invitrogen™

Tango™ Assay Development Protocol

Part # K1823

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TABLE OF CONTENTS

1.0 Overview of Tango™ Assay Technology

2.0 Overview of Beta-Lactamase Technology

3.0 Materials Supplied

4.0 Materials Required, but Not Supplied

5.0 Tango™ Vectors Overview
- 5.1 "Bait" Vectors
- 5.2 "Prey" Vectors
- 5.3 Reporter Vector

6.0 Tango™ Cloning Design and Vector Use
- 6.1 Experimental Outline
- 6.2 Using the Vectors
- 6.3 Propagating the Vectors
- 6.3 Cloning Sites for for pcDNA3 Tango Bait n-CD8 M and pcDNA3 Tango Bait n-CD8 Y
- 6.4 Cloning Sites for pcDNA5 Tango Prey c-TEV
- 6.5 Cloning Sites for pcDNA5 Tango Prey n-TEV
- 6.6 E. Coli Transformation

7.0 Setting up Tango™ Assays in Mammalian Cells
- 7.1 General Considerations in Tango™ Assay Development
- 7.2 Utilizing the Tango™ UAS-*bla* U2OS Cell Line
- 7.3 Detailed Cell Handling Procedures
- 7.4 Transient Transfection and Analysis of Mammalian Cells
- 7.5 Stable Cell Line Engineering
- 7.6 Tango™ Assay Substrate Loading and Detection
- 7.7 Going Beyond the Tango™ UAS-*bla* U2OS cell line

8.0 Appendix (Vector Maps & Sequences)
- pcDNA3 Tango Bait n-CD8 M
- pcDNA3 Tango Bait n-CD8 Y
- pcDNA5 Tango Prey c-TEV
- pcDNA5 Tango prey n-TEV
- pLenti-zeo/UAS-bla

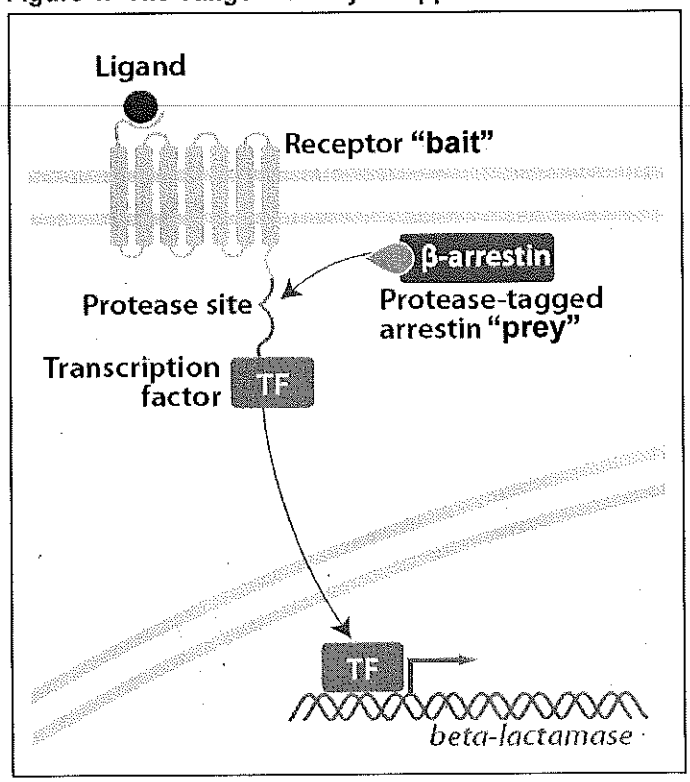
9.0 References

10.0 Purchaser Notification

1.0 OVERVIEW OF TANGO™ ASSAY TECHNOLOGY

The Tango™ Assay technology combines the benefits of the Tango™ assay platform with the highly accurate, sensitive, and live cell Beta-lactamase reporter system. The Tango™ assay measures protein-protein interactions in live mammalian cells. One of the proteins of interest is anchored to cell surface either by its own transmembrane domains or through fusion to CD8 using a "bait" vector provided in this kit. The "bait" vector also provides at its intracellular C-terminus, an in-frame fusion to an exogenous transcription factor, GAL4-VP16. Interposed between the protein of interest and the transcription factor is a specific cleavage sequence for a non-native protease. This chimeric receptor protein is expressed in a cell line containing the beta-lactamase reporter gene responsive to the transcription factor. The "prey" vectors allow an interacting protein to be expressed as a protease fusion protein that recognizes and cleaves the site between the anchored membrane protein and transcription factor. The assay is performed by adding a stimulus to the growing cells for a defined period and measuring the activity of the reporter gene. Activation of the reporter gene provides a quantifiable measurement of the degree of interaction between the anchored membrane protein and the protease-tagged interaction partner and is unaffected by other signaling pathways in the cell, providing an exquisitely specific readout of protein-protein interaction.

Figure 1. The Tango™ Assay as applied to GPCRs.



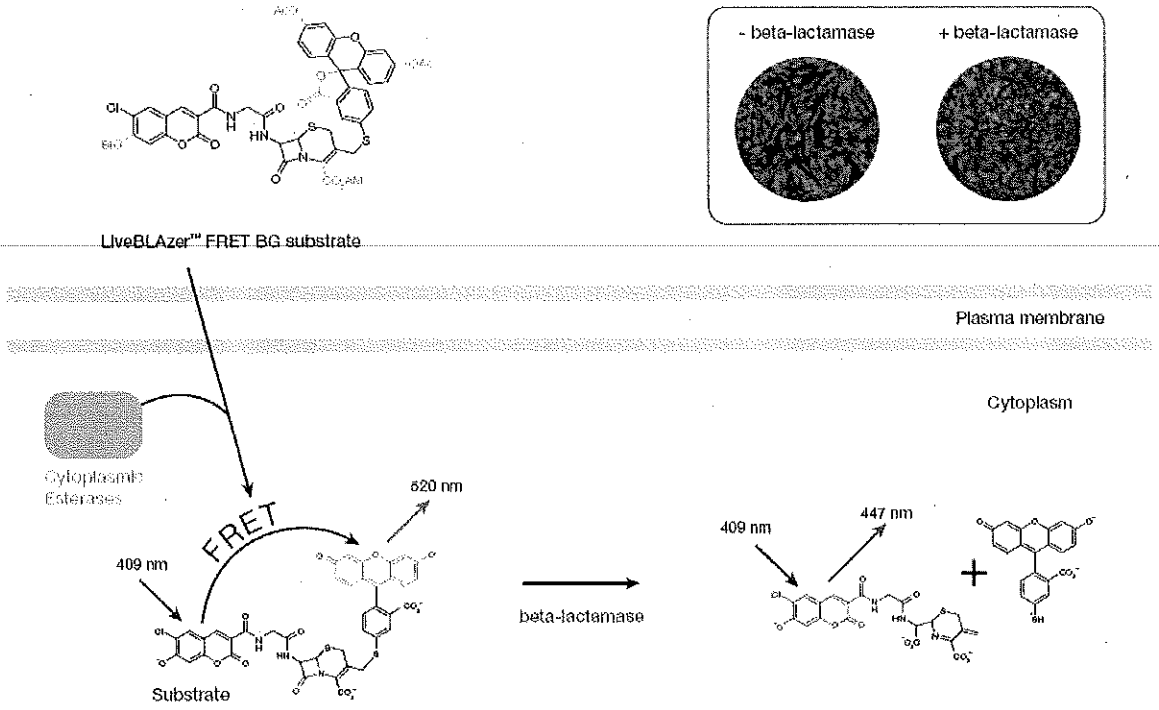
Upon ligand binding and receptor activation, a protease-tagged beta-arrestin is recruited to the GPCR that has been modified at the C-terminus to include a transcription factor linked via the respective protease cleavage site. The protease in turn cleaves the transcription factor from the GPCR, the transcription factor translocates to the nucleus, and beta-lactamase expression occurs.

The Tango™ techonology can similarly be applied to other protein-protein interactions by anchoring one protein fused to transcription factor separated by a protease cleavage site (the bait) to the cell surface and fusing the other protein (the prey) to the protease.

2.0 OVERVIEW OF BETA-LACTAMASE TECHNOLOGY

The Beta-Lactamase Technology uses a mammalian-optimized beta-lactamase reporter gene (bla) combined with a FRET-enabled substrate to provide reliable and sensitive detection in cells (Zlokarnik, et al. 1998) (Figure 2). Cells are loaded with a simple engineered fluorescent substrate containing two fluorophores, coumarin and fluorescein. In the absence of bla expression, the substrate molecule remains intact. In this state, excitation of the coumarin results in fluorescence resonsance energy transfer to the fluorescein moiety and emission of green fluorescent light. However, in the presence of bla expression, the substrate is cleaved separating the fluorphores and disrupting energy transfer. Excitation of the coumarin in the presence of bla enzyme activity results in a blue fluorescence signal. The resulting coumarin:fluorescein ratio provides a normalized reporter response which can minimize experimental noise that can mask subtle underlying biological responses of interest. The Beta-lactamase Reporter Technology has been proven in high-throughput screening (HTS) campaigns for a range of target classes, including GPCRs (Kunapuli, et al. 2003 and Xing, et al 2000), nuclear receptors (Qureshi, et al. 2003, Peekhaus, et al. 2003, and Chin, et al. 2003) and kinase signaling pathways (Whitney, et al. 1998).

Figure 2.



Fluorescent detection of beta-lactamase reporter gene response using the Beta-lactamase technology. After substrate loading, cells absent beta-lactamase expression appear green fluorescent. In the presence of beta-lactamase expression, the substrate is cleaved and cells appear blue fluorescent.

3.0 MATERIALS SUPPLIED

Vectors

Vector Name: Tango™ pcDNA3-Bait n-CD8 M (K1824A)

Description: The Tango™ pcDNA3-Bait n-CD8 M vector is a "bait" vector used to express one of the protein interaction partners in a membrane-anchored form. It contains a TEV protease cleavage site with a Methionine (M) in the P1′ position preceding a Gal4-VP16 transcription factor. The TEV protease exhibits a high level of catalytic activity when an M is present in the P1′ position (Kapust, et al. 2002). A MluI restriction site, preceding the TEV protease site, allows for a gene of interest to be cloned in-frame with the TEV protease site and Gal4-VP16 transcription factor to create a fusion protein. In addition, this vector contains a CD8 coding sequence upstream of the MluI site to allow the gene of interest to be expressed as a transmembrane fusion protein to be anchored on the cell membrane. Alternatively, if the gene of interest encodes a transmembrane protein, the gene can be cloned in frame with the TEV protease cleavage site and the Gal4-VP16 transcription factor by removing the CD8 using a combination of restriction sites AgeI and MluI.

Shipping Condition: Dry Ice

Storage Condition: -20° C

Quantity: 20 µg

Application: A "bait" vector for the development of Tango™ Protein-Interaction Assays with strong affinity for the "prey" TEV protease

Selection Marker: Geneticin® (Use at 200 µg/mL when utilizing the Tango™ UAS-*bla* U2OS cell line. If other cell types are used, a titration should be performed to determine the optimal amount of antibiotic to use.)

Vector Name: Tango™ pcDNA-Bait n-CD8 Y (K1825A)

Description: The Tango™ pcDNA-Bait n-CD8 Y vector is a "bait" vector used to express one of the protein interaction partners in a membrane-anchored form. It contains a TEV protease cleavage site with a Tyrosine (Y) in the P1′ position preceding a Gal4-VP16 transcription factor. The TEV protease exhibits an intermediate level of catalytic activity when a Y is present in the P1′ position (Kapust, et al. 2002). A MluI restriction site, preceding the TEV protease site, allows for a gene of interest to be cloned in-frame with the TEV protease site and Gal4-VP16 transcription factor to create a fusion protein. In addition, this vector contains a CD8 coding sequence upstream of the MluI site to allow the gene of interest to be expressed as a transmembrane fusion protein to be anchored on the cell membrane. Alternatively, if the gene of interest encodes a transmembrane protein, the gene can be cloned in frame with the TEV protease cleavage site and the Gal4-VP16 transcription factor by removing the CD8 using a combination of restriction sites AgeI and MluI.

Shipping Condition: Dry Ice

Storage Condition: -20° C

Quantity: 20 µg

Application: A "bait" vector for the development of Tango™ Protein-Interaction Assays with intermediate affinity for the "prey" TEV protease

Selection Marker: Geneticin® (Use at 200 µg/mL when utilizing the Tango™ UAS-*bla* U2OS cell line. If other cell types are used, a titration should be performed to determine the optimal amount of antibiotic to use.)

Vector Name:	**Tango™ pcDNA5-Prey c-TEV (K1826A)**
Description:	The Tango™ pcDNA5-Prey c-TEV vector is a "prey" vector that contains a multiple cloning site (MCS) allowing in frame c-terminal fusion of a gene with a TEV protease for use in the Tango™ Assay System. Interaction of the protein encoded by this gene with the protein expressed by a "bait" vector leads to the TEV protease mediated release of GAL4-VP16 into nucleus and activation of beta-lactamase reporter in the Tango™ UAS-*bla* U2OS cell line included in this kit.
Shipping Condition:	Dry Ice
Storage Condition:	-20° C
Quantity:	20 µg
Application:	A "prey" vector with c-terminal fusion of TEV protease for the development of Tango™ Protein-Interaction Assays
Selection Marker:	Hygromycin (The optimal amount of antibiotic to be utilized for cell line development is cell type dependent and should be determined by a titration of the antibiotic.)

Vector Name:	**Tango™ pcDNA5-Prey n-TEV (K1827A)**
Description:	The Tango™ pcDNA5-Prey n-TEV vector is an alternative "prey" vector that contains a multiple cloning site (MCS) allowing in frame n-terminal fusion of a gene with a TEV protease for use in the Tango™ Assay System. Interaction of the protein encoded by this gene with the protein expressed by a "bait" vector leads to the TEV protease mediated release of GAL4-VP16 into nucleus and activation of beta lactamase reporter in the Tango™ UAS-*bla* U2OS cell line included in this kit.
Shipping Condition:	Dry Ice
Storage Condition:	-20° C
Quantity:	20 µg
Application:	A "prey" vector with n-terminal fusion of TEV protease for the development of Tango™ Protein-Interaction Assays
Selection Marker:	Hygromycin (The optimal amount of antibiotic to be utilized for cell line development is cell type dependent and should be determined by a titration of the antibiotic.)

Vector Name:	**pLenti-zeo/UAS-bla (K1860A)**
Description:	The pLenti-zeo/UAS-bla vector contains the upstream activating sequence (UAS) which binds the Gal4-VP16 transcription factor upstream of the beta-lactamase gene.
Shipping Condition:	Dry Ice
Storage Condition:	-20° C
Quantity:	20 µg
Application:	The development of Tango™ Protein-Interaction Assays.
Selection Marker:	Zeocin (The optimal amount of antibiotic to be utilized for cell line development is cell type dependent and should be determined by a titration of the antibiotic.)

Cell Lines

Cell Line Name:	**Tango™ UAS-*bla* U2OS Cell Line (K1828A)**
Description:	In this Open-Model kit, Tango™ UAS-*bla* U2OS cells contain a beta-lactamase reporter gene under control of the UAS response element stably integrated into U2OS cells. This cell line can be used to produce Tango™ protein-interaction assays.
Shipping Condition:	Dry Ice
Storage Condition:	Liquid nitrogen. Immediately upon receipt, cells must be stored in liquid nitrogen. Cells cannot be stored at -80°C, as they may lose viability.
Quantity:	>8,000,000 cells (8 x 10^6 cells/ml)
Application:	The development of Tango™ Protein-Interaction Assays.
Growth Properties:	Adherent
Cell Phenotype:	Epithelial
Selection Marker(s):	Zeocin (200 µg/mL)
Mycoplasma Testing:	Negative
BioSafety Level:	1

Cell Line Name:	**Tango™ Beta-arrestin2-UAS-*bla* U2OS Cell Line (K1821A)**
Description:	In this Open-Model kit, Tango™ Beta-arrestin2-UAS-*bla* U2OS cells contain a beta-lactamase reporter gene under control of the UAS response element stably integrated into U2OS cells. This cell line also stably expresses a Beta-arrestin2-TEV protease fusion protein. The Tango™ Beta-arrestin2-UAS-*bla* U2OS cell line can be utilized for developing GPCR assays for arrestin recruitment.
Shipping Condition:	Dry Ice
Storage Condition:	Liquid nitrogen. Immediately upon receipt, cells must be stored in liquid nitrogen. Cells cannot be stored at -80°C, as they may lose viability.
Quantity:	>8,000,000 cells (8 x 10^6 cells/ml)
Application:	The development of Tango™ GPCR Assays.
Growth Properties:	Adherent
Cell Phenotype:	Epithelial
Selection Marker(s):	Zeocin (200 µg/mL), Hygromycin (50 µg/mL)
Mycoplasma Testing:	Negative
BioSafety Level:	1

4.0 MATERIALS REQUIRED, BUT NOT SUPPLIED

Media/Reagents	Recommended Source	Cat. no.
LiveBLAzer® Loading Kit LiveBLAzer™ FRET B/G Substrate (CCF4-AM) substrate DMSO for Solution A Solution B Solution C	Invitrogen	 K1427 (70 µg) K1095 (200 µg) K1096 (1 mg) K1030 (5 mg)
Recovery™ Cell Culture Freezing Medium	Invitrogen	102648-010
McCoy's 5A Medium (modified) (1X)	Invitrogen	16600-082
Freestyle Expression Medium	Invitrogen	12338-018
Fetal bovine serum, (FBS), dialyzed, tissue-culture grade	Invitrogen	26400-036
Non-essential amino acids (NEAA)	Invitrogen	11140-050
Penicillin/Streptomycin	Invitrogen	15140-122
Phosphate-buffered saline without calcium and magnesium [PBS(-)]	Invitrogen	14190-136
HEPES (1M, pH 7.3)	Invitrogen	15630-80
Sodium pyruvate	Invitrogen	11360-070
0.05% Trypsin/EDTA	Invitrogen	25300-054
Hygromycin B	Invitrogen	10687-010
Zeocin™ Selective Antibiotic	Invitrogen	R250-05
Geneticin® Selective Antibiotic	Invitrogen	10131-035
Ampicillin	Invitrogen	11593-027
Lipofectamine™ LTX Plus Transfection Reagent	Invitrogen	15338-100
Plus™ Reagent	Invitrogen	11514-015
MluI Restriction Enzyme	Invitrogen	15432-016
AgeI Restriction Enzyme	NEB	R0552S
T4 DNA Ligase	Invitrogen	15224-017
One Shot® TOP10 Cells	Invitrogen	C4040-10 *or* C4040-50
One Shot® MAX Efficiency® DH5™-T1R Cells	Invitrogen	12297-016
One Shot® Stbl3™ Chemically Competent *E. coli*	Invitrogen	C7373-03
PureLink™ Quick Gel Extraction Kit	Invitrogen	K2100-12
PureLink™ HQ Mini Plasmid Purification Kit	Invitrogen	K2100-01

Consumables	Recommended Source	Cat. no.
Black-wall, clear-bottom, 384-well assay plates (with low fluorescence background)	Costar	3712
Black-wall, clear-bottom, 96-well assay plates	Corning	3703
Compressed Air	Various	—

Equipment	Recommended Source
Fluorescence plate reader with bottom-read capabilities	Various
Filters if required for plate reader	Chroma Technologies

Optional Equipment and Materials

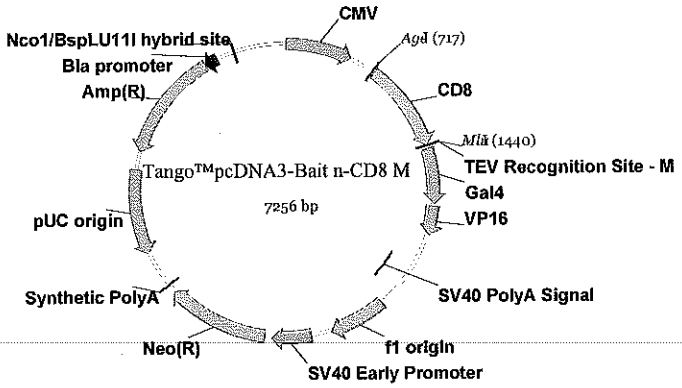
- Epifluorescence or fluorescence-equipped microscope, equipped with appropriate filters
- Microplate centrifuge

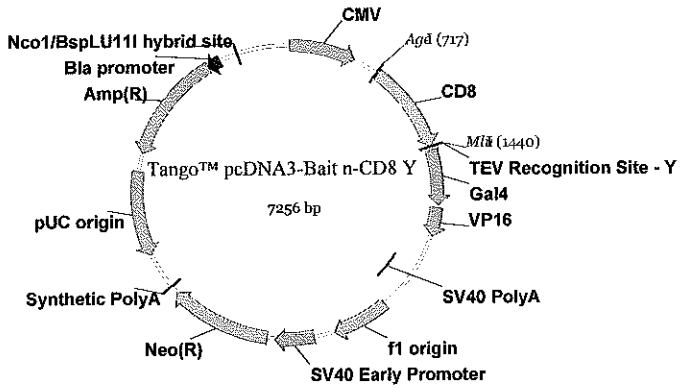
Reagents	Recommended Source	Catalog #
Dynal® CD8 Positive Isolation Kit	Invitrogen	113-33D
Dynabeads® CD8	Invitrogen	111-47D
DETACHaBEAD® CD4/CD8	Invitrogen	12504D
Phosphate-buffered saline without calcium and magnesium [PBS(-)]	Invitrogen	14190-136
Bovine Serum Albumin, 10%	Invitrogen	P2046
Choose size for application (cell number): ▪ Dynal MPC®-9600 (Optimal working volume: 5 µl – 200 µl) ▪ Dynal MPC®-L (Optimal working volume: 1 ml – 8 ml) ▪ Dynal MPC®-50 (Optimal working volume: 15 – 50 ml) ▪ DynaMag™-50	Invitrogen	120-06D 120-21D 120-24 123-02D
Hind III Restriction Enzyme	Invitrogen	15207-012

5.0 TANGO™ VECTORS OVERVIEW

5.1 "Bait" Vectors

Tango™ pcDNA3-Bait n-CD8 M and Tango™ pcDNA3-Bait n-CD8 Y are two versions of "bait" vectors that allow expression of the gene of interest in mammalian cells, with the difference of P1′ position of the TEV protease site and the resulting fusion protein having different substrate efficiency for TEV protease. The TEV protease exhibits higher catalytic efficiency when an M is present in the P1′ position than a Y (Kapust, et al. 2002). In some instances, higher catalytic efficiency is needed to boost assay signals, however in other instances, this increased catalytic efficiency may lead to higher background signals reducing assay windows. The appropriate vector configuration for a particular interaction pairs needs to be determined empirically and may depend upon several factors such as the strength of protein-protein interactions.





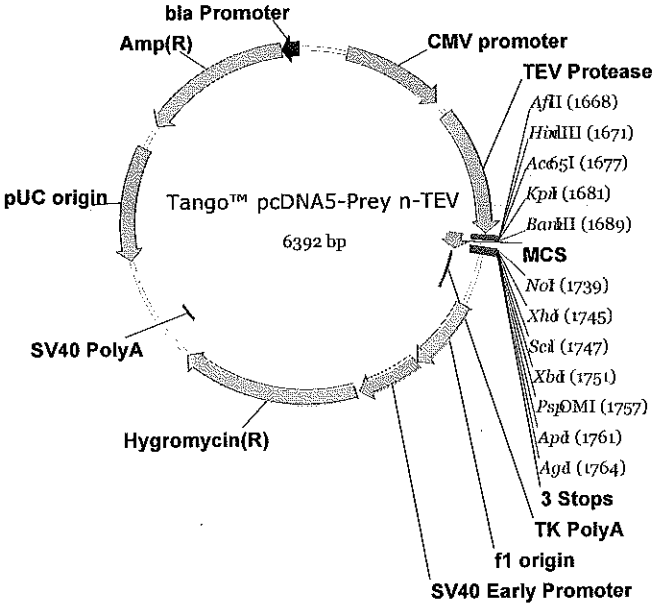
The vectors contain the following elements:

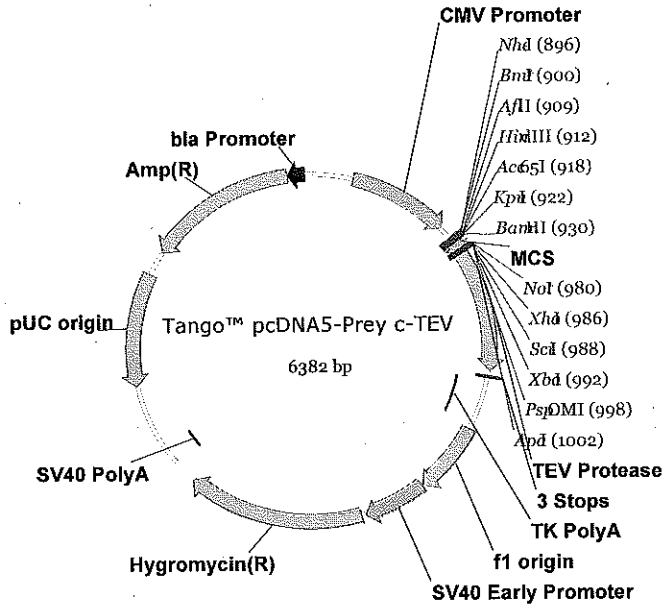
- Human cytomegalovirus immediate-early (CMV) promoter/enhancer for active expression in most mammalian cell types
- Options of using MluI site for in-frame fusion of the gene of interest with CD8 at N-terminus and Gal4-VP16 at C-terminus (recommended for assays of cytoplasmic protein-protein interactions) or using AgeI and MluI sites for in-frame fusion of the gene of interest with Gal4-VP16 at C-terminus (recommended for assays wherein one protein interaction partner is a transmembrane protein)
- Options of TEV Protease P1′ site: Methionine for enhanced substrate efficiency in Tango™ pcDNA3-Bait n-CD8 M and Tyrosine for intermediate substrate efficiency in Tango™ pcDNA3-Bait n-CD8 Y

- SV40 polyadenylation sequence for proper termination and processing of the transcript f1 intergenic region for production of single-strand DNA in F plasmid-containing *E. coli*
- SV40 early promoter and origin for expression of the neomycin resistance gene and stable propagation of the plasmid in mammalian hosts expressing the SV40 large T antigen
- Neomycin resistance gene for selection of stable cell lines
- The ampicillin (*bla*) resistance gene for selection in *E. coli*
- The pUC origin for high copy replication and maintenance of the plasmid in *E. coli*
- Gal4-VP16 sequence for expression of Gal4 transcriptional activator capable of binding to UAS and turning on genes placed downstream of UAS promoter

5.2 "Prey" Vectors

Tango™ pcDNA5-Prey c-TEV and Tango™ pcDNA5-Prey n-TEV are two versions of "prey" vectors that allow for stable expression of an interaction partner in the form of C-terminal or N-terminal fusion of TEV protease.





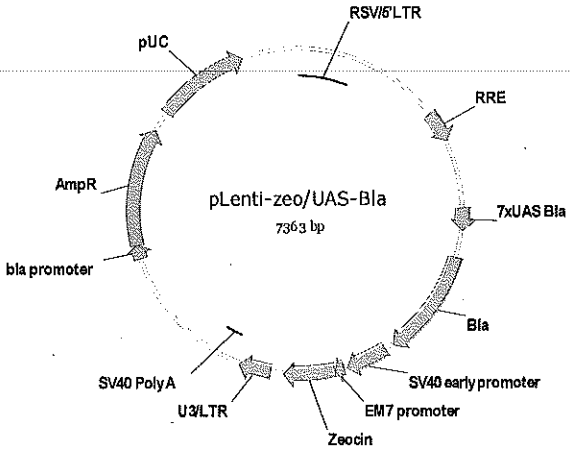
The vectors contain the following elements:

- Human cytomegalovirus immediate-early (CMV) promoter/enhancer for stable expression of the TEV protease fusion protein
- TEV protease (Tobacco Etch Virus) gene for cleavage of the Gal4-VP16 transcription factor from your gene of interest

- SV40 polyadenylation sequence for proper termination and processing of the transcript
- The pUC origin for high copy replication and maintenance of the plasmid in *E. coli*
- The ampicillin (*bla*) resistance gene for selection in *E. coli*
- Hygromycin resistance gene for selection of stable cell lines
- SV40 early promoter for high level expression of the hygromycin resistance gene

5.3 Reporter Vector

pLenti-zeo/UAS-bla contains the beta-lactamase reporter gene under the transcriptional control of the Upstream Activator Sequence (UAS) response element. This vector can be used with the ViraPower™ Lentiviral Expression System to create a lentiviral stock. The lentiviral stock can then be used to transduce any cell line of interest to become a UAS-bla cell line. Alternatively, this vector may be used with lipid based transfection methods such as Lipofectamine™ LTX Plus. The UAS-bla reporter can be activated by Gal4-VP16, a fusion of Gal4 DNA-binding domain and transcription activation domain of Herpes Simplex Virus Protein 16. Gal4 is a commonly-used yeast transcriptional activator, and the Gal4 DNA-binding domain (DBD) is capable of binding to UAS. Beta-lactamase is a powerful reporter gene that allows for ratiometric reporter responses with minimal experimental noise, and is expressed when the Gal4 DBD binds to the UAS response element.



The pLenti-zeo/UAS-bla vector contains the following elements:

- UAS - Upstream Activator Sequence (UAS) response element. A gene of interest fused to the Gal4 DNA binding domain can bind to the UAS and drive the expression of beta-lactamase
- Beta-lactamase reporter gene allows for ratiometric reporter responses with minimal experimental noise
- SV40 early promoter and origin allows high-level expression of the selection marker and episomal replication in cells expressing the SV40 large T antigen.
- EM7 promoter synthetic prokaryotic promoter for expression of the selection marker in E. coli.
- Zeocin resistance gene permits selection of stably transduced mammalian cell lines
- ΔU3/HIV-1 truncated 3′ LTR allows viral packaging but self-inactivates the 5′ LTR for biosafety purposes (Dull et al., 1998). The element also contains a polyadenylation signal for transcription termination and polyadenylation of mRNA in transduced cells.

- SV40 polyadenylation signal allows transcription termination and polyadenylation of mRNA.
- *bla* promoter allows expression of the ampicillin resistance gene.
- Ampicillin resistance gene (*bla*) allows selection of the plasmid in E. coli.
- pUC origin permits high-copy replication and maintenance in E. coli.
- Rous Sarcoma Virus (RSV) enhancer/promoter allows Tat-independent production of viral mRNA (Dull et al., 1998).
- HIV-1 truncated 5′ LTR permits viral packaging and reverse transcription of the viral mRNA (Luciw, 1996).
- 5′ splice donor and 3′ acceptors enhances the biosafety of the vector by facilitating removal of the ψ packaging sequence and RRE such that expression of the gene of interest in the transduced host cell is no longer Rev-dependent (Dull et al., 1998).
- HIV-1 psi (ψ) packaging signal allows viral packaging (Luciw, 1996).
- HIV-1 Rev response element (RRE) permits Rev-dependent nuclear export of unspliced viral mRNA (Kjems et al., 1991; Malim et al., 1989).

6.0 TANGO™ CLONING DESIGN AND VECTOR USE

6.1 Experimental Outline

Use the following outline to clone and express your gene of interest in the cloning vectors provided in this kit.

General Considerations:

1. Use of the Tango™ technology requires a "bait" vector and a "prey" vector. If one of the interaction partner proteins is a transmembrane protein, this protein should be considered to be cloned into the "bait" vectors. Using the AgeI and MluI site, one can replace the CD8 coding sequence with the coding sequence of the transmembrane protein. PCR cloning is the best way to ensure in-frame fusion of the gene of interest with GAL4-VP16. The 5′ PCR primer should include the AgeI site, encode the initial amino acid sequences and conform to the Kozak sequence. The 3′ PCR primer should have the stop codon removed and have a MluI restriction site in-frame with the Gal4-VP16 to the C-terminus.
2. If both interaction partner proteins are cytosolic proteins, one of them should be chosen to be anchored to the cell membrane through CD8 fusion as a "bait" protein. PCR cloning is the best way to ensure in-frame fusion of the gene of interest with both CD8 and GAL4-VP16. The 5′ PCR primer should include the MluI site and encode the initial amino acid sequences in frame with the CD8 to the N-terminus. The 3′ PCR primer should have the stop codon removed and have a MluI restriction site in-frame with the Gal4-VP16 to the C-terminus.
3. Two versions of the "bait" vectors are offered with the difference of P1′ site of the TEV protease site being Methionine or Tyrosine. We recommend that you clone you gene of interest into both versions and determine empirically if enhanced TEV cleavage site (Methionine at P1′) or intermediate strength TEV cleavage site (Tyrosine at P1′) works best for the interaction assay.
4. The interaction partner protein is expressed by the "prey" vectors as a TEV fusion protein. If structural information of the interaction proteins is known, one may be able to choose from the two options: N-terminal fusion of TEV or C-terminal fusion of TEV. Otherwise, we advise that one starts with the C-terminal fusion of TEV using pcDNA5 Tango Prey c-TEV.
5. Consult the restriction cloning sites described in this manual to design a strategy to clone your gene.
6. Digest your vector with the appropriate restriction enzymes, treat with alkaline phosphatase if desired, and gel purify your vector backbone.
7. Prepare your insert containing your gene of interest via PCR or other method. Purify any PCR amplicons or digest products as needed.
8. Ligate your prepared insert into the prepared vector and transform into E. coli. Select transformants on LB plates containing 100 µg/ml ampicillin.
9. Analyze your transformants for the presence of insert by restriction digestion.
10. Select a transformant with the correct restriction pattern and use sequencing to confirm that your gene is cloned in the proper orientation.

6.2 Using the Vectors

All vectors in this kit are supplied as supercoiled plasmids. Although Invitrogen has previously recommended using a linearized destination vector for more efficient recombination, further testing has found that linearization of these vectors is **NOT** required to obtain optimal results for any downstream application.

6.3 Propagating the Vectors

Important: We recommend using *E. coli* that is wild type for endonuclease 1 (*end*A1+). When performing plasmid DNA isolation with commercially available kits, ensure that Solution I of the Lysis buffer (often called Resuspension Buffer) contains 10 mM EDTA to inactivate the endonuclease to avoid DNA nicking and vector

degradation. Alternatively, follow the instructions included in the plasmid purification kits for *end*A1+ *E. coli* strains. We recommend using the PureLink™ HQ Mini Plasmid Purification Kit.

6.3.1 Propagating Tango™ pcDNA3-Bait n-CD8 M and Tango™ pcDNA3-Bait n-CD8 Y

If you wish to propagate and maintain the bait vectors, we recommend using TOP10 or DH5α from Invitrogen for transformation. Select for transformants using LB agar with Ampicillin (100µg/mL).

6.3.2 Propagating Tango(TM) pcDNA5-Prey c-TEV and Tango™ pcDNA5-Prey n-TEV

If you wish to propatate and maintain the prey vectors, we recommend using TOP10 or DH5α from Invitrogen for transformation. Select for transformants using LB agar with Ampicillin (100µg/mL).

6.3.3 Propagating pLenti-*Zeo*/UAS-*bla*

To propagate and maintain the pLenti-*Zeo*/UAS-*bla* vector, we recommend using One Shot® Stbl3™ chemically competent *E. coli* (available separately from Invitrogen) to reduce the likelihood of unwanted recombination. Select for transformants on LB agar plates containing 100 µg/ml ampicillin, and analyze the transformants as described below. We recommend analyzing the transformants using restriction digestion analysis. This ensures that aberrant recombination has not occurred between the plasmid LTRs of the pLenti-*zeo*/UAS-*bla* vector. You will screen colonies by performing miniprep DNA isolation and restriction analysis to validate the clones. After verifying the correct clones, you will use the miniprep DNA to re-transform *E. coli*. You will then isolate plasmid DNA for transfection and lentivirus production. Plasmid DNA for transfection must be very clean and free from contaminants and salts, and should be isolated using a PureLink™ HQ Mini Plasmid Purification Kit.

Important: To confirm that no rearrangement in the LTR regions of the pLenti-Zeo/UAS-bla vector has taken place, perform a restriction digest using the *Hind* III restriction enzyme. After digestion with this enzyme, 5 DNA fragments should be generated (556 bp, 584 bp, 670 bp, 1897 bp, and 3344 bp). Any unexpected DNA fragments are a result of LTR recombination.

Notes on Isolating Lentiviral Plasmid DNA

This protocol provides general steps to retransform *E. coli* and perform isolation of plasmid DNA for lentivirus production. pLenti plasmid DNA midipreps often have lower yields; therefore, a 100 ml volume of culture must be used for one DNA midiprep.

1. Dilute 1 µl of miniprep plasmid DNA from a positive clone 1:500 in TE.
2. Use 1 µl of this diluted DNA to retransform competent *E. coli*. Recommend using One Shot® Stbl3 chemically competent E. *coli*.
3. Plate approximately one-tenth of the transformation on LB plates containing 100 µg/ml ampicillin and incubate at 37°C overnight.
4. Pick 1 colony and culture in 2-3 ml LB medium containing 100 µg/ml ampicillin for 6-8 hours at 37°C to obtain a starter culture.
5. Inoculate the entire volume of the starter culture into LB medium containing 100 µg/ml ampicillin and culture at 37°C overnight.

   Note: Use a 100 ml volume for large scale or midiprep isolation of DNA.
6. Isolate plasmid DNA using S.N.A.P.™ MidiPrep Kit or equivalent.

   Note: For best results using the S.N.A.P.™ MidiPrep Kit, split the 100 ml culture into two 50-ml tubes and process as if they were separate samples. Run both samples over the same DNA binding column B, (*i.e.* perform two spins) and treat as a single DNA prep in subsequent steps.
7. Perform restriction analysis as described on the previous page.
8. Use the purified plasmid DNA from the positive clone for producing the lentivirus.

   Note: Typical DNA yield should be ~300-400 µg and the O.D. $_{260/280}$ ratio should be between 1.8 and 2.1.

6.4 General Molecular Biology Techniques

For help with DNA ligations, E. coli transformations, restriction enzyme analysis, purification of single-stranded DNA, DNA sequencing, and DNA biochemistry, please refer to Molecular Cloning: A Laboratory Manual (Sambrook et al., 1989) or Current Protocols in Molecular Biology (Ausubel et al., 1994).

6.5 Cloning Sites for Tango™ pcDNA3-Bait n-CD8 M and Tango™ pcDNA3-Bait n-CD8 Y

The following figure illustrates the location of cloning sites for Tango™ pcDNA3-Bait n-CD8 M and Tango™ pcDNA3-Bait n-CD8 Y. These two vectors are identical, except for the TEV protease site P1′ position (refer to section 5.1 for additional information). Restriction sites are highlighted. These sites have been confirmed by sequencing and functional testing. For a map, description and complete sequence of the features of Tango™ pcDNA3-Bait n-CD8 M and Tango™ pcDNA3-Bait n-CD8 Y, please refer to the **Appendix**.

- For cloning of a transmembrane protein, use the Age1 site (5′) and Mlu1 site (3′) to replace CD8 coding sequence.
- For cloning of a cytosolic protein, use the Mlu1 site to insert the gene of interest as in-frame fusion to CD8 and Gal4-VP16.

Tango™ pcDNA3-Bait n-CD8 M and Tango™ pcDNA3-Bait n-CD8 Y contain identical cloning sites. Age1 and Mlu1 sites are highlighted in grey. The Kozak sequence is underlined.

```
                                              AgeI                               CD8
                                            ~~~~~~~          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                              MetAla LeuProVal ThrAlaLeuLeu LeuProLeu·
 641   CAAGTTTGTA CAAAAAAGCA GGCTGGTACC GGTCCGGAAT TCACCATGGC CTTACCAGTG ACCGCCTTGC TCCTGCCGCT

                                         CD8
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       ·AlaLeuLeu LeuHisAlaAla ArgProSer GlnPheArg ValSerProLeu AspArgThr TrpAsnLeu GlyGluThrVal·
 721   GGCCTTGCTG CTCCACGCCG CCAGGCCGAG CCAGTTCCGG GTGTCGCCGC TGGATCGGAC CTGGAACCTG GGCGAGACAG
/////////////////////////////////////////////////////////////////////////////////////////////////
       /

                                         CD8
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
       ·SLeuValIle ThrLeuTyr CysAsnHisArg AsnArgArg ArgValCys LysCysProArg ProValVal LysSerGly
1281   CACTGGTTAT CACCCTTTAC TGCAACCACA GGAACCGAAG ACGTGTTTGC AAATGTCCCC GGCCTGTGGT CAAATCGGGA

                 CD8                      MluI TEV Recognition Site - M           Gal4
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~    ~~~~~~~~~~~~~~~~~~~~
       AspLysProSer LeuSerAla ArgTyrVal ThrArgGluAsn LeuTyrPhe GlnMetArg LysLeuLeuSer SerIleGlu·
1361   GACAAGCCCA GCCTTTCGGC GAGATACGTC ACGCGTGAGA ACCTGTACTT CCAGATGAGA AAGCTACTGT CTTCTATCGA
```

6.5.1 Points to Consider Before Cloning into Tango™ pcDNA3-Bait n-CD8 M and Tango™ pcDNA3-Bait n-CD8 Y

Translation Initiation

Your insert should contain a Kozak consensus sequence with an ATG initiation codon for proper initiation of translation if you are to replace the CD8 coding sequence within the two restriction sites, AgeI and MluI (Kozak, 1987; Kozak, 1991; Kozak, 1990). An example of a Kozak consensus sequence is provided below. Other sequences are possible, but the G or A at position -3 and the G at position +4 are the most critical for function (shown in bold). The ATG initiation codon is shown underlined.

(G/A)NN<u>ATG</u>**G**

Stop Codon Removal and Correct Reading Frame

Tango™ pcDNA-Bait n-CD8 M and Tango™ pcDNA3-Bait n-CD8 Y are fusion vectors. Your gene should be in-frame with the TEV protease recognition site, and should <u>NOT contain a stop codon</u>.

6.5.2 PCR Primer Design

If needed, you may amplify your gene of interest with appropriate PCR primers in order to add on the restriction sites or remove the stop codon from your gene. The primers should include 18-25bp of gene specific sequence, followed by the restriction site sequence. Include 6-8bp of GC-rich sequence on the 5′ end of both primers to allow for efficient digestion by the restriction enzyme. Some examples are shown on the following page.

Forward amplification primer (Age1) for a transmembrane protein coding gene, Kozak sequence underlined:
5′-[6-8bp GC-rich sequence]ACCGGTCACCATG[18-25bp gene-specific sequence)

Forward amplification primer (MluI) for a cytosolic protein coding gene, no Kozak sequence needed:
5′-[6-8bp GC-rich sequence]ACGCGTATG[18-25bp gene-specific sequence]

Reverse amplification primer at MluI site:
5′-[6-8bp GC-rich sequence]ACGCGT[18-25bp gene-specific reverse strand sequence]

If your gene of interest contains an internal AgeI site, it may still be possible to use the AgeI restriction site of the "Bait" vectors. The following restriction enzymes contain a compatible 5′overhang sequence: BsaWI (W/CCGGW), BspEI (T/CCGGA), BsrF1 (R/CCGGY), NgoMIV (G/CCGGC), XmaI (C/CCGGG), SgrAI (CR/CCGGYG). Amplify your gene of interest as described above, but rather than AgeI, use the compible enzyme of your choosing in your 5′-primer sequence.

If your gene of interest contains an internal MluI site, BssHII (G/CGCGC) contains a compatible overhang sequence and can be substituted for MluI in your Reverse Amplification Primer.
Note: Remember to keep your gene in-frame with the TEV cleavage site, and do NOT include a stop codon.

6.6 Cloning Sites for Tango™ pcDNA5-Prey c-TEV

The following figure illustrates the location of cloning sites for Tango™ pcDNA5-Prey c-TEV. Restriction sites are labeled to indicate the cleavage site. These sites have been confirmed by sequencing. For a map, description and complete sequence of the features of Tango™ pcDNA5-Prey c-TEV, please refer to the **Appendix**.

The first amino acid of TEV Protease is highlighted in grey, and the multiple cloning site sequences are translated in-frame with TEV Protease. Stop codons in the MCS are indicated with asterisks (***). Stop codons which are out-of-frame with TEV Protease are underlined.

```
        CMV promoter
      ~~~~~~~~~~~~~~~~~~~~
801   GTCTATATAA GCAGAGCTCT CTGGCTAACT AGAGAACCCA CTGCTTACTG GCTTATCGAA ATTAATACGA CTCACTATAG
      CAGATATATT CGTCTCGAGA GACCGATTGA TCTCTTGGGT GACGAATGAC CGAATAGCTT TAATTATGCT GAGTGATATC

                                                    MCS
                      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                     HindIII
                                     ~~~~~~
                       NheI                    KpnI
                       ~~~~~~~                 ~~~~~~~
                       BmtI          AflII     Acc65I       BamHI
                       ~~~~~~~       ~~~~~~~   ~~~~~~~      ~~~~~~~
                      AlaSer Val***Thr*** AlaTrpTyr ArgAlaArg IleHis***Ser SerValVal GluPheCys
881   GGAGACCCAA GCTGGCTAGC GTTTAAACTT AAGCTTGGTA CCGAGCTCGG ATCCACTAGT CCAGTGTGGT GGAATTCTGC
      CCTCTGGGTT CGACCGATCG CAAATTTGAA TTCGAACCAT GGCTCGAGCC TAGGTGATCA GGTCACACCA CCTTAAGACG

                        MCS                                          TEV Protease
      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                SciI          PspOMI
                               ~~~~~~~        ~~~~~~~
                                XhoI   XbaI
                               ~~~~~~~ ~~~~~~
                          NotI                 ApaI
                         ~~~~~~~~~             ~~~~~~~
      ArgTyrProAla GlnTrpArg ProLeuGlu  SerArgGlyPro GlySerLeu PheLysGly ProArgAspTyr AsnProIle·
961   AGATATCCAG CACAGTGGCG GCCGCTCGAG  TCTAGAGGGC CCGGTAGCTT GTTTAAGGGA CCACGTGATT ACAACCCGAT
      TCTATAGGTC GTGTCACCGC CGGCGAGCTC  AGATCTCCCG GGCCATCGAA CAAATTCCCT GGTGCACTAA TGTTGGGCTA
```

6.6.1 Points to Consider Before Cloning into Tango™ pcDNA5-Prey c-TEV

Translation Initiation

Your insert should contain a Kozak consensus sequence with an ATG initiation codon for proper initiation of translation. An example of a Kozak consensus sequence is provided below. Other sequences are possible, but the G or A at position -3 and the G at position +4 are the most critical for function (shown in bold). The ATG initiation codon is shown underlined.

(G/A)NNATGG

Minimizing Length of Linker

While the MCS of this vector was designed for your cloning convenience, we recommend using a cloning strategy that will minimize the number of linker amino acids between your gene of interest and the TEV Protease gene. A suitable cloning strategy is provided below, under "PCR Primer Design".

Stop Codon Removal and Correct Reading Frame

Tango™ pcDNA5-Prey c-TEV is a fusion vector. Your gene be in-frame with TEV protease, and should NOT contain a stop codon. Note that the MCS contains some stop codons in-frame with TEV Protease. Design your cloning strategy so that these stop codons will either not be present in the final construct, or will be out of frame with your fusion protein. If the Xba I or Nhe1 sites are used, please note that these sites contain an internal stop codon (TCTAGA, GCTAGC). Genes should be cloned in-frame with the Xba1 site and/or Nhe1 site to avoid these stop codons and be in-frame with the TEV protease. An example cloning strategy is provided below under "PCR Primer Design".

6.6.2 PCR Primer Design for Cloning PCR Products into Tango™ pcDNA5-Prey c-TEV

If needed, you may amplify your gene of interest with appropriate PCR primers in order to add on the restriction sites or remove the stop codon from your gene. The primers should include 18-25bp of gene specific sequence, followed by the restriction site sequence. Include 6-8bp of GC-rich sequence on the 5′ end of both primers to allow for efficient digestion by the restriction enzyme.

Example: Clone the gene of interest into the 5′ HindIII site and the 3′ Apa1 site.

The following example primers include a Kozak sequence for proper initiation of translation, minimizes the length of the linker between the gene of interest and TEV Protease, and removes the stop codon for the end of the gene of interest.

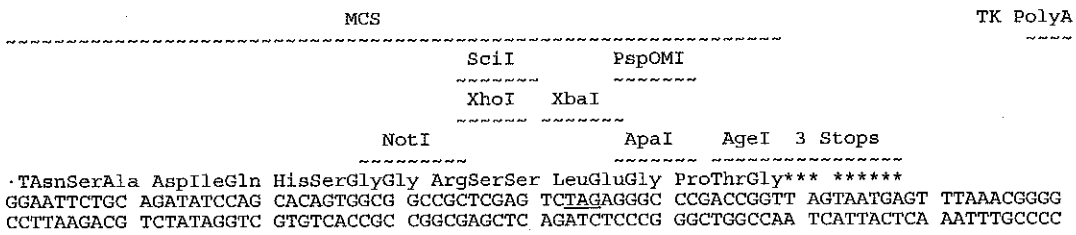
Forward amplification primer (HindIII), Kozak sequence underlined:
5′-[6-8bp GC-rich sequence]AAGCTTCACCATG[18-25bp gene-specific sequence]-3′

Reverse amplification primer with no stop codon (Apa1):
5′-[6-8bp GC-rich sequence]GGGCCC[18-25bp gene-specific reverse strand sequence]-3′

6.7 Cloning Sites for Tango™ pcDNA5-Prey n-TEV

The following figure illustrates the location of cloning sites for Tango™ pcDNA5-Prey n-TEV. Restriction sites are labeled to indicate the cleavage site. These sites have been confirmed by sequencing. For a map, description and complete sequence of the features of Tango™ pcDNA5-Prey n-TEV, please refer to the **Appendix**. The last amino acid of TEV Protease is highlighted in grey. Stop codons which are out-of-frame with TEV Protease are underlined.

```
          TEV Protease                                  MCS
       ~~~~~~~~~~~~~~~~~~~~~~~~~        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                   HindIII
                                                   ~~~~~~
                                                          KpnI
                                                          ~~~~~~~
                                               AflII      Acc65I       BamHI
                                               ~~~~~~~    ~~~~~~~      ~~~~~~~
      ·TyrAspVal ProAspTyr AlaSerLeuAla PheLysLeu LysLeuGlyThr GluLeuGly SerThrSer ProValTrpTrp·
1601   ATATGATGTT CCAGATTACG CTTCGCTAGC GTTTAAACTT AAGCTTGGTA CCGAGCTCGG ATCCACTAGT CCAGTGTGGT
       TATACTACAA GGTCTAATGC GAAGCGATCG CAAATTTGAA TTCGAACCAT GGCTCGAGCC TAGGTGATCA GGTCACACCA
```



6.7.1 Points to Consider Before Cloning into Tango™ pcDNA5-Prey n-TEV

Translation Initiation
Your gene does not require a Kozak sequence or an ATG for proper translation initiation.

Minimizing Length of Linker
While the MCS of this vector was designed for your cloning convenience, we recommend using a cloning strategy that will minimize the number of linker amino acids between your gene of interest and the TEV Protease gene. A suitable cloning strategy is provided below, under "PCR Primer Design".

Stop Codons and Correct Reading Frame
Tango™ pcDNA5-Prey n-TEV is a fusion vector. Your gene should be in-frame with TEV protease. You may choose to include a stop codon for your gene. There are 3 tandem stop codons provided in the vector immediately following the multiple cloning site. Note that your gene must be in-frame with these stop codons in order to take advantage of them. A suitable cloning strategy is provided below, under "PCR Primer Design".

6.7.2 PCR Primer Design for Cloning PCR Products into Tango™ pcDNA5-Prey n-TEV

If needed, you may amplify your gene of interest with appropriate PCR primers in order to add on the restriction sites or remove the stop codon from your gene. The primers should include 18-25bp of gene specific sequence, followed by the restriction site sequence. Include 6-8bp of GC-rich sequence on the 5′ end of both primers to allow for efficient digestion by the restriction enzyme.

Example 1: Clone the gene of interest into the 5′ AflII site and the 3′ AgeI sites, with or without a stop codon.

Forward amplification primer (AflII):
5′-[6-8bp GC-rich sequence]CTTAAG[18-25bp gene-specific sequence]-3′

Reverse amplification primer (AgeI), stop codon underlined:
5′-[6-8bp GC-rich sequence]ACCGGTCTA[18-25bp gene-specific reverse strand sequence]-3′

Reverse amplification primer (AgeI), without stop codon:
5′-[6-8bp GC-rich sequence]ACCGGT[18-25bp gene-specific reverse strand sequence]-3′

6.8 E. coli Transformation of the "Bait" and "Prey" Vectors

See Section 6.2.2 for transformation and isoloation of the pLenti-Zeo/UAS-bla Vector
Use TOP10 or DH5α competent E. coli from Invitrogen for transforming your ligation mix. Select transformants on LB agar with ampicillin (100ug/mL).

You may use any method of your choice for transformation. Chemical transformation is the most convenient for most researchers. Electroporation is the most efficient and the method of choice for large plasmids.

Select 10-20 clones and analyze for the presence and orientation of your insert. We recommend that you sequence your construct to confirm that your gene is in the correct orientation for expression, contains an ATG or Kozak sequence as needed, and is in frame with the appropriate fusion partner. Please refer to the cloning site and plasmid diagrams for the sequences and location of the priming sites.

Preparing a Glycerol Stock

Once you have identified the correct clone, purify the colony and make a glycerol stock for long-term storage. You should keep a DNA stock of your plasmid at -20°C.

1. Streak the original colony out on an LB plate containing 100 ug/ml ampicillin. Incubate the plate at 37°C overnight.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 100 µg/ml ampicillin.
3. Grow the culture to mid-log phase (OD600 = 0.5-0.7).
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C.

Plasmid Preparation

Once you have generated your expression clone, you must isolate plasmid DNA for transfection. Plasmid DNA for transfection into eukaryotic cells must be very clean and free from phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using the S.N.A.P.. MiniPrep Kit (10-15 µg DNA, Catalog no. K1900-01), the S.N.A.P.. MidiPrep Kit (10-200 µg DNA, Catalog no. K1910-01), or CsCl gradient centrifugation.

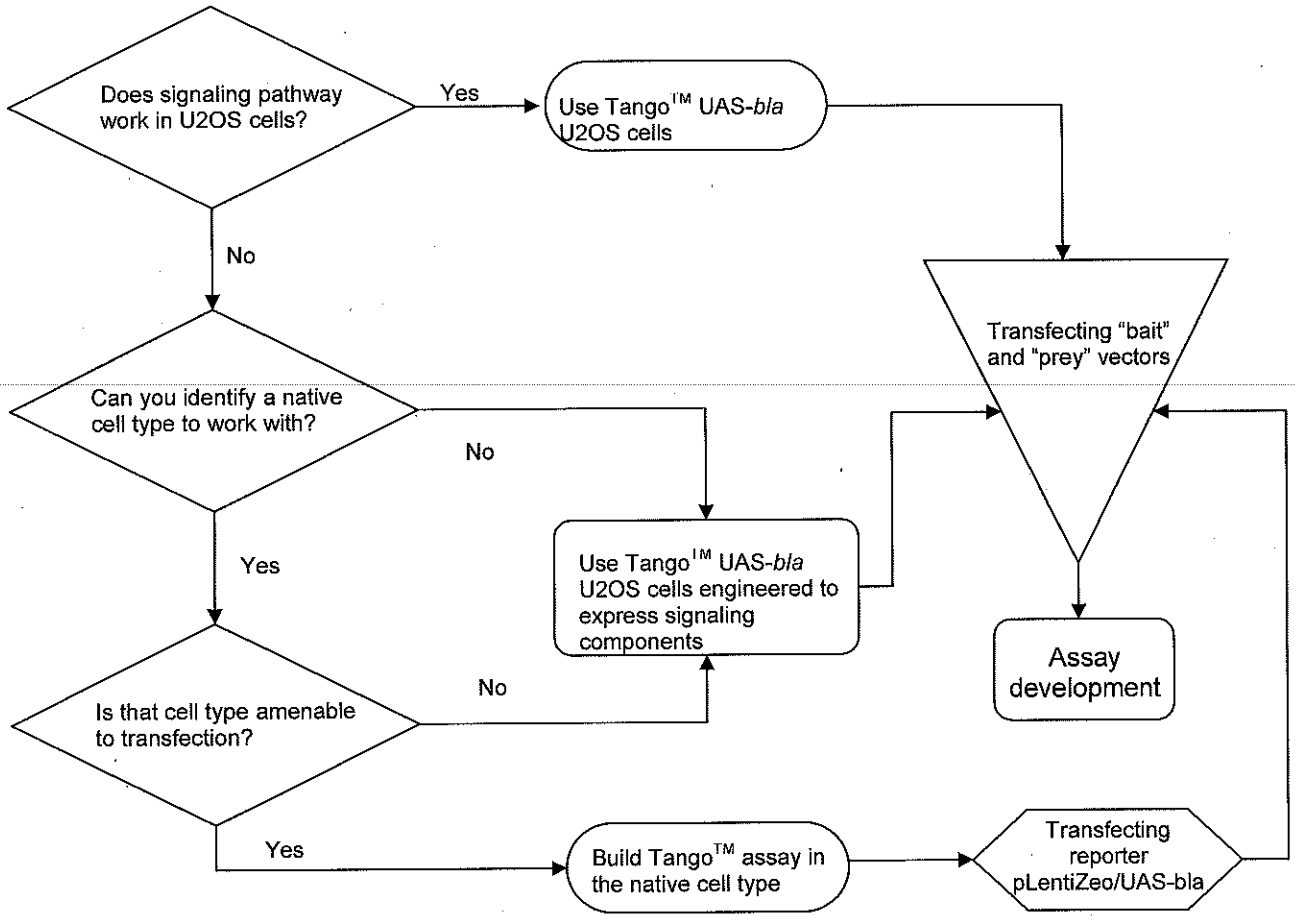
Transfection

Once you have verified that your gene is cloned properly (in the correct orientation and contains an initiation ATG codon), you are ready to transfect your cell line of choice. We recommend that you include the positive control vector and a mock transfection (negative control) to evaluate your results.

7.0 SETTING UP TANGO™ ASSAYS IN MAMMALIAN CELLS

7.1 General considerations in Tango™ assay development:

There are three key components in a Tango™ assay: the reporter, the interaction porters and the signaling components that modulate the interaction. The choice of cell line is largely dependent on the presence of signaling components that modulate the interaction. It is desirable to measure that signaling event in the native cell background that the signaling event happens and leads to the changes of the protein-protein interaction. Attached is a decision tree that captures the guidelines of Tango™ assay development.



Note: If the pathway of interest is beta-arrestin recruitment to a GPCR and U2OS is a suitable cell background, the Tango™ Beta-arrestin2-UAS-*bla* U2OS cells should be utilized.

7.2 Utilizing the Tango™ UAS-*bla* U2OS or the Tango™ Beta-arrestin2-UAS-*bla* U2OS Cell Lines

7.2.1 Media Required for Tango™ UAS-*bla* U2OS

Component	Growth Medium (-)	Growth Medium (+)	Assay Medium	Freeze Medium
McCoy's 5a Media	90%	90%	—	—
Dialyzed FBS	10%	10%	—	—
Freestyle Expression Medium	—	—	100%	—
NEAA	0.1 mM	0.1 mM	—	—
HEPES (pH 7.3)	25 mM	25 mM	—	—
Penicillin	100 U/ml	100 U/ml	—	—
Streptomycin	100 µg/ml	100 µg/ml	—	—
Zeocin antibiotic	—	200 µg/ml	—	—
Recovery™ Cell Culture	—	—	—	100%

Note: Unless otherwise stated, all media and solutions should be at least room temperature (37°C is best) before adding them to the cells.

7.2.2 Media Required for the Tango™ Beta-arretin2-UAS-*bla* U2OS Cell Line

Component	Growth Medium (-)	Growth Medium (+)	Assay Medium	Freeze Medium
McCoy's 5a Media	90%	90%	—	—
Dialyzed FBS	10%	10%	—	—
Freestyle Expression Medium	—	—	100%	—
NEAA	0.1 mM	0.1 mM	—	—
HEPES (pH 7.3)	25 mM	25 mM	—	—
Penicillin	100 U/ml	100 U/ml	—	—
Streptomycin	100 µg/ml	100 µg/ml	—	—
Zeocin antibiotic	—	200 µg/ml	—	—
Hygromycin antibiotic	—	50 µg/ml	—	—
Recovery™ Cell Culture Freezing Media	—	—	—	100%

Note: Unless otherwise stated, all media and solutions should be at least room temperature (37°C is best) before adding them to the cells.

7.3 Detailed Cell Handling Procedures

7.3.1 Thawing Method

1. Place 44 ml of Growth Medium (-) into a T225 flask.
2. Place the flask in a 37°C/5% CO2 incubator for 15 minutes to allow medium to equilibrate to the proper pH and temperature.
3. Remove vial of cells to be thawed from liquid nitrogen and rapidly thaw by placing at 37°C in a water bath with gentle agitation for 1-2 minutes. Do not submerge vial in water.
4. Decontaminate the vial by wiping with 70% ethanol before opening in a Class II biological safety cabinet.
5. Transfer the vial contents dropwise into 10 ml of Growth Medium (-) in a sterile 15 ml conical tube.
6. Centrifuge cells at 200 x *g* for 5 minutes.
7. Aspirate supernatant and resuspend the cell pellet in 1 ml of fresh Growth Medium (-).
8. Transfer contents to the T225 tissue culture flask containing pre-equilibrated Growth Medium (-) and place flask in the 37°C/5% CO_2 incubator.
9. At first passage switch to Growth Medium (+).

7.3.2 Propagation Method & Growth conditions

1. Cells should be thawed in Growth Medium (-) and grown in Growth Medium (+). Cells should be passaged or fed at least twice a week and maintained in a 37°C/5% CO_2 incubator. Cells should be maintained between 10% and 90% confluence. Do not allow cells to reach confluence.
2. Cells should be frozen at $8x10^6$ cells/ml in Freeze Medium..
3. To passage cells, aspirate medium, rinse once in PBS, add Trypsin/EDTA (3 ml for a T75 flask and 5 ml for a T175 flask, and 7 ml for T225 flask) and swirl to coat the cells evenly. Cells usually detach after ~2–5 minutes exposure to Trypsin/EDTA. Add an equal volume of growth medium to inactivate Trypsin.
4. Verify under a microscope that cells have detached and clumps have completely dispersed.
5. Centrifuge cells at 200 x g for 5 minutes and resuspend in Growth Medium (+).

7.3.3 Freezing Method

1. Harvest the cells as described above in the Propagation Methods step 4. After detachment, count the cells, then spin cells down and resuspend in 4°C Freeze Medium at 8×10^6 cells/ml.
2. Dispense 1.0 ml aliquots into cryogenic vials.
3. Place in an insulated container for slow cooling and store overnight at -80°C.
4. Transfer to liquid nitrogen the next day for storage.

7.4 Transient Transfection and Analysis of Mammalian Cells

If you start with the Tango™ UAS-*bla* U2OS cells or have engineered the UAS-*bla* reporter in the cell line of your choice, it is often desirable to transiently transfect the "bait" and "prey" vectors and test if the Tango™ assay works before committing the considerable amount of time required for stable cell line generation.

This protocol uses Lipofectamine™ LTX Plus, and uses a 96-well, black-walled, clear bottom assay plate. The transfection process is similar to that recommended in the Lipofectamine™ LTX Plus protocol, so we also recommend using that as a guide. For alternate cell types, Lipofectamine™ 2000 may give better results.

Note: One column of the 96-well plate should be media only (no cell control wells) for background subtraction.

1. On the day before transfection, seed cells in Growth medium without antibiotics so that cells will be ~90% confluent at the time of transfection (seed ~40K cells/well). For a 96-well assay plate, use 100 µl of growth media per well.

2. Prepare transfection complexes (volumes and concentrations are given for a full 96-well plate):
   - Dilute 7 µg of DNA in 1.4 mL Opti-Mem® I, mix gently.
   - Add 7 µL of Plus™ Reagent and incubate for 5 minutes at room temperature.
   - Add 17.5 µL of Lipofectamine™ LTX Reagent and incubate at RT for 30 minutes.
   - Add 5.6 mL of Growth Medium without antibiotics, mix gently.

3. Aspirate the Growth Media out of the 96-well plate.

4. Add 70 µL of the transfection mixture (Lipofectamine™ LTX Plus /DNA/Media) prepared in step 2 to each well in the 96-well plate.

5. The following day, exchange the media on the cells with 90 µL of fresh assay media. Add 10 µL of a 10X stock of your test/reference compound (e.g. agonist) (generally do not exceed a DMSO concentration of 1%), incubate for 16 hours* at 37°C/ 5% CO_2, and assay the cells for beta-lactamase activity by adding 20 µL of 6X LiveBLAzer™-FRET B/G Substrate Mixture and incubating cells for 2 hours at room temperature (for reagent preparation and beta-lactamase detection guidelines, please refer to section 7.6.

Note: This protocol is for a 24 hour transfection. Greater sensitivity may also be obtained with a 48 hour transfection or alternative assay media formulations. For a 48 hour transfection, exchange the media 24 hours after transfection with growth media without antibiotics, and then wait an additional day prior to performing the assay. Our first recommendations for alternative assay medias include Growth Media or Growth Media altered to include 1% dFBS instead of 10% dFBS. This protocol also outlines the procedures for using a 96 well plate. The same assay can also be performed in a 384 well plate. To perform the assay in a 384 well plate we recommend plating cells at ~14K cells/well, using 30 µl of transfection mixture/well, and loading the cells with 6 µL of of 6X LiveBLAzer™-FRET B/G Substrate Mixture.

* We recommend beginning with a stimulation time of 16 hours at 37°C/ 5% CO_2 prior to addition of LiveBLAzer™-FRET B/G Substrate. This incubation time in the final assay format should be able to be reduced to 5 hours.

7.5 Stable Cell Line Engineering in the Tango™ UAS-*bla* U2OS Cells

7.5.1 Getting Started

Day 1

1. Plate Tango™ UAS-*bla* U20S cells in a 6 well dish at 1 million cells/well the night before the assay in Growth Medium (-). Need one well for each receptor and an extra well for the negative control (no DNA).

Day 2

2. Transfect the cells using Lipofectamine™ LTX Plus.
   a. 500 µL Opti-MEM® + 2.5 µg of each of the bait and prey vector DNA (mix).
   Note: If using the Tango™ Beta-arretin2-UAS-*bla* U2OS cells, use 2.5 µg of the "prey" vector, and the "bait" vector is not used.
   b. Add 2.5 uL PLUS reagent to the diluted DNA (mix) and incubate 5 min. at RT.
   c. Add 6.25 uL Lipofectamine™ LTX to the diluted DNA (mix) and incubate at RT for 30 min.
   d. Add the Lipofectamine/DNA complexes to the well containing cells (the well should have 2 mL of media in it and gently rock the plate back and forth.
   e. Place the plate at 37°C 5% CO_2 overnight.

Day 3

3. Passage the cells. Each well of the 6 well plate goes into 1xT-75 flask.
4. Place the flasks at 37°C 5% CO_2 overnight.

Day 4

5. Add Geneticin® selection antibiotic (200µg/mL) and Hygromycin selection antibiotic (50 µg/mL).

Next 2-3 Weeks

6. Exchange media every 3-4 days. The non-stably transfected cells should die off in within 5 days. The stably transfected cells should grow colonies and expand. After the 1st passage of the transfected cell line you will likely see an additional die off of the cell culture. It is strongly suggested that you maintain the Geneticin® and Hygromycin selection for a full 2 weeks prior to assaying the cell line for function.

7.5.2 Testing the antibiotic selected pool for a response to the primary agonist

General Assay Protocol

1. Plate the cells at 10,000 – 15,000 cells/well in a black walled, clear bottom 384-well plate in Assay Media (Freestyle Expression Media) 16-24 hours in advance of the assay. We generally plate in 36 µL of Assay Media/Well.

2. On the day of the assay, stimulate the cells for 5-16 hours at 37^0C/5% CO_2 with 4 uL of the 5x concentration primary agonist (generally do not exceed a DMSO concentration of 1%).
3. Following the stimulation, load the cells with 8 uL of the 6X beta-lactamase substrate for 2 hours at room temperature. For instructions on preparing the loading solution see section 7.6.1.
4. Troubleshooting/Assay Considerations:
   a. If no/low signals or a shift in the expected potency of the primary agonist are observed, several alterations in experimental protocol should be tested. This includes testing a stimulation time of 16 hours instead of 5 hours and/or testing alternative assay medias. Our first recommendations for alternative assay medias include Growth Media (+) or Growth Media (+) altered to include 1% dFBS instead of 10% dFBS without Zeocin, Hygromycin, or Geneticin selection.
   b. If a low signal is observed, the performance of the assay can be improved through functional sorting by flow cytometry.

7.5.3 CD8 Enriched Pools of Cells

If the "Bait" vector was used to create a CD8 fusion protein, a pool of cells that is enriched for the CD8 fusion protein can be created prior to isolating stable cell clones by utilizing the following protocol. If a CD8 fusion protein was not utilized, skip to section 7.5.4.

1. Prepare Enrichment Buffer: D-PBS + 0.1% BSA + 2mM EDTA, pH 7.4.
2. Harvest and centrifuge at 100xG for 5 min.
3. Resuspend to $5x10^6$ cells/mL in Enrichment buffer.
4. Add Dynabeads® CD8 to each tube in a 4:1 (beads:cells) ratio.

Note: Dynabeads® CD8 are prepackaged at $4x10^8$ beads/mL in PBS + 0.02% sodium azide (NaN3). Example: If a total of $1x10^7$ cells will be enriched, $4x10^7$ beads or 100 µL of beads will be added to the cells.

5. Incubate on nutator at 4^0C for 20 min.
6. Enrich cells:
   a. Place the tube in a Dynal MPC® or DynaMag™-50 magnet for 1 min.
   b. Remove supernatant (may save for analysis)
   c. Add equal volume of Enrichment Buffer (same volume as is step #3).
   d. Wash the beads, carefully re-suspending the cells by gently tapping and loosening up the bead clumps.
   e. Repeat wash.
   f. Remove the supernatant.
7. Re-suspend the bead bound cells to $2x10^7$ cells/mL in Enrichment buffer.
8. Add 10 µL DETACHaBEAD® CD4/CD8 per $1x10^7$ Dynabeads® CD8.

Note: Do not use less than 10 µL DETACHaBEAD® CD4/CD8.

9. Incubate 40-45 min. on the nutator at room temperature.
10. Place the tube in a Dynal MPC® or DynaMag™-50 magnet for 1 min.
11. Transfer the supernatant (detached cells) to a fresh tube.
12. Obtaine residual cells by washing 2-3 times with Enrichment Buffer (same volume as in step #3 and Step #6).
13. Combine detached cells together and centrifuge at 100xG for 5 min.
14. Resuspend cells in growth media.

7.5.4 Isolation of Stable Cell Clones

Stable cell clones can be isolated via a variety of methods. If a flow cytomer with sorting capabilities is available, we recommend following the method outlined in sections 7.5.5 and 7.5.6 for isolating stable clones. If a flow cytometer with sorting capabilities is not available, standard alternative methods for clonal isolation such as limiting dilutions or cloning rings may be utilized and the resultant clones tested for a response to the primary agonist.

7.5.5 Preparing Cells for Sorting by Flow Cytometry

1. At least one day before the sort, split cells into 1 T75 (which should be ~80% confluent at the time stimulation for the "stimulated" cells), and 1 T25 (for the un-stimulated cells, can use a T75 if you want), and 1 T75 to keep passing (just need enough cells to keep the cell line growing).

   For a 5 hour stimulation:

   a. On the morning of the sort, stimulate the cells in the T75 flask with the primary agonist. (If you have run a dose response for this agonist, use the lowest concentration of agonist that gives you a full response.) If there has been an apparent shift in potency, we recommend sorting with an EC_{50} concentration of the agonist.

   b. Stimulate the cells for 5 hrs at 37°C 5% CO_2 then move on to step 2.

   For a 16-20 hour stimulation:

   c. On the night prior to the sort, stimulate the cells in the T75 flask with the primary agonist. (If you have run a dose response for this agonist, use the lowest concentration of agonist that gives you a full response.) If there has been an apparent shift in potency, we recommend sorting with an EC50 concentration of the agonist.

   d. Stimulate the cells for ~16 to 20 hrs at 37°C 5% CO_2 then move on to step 2.

2. After stimulation, trypsinize the stimulated T75 flask and the un-stimulated (T25 or T75) flask. Spin cells down and re-suspend in FACS Loading solution.

   - 6 µL CCF4-AM
   - 60 µL Solution B
   - Bring up to 6 mL with FACS Sort Buffer
     Note: Scale recipe as needed. Cells should load at 1-2 million cells/ml.

   FACS Sort Buffer: PBS without Calcium and Magnesium (Gibco 14190-144)+ 1.0% glucose(Sigma G5767) + 1mM EDTA(Sigma E6758) + 25mM HEPES(Gibco 15630-080)@ pH 7.4

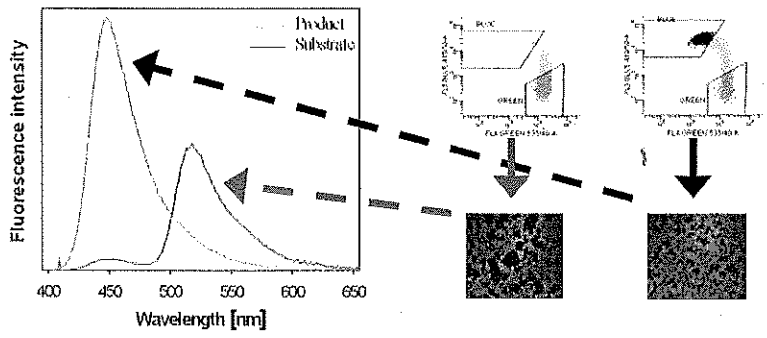
7.5.4 Clone Selection by FACS Sorting

Instrument Set Up

Requirements: Flow cytometer with sorting capabilities. (VantageSE, Aria, MoFlow, Epics), equipped with a violet laser (407nm, 409nm, 413nm or multiline 407-415nm).

Note: It is possible to use UV excitation (337.5-356.4nm). However, resolution between the green and blue emission signal will be closer together and harder to resolve on cell lines with low response.

We recommend the following detection filters: HQ460/50m (blue) and HQ535/40m (green) bandpass filters separated by a 490nm dichroic mirror. Filter sets are available from Chroma Technologies (1-800-824-7662, www.chroma.com). Chroma Set # 41031



Suggested nozzle tip sizes: Jurkat or lymphocyte like cell types - 70µm, HEK, Hela or CHO - 90 or 100µm.

Alignment

Follow instrument manufacturer's alignment procedures. Purchase of a 2µm yellow/green bead (Fluoresbrite® YG Microspheres 2.00µm Cat. 18338 from Polysciences) can be used as an alignment bead. These beads are excited by the violet laser and bead emission is detectable in both the green and blue emission filters/detectors.

Control cells are available from Invitrogen. GeneBLAzer® Jurkat Control Kit catalog no.K1206 and GeneBLAzer® CHO-K1 Control Kit catalog no. K1207. These cells should only be used as confirmation that the flow cytometer or microscope is set up properly for GeneBLAzer® detection. They should not be used for detector setting prior to running the cell line to be sorted. They should not be used as a comparison of fluorescence intensities of developing cell lines.

Substrate Loading

- Substrate Loading for FACS sorting consists of 1mM stock solution A (CCF4) and solution B supplied in the LiveBLAzer™-FRET B/G loading kits.
- Sort buffer consists of PBS without Calcium and Magnesium (Gibco 14190-144) + 1.0% glucose (Sigma G5767) + 1mM EDTA (Sigma E6758) + 25mM HEPES (Gibco 15630-080) @ pH 7.4
- For every $1.0x10^6$ cells, use the chart on the next page. The chart is a general guideline for the most common cell types.

Cells	Soln A (1mM Stock)	Soln B	Sort Buffer	Load Time
Jurkat	2µl	10µl	1ml	60 min
HEK/Hela	2µl	16µl	1ml	90 min
CHO* and Others	2µl	10µl	1ml	90-120 min

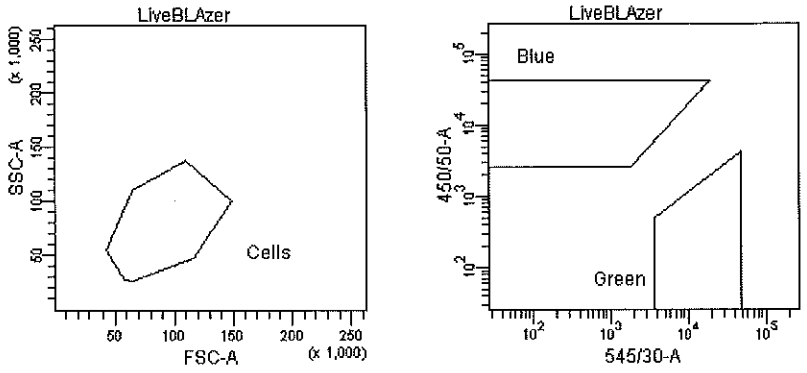
*CHO cells can actively pump out the substrate and load poorly. Adding solution D (1mM Probenecid), catalog no. K1156 can correct this. However, adding Probenecid may affect sort survivability. The general practice to counter this effect is to sort more plates and increase the amount of cells collected in a pooled sort.

Harvest and count cells. Using a 15ml or 50ml conical tube, add the appropriate amounts of substrate and sort buffer to the cells. Incubate at room temperature, gently shaking, covered on a platform shaker.

After completion of substrate loading, spin cells down cells and resuspend cells in sort buffer. Pipette cells through a 40µm mesh cell strainer and into 12x75 FACS tubes. Samples are now ready for sorting.

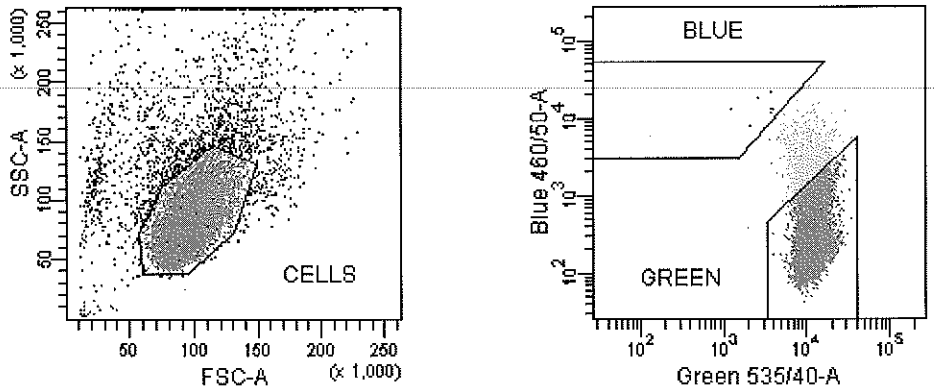
Experiment Set Up

1. Create a FSC vs. SSC dot plot. Draw a gate for the cell population.
2. Create a Green vs. Blue dot plot. Format this dot plot to look at the cell population. Draw gate for the green and blue populations.

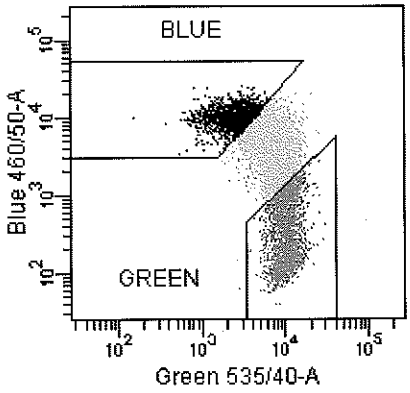


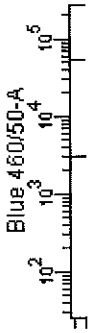
Running Cell Samples

1. First run the loaded unstimulated cells on the instrument. Increase the voltage to the FSC/SSC detectors so that the cell population is separated from debris. Draw a tight gate around the cell population.
2. Increase/decrease the voltage to the green and blue detectors so the fluorescent cell population falls into the green gated region. Make certain that events are not lost on the Green (x) axis.



3. Increase compensation values to lower the green population from any blue or turquoise background. Record data, stopping the file on 10,000 events in the cell gate.
4. Run a tube of sterile PBS to rinse the sample injection port (SIP) before running stimulated sample for sorting.
5. Run stimulated sample on instrument. Detector values should not change. As a general rule, the values should not be changed unless stimulated sample cells fall outside the cell gate. If cell loading is poor, move the gate to adjust for signal.





Tube: UNSTIM

Population	#Events	%Parent	%Total
All Events	12,393		100.0
CELLS	10,489	84.6	84.6
GREEN	9,141	87.1	73.8
BLUE	10	0.1	0.1

Tube: STIM

Population	#Events	%Parent	%Total
All Events	15,951		100.0
CELLS	10,771	67.5	67.5
GREEN	2,202	20.4	13.8
BLUE	1,874	17.4	11.7

% Blue response =17.4%

6. At this time increase compensation values to separate the blue population from the green signal. Make certain that events are not lost on the Blue (y) axis. Record data, stopping the file on 10,000 events in the cell gate.
7. Calculate % blue response by subtracting the unstimulated % blue response from the stimulated % blue response.

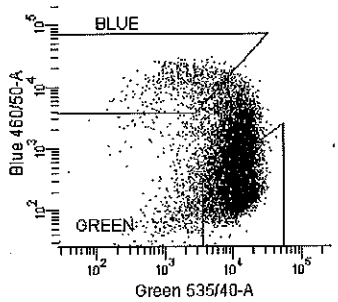
Sorting Strategies

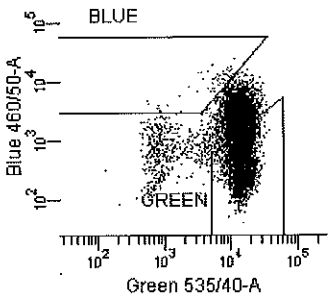
If the stimulated sample has a blue response, sort at least three 96-well plates of clones at one cell per well. Sort the remainder of the blue response sample in a large pooled sort.

Test the clones for response after sorting and re-sort if needed to enrich for blue response. Or if the blue background is high re-sort unstimulated green responsive cells in pooled sort.

Identifying Poor loading Conditions

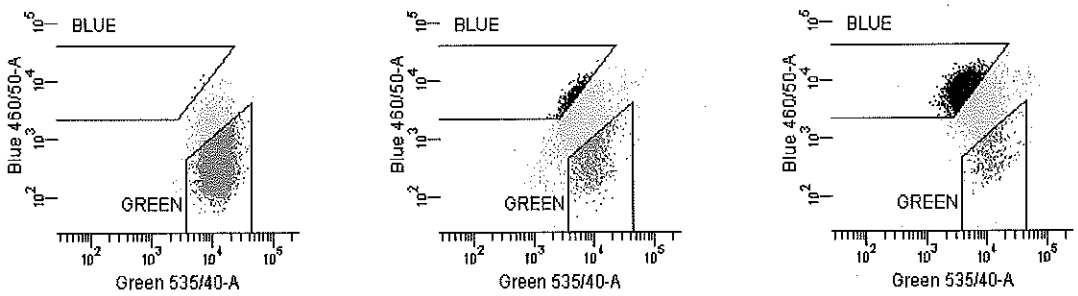
Examples of poor loading caused by shortened loading time or insufficient amount of substrate for cell concentration:



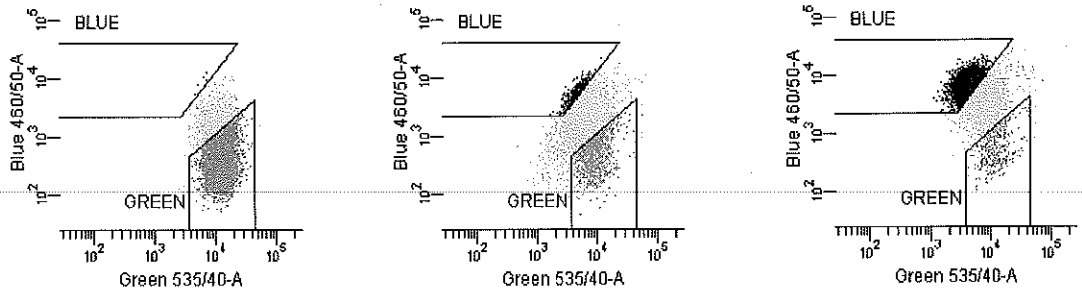


Examples of poor loading with CHO cells and correction with adding solution D (1mM Probenecid):

CHO Unstim | CHO Stim - probenecid | CHO Stim + probenecid



CHO Unstim | CHO Stim - probenecid | CHO Stim + probenecid



Improving Survivability

Techniques to improve cell survivability during single cell cloning include:

- Use a lower sheath pressure 10-12 psi.
- Keep sample cold (4°C) during collection.
- Collection plates (96-well) with growth media supplemented with 25mM HEPES acclimated in 37° incubator.
- For long pooled sorts, collect into straight serum (Gibco 26400-044). It is not recommended to collect a large pooled sort into growth media; eventually salts will form between the carbonate in the media and the phosphate in the buffer. This will impact cell survivability.

7.6 Tango™ Assay Substrate Loading and Detection

7.6.1 Substrate Loading and Incubation

This protocol is designed for loading cells with LiveBLAzer™ FRET B/G Substrate (CCF4-AM). If alternative substrates are used please follow the loading protocol provided with the substrate.

1. Prepare Solution A: 1 mM LiveBLAzer™ FRET B/G Substrate stock solution in dry DMSO. Store the aliquots of the stock solution at -20°C until use.
2. Prepare 6X LiveBLAzer™ FRET B/G Substrate (CCF4-AM)
   - Add 6 µl of Solution A to 60 µl of Solution B and vortex.
   - Add 934 µl Solution C to the above solution and vortex.
3. Remove assay plate from the incubator.

4. Add the 6X Loading Solution to each well (i.e. 8 uL of loading solution added to a 40 µL sample volume).
5. Cover the plate to protect it from light and evaporation.
6. Incubate at room temperature for 120 minutes.

   Note: Handle the plate gently and do not touch the bottom.

7.6.2 Detection

All measurements are made at room temperature from the bottom of the wells, preferably in 384-well black-wall, clear-bottom assay plates with low fluorescence background. Before reading the plate, remove dust from the bottom with compressed air.

Instrumentation, Filters, and Plates

1. Fluorescence plate reader with bottom reading capabilities.
2. Recommended filters for a filter based fluorescence plate reader:

   Excitation filter: 409/20 nm
   Emission filter: 460/40 nm
   Emission filter: 530/30 nm

3. Recommended settings for a monochrometer based instrument:

   Excitation: 409 nM
   Emission: 460 nM
   Emission 530 nM

IMPORTANT:

Particular settings for your instrument of choice may need to be optimized for that instrument. These setting may include things such as gain settings, sensitivity settings, band-widths, and others. When first setting up beta-lactamase assays for your system, we also recommend visualizing the assay on a fluorescent microscope to determine if the cells have responded appropriately.

Reading an Assay Plate

1. Set the fluorescence plate reader to bottom-read mode.
2. Allow the lamp in the fluorescence plate reader to warm up for at least 10 min. before making measurements.
3. Use the following filter selections:

	Scan 1	Scan 2
Purpose:	Measure fluorescence in the blue channel	Measure FRET signal in the green channel
Excitation filter:	409/20 nm	409/20 nm
Emission filter:	460/40 nm (using the gain determined during calibration)	530/30 nm (using the gain determined during calibration)

Visual Observation of Intracellular LiveBLAzer™ FRET B/G Substrate (CCF4-AM)

Note: Microscopic visualization of cells will cause photobleaching. Always read the assay plate in the fluorescence plate reader before performing microscopic visualization.

An inverted microscope equipped for epifluorescence and either a xenon or mercury excitation lamp may be used to view the LiveBLAzer™ FRET B/G Substrate (CCF4-AM) signal in cells. Recommended filter sets for observing beta-lactamase activity are described below and are available from Chroma Technologies (800-824-7662).

Chroma Set # 41031
Excitation filter: HQ405/20x (405 ±10)
Dichroic mirror: 425 DCXR
Emission filter: HQ435LP (435 long-pass)

FILTER SIZES VARY FOR SPECIFIC MICROSCOPES AND NEED TO BE SPECIFIED WHEN THE FILTERS ARE ORDERED.

7.6.3 Data Analysis

Background Subtraction and Ratio Calculation

Background subtraction for both emission channels (460 nm and 530 nm) is recommended.

1. Use the assay plate layout to identify the location of the Cell-free Control wells. These control wells are used for background subtraction.
2. Determine the average emission from the Cell-free Control wells at both 460 nm (Average Blue Background) and 530 nm (Average Green Background).
3. Subtract the Average Blue background from all of the Blue emission data.
4. Subtract the Average Green background from all of the Green emission data.
5. Calculate the Blue/Green Emission Ratio for each well, by dividing the background subtracted blue emission values by the background subtracted green emission values.

7.7 Going Beyond the Tango™ U2OS cell line

7.7.1 Considerations:

Please consult the decision tree diagram at the beginning of Section 7.0 for the decision of using a host cell line other than the TangoTM U2OS cells included in this kit. The cell host to be used must be amenable to cell transfection and sorting. It may be necessary to test if probenecid is required for optimal beta-lactamase substrate loading. In addition, it may also be necessary to optimize the antibiotic selection by testing the "kill curve" by the selection agent.

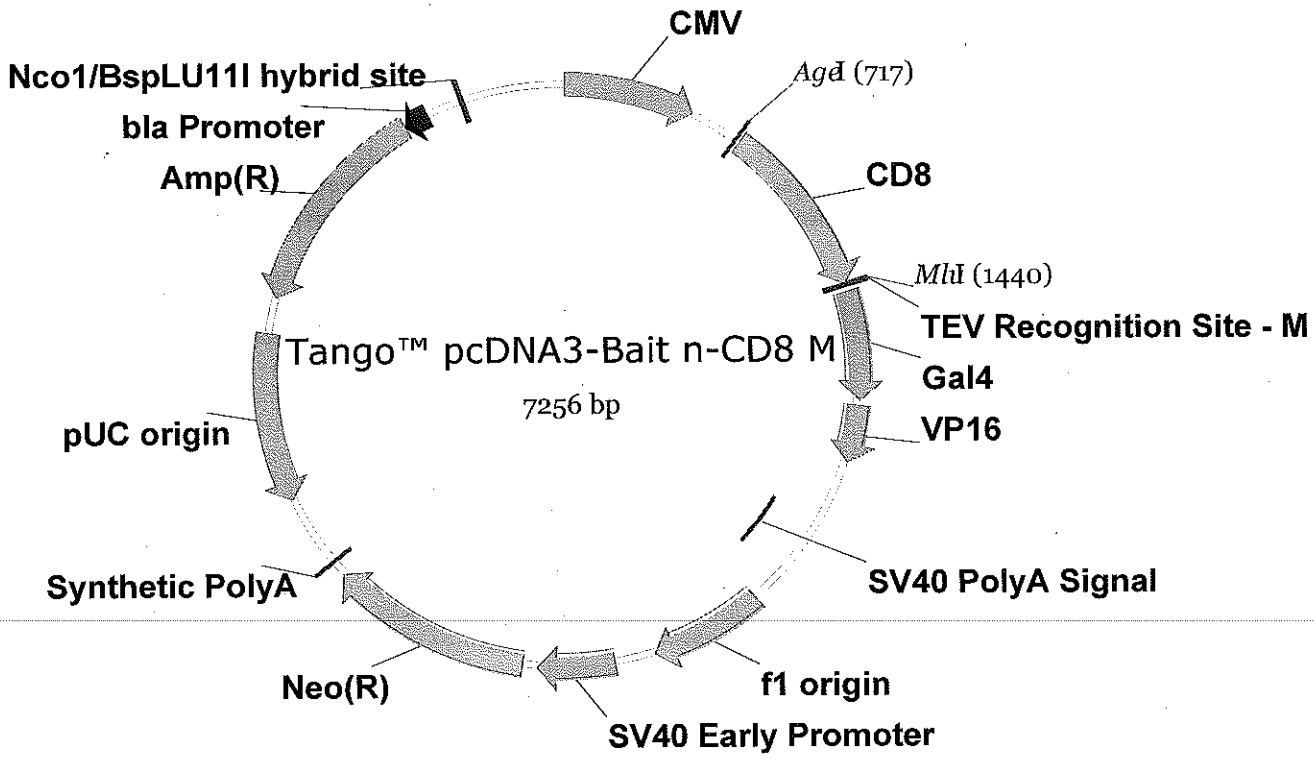
7.7.2 Engineering the UAS-bla reporter in the cell host

Note: The vector maps and sequences for the pLenti-zeo/UAS-bla vectors are located in the **Appendix** .

1. Perform a zeocin kill curve on your cell line of interest prior to beginning the experiment.
2. Transfect the pLenti-zeo/UAS-bla into the cell line of interest utilizing a lipid based transfection method (such as Lipofectamine LTX Plus) following the manufacturer's instructions, electroportation, Lentiviral transduction or your gene delivery method of choice.
3. Carry out antibiotic selection on the cells using the pre-determined level of zeocin as determined by the kill curves. We recommend including a mock transfected or untransfected control to ensure that the antibiotics are killing the untransfected cells as expected.
4. Selection is generally carried out for at least 2 weeks prior to assaying the cells.
5. The function of the pLenti-zeo/UAS-bla plasmid should be determined via transient tranfection using a control plasmid such as a CMV-Gal4/VP16 construct.
6. Individual clones may be sorted out using methods such as flow cytometry or limiting dilution to obtain a clone exhibiting large assay windows.
7. In general, to obtain the best performing beta-lactamase clonal cell lines, a cell line that exhibits low constitutive activity (low/no blue signal) should be selected.

8.0 APPENDIX (VECTOR MAPS & SEQUENCES)

Tango™ pcDNA3-Bait n-CD8 M



General Description

DNA Plasmid Tango™ pcDNA3-Bait n-CD8 M
Entire molecule length: 7256 bp

Feature Map

CD8	Start: 734 End: 1438
Neo(R)	Start: 3793 End: 4587
TEV Recognition Site - M	Start: 1445 End: 1465
Gal4	Start: 1469 End: 1906
VP16	Start: 1928 End: 2158
pUC origin	Start: 4941 End: 5614 (Complementary)
Amp(R)	Start: 5759 End: 6619 (Complementary)
SV40 PolyA Signal	Start: 2419 End: 2685
Synthetic PolyA	Start: 4651 End: 4699
CMV Promoter	Start: 1 End: 517
SV40 Early Promoter	Start: 3426 End: 3734
bla Promoter	Start: 6620 End: 6718 (Complementary)
f1 origin	Start: 2811 End: 3266

Restriction/Methylation Map

Enzyme	# of cuts	Positions
AgeI	1	717
MluI	1	1440

Sequence

```
1     atgcatgtcg ttacataact tacggtaaat ggcccgcctg gctgaccgcc caacgacccc cgcccattga
71    cgtcaataat gacgtatgtt cccatagtaa cgccaatagg gactttccat tgacgtcaat gggtggagta
141   tttacggtaa actgcccact tggcagtaca tcaagtgtat catatgccaa gtacgccccc tattgacgtc
211   aatgacggta aatggcccgc ctggcattat gcccagtaca tgaccttatg ggactttcct acttggcagt
281   acatctacgt attagtcatc gctattacca tggtgatgcg gttttggcag tacatcaatg ggcgtggata
351   gcggtttgac tcacggggat ttccaagtct ccaccccatt gacgtcaatg ggagtttgtt ttggcaccaa
421   aatcaacggg actttccaaa atgtcgtaac aactccgccc cattgacgca aatgggcggt aggcgtgtac
491   ggtgggaggt ctatataagc agagctcgtt tagtgaaccg tcagatcgcc tggagacgcc atccacgctg
561   ttttgacctc catagaagac accgggaccg atccagcctc cggactctag cctaggccgc gggacggata
631   acaatttcac acaggaaaca gctatgacca ttaggcctat ttaggtgaca ctatagaaca agtttgtaca
701   aaaaagcagg ctggtaccgg tccggaattc accatggcct taccagtgac cgccttgctc ctgccgctgg
771   ccttgctgct ccacgccgcc aggccgagcc agttccgggt gtcgccgctg gatcggacct ggaacctggg
841   cgagacagtg gagctgaagt gccaggtgct gctgtccaac ccgacgtcgg gctgctcgtg gctcttccag
911   ccgcgcggcg ccgccgccag tcccaccttc ctcctatacc tctcccaaaa caagcccaag gcggccgagg
981   ggctggacac ccagcggttc tcgggcaaga ggttggggga caccttcgtc ctcaccctga gcgacttccg
1051  ccgagagaac gagggctact atttctgctc ggccctgagc aactccatca tgtacttcag ccacttcgtg
1121  ccggtcttcc tgccagcgaa gcccaccacg acgccagcgc cgcgaccacc aacaccggcg cccaccatcg
1191  cgtcgcagcc cctgtccctg cgcccagagg cgtgccggcc agcggcgggg ggcgcagtgc acacgagggg
1261  gctggacttc gcctgtgata tctacatctg ggcgcccctg gccgggactt gtggggtcct tctcctgtca
1331  ctggttatca ccctttactg caaccacagg aaccgaagac gtgtttgcaa atgtccccgg cctgtggtca
1401  aatcgggaga caagcccagc ctttcggcga gatacgtcac gcgtgagaac ctgtacttcc agatgagaaa
1471  gctactgtct tctatcgaac aagcatgcga tatttgccga cttaaaaagc tcaagtgctc caaagaaaaa
1541  ccgaagtgcg ccaagtgtct gaagaacaac tgggagtgtc gctactctcc caaaaccaaa aggtctccgc
1611  tgactagggc acatctgaca gaagtggaat caaggctaga aagactggaa cagctatttc tactgatttt
1681  tcctcgagaa gaccttgaca tgattttgaa aatggattct ttacaggata taaaagcatt gttaacagga
1751  ttatttgtac aagataatgt gaataaagat gccgtcacag atagattggc ttcagtggag actgatatgc
1821  ctctaacatt gagacagcat agaataagtg cgacatcatc atcggaagag agtagtaaca aaggtcaaag
1891  acagttgact gtatcgccgg aattcccggg gatctgggcc ccccgaccg atgtcagcct gggggacgag
1961  ctccacttag acggcgagga cgtggcgatg gcgcatgccg acgcgctaga cgatttcgat ctggacatgt
2031  tgggggacgg ggattccccg ggtccgggat ttacccccca cgactccgcc ccctacggcg ctctggatat
2101  ggccgacttc gagtttgagc agatgtttac cgatgccctt ggaattgacg agtacggtta gtaatgagct
2171  agcttgggat ctttgtgaag gaaccttact tctgtggtgt gacataattg gacaaactac ctacagagat
2241  ttaaagctct aaggtaaata taaaattttt aagtgtataa tgtgttaaac tagctgcata tgcttgctgc
2311  ttgagagttt tgcttactga gtatgattta tgaaaatatt atacacagga gctagtgatt ctaattgttt
2381  gtgtatttta gattcacagt cccaaggctc atttcaggcc cctcagtcct cacagtctgt tcatgatcat
2451  aatcagccat accacatttg tagaggtttt acttgcttta aaaaacctcc cacacctccc cctgaacctg
2521  aaacataaaa tgaatgcaat tgttgttgtt aacttgttta ttgcagctta taatggttac aaataaagca
2591  atagcatcac aaatttcaca aataaagcat ttttttcact gcattctagt tgtggtttgt ccaaactcat
2661  caatgtatct tatcatgtct ggatcgatcc tgcattaatg aatcggccaa cgcgcgggga gaggcggttt
2731  gcgtattggc tggcgtaata gcgaagaggc ccgcaccgat cgcccttccc aacagttgcg cagcctgaat
2801  ggcgaatggg acgcgccctg tagcggcgca ttaagcgcgg cgggtgtggt ggttacgcgc agcgtgaccg
2871  ctacacttgc cagcgcccta gcgcccgctc ctttcgcttt cttcccttcc tttctcgcca cgttcgccgg
2941  ctttccccgt caagctctaa atcgggggct ccctttaggg ttccgattta gtgctttacg gcacctcgac
3011  cccaaaaaac ttgattaggg tgatggttca cgtagtgggc catcgccctg atagacggtt tttcgccctt
3081  tgacgttgga gtccacgttc tttaatagtg gactcttgtt ccaaactgga acaacactca accctatctc
3151  ggtctattct tttgatttat aagggatttt gccgatttcg gcctattggt taaaaaatga gctgatttaa
3221  caaatattta acgcgaattt taacaaaata ttaacgttta caatttcgcc tgatggcggta ttttctcctt
3291  acgcatctgt gcggtatttc acaccgcata cgcggatctg cgcagcacca tggcctgaaa taacctctga
3361  aagaggaact tggttaggta ccttctgagg cggaaagaac cagctgtgga atgtgtgtca gttagggtgt
3431  ggaaagtccc caggctcccc agcaggcaga agtatgcaaa gcatgcatct caattagtca gcaaccaggt
3501  gtggaaagtc cccaggctcc ccagcaggca gaagtatgca aagcatgcat ctcaattagt cagcaaccat
3571  agtcccgccc ctaactccgc ccatcccgcc cctaactccg cccagttccg cccattctcc gccccatggc
3641  tgactaattt tttttattta tgcagaggcc gaggccgcct cggcctctga gctattccag aagtagtgag
3711  gaggcttttt tggaggccta ggcttttgca aaaagcttga ttcttctgac acaacagtct cgaacttaag
```

```
3781  gctagagcca ccatgattga acaagatgga ttgcacgcag gttctccggc cgcttgggtg gagaggctat
3851  tcggctatga ctgggcacaa cagacaatcg gctgctctga tgccgccgtg ttccggctgt cagcgcaggg
3921  gcgcccggtt ctttttgtca agaccgacct gtccggtgcc ctgaatgaac tgcaggacga ggcagcgcgg
3991  ctatcgtggc tggccacgac gggcgttcct tgcgcagctg tgctcgacgt tgtcactgaa gcgggaaggg
4061  actggctgct attgggcgaa gtgccggggc aggatctcct gtcatctcac cttgctcctg ccgagaaagt
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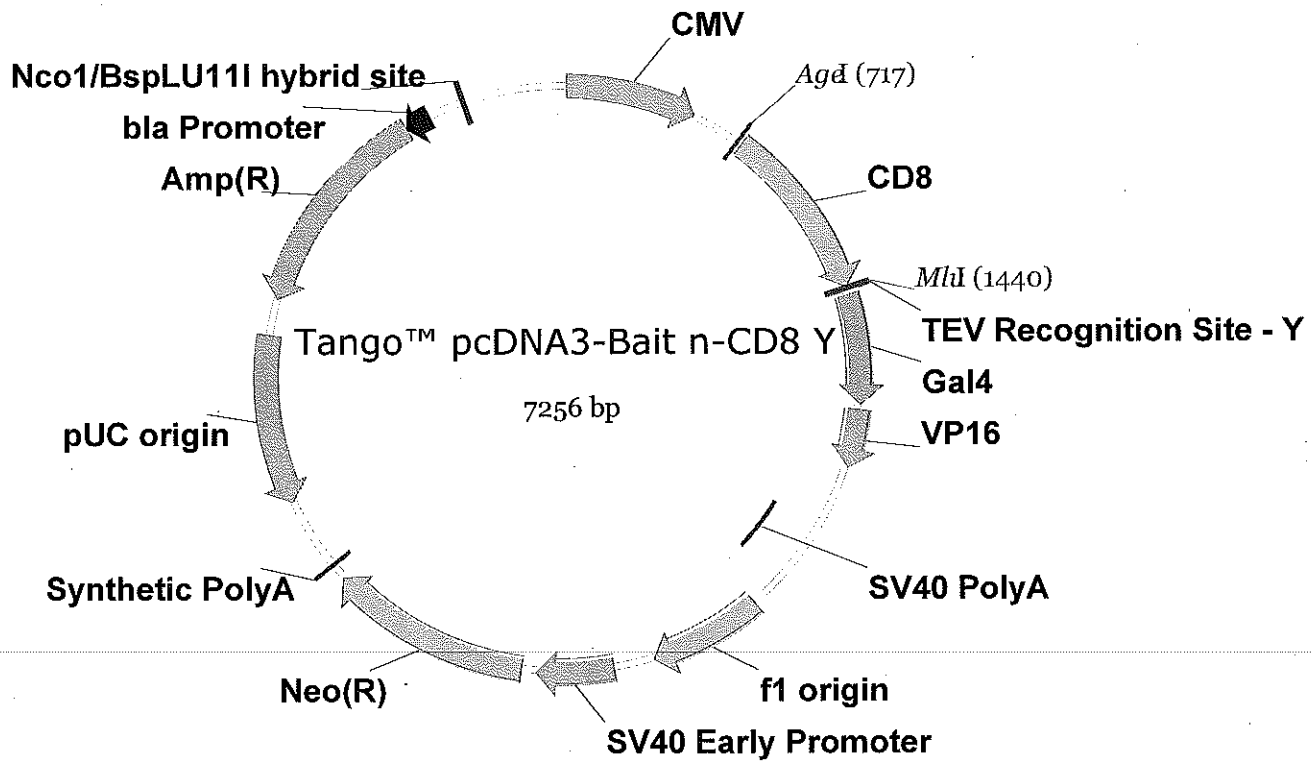
Tango™ pcDNA3-Bait n-CD8 Y



General Description

DNA Plasmid pcDNA3 Tango Bait n-CD8 Y
Entire molecule length: 7256 bp

Feature Map

CD8	Start: 734 End: 1438
Neo(R)	Start: 3793 End: 4587
TEV Recognition Site - M	Start: 1445 End: 1465
Gal4	Start: 1469 End: 1906
VP16	Start: 1928 End: 2158
pUC origin	Start: 4941 End: 5614 (Complementary)
Amp(R)	Start: 5759 End: 6619 (Complementary)
SV40 PolyA Signal	Start: 2419 End: 2685
Synthetic PolyA	Start: 4651 End: 4699
CMV Promoter	Start: 1 End: 517
SV40 Early Promoter	Start: 3426 End: 3734
bla Promoter	Start: 6620 End: 6718 (Complementary)
f1 origin	Start: 2811 End: 3266

Restriction/Methylation Map

Enzyme	# of cuts	Positions
AgeI	1	717
MluI	1	1440

Sequence

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141   tttacggtaa actgcccact tggcagtaca tcaagtgtat catatgccaa gtacgccccc tattgacgtc
211   aatgacggta aatggcccgc ctggcattat gcccagtaca tgaccttatg ggactttcct acttggcagt
281   acatctacgt attagtcatc gctattacca tggtgatgcg gttttggcag tacatcaatg ggcgtggata
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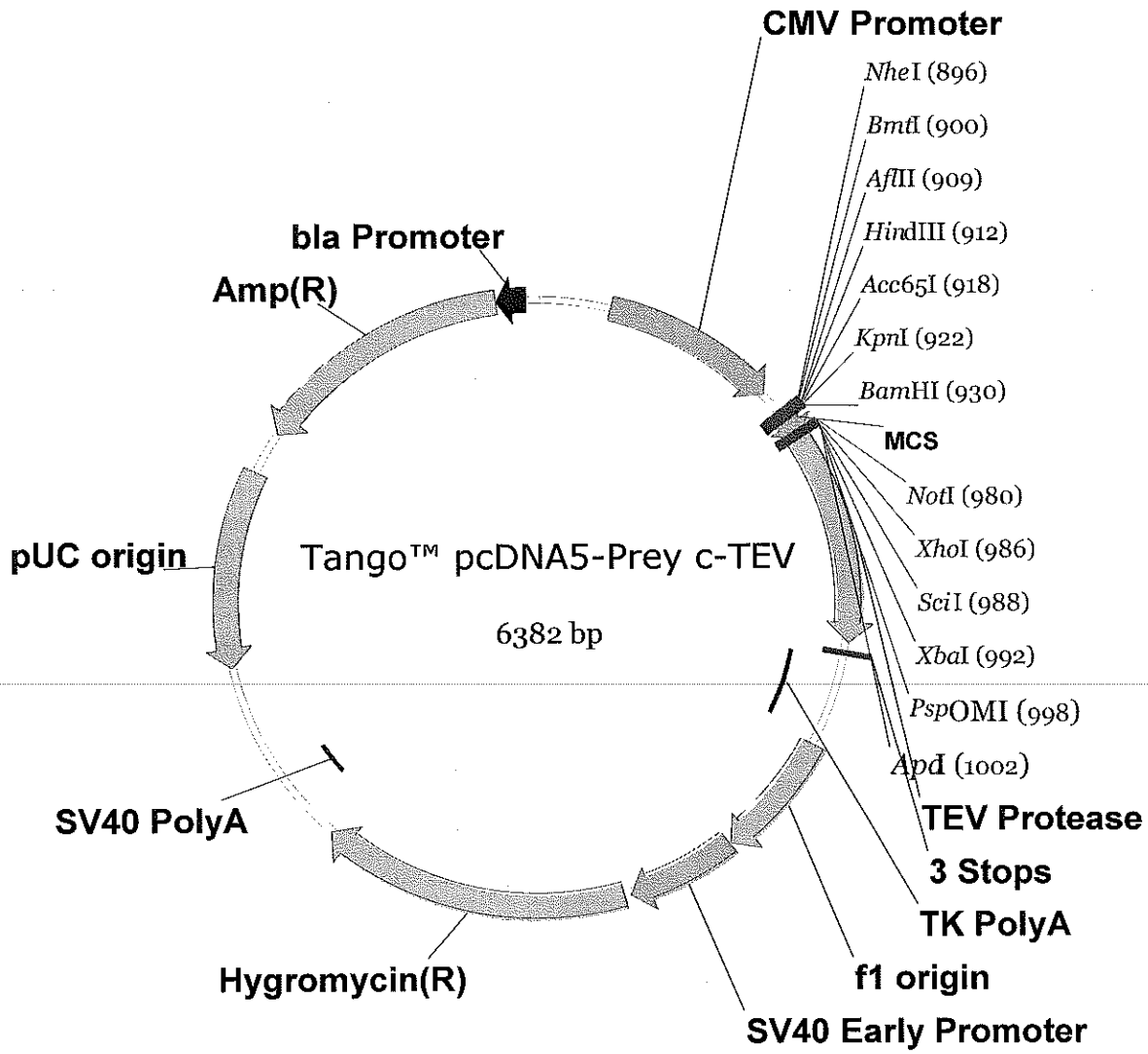
Tango™ pcDNA5-Prey c-TEV



General Description

DNA pcDNA5 Tango Prey c-TEV
Entire molecule length: 6382 bp

Feature Map

TEV Protease	Start: 1006 End: 1722
3 Stops	Start: 1759 End: 1767
Hygromycin(R)	Start: 2904 End: 3929
Amp(R)	Start: 5386 End: 6246 (Complementary)
MCS	Start: 895 End: 1002
TK PolyA	Start: 1776 End: 2047
SV40 PolyA	Start: 4058 End: 4188
CMV Promoter	Start: 232 End: 819
SV40 Early Promoter	Start: 2516 End: 2885
bla Promoter	Start: 6247 End: 6345 (Complementary)
f1 origin	Start: 2083 End: 2511
pUC origin	Start: 4571 End: 5241 (Complementary)

Sequence

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1821  cccgcgctat gacggcaata aaaagacaga ataaaacgca cgggtgttgg gtcgtttgtt cataaacgcg
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1961  ttcttccttt tccccacccc acccccaaag ttcgggtgaa ggcccagggc tcgcagccaa cgtcgggggcg
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2451  ttggggattt cggcctattg gttaaaaaat gagctgattt aacaaaaatt taacgcgaat taattctgtg
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4201  ctctagctag agcttggcgt aatcatggtc atagctgttt cctgtgtgaa attgttatcc gctcacaatt
4271  ccacacaaca tacgagccgg aagcataaag tgtaaagcct ggggtgccta atgagtgagc taactcacat
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4621  gctggcgttt ttccataggc tccgcccccc tgacgagcat cacaaaaatc gacgctcaag tcagaggtgg
4691  cgaaacccga caggactata aagataccag gcgtttcccc ctggaagctc cctcgtgcgc tctcctgttc
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5881  actgcataat tctcttactg tcatgccatc cgtaagatgc ttttctgtga ctggtgagta ctcaaccaag
5951  tcattctgag aatagtgtat gcggcgaccg agttgctctt gcccggcgtc aatacgggat aataccgcgc
6021  cacatagcag aactttaaaa gtgctcatca ttggaaaacg ttcttcgggg cgaaaactct caaggatctt
6091  accgctgttg agatccagtt cgatgtaacc cactcgtgca cccaactgat cttcagcatc ttttactttc
6161  accagcgttt ctgggtgagc aaaaacagga aggcaaaatg ccgcaaaaaa gggaataagg gcgacacgga
6231  aatgttgaat actcatactc ttcctttttc aatattattg aagcatttat cagggttatt gtctcatgag
6301  cggatacata tttgaatgta tttagaaaaa taaacaaata ggggttccgc gcacatttcc ccgaaaagtg
6371  ccacctgacg tc
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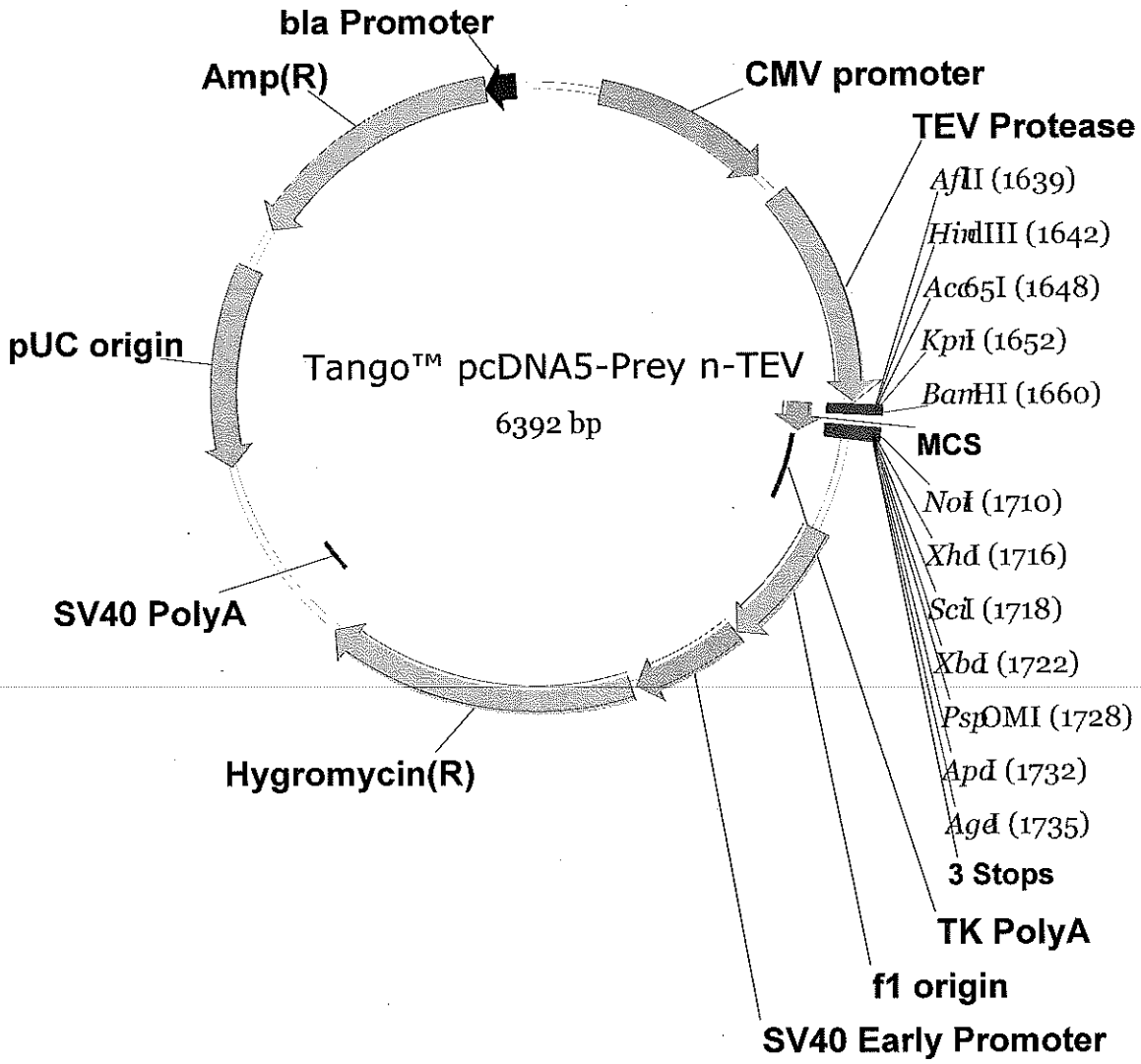
Tango™ pcDNA5-Prey n-TEV



General Description

DNA pcDNA5 Tango Prey n-TEV
Entire molecule length: 6392 bp

Feature Map

TEV Protease	Start: 879 End: 1622
3 Stops	Start: 1740 End: 1748
Hygromycin(R)	Start: 2885 End: 3910
Amp(R)	Start: 5367 End: 6227 (Complementary)
MCS	Start: 1631 End: 1739
TK PolyA	Start: 1757 End: 2028
SV40 PolyA	Start: 4039 End: 4169
CMV promoter	Start: 203 End: 790
SV40 Early Promoter	Start: 2497 End: 2866
bla Promoter	Start: 6228 End: 6326 (Complementary)
f1 origin	Start: 2064 End: 2492
pUC origin	Start: 4552 End: 5222 (Complementary)

Sequence

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141   gaagaatctg cttagggtta ggcgttttgc gctgcttcgc gatgtacggg ccagatatac gcgttgacat
211   tgattattga ctagttatta atagtaatca attacggggt cattagttca tagcccatat atggagttcc
281   gcgttacata acttacggta aatggcccgc ctggctgacc gcccaacgac cccgcccat tgacgtcaat
351   aatgacgtat gttcccatag taacgccaat agggactttc cattgacgtc aatgggtgga gtatttacgg
421   taaactgccc acttggcagt acatcaagtg tatcatatgc caagtacgcc ccctattgac gtcaatgacg
491   gtaaatggcc cgcctggcat tatgcccagt acatgacctt atgggacttt cctacttggc agtacatcta
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631   gactcacggg gatttccaag tctccaccc attgacgtca atgggagttt gttttggcac caaaatcaac
701   gggactttcc aaaatgtcgt aacaactccg ccccattgac gcaaatgggc ggtaggcgtg tacggtggga
771   ggtctatata agcagagctc tctggctaac tagagaaccc actgcttact ggcttatcga aattaatacg
841   actcactata gggagaccca agctggctag ccaccatgag cttgtttaag ggaccacgtg attacaaccc
911   gatatcgagc accatttgtc atttgacgaa tgaatctgat gggcacacaa catcgttgta tggtattgga
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1051  tacatggtgt attcaaggtc aagaacacca cgactttgca acaacacctc attgatggga gggacatgat
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1191  gagcgcatat gtcttgtgac aaccaacttc caaactaaga gcatgtctag catggtgtca gacactagtt
1261  gcacattccc ttcatctgat ggcatattct ggaagcattg gattcaaacc aaggatgggc agtgtggcag
1331  tccattagta tcaactagag atgggttcat tgttggtata cactcagcat cgaatttcac caacacaaac
1401  aattatttca caagcgtgcc gaaaaacttc atggaattgt tgacaagtca ggaggcgcag cagtgggtta
1471  gtggttggcg attaaatgct gactcagtat tgtgggggggg ccataaagtt ttcatgagca aacctgaaga
1541  gccttttcag ccagttaagg aagcgactca actcatgaat gaattggtgt actcgtaccc atatgatgtt
1611  ccagattacg cttcgctagc gtttaaactt aagcttggta ccgagctcgg atccactagt ccagtgtggt
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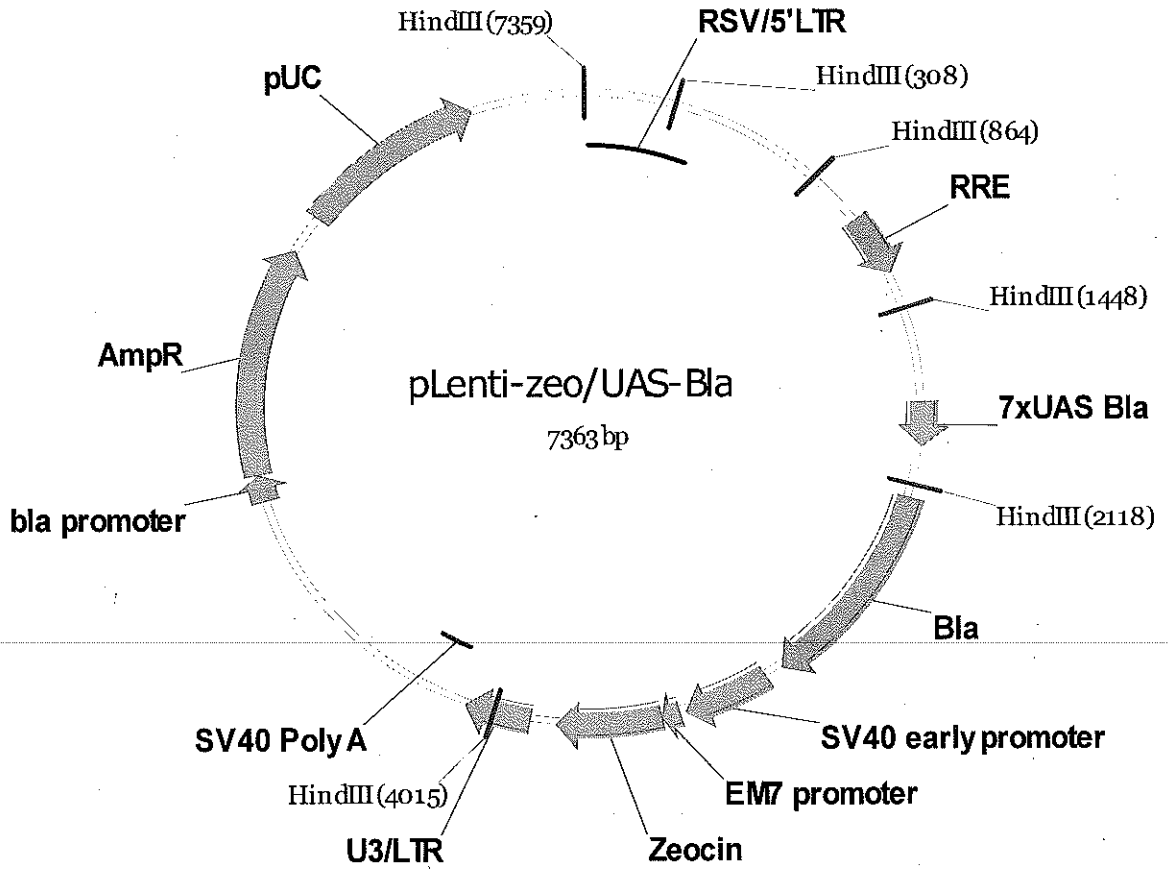
pLenti-zeo/UAS-bla



General Description

DNA pLenti-zeo/UAS-Bla
Entire molecule length: 7363 bp

RRE	Start: 1075	End: 1308
UAS Bla	Start: 1805	End: 1972
Bla	Start: 2169	End: 2956
SV40 early promoter	Start: 3022	End: 3331
EM7 promoter	Start: 3350	End: 3416
Zeocin	Start: 3417	End: 3791
U3/LTR	Start: 3883	End: 4117
bla promoter	Start: 5179	End: 5277
AmpR	Start: 5278	End: 6138
pUC	Start: 6283	End: 6956
SV40 Poly A	Start: 4189	End: 4320
RSV/5'LTR	Start: 1	End: 410

Sequence pLenti-*zeo*/UAS-*bla*

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