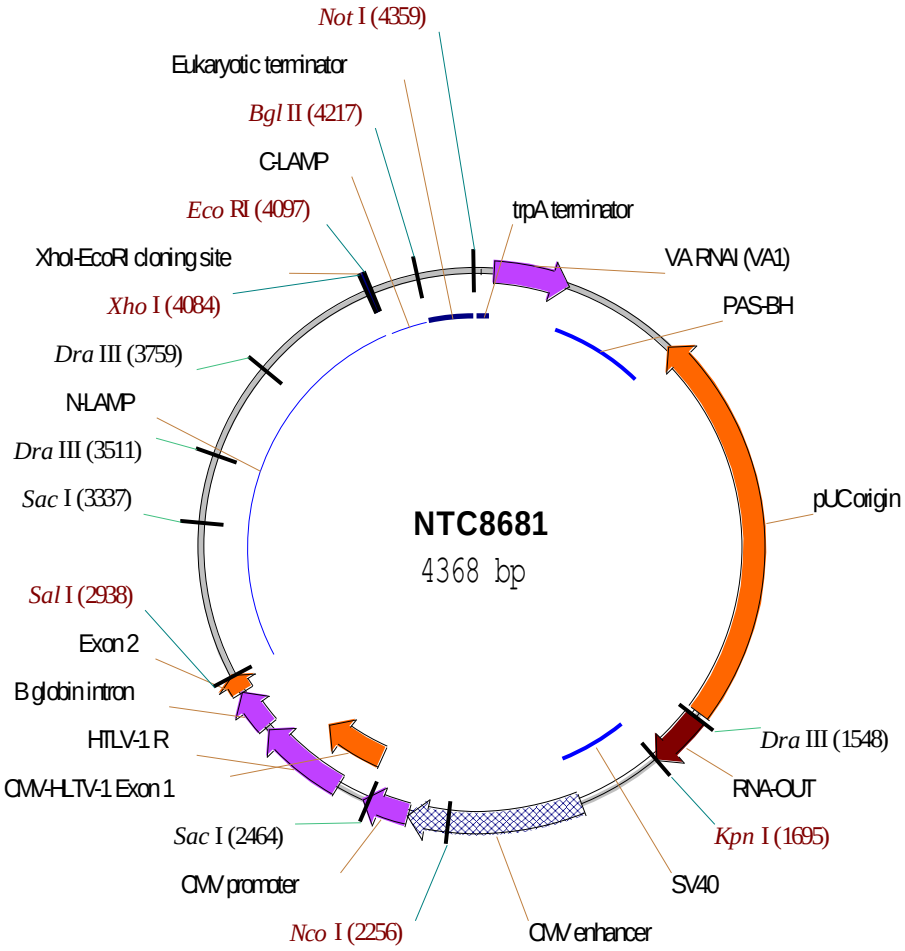
Telephone: (402) 323-6289, **Fax**: (402) 323-6292, **Email:** natx@natx.com.

NTC8681 (Endosomal targeting)



Vector Features

trpA prokaryotic terminator: 4367-28
Adenovirus serotype 5 VA RNAI (VA1): 32-225
Primosomal assembly site (PAS-BH) extended origin: 235-519
pUC replication origin: 520-1534
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SV40 enhancer: 1696-1915
CMV enhancer: 1916-2361
CMV promoter: 2362-2481
Untranslated leader (exon 1): 2482-2668
HTLV-1 R: 2553-2778
Synthetic Rabbit β-globin-based 3' intron: 2787-2893
Intron: 2669-2893
Exon 2 (SR-protein binding sites-Kozak):2894-2942
LAMP1 N-terminal endosome targeting tag: 2943-4082
*Xho*I-*Eco*RI cloning cassette: 4083-4101
LAMP1 C-terminal membrane anchor: 4102-4212
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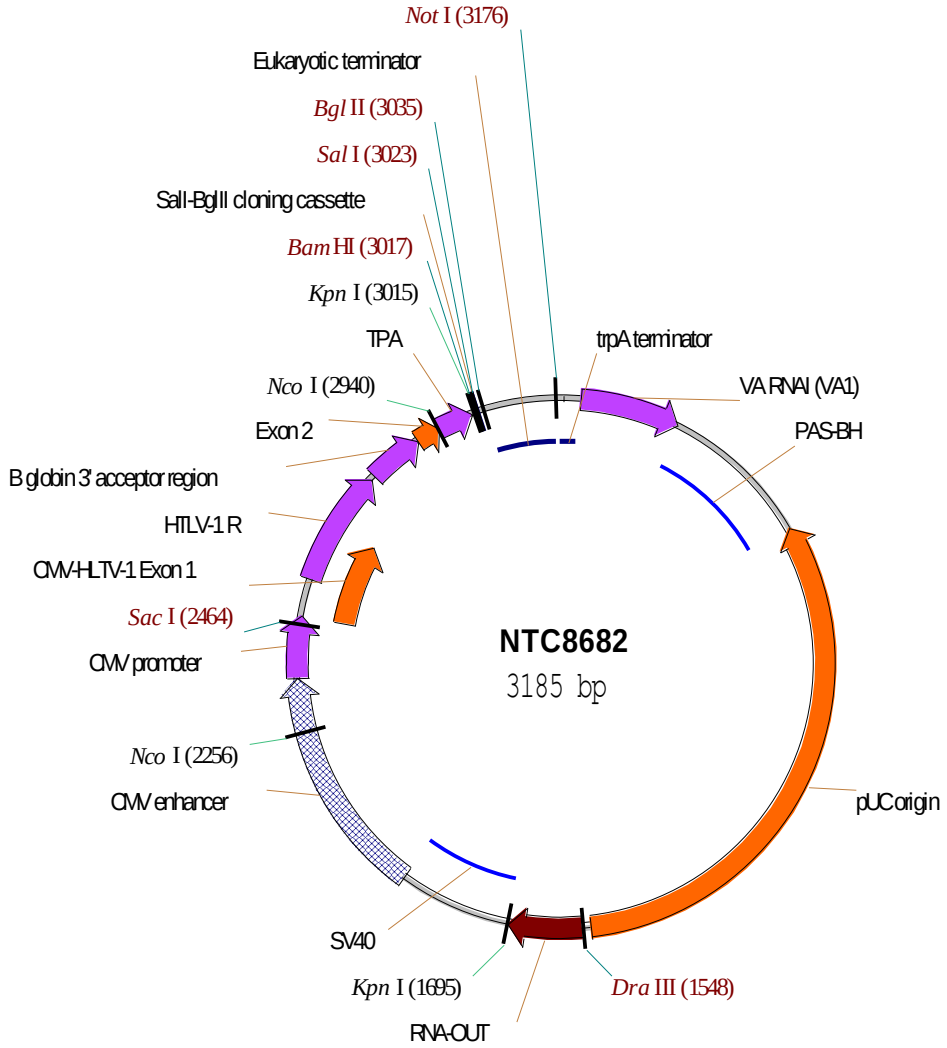

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CMV enhancer: 1916-2361
CMV promoter: 2362-2481
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HTLV-1 R: 2553-2778
Synthetic Rabbit β-globin-based 3' intron: 2787-2893
Intron: 2669-2893
Exon 2 (SR-protein binding sites-Kozak):2894-2940
TPA N-terminal targeting tag: 2941-3009
*Sal*I-*Bgl*II cloning cassette: 3022-3039
Eukaryotic terminator: 3040-3174

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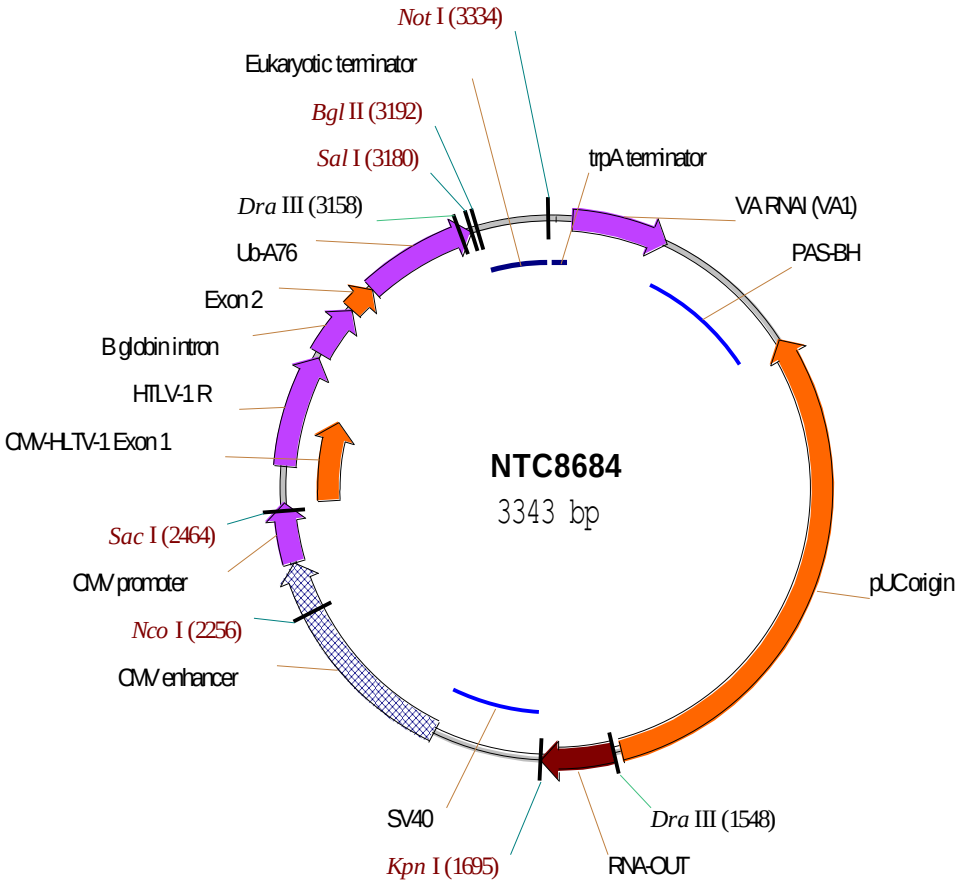
NTC8684 (Proteosomal targeting)



Vector Features
trpA prokaryotic terminator: 3342-28
Adenovirus serotype 5 VA RNAI (VA1): 32-225
Primosomal assembly site (PAS-BH) extended origin: 235-519
pUC replication origin: 520-1534
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Intron: 2669-2893
Exon 2 (SR-protein binding sites-Kozak):2894-2950
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*Sal*I-*Bgl*II cloning cassette: 3179-3196
Eukaryotic terminator: 3197-3331

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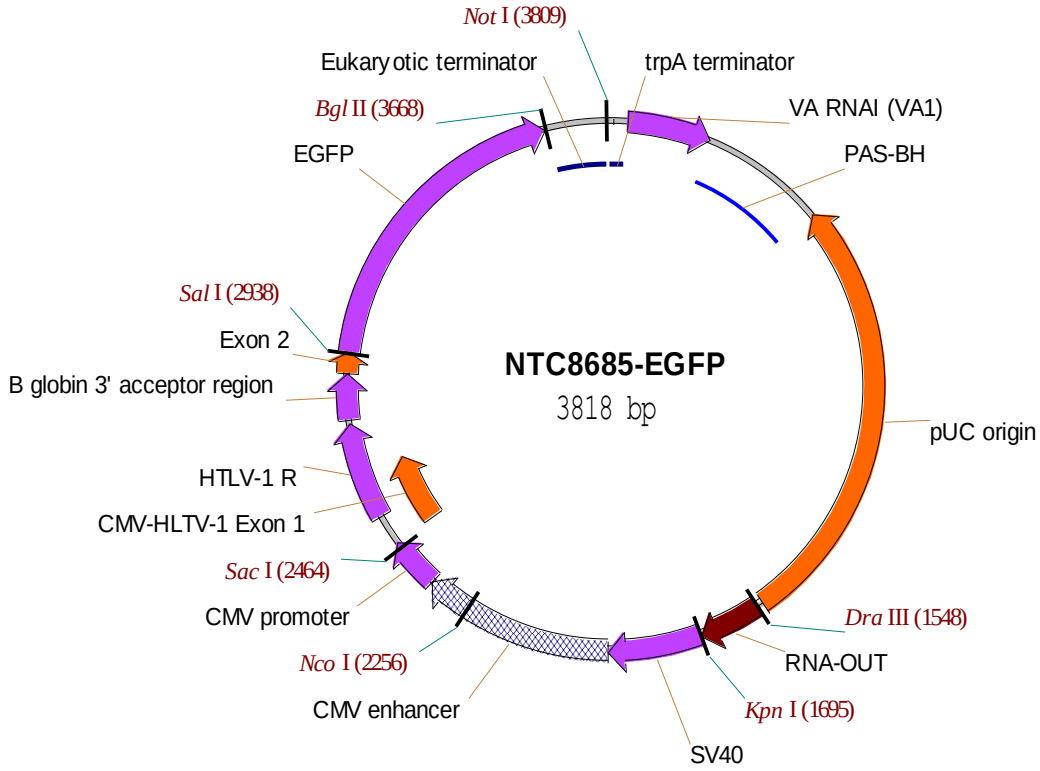

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Exon 2 (SR-protein binding sites-Kozak):2894-2942
EGFP: 2943-3662
*Sal*I-*Bgl*II cloning cassette: 2937-3672
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