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1. Description

The Tango™ GPCR Enabling Kit provides the vector, cell line, and instructions necessary for creating Tango™ beta-arrestin recruitment assays for GPCRs in a U2OS cell background.

1.1 Tango™ GPCR Cell-Based Assays

Tango™ GPCR Assay technology combines the benefits of the Tango™ assay platform with the highly accurate, sensitive, and easy-to-use GeneBLAzer® beta-lactamasereporter system. The Tango™ assay platform is based upon ligand binding to G-Protein Coupled Receptors (GPCRs) that triggers desensitization, a process mediated by the recruitment of intracellular arrestin proteins to the activated receptor. As a result, the ligand-induced activation of GPCRs may be assayed by monitoring the interaction of arrestin with the test GPCR. A major advantage of this approach is that it does not depend on knowledge of the G-protein signaling specificity of the target receptor.

The design of the Tango™ GPCR assay is shown in Figure 1. The target GPCR is fused at its intracellular C-terminus to an exogenous transcription factor. Interposed between the receptor 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 *bla* reporter gene responsive to the transcription factor. The cell line also expresses an arrestin-protease fusion protein that recognizes and cleaves the site between the receptor and transcription factor. The assay is performed by adding a ligand 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 target receptor and the protease-tagged arrestin partner. Additionally, it is unaffected by other signaling pathways in the cell, thus providing a highly selective readout of target receptor activation.

Figure 1.

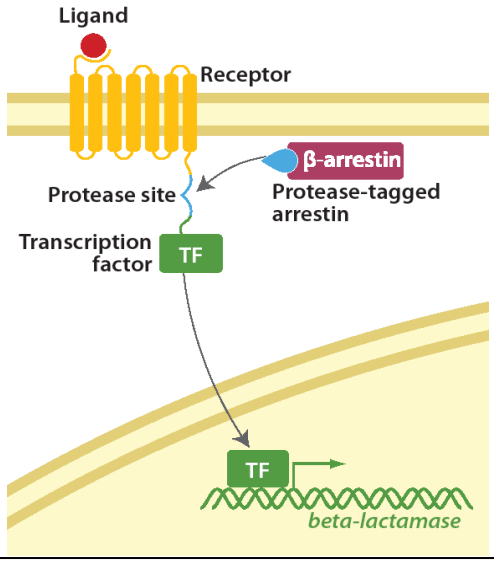


Figure 1—Tango™ GPCR cell-based beta-arrestin signaling. Upon ligand binding and receptor activation, a protease-tagged beta-arrestin molecule is recruited to the GPCR that has been modified at the C-terminus to include a transcription factor linked by a protease cleavage site. The protease cleaves the transcription factor from the GPCR, which translocates to the nucleus and activates the expression of beta-lactamase.

Figure 2.

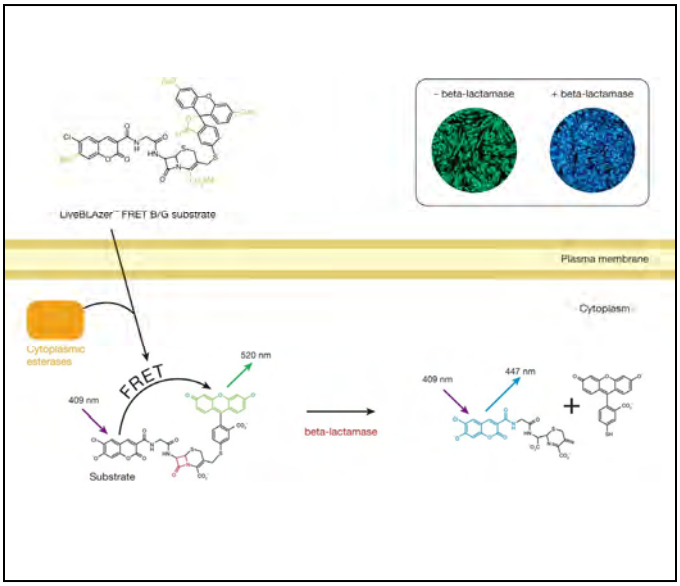


Figure 2—Sensitive fluorescent detection of beta-lactamase reporter gene response using GeneBLAzer® technology. After loading the cell permeable LiveBLAzer™ FRET B/G substrate, cellular fluorescence is measured. In the absence of beta-lactamase expression, cells generate green fluorescence. In the presence of beta-lactamase expression, the substrate is cleaved and the cells generate blue fluorescence.

The Tango™ assay technology uses a mammalian-optimized beta-lactamase (*bla*) reporter gene combined with a FRET-enabled substrate to provide reliable and sensitive detection in cells (1) (Figure 2). Cells are loaded with an 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 resonance 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 fluorophores 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 that can minimize experimental noise that masks subtle underlying biological responses of interest. The Tango™ assay technology has been proven in high-throughput screening (HTS) campaigns for a range of target classes, including G-protein coupled receptors (GPCRs) (2, 3), nuclear receptors (4–6) and kinase signaling pathways (7).

1.2 Tango™ Vector Overview

The Tango™ pDEST-Gal4VP16-M vector allows expression of the GPCR (or gene of interest) in mammalian cells, with the option of tetracycline-regulated expression in cell lines expressing the Tet repressor. The vector contains the following elements (see **Section 12** for a vector map; the complete vector sequence is available on the web at www.invitrogen.com).

- Hybrid promoter consisting of human cytomegalovirus immediate-early (CMV) promoter/enhancer and tetracycline operator 2 (TetO2) sites for active expression in most mammalian cell types, except in cells expressing a tetracyclin-regulated transcription factor wherein the promoter activity will be modulated by the presence of tetracycline (see the next section for details)
- 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*
- Age1 and Mlu1 sites for cloning in-frame with the TEV recognition site and Gal4-VP16 transcription factor
- TEV recognition cleavage sites (ENLYFQM for enhanced cleavage efficiency) for cleavage of the Gal4-VP16 transcription factor sequence
- Gal4-VP16 sequence for expression of Gal4 transcriptional activator capable of binding to UAS and turning on genes placed downstream of UAS promoter
- Gateway® cassette, containing chloramphenicol resistance and ccdB gene, flanked by attR1 and attR2 recombination sites, for optional Gateway® recombinational cloning of gene of interest from an entry clone

1.3 T-REx™ System Overview

The Tango™ pDEST-Gal4VP16-M vector may be used in conjunction with the T-REx™ System. These vectors contain two tetracycline operator 2 (TetO2) sites within the human CMV promoter for tetracycline-regulated expression of your gene of interest (Yao et al., 1998). The TetO2 sequences serve as binding sites for four Tet repressor molecules (comprising two Tet repressor homodimers) and confer tetracycline-responsiveness to your gene of interest. In the T-REx™ System, the Tet repressor is expressed from the pcDNA™6/TR plasmid. In the absence of tetracycline, expression of your gene of interest is repressed by the binding of Tet repressor homodimers to the TetO2 sequences. Addition of tetracycline to the cells derepresses the hybrid CMV/TetO2 promoter and allows expression of your gene of interest. For more information about pcDNA™6/TR and the Tet repressor, refer to the T-REx™ System manual. All Invitrogen manuals are available for downloading at www.invitrogen.com or by contacting Technical Support.

Tet Operator Sequences

The promoters of bacterial *tet* genes contain two types of operator sequences, O1 and O2, that serve as high affinity binding sites for the Tet repressor (Hillen and Berens, 1994; Hillen *et al.*, 1983). Each O1 and O2 site binds to one Tet repressor homodimer. While Tet repressor homodimers bind to both *tet* operators with high affinity, studies have shown that the affinity of the Tet repressor homodimer for O2 is three- to five-fold higher than for O1 (Hillen and Berens, 1994).

Tet operators have been incorporated into heterologous eukaryotic promoters to allow tetracycline-regulated gene expression in mammalian cells (Gossen and Bujard, 1992; Yao *et al.*, 1998). In the T-REx™ System, two copies of the O2 operator sequence (TetO2) are inserted into the strong CMV promoter of the pT-REx-DEST vectors to allow regulated expression of your gene of interest by tetracycline. For more information about *tet* operators, refer to Hillen and Berens (1994).

Yao *et al.* (1998) have recently demonstrated that the location of *tet* operator sequences in relation to the TATA box of a heterologous promoter is critical to the function of the *tet* operator. Regulation by tetracycline is only conferred upon a heterologous promoter by proper spacing of the TetO2 sequences from the TATA box (Yao *et al.*, 1998). For

this reason, the first nucleotide of the TetO2 operator sequence has been placed 10 nucleotides after the last nucleotide of the TATA element in the CMV promoter for the Tango™ pDEST-Gal4VP16-M Vector.

In other tetracycline-regulated systems, the TetO2 sequences are located upstream of the TATA element in the promoter of the inducible expression vector (Gossen and Bujard, 1992). These systems differ substantially from the T-REx™ System in that they use regulatory molecules composed of the Tet repressor fused to a viral transactivation domain. The presence of viral transactivation domains appears to overcome the requirement for specific positioning of the TetO2 sequences in relation to the TATA box of the heterologous promoter. However, the presence of viral transactivation domains has been found to have deleterious effects in some mammalian cell lines.

2. Materials Supplied

Vector:	The Tango™ pDEST-Gal4VP16-M vector contains a TEV protease cleavage site preceding a Gal4-VP16 transcription factor. Mlu1 and AgeI restriction sites preceding the TEV protease site allow for a GPCR to be cloned in frame with the TEV protease site and Gal4-VP16 transcription factor to create a fusion protein. Alternatively, a Gateway® pDONR™ 221 entry vector containing the GPCR of interest without a stop codon can be created and transferred into the Tango™ pDEST-Gal4VP16-M Vector via an LR reaction.
Shipping Condition:	Dry ice
Storage Condition:	–20°C
Quantity:	20 µg
Application	The development of Tango™ Assays for GPCRs
Selection Marker(s):	Geneticin® (Use at 200 µg/ml when utilizing the Tango™ Beta-arrestin2-UAS-*bla* U20S cell line for initial selection). Maintain cell line in 100 µg/ml Geneticin®.

Cell Line:	Tango™ Beta-arrestin2-UAS-*bla* U20S cells contain a stably integrated beta-lactamase reporter gene under control of the UAS response element. This cell line constitutively expresses a fusion protein where beta-arrestin is fused to the TEV protease. This cell line can be used to produce Tango™ assays for GPCRs.
Shipping Condition:	Dry ice
Storage Condition:	Liquid nitrogen. **Immediately upon receipt, cells must be stored in liquid nitrogen or thawed for immediate use. Cells stored at –80°C can quickly lose viability.**
Quantity:	>8,000,000 cells (8×10^6 cells/ml)
Application	The development of Tango™ Assays for GPCRs.
Growth Properties of cells	Adherent
Cell Phenotype	Epithelial
Selection Marker(s):	Zeocin™ (200 µg/ml), Hygromycin (50 µg/ml)
***Mycoplasma* level**	Negative
BioSafety Level:	1

3. Materials Required

Use the following table to determine the additional media and reagents required for use with each kit:

Media/Reagents	Recommended Source	Part no.	Restriction Digest	Gateway® Cloning
LiveBLAzer™-FRET B/G Loading Kit (LiveBLAzer™-FRET B/G Substrate (CCF4-AM), DMSO for Solution A, Solution B, Solution C)	Invitrogen	K1095 (200 µg) K1096 (1 mg) K1030 (5 mg)	✓	✓
Recovery™ Cell Culture Freezing Medium	Invitrogen	12648–010	✓	✓
McCoy's 5A Medium (modified) (1X)	Invitrogen	16600–082	✓	✓
Opti-MEM® I	Invitrogen	31985–070	✓	✓
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 (1 M, pH 7.3)	Invitrogen	15630–080	✓	✓
0.05% Trypsin/EDTA	Invitrogen	25300–054	✓	✓
Zeocin™	Invitrogen	R250–01	✓	✓
Hygromycin	Invitrogen	10687–010	✓	✓
Geneticin®	Invitrogen	10131–027	✓	✓
Ampicillin	Invitrogen	11593–027	✓	✓
Lipofectamine™ LTX Plus Transfection Reagent	Invitrogen	15338–100	✓	✓
Plus™ Reagent	Invitrogen	11514–015	✓	✓
Glucose	Sigma	G5767	✓	✓
EDTA	Sigma	E6758	✓	✓
*Mlu*I Restriction Enzyme	Invitrogen	15432–016	✓	
*Age*I Restriction Enzyme	NEB	R0552S	✓	
T4 DNA Ligase	Invitrogen	15224–017	✓	
One Shot® MAX Efficiency® DH5-α™-T1R Cells	Invitrogen	12297–016	✓	✓
One Shot® TOP10 Competent Cells	Invitrogen	C4040–10 C4040–50	✓	✓
One Shot® ccdB Survival™ 2 T1^R Competent Cells	Invitrogen	A10460	✓	✓
pDONR™ 221	Invitrogen	12536–017		✓
Gateway® LR Clonase™ II Enzyme Mix	Invitrogen	11791–020		✓
Gateway® BP Clonase™ II Enzyme Mix	Invitrogen	11789–020		✓
S.N.A.P.™ Gel Purification Kit	Invitrogen	K1999–25	✓	✓
PureLink™ Quick Gel Extraction System	Invitrogen	K2100-12	✓	✓
PureLink™ HQ Mini Plasmid Purification Kit	Invitrogen	K2100–01	✓	✓
*Xba*I Restriction Enzyme	Invitrogen	15226–012	✓	
*Bam*HI Restriction Enzyme	Invitrogen	15201–023	✓	
pcDNA™6/TR	Invitrogen	V1025–20	For inducible expression of GPCR	

Use the following tables to determine the additional materials required for use with each kit:

Consumables	Recommended Source	Part no.
Black-wall, clear-bottom, 384-well assay plates	Corning	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 (see **Section 10.4**)	Chroma Technologies
Optional: Epifluorescence- or fluorescence-equipped microscope, with appropriate filters	Various
Optional: Microplate centrifuge	Various

4. Tango™ Cloning Design and Vector Use

4.1 Cloning Options

Consult the restriction cloning sites described in this manual to design a strategy to clone your gene of interest in Tango™ pDEST-Gal4VP16-M, or consult the section on Gateway® cloning to design PCR primers for recloning your gene of interest into the **Gateway® pDONR™ 221 vector**.

If you are using a restriction enzyme cloning strategy:

1. Digest your vector with the appropriate restriction enzymes, treat with alkaline phosphatase (if desired), and gel purify your vector backbone.
2. Ligate your prepared insert into the prepared vector and transform into *E. coli*. Select transformants on LB plates containing 100 µg/ml ampicillin.

If you are using a Gateway® cloning strategy:

1. Amplify your gene (without a stop codon) using primers containing the *att*B1 and *att*B2 sites, and clone into the **Gateway® pDONR™ 221 vector** in a Gateway® *att*B × *att*P recombination reaction to create your Gateway® ENTRY vector (see section on Gateway® cloning). Confirm the Gateway® ENTRY vector via restriction digest and sequencing.
2. Subclone your gene from the Gateway® ENTRY vector to Tango™ pDEST GAL4VP16-M in a Gateway® *att*L × *att*R recombination reaction.
3. Analyze your transformants for the presence of insert by restriction digestion.
4. Select a transformant with the correct restriction pattern and sequence to confirm that your gene is cloned in the proper orientation.
5. Transfect your construct into the supplied Tango™ Beta-arrestin2-UAS-bla U2OS cells using your method of choice. If you desire Tetracycline-regulated expression, make sure that the master cell line also expresses the Tetracycline repressor protein from a plasmid such as pcDNA™6/TR. Generate a stable cell line, if desired.
6. Test for recombinant gene expression by western blot analysis or Tango™ GPCR assay.

4.2 Using Tango™ pDEST-Gal4VP16-M

The Tango™ pDEST-Gal4VP16-M vector is supplied as supercoiled plasmid. 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.

If you wish to propagate and maintain the Tango™ pDEST-Gal4VP16-M vector, we recommend using Library Efficiency® DB3.1. Competent Cells (Catalog no. 11782-018) from Invitrogen for transformation. The DB3.1. *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the ccdB gene. Select for transformed colonies using LB agar containing Ampicillin (100 µg/ml) and Chloramphenicol (34 µg/ml).

Note: Do **NOT** use general *E. coli* cloning strains including TOP10 or DH5α for propagation and maintenance of Tango™ pDEST-Gal4VP16-M as these strains are sensitive to ccdB effects.

4.3 Restriction Enzyme Cloning into Tango™ pDEST-Gal4VP16-M

Consult the diagrams provided on the following pages to design a strategy for cloning your gene of interest. General considerations for cloning and transformation are listed below.

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

4.3.1 Points to Consider Before Cloning into Tango™ pDEST-Gal4VP16-M

Your insert should contain a Kozak consensus sequence with an ATG initiation codon for proper initiation of translation (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)NNATG**G**

Important Note:

Tango™ pDEST-Gal4VP16-M Vector is a fusion vector. **Your gene should *NOT* contain a stop codon**.

4.3.2 Cloning Site of Tango™ pDEST-Gal4VP16-M

Cloning site of the Tango™ pDEST-Gal4VP16-M vector are shown below. Restriction sites are labeled to indicate the cleavage sites. These sites have been confirmed by sequencing and functional testing. For a map, and a description of the features of Tango™ pDEST-Gal4VP16-M vector, refer to the **Appendix**.

- For restriction enzyme cloning, use *Age*I for the 5′ and *Mlu*I for the 3′ ends of your gene of interest.
- Ensure that your gene is in-frame with the TEV cleavage recognition site (ENLYFQM) for efficient fusion protein production.

```
5036    TCGCCTGGAG ACGCCATCCA CGCTGTTTTG ACCTCCATAG AAGACACCGG

                                  AgeI
                                  ~~~~~~~
5086    GACCGATCCA GCCTCCGGAC TCTAGTACCG GTGATATCCT CGAGCCCATC

                               attR1
        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
5136    AACAAGTTTG TACAAAAAAG CTGAACGAGA AACGTAAAAT GATATAAATA

                               attR1
        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
5186    TCAATATATT AAATTAGATT TTGCATAAAA AACAGACTAC ATAATACTGT

               attR1
        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~
5236    AAAACACAAC ATATCCAGTC ACTATGGCGG CCGCATTAGG CACCCCAGGC
        //////////////////////////////////////////////////////

                               attR2
        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
6717    CATAGTGACT GGATATGTTG TGTTTTACAG TATTATGTAG TCTGTTTTTT

                               attR2
        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
6767    ATGCAAAATC TAATTTAATA TATTGATATT TATATCATTT TACGTTTCTC


                 attR2                  MluI   TEV Recognition Site
         ~~~~~~~~~~~~~~~~~~~~~~~~~~~  ~~~~~~~  ~~~~~~~~~~~~~~~~~
                                                GluAsnLeu TyrPheGln
6817    GTTCAGCTTT CTTGTACAAA GTGGTCCACG CGTGAGAACC TGTACTTCCA

                              Gal4
        ~~~~    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
        Met  Lys LeuLeuSerSer IleGluGln AlaCysAsp IleCysArgLeu
6867    GATGAGAAAG CTACTGTCTT CTATCGAACA AGCATGCGAT ATTTGCCGAC

                              Gal4
        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
        LLysLysLeu LysCysSer LysGluLysPro LysCysAla LysCysLeu
6917    TTAAAAAGCT CAAGTGCTCC AAAGAAAAAC CGAAGTGCGC CAAGTGTCTG
```

4.3.3 PCR Primer Design for Cloning PCR Products into Tango™ pDEST-Gal4VP16-M

If needed, you may amplify your gene of interest with the appropriate PCR primers to add on the restriction sites or to remove the stop codon from your gene. The primers should include 18–25bp of gene specific sequence, followed by the restriction site sequence (*i.e. Age*I for the 5′ and *Mlu*I for the 3′ ends of your gene of interest). Include 6–8bp of GC-rich sequence on the 5′ end of both primers to allow for efficient digestion by the restriction enzyme.

Example:

Forward amplification primer (*Age*I), Kozak sequence underlined:
5′-[6–8bp GC-rich sequence]ACCGGTCACCATG[18–25bp gene-specific sequence)-3′

Reverse amplification primer (*Mlu*I):
5′-[6–8bp GC-rich sequence]ACGCGT[18–25bp gene-specific reverse strand sequence)-3′

PCR Amplification of Gene of Interest Containing an Internal *Age*I or *Mlu*I Site

If your gene of interest contains an internal *Age*I site, it may still be possible to use the *Age*I restriction site of the pDEST-Gal4VP16-M vector. The following restriction enzymes produce *Age*I-compatible 5′-overhang sequences: *Bsa*WI (W/CCGGW), *Bsp*EI (T/CCGGA), *Bsr*F1 (R/CCGGY), *Ngo*MIV (G/CCGGC), *Xma*I (C/CCGGG), *Sgr*AI (CR/CCGGYG). Amplify your gene of interest as described above, but rather than *Age*I, use the compatible enzyme of your choice in your 5′-primer sequence.

If your gene of interest contains an internal *Mlu*I site, substitute the *Mlu*I restriction sequence in your Reverse Amplification Primer with that of *Bss*HII (G/CGCGC) which produces an *Mlu*I-compatible overhang sequence.

Note: Remember to keep your gene in-frame with the TEV cleavage site, and do **NOT** include a stop codon.

4.3.4 E. coli *Transformation*

Transform TOP10 or DH5α competent *E. coli* from Invitrogen with your ligation mix. Select transformants on LB agar plates with ampicillin (100 µg/ml).

Note: Do not use chloramphenicol for selection of the ligated plasmid, as the Gateway® cassette containing the chloramphenicol resistance gene is removed during plasmid digestion in preparation for cloning.

You may use any transformation method of your choice. While chemical transformation is the most convenient for most researchers, electroporation is the most efficient method for large plasmids.

Pick 10–20 clones and analyze for the presence and orientation of your insert. We recommend that you sequence your construct with CMV Forward and SV40 PolyA Reverse primers to confirm that your gene is in the correct orientation for expression and contains an ATG. Refer to the cloning site and plasmid diagrams for the sequence and location of the priming sites.

4.4 Gateway® Cloning into Tango™ pDEST-Gal4VP16-M

The Gateway® Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (1) to provide a rapid and highly efficient way to move DNA sequences into multiple vector systems. The Gateway® Technology is schematically represented below.

*att*B1-gene-*att*B2 × *att*P1-ccdB-*att*P2 ⇔ *att*L1-gene-*att*L2 × *att*R1-ccdB-*att*R2
(expression clone) (pDONR™) (entry clone) (destination vector)

The *att*B × *att*P recombination reaction is mediated by the Gateway® BP Clonase™ II enzyme mix and the *att*L × *att*R recombination reaction is catalyzed by the Gateway® LR Clonase™ II enzyme mix. ccdB is the F plasmid-encoded gene that inhibits growth of E. coli (2,3) and "gene" represents any DNA segment of interest (*e.g.* PCR product, cDNA, genomic DNA).

Tango™ pDEST-Gal4VP16-M contains the *att*R1 and *att*R2 sequences, and is a Gateway® Destination vector compatible with the Gateway® pENTR221 vector containing your gene of interest and the *att*L1 and *att*L2 sequences. To recombine your gene of interest into Tango™ pDEST-Gal4VP16-M, make sure that your gene does **NOT** contain a stop codon, and is in the proper reading frame for expression as a C-terminal fusion. Note that the Gateway® *att* sites add on 7 amino acids of extra spacer sequence between the gene of interest and the TEV recognition site. We have successfully developed assays utilizing the Tango™ pDEST-Gal4VP16-M vector as a Gateway® Destination vector for GPCR expression. General considerations for cloning and transformation are listed below.

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

4.4.1 Constructing Your Gateway® ENTRY Vector

We recommend using pDONR™ 221 to construct your Gateway® ENTRY vector. An ENTRY vector made by recombining your GPCR with pDONR™ 221 will have minimal extra sequence between the gene of interest and the *att*L sites, and thus a minimal distance between the C-terminus of the GPCR and the TEV cleavage site after the *att*L × *att*R recombination reaction between the ENTRY clone and Tango™ pDEST-Gal4VP16-M. We have found that minimizing this distance may help to increase assay sensitivity.

Refer to the Gateway® Technology with Clonase™ II and Gateway® pDONR™ Vectors manuals (part nos. 25-0749 and 25-0531, respectively) for detailed instructions on constructing your Gateway® ENTRY vector with your gene of interest. These manuals are available for downloading at www.invitrogen.com or by contacting Technical Support. Keep in mind the cloning considerations below when designing your PCR primers.

4.4.2 Designing attB PCR Primers for Tango Enabling Model ENTRY Vectors

To generate PCR products suitable for use as substrates in a Gateway® BP recombination reaction with the donor vector pDONR™221, you will need to incorporate *att*B sites into your PCR products. Guidelines are provided below to help you design your PCR primers.

Important: Kozak Consensus Sequence

Your insert should contain a Kozak consensus sequence with an ATG initiation codon for proper initiation of translation (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**

In the following example, we design the forward *att*B PCR primer to allow the expression of a native protein of interest. The *att*B1 site is indicated in bold and the ATG initiation codon for the protein of interest is underlined. Inclusion of the Kozak consensus sequence allows protein expression in mammalian cells.

5′-GGGG **ACA AGT TTG TAC AAA AAA GCA GGC** ACC <u>ATG</u> (18–25bp gene-specific nucleotides)-3′

Important: Remove Stop Codon from Your Gene of Interest

Tango™ pDEST-Gal4VP16-M is a fusion vector. **Your gene should *NOT* contain a stop codon**. When designing primers for subcloning your gene of interest into a Gateway® pDONR™ vector, design them such that the resulting PCR product will **NOT** contain a stop codon for your gene of interest.

Important: Reverse Primer Design for C-Terminal Fusions!

In order to maintain the proper reading frame for fusion protein expression, design the *att*B reverse primer such that the reading frame of the GPCR matches the reading frame of the TEV protease recognition site.

Note: The design of the *att*B reverse primer used for constructing the ENTRY clones compatible with Tango™ pDEST-Gal4VP16-M is slightly different than the one outlined in the Gateway® Technology manual. It differs by the exclusion of one nucleotide on the 3′ end of the *att*B2 sequence. This modification in design is necessary to retain the correct reading frame between the GPCR and the TEV protease recognition site.

In the following example, we design the *att*B reverse PCR primer to allow the expression of a C-terminal fusion protein of interest. The *att*B2 site is indicated in bold. Remember that the gene-specific nucleotides need to be in frame with the *att*B2 sequence and that the stop codon must be removed.

Tip: Keep the TCT-TGT (TGT-ACA on the complementary strand) triplets in the *att*R2 site in frame with the translation reading frame of the GPCR.

5′-GG GG**A CCA CTT TGT ACA AGA AAG CTG GGT** (18–25bp gene-specific nucleotides)-3′

After amplification of your PCR product and cloning into pDONR™ 221 Gateway® vector, the recombined sequence of the ENTRY vector should be as follows.

```
                                  attL1
                        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
AGTCT TAAGC TCGGG CCCCA AATAA TGATT TTATT TTGAC TGATA GTGAC
TCAGA ATTCG AGCCC GGGGT TTATT ACTAA AATAA AACTG ACTAT CACTG
                           attL1
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
CTGTT CGTTG CAACA AATTG ATGAG CAATG CTTTT TTATA ATGCC AACTT
GACAA GCAAC GTTGT TTAAC TACTC GTTAC GAAAA AATAT TACGG TTGAA
      attL1
~~~~~~~~~~~~~~~~~~~~~
TGTAC AAAAA AGCAG GCACC ---GENE-----ACCCA GCTTT CTTGT ACAAA
ACATG TTTTT TCGTC CGTGG ------------TGGGT CGAAA GAACA TGTTT
                                      ~~~~~~~~~~~~~~~~~~~~~~~~
                                             attL2
GTTGG CATTA TAAGA AAGCA TTGCT TATCA ATTTG TTGCA ACGAA CAGGT
CAACC GTAAT ATTCT TTCGT AACGA ATAGT TAAAC AACGT TGCTT GTCCA
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                           attL2
CACTA TCAGT CAAAA TAAAA TCATT ATTTG CCATC CAGCT GATAT CCCCT
GTGAT AGTCA GTTTT ATTTT AGTAA TAAAC GGTAG GTCGA CTATA GGGGA
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
             attL2
```

4.4.3 Ultimate™ ORF Collection

The Ultimate™ ORF Clones are human and mouse open reading frames (ORFs) provided in the Gateway® entry vector pENTR™221. The Ultimate™ ORF clones have been fully insert sequenced, and can be readily identified and ordered via an online search using the Ultimate™ ORF Browser at

http://orf.invitrogen.com/cgi-bin/ORF_Browser.

Any GPCR clone ordered from the Ultimate™ ORF Collection will contain a stop codon at the C-terminus, and an additional nucleotide after the gene of interest, which must both be removed before use in an *att*L × *att*R recombination reaction with the Tango™ pDEST-Gal4VP16-M. The stop codon and the extra nucleotide may be removed by site-directed mutagenesis, or by re-cloning the gene of interest into a Gateway® pDONR™ vector without a stop codon.

4.4.4 Recombining Your Gene of Interest into Tango™ pDEST-Gal4VP16-M

Each entry clone contains *att*L sites flanking the gene of interest. Genes in an entry clone are transferred to the destination vector backbone in a recombination reaction catalyzed by the Gateway® LR Clonase® II enzyme mix. The resulting recombinant DNA is then transformed into *E. coli* and the expression clone selected. Recombination between the *att*R sites on the destination vector and the *att*L sites on the entry clone replaces the ccdB gene and the chloramphenicol (CmR) gene with the gene of interest and results in the formation of *att*B sites in the expression clone.

Follow the instructions in the Gateway® Technology Manual (part no. 25-0522) to set up the LR Clonase® II reaction, transform *E. coli*, and select for the expression clone. All Invitrogen manuals are available for downloading at www.invitrogen.com or by contacting Technical Support.

After the *att*L × *att*R reaction between the ENTRY clone and Tango™ pDEST-Gal4VP16-M, the recombined sequence of the expression vector will be as follows. Note that the gene of interest, starting with ATG, is in-frame with the TEV protease cleavage site (ENLYFQM):

```
                                        attB1
                                  ~~~~~~~~~~~~~~~~~~~~~~~~
ACCGG TGATA TCCTC GAGCC CATCA ACAAG TTTGT ACAAA AAAGC AGGCA
TGGCC ACTAT AGGAG CTCGG GTAGT TGTTC AAACA TGTTT TTTCG TCCGT
                                                    ENLYFQM
                                                      ~~
  Met             ThrGln LeuSer Cys ThrLys TrpSer Thr ArgGlu
CCATG ----GENE----ACCCA GCTTT CTTGT ACAAA GTGGT CCACG CGTGA
GGTAC ------------TGGGT CGAAA GAACA TGTTT CACCA GGTGC GCACT
                  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                            attB2
 ENLYFQM                     Gal4
~~~~~~~~~~~~~~~~~~~~~~    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
AsnLeu Tyr PheGln MetArg Lys LeuLeu SerSer Ile GluGln AlaCys
GAACC TGTAC TTCCA GATGA GAAAG CTACT GTCTT CTATC GAACA AGCAT
CTTGG ACATG AAGGT CTACT CTTTC GATGA CAGAA GATAG CTTGT TCGTA
                       Gal4
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Asp IleCys·ArgLeu Lys LysLeu LysCys Ser LysGlu LysPro Lys
GCGAT ATTTG CCGAC TTAAA AAGCT CAAGT GCTCC AAAGA AAAAC CGAAG
CGCTA TAAAC GGCTG AATTT TTCGA GTTCA CGAGG TTTCT TTTTG GCTTC
```

4.4.5 E. coli Transformation

Use TOP10 or DH5α competent E. coli from Invitrogen for transforming your ligation mix. Select transformants on LB agar with ampicillin (100ug/ml). **Note:** Do not use chloramphenicol for selection of the ligated plasmid, as the Gateway cassette containing Chloramphenicol resistance gene will be removed during the Gateway *att*L × *att*R reaction.

You may use any transformation method of your choice. While chemical transformation is the most convenient for most researchers, electroporation is the most efficient method for large plasmids.

Select 10–20 clones and analyze for the presence and orientation of your insert. We recommend that you sequence your construct with CMV Forward and SV40 PolyA Reverse primers to confirm that your gene is in the correct orientation for expression and contains an ATG. Refer to the cloning site and plasmid diagrams for the sequences and location of the priming sites.

4.5 Plasmid Preparation

4.5.1 Preparing a Glycerol Stock

Once you have identified the correct Tango™ pDEST-Gal4VP16-M clone containing your gene of interest, prepare a glycerol stock for long-term storage. You should also keep a DNA stock of your plasmid at –20°C.

1. Streak the original colony out on an LB plate containing 50 µg/ml ampicillin. Incubate the plate at 37°C overnight.
2. Isolate a single colony and inoculate into 1–2 ml of LB containing 50 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.

4.5.2 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 PureLink™ HiPure Plasmid Prep Kits (available separately from Invitrogen), or CsCl gradient centrifugation.

4.5.3 Transfection

Once you have verified that your gene is cloned properly (in the correct orientation and containing an ATG initiation 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.

5. Cell Growth and Handling

Note: Refer to **Section 6, Media Requirements** for specific media recipes.

5.1 Thawing Method

1. Place 44 ml of Thawing Medium (see **Section5**, **Media Requirements**) into a T225 flask.
2. Place the flask in a 37°C/5% CO_2 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 Thawing Medium in a sterile 15 ml conical tube.
6. Centrifuge cells at 200 × *g* for 5 minutes.
7. Aspirate supernatant and resuspend the cell pellet in 1 ml of fresh Thawing Medium.
8. Transfer contents to the T225 tissue culture flask containing pre-equilibrated Thawing Medium and place flask in the 37°C/5% CO_2 incubator.
9. At first passage switch to Growth Medium containing antibiotics (see **Section 5**, **Media Requirements**).

5.2 Cell Propagation

1. Passage or feed cells at least twice a week. Maintain cells between 10% and 95% confluence. Do **NOT** allow cells to reach confluence.
2. To passage cells, aspirate medium, rinse once in PBS, add 0.05% Trypsin/EDTA (3 ml for a T75 flask, 5 ml for a T175 flask, and 7 ml for T225 flask) and swirl to coat the cells evenly. Cells usually detach ~2–5 minutes after exposure to 0.05% Trypsin/EDTA. Add an equal volume of Growth Medium (pre-warmed to 37°C) to inactivate Trypsin.
3. Verify under a microscope that cells have detached and clumps have completely dispersed.
4. Centrifuge cells at 200 × g for 5 minutes and resuspend in Growth Medium (pre-warmed to 37°C).

   Note: After pDEST-GAL4VP16-M has been transfected into the Tango™ Beta-arrestin2-UAS-bla U2OS cell line, 100 µg/ml Geneticin® should also be added to the Growth Media composition.

5.3 Freezing Method

1. Harvest the cells as described in **Section 5.2** (above), Step 2. After detachment, count the cells, centrifuge cells at 200 × *g* for 5 minutes, and resuspend in Freeze Medium at 4°C to a density of 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.

6. Media Requirements

Note: Unless otherwise stated, have all media and solutions at least at room temperature (we recommend 37°C for optimal performance) before adding to cells.

Note: Make **NO MEDIA SUBSTITUTIONS**, as these cell lines have been specifically validated for optimal assay performance with these media. For dividing cells, we recommend that you create and store an aliquot for back up.

***Note:* After pDEST-GAL4VP16-M has been transfected into the Tango™ Beta-arrestin2-UAS-bla U2OS cell line, 100 µg/ml Geneticin® should also be added to the Growth Media composition.

Component	Thawing 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 mg/ml	100 µg/ml	—	—
Zeocin antibiotic	—	200 µg/ml	—	—
Hygromycin antibiotic	—	50 µg/ml	—	—
Geneticin®	—	**	—	—
Recovery™ Cell Culture Freezing Media	—	—	—	100%

7. Transient Transfection and Analysis of Cells

7.1 Transient Transfection and Analysis Protocol

It is often desirable to transiently transfect your cells with Tango™ modified GPCR containing vectors to test if the Tango™ assay works before committing the considerable amount of time required for stable cell line generation.

This protocol uses Lipofectamine™ LTX and PLUS™ Reagents, and a 96-well black-walled clear bottom assay plate. The transfection process is similar to that recommended in the Lipofectamine™ LTX and PLUS™ Reagents protocol, which can be used as a guide.

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 ~40,000 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 OptiMem® 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 room temperature 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 and PLUS™ Reagents/DNA/Media) prepared in step 2 to each well in the 96-well plate.
5. The following day, exchange the media with 90 µl of fresh assay media. Add 10 µl of a 10X stock of your test/reference compound (*e.g.* agonist) (do not exceed a DMSO concentration of 1%), and incubate for 16 hours at 37°C/5% CO_2. Assay the cells for beta-lactamaseactivity by adding 20 µl of 6X LiveBLAzer™-FRET B/G Substrate Mixture and incubating for 2 hours at room temperature. For reagent preparation and beta-lactamase detection guidelines, refer to **Section 10**.

Note: We recommend beginning with a stimulation time of 16 hours at 37°C/5% CO_2 before adding the LiveBLAzer™-FRET B/G Substrate. You may reduce this incubation time in the final assay format to 5 hours.

Note: This protocol is for a 24-hour transfection. You may be able to obtain greater sensitivity with a 48 hour transfection or by using alternative assay media formulations. For a 48-hour transfection, exchange the media 24 hours post-transfection with growth media without antibiotics, and wait an additional day before performing the assay. See **Section 10.2** for recommended alternative assay media formulations. This assay may also be performed in a 384-well plate by plating cells at ~14,000 cells/well, using 30 µl of transfection mixture/well, and loading the cells with 6 µl of 6X LiveBLAzer™-FRET B/G Substrate Mixture.

8. Stable Cell Line Generation

Once you have confirmed that the Tango™ assay works in transiently transfected cells, proceed with stable cell line generation.

This protocol uses Lipofectamine™ LTX and PLUS™ Reagents, and a 96-well black-walled clear bottom assay plate. The transfection process is similar to that recommended in the Lipofectamine™ LTX and PLUS™ Reagents protocol, which can be used as a guide.

8.1 Getting Started

Day 1:

1. Plate Tango™ Beta-arrestin2-UAS-bla U20S cells the night before the assay in a 6-well plate at 1 million cells per well in 2 ml of Growth Media without antibiotics. Use one well for each receptor and an extra well for the negative control (no DNA or the empty vector).

Day 2

2. Transfect the cells using Lipofectamine™ LTX Plus in a 6-well plate. All amounts are given on a per well basis.
   a. Dilute 2.5 µg of DNA in 500 µl OptiMEM®. Mix gently.
   b. Add 2.5 µl of Plus™ Reagent to the diluted DNA, mix gently, and incubate for 5 minutes at room temperature.
   c. Add 6.25 µl Lipofectamine™ LTX to the diluted DNA, mix gently, and incubate at room temperature for 30 minutes.
   d. Add the Lipofectamine-DNA complexes to the well containing the cells. Gently rock the plate back and forth to mix.
   e. Place the plate in the 37ºC, 5% CO_2 incubator overnight.

Day 3

3. Passage the cells in each well of the 6-well plate into a separate T75 flask. Incubate at 37ºC, 5% CO_2 overnight.

Day 4

5. Add Geneticin® selection antibiotic at a final concentration of 200 µg/ml.

Next ~2–3 Weeks

6. Exchange media every 3–4 days. The non-stably transfected cells should die off within 5 days, while the stably transfected cells should grow colonies and expand. After the first passage of the transfected cell line you will likely see an additional die-off of the cell culture. We strongly recommend that you maintain the Geneticin® selection for two full weeks before proceeding to functional assays for your cell line.

8.2 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 per well in a black walled, clear bottom 384-well plate in Assay Media (Freestyle™ Expression Medium) 16–24 hours before the assay. We generally use 36 µl of Assay Medium per well.
2. On the day of the assay, stimulate the cells for 5 hours at 37ºC/5% CO_2 with the primary agonist. Do not exceed a DMSO concentration of 1%.
3. Following the stimulation, load the cells with beta-lactamase substrate for 2 hours at room temperature. For instructions on preparing the loading solution, see **Section 10.3**.

Troubleshooting/Assay Considerations:

If you observe no or low signal, or a shift in the expected potency of the primary agonist, you should increase the stimulation time from 5 hours to 16 hours. You may also try alternative assay formulations. Our recommendations for alternative assay media include Growth Media alone or Growth Media containing 1% FBS (instead of 10% FBS). You may also improve the assay performance through functional sorting by flow cytometry.

8.3 Isolation of Stable Cell Clones

Stable cell clones can be isolated via a variety of methods. If a flow cytometer with sorting capabilities is available, we recommend following the method outlined in **Section 9** for isolating stable clones. If a flow cytometer with sorting capabilities is not available, you may utilize standard methods for clonal isolation such as limiting dilutions or cloning rings and test the resultant clones for a response to the primary agonist.

9. FACS Sorting

9.1 Preparing Cells for Sorting by Flow Cytometry

1. At least one day before the sort, split cells into two T75 flask (one for continual growth as the "unsorted" sample, the other for the "stimulated" cells, which should be ~80–90% confluent at the time stimulation), and a T25 flask (for the "unstimulated" cells).

   For 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 37ºC 5% CO_2 then move on to FACS sorting.

   For 16–20 hour stimulation:

   a. 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 EC_{50} concentration of the agonist.

   b. Stimulate the cells for ~16–20 hrs at 37ºC 5% CO_2 then move on to FACS sorting.

2. After stimulation, trypsinize the stimulated T75 flask and the unstimulated T25 flask. Spin cells down and re-suspend in FACS Sort Buffer. Load the cells at 1–2 × 10^6 cells/ml. You may scale recipe as needed.

   FACS Sort Buffer: PBS without Calcium and Magnesium, 1.0% glucose, 1 mM EDTA, 25 mM HEPES pH 7.4

   a. 6 µl CCF4-AM (Solution A)

   b. 60 µl Solution B

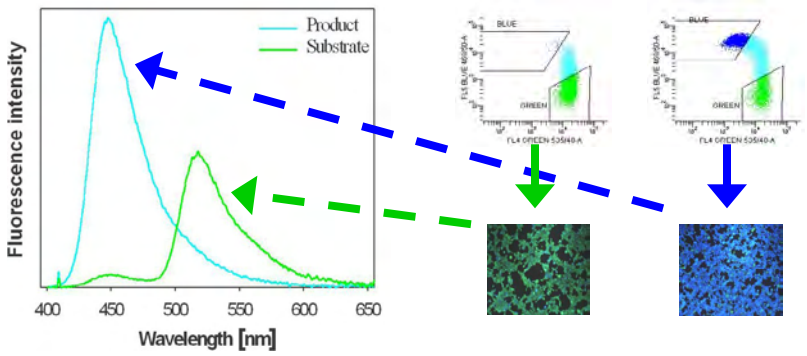
   c. Bring up to 6 ml with FACS Sort Buffer

9.2 Instrument Setup

1. **Requirements:** Flow cytometer with sorting capabilities (*e.g.*, VantageSE, Aria, MoFlow, Epics) equipped with a violet laser (407 nm, 409 nm, 413 nm, or multiline 407–415 nm). **Note:** It is possible to use UV excitation (337.5–356.4 nm). However, resolution between the green and blue emission signal will be closer together and harder to resolve on cell lines with low response.

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

3. **Suggested nozzle tip sizes:** 70 µm for Jurkat or lymphocyte-like cell types, and 90 or 100 µm for HEK, HeLa or CHO cells.

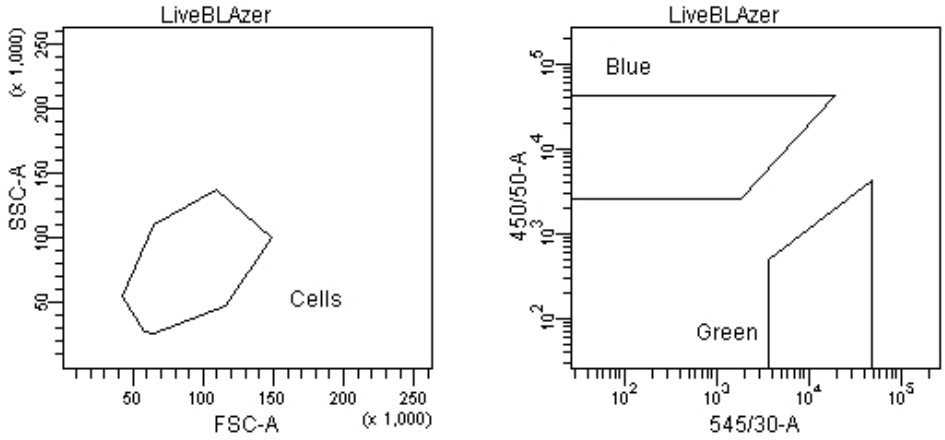


9.3 Alignment

1. Follow instrument manufacturer's alignment procedures. Use a 2 µm yellow/green bead as an alignment bead (*e.g.* Fluoresbrite® YG Microspheres 2.00 µm, Cat. no. 18338 from Polysciences). These beads are excited by the violet laser and bead emission is detectable in both the green and blue emission filters/detectors.
2. Control cells are available from Invitrogen (GeneBLAzer® Jurkat Control Kit, Cat. no.K1206, and GeneBLAzer® CHO-K1 Control Kit, Cat. no.K1207). Use these cells only as confirmation that the flow cytometer or the microscope is properly set up for GeneBLAzer® detection. Do **NOT** use these cells for setting the detector prior to running the cell line to be sorted. Do **NOT** use these cells for comparing the fluorescence intensities of developing cell lines.

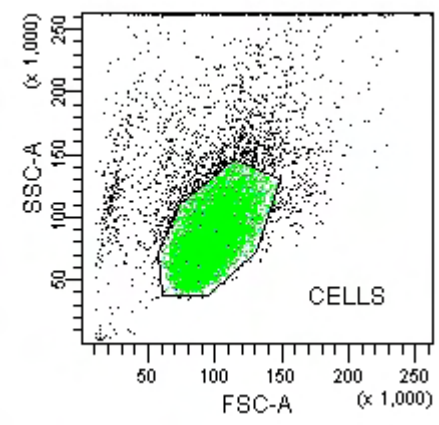
9.4 Experimental Setup

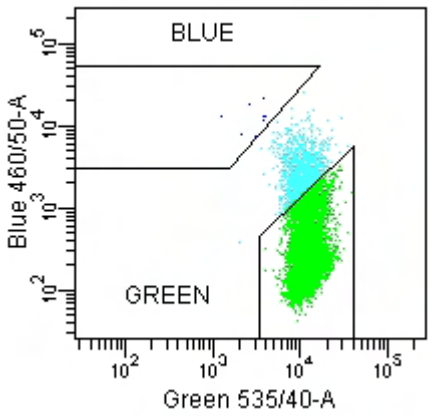
1. Create a FSC vs. SSC dot plot. Draw a gate for the cell population
2. Create a Green vs. Blue dot plot. Format this dot plot to look at the cell population. Draw gate for the green and blue populations.



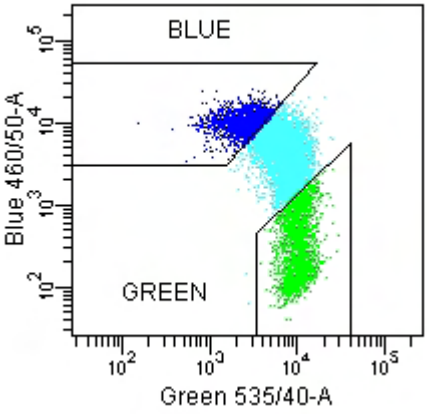
9.5 Running Cell Samples

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, cell loading is poor and then move the gate to adjust for signal.



% Blue response =17.4%

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

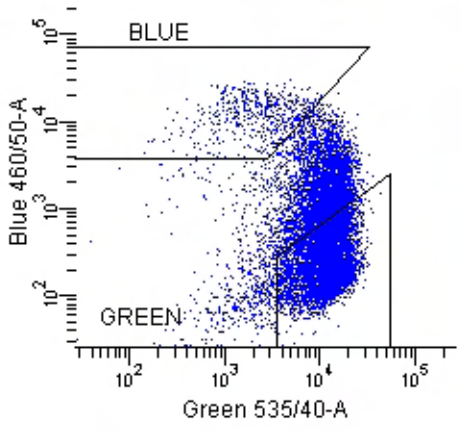
6. At this time increase compensation values to separate the blue population from the green signal. Make certain that events are not lost on the Blue (y) axis. Record data, stopping the file on 10,000 events in the cell gate.
7. Calculate % blue response by subtracting the unstimulated % blue response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

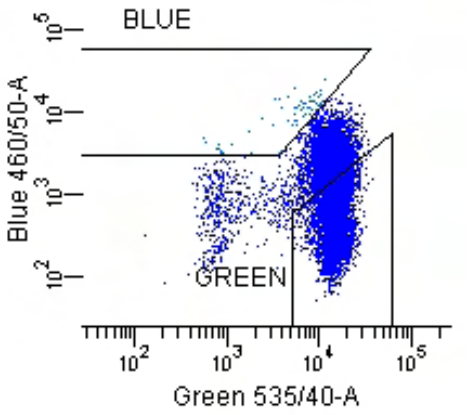
9.5.1 Sorting Strategies

- 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. If the blue background is high, re-sort unstimulated green responsive cells in pooled sort.

9.5.2 Identifying Poor Loading Conditions

- Below are examples of poor loading caused by shortened loading time or insufficient amount of substrate for cell concentration.





9.5.3 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 25 mM HEPES acclimated in a 37°C incubator.
- For long pooled sorts, collect into straight dialyzed serum (*e.g.* GIBCO® Fetal Bovine Serum, dialyzed, Cat. no. 26400-044). It is not recommended to collect a large pooled sort into growth media as salts will eventually form between the carbonate in the media and the phosphate in the buffer, impacting cell survivability.

10. Assay Procedure

The following instructions outline the recommended procedure for determining activity of compounds as modulators of CXCR3 using LiveBLAzer™-FRET B/G Substrate as the readout. If alternative substrates are used (*e.g.* ToxBLAzer™ DualScreen or LyticBLAzer™ Loading kits), follow the loading protocol provided with the product.

10.1 Quick Assay Reference Guides

For a more detailed assay protocol, see **Section 10.2.**

Agonist Assay Quick Reference Guide

	Unstimulated Wells	Stimulated Wells	Cell-free Wells	Test Compound Wells
Step 1 Plate cells	32 µl cells in Assay Medium (10,000 cells/well)	32 µl cells in Assay Medium (10,000 cells/well)	32 µl Assay Medium (no cells)	32 µl cells in Assay Medium (10,000 cells/well)
Step 2 Add Agonist or Test Compounds	8 µl Assay Medium with 0.5% DMSO	8 µl 5X agonist in Assay Medium with 0.5% DMSO	8 µl Assay Medium with 0.5% DMSO	8 µl 5X Test Compounds in 0.5% DMSO
Step 3 Incubate cells	Incubate in a humidified 37°C/5% CO_2 incubator for 16 hours			
Step 4 Prepare 6X Substrate Mix	6 µl of 1 mM LiveBLAzer™-FRET B/G (CCF4-AM) Substrate + 60 µl of solution B, mix. Add 904 µl of Solution C, mix. Add 30 µl of Solution D, mix.			
Step 5 Add Substrate Mixture	8 µl per well			
Step 6 Incubate Substrate Mix + cells	2 hours at room temperature in the dark			
Step 7 Detect activity	See **Section 10.4**			
Step 8 Analyze data	See **Section 11**			

Antagonist Assay Quick Reference Guide

	Unstimulated Wells	Stimulated Wells	Antagonist Control Wells	Cell-free Wells	Test Compound Wells
Step 1 Plate cells, incubate	32 µl cells in Assay Medium (10,000 cells/well)	32 µl cells in Assay Medium (10,000 cells/well)	32 µl cells in Assay Medium (10,000 cells/well)	32 µl Assay Medium (no cells)	32 µl cells in Assay Medium (10,000 cells/well)
Step 2 Add Antagonist or Test Compounds, incubate	4 µl Assay Medium with 0.5% DMSO	4 µl Assay Medium with 0.5% DMSO	4 µl 10X antagonist in Assay Medium with 0.5% DMSO	4 µl Assay Medium with 0.5% DMSO	4 µl 10X Test Compounds in Assay Medium with 0.5% DMSO
	Incubate plate with Antagonist for 30 minutes before proceeding				
Step 3 Add Agonist	4 µl Assay Medium with 0.5% DMSO	4 µl 10X agonist in Assay Medium with 0.5% DMSO	4 µl 10X agonist in Assay Medium with 0.5% DMSO	4 µl 10X agonist in Assay Medium with 0.5% DMSO	4 µl 10X agonist in Assay Medium with 0.5% DMSO
Step 4 Incubate cells	Incubate in a humidified 37°C/5% CO_2 incubator for 16 hours				
Step 5 Prepare 6X Substrate Mix	6 µl of 1 mM LiveBLAzer™-FRET B/G (CCF4-AM) Substrate + 60 µl of solution B, mix. Add 904 µl of Solution C, mix. Add 30 µl of Solution D, mix.				
Step 6 Add Substrate Mixture	8 µl per well				
Step 7 Incubate Mixture	2 hours at room temperature in the dark				
Step 8 Detect activity	See **Section 10.4**				
Step 9 Analyze data	See **Section 11**				

10.2 Detailed Assay Protocol

Plate layouts and experimental outlines will vary; in screening mode, we recommend using at least three wells for each control: Unstimulated Control, Stimulated Control, and Cell-free Control.

Note: Some solvents may affect assay performance. Assess the effects of solvent before screening. The cell stimulation procedure described below is carried out in the presence of 0.1% DMSO to simulate the effect that a Test Compound's solvent might have on the assay. If you use other solvents and/or solvent concentrations, optimize the following assay conditions appropriately.

10.2.1 Precautions

- Work on a dust-free, clean surface. Always handle the 384-well, black-wall, clear-bottom assay plate by the sides; do not touch the clear bottom of the assay plate.
- If pipetting manually, you may need to centrifuge the plate briefly at room temperature (for 1 minute at 14 × *g*) after additions to ensure all assay components are on the bottom of the wells.

10.2.2 Plating Cells

1. Harvest cells and resuspend in Assay Medium to a density of 312,500 cells/ml.
2. Add 32 µl per well of the Assay Medium to the Cell-free Control wells. Add 32 µl per well of the cell suspension to the Test Compound wells, the Unstimulated Control wells, and Stimulated Control wells. Proceed to **Section 10.2.3** for an Agonist assay or **Section 10.2.4** for an Antagonist assay.

10.2.3 Agonist Assay Plate Setup

Note: This subsection provides directions for performing an Agonist assay. See **Section 10.2.4** for directions for performing an Antagonist assay.

1. Prepare a stock solution of 0.5% DMSO in Assay Medium.
2. Prepare a 5X stock of Test Compounds in Assay Medium with 0.5% DMSO.
3. Prepare a 5X stock of agonist in Assay Medium with 0.5% DMSO. We recommend running a dose response curve to determine the optimal concentration of the agonist solution.
4. Add 8 µl of the stock solution of 0.5% DMSO in Assay Medium to the Unstimulated Control and Cell-free Control wells.
5. Add 8 µl of the 5X stock solution of agonist to the Stimulated Control wells.
6. Add 8 µl of the 5X stock of Test Compounds to the Test Compound wells.
7. Incubate the Agonist assay plate in a humidified 37°C/5% CO_2 incubator for 16 hours. Then proceed to **Section 10.3** for Substrate Loading and Incubation.

10.2.4 Antagonist Assay Plate Setup

Note: This subsection provides directions for performing an Antagonist assay. See **Section 10.2.3** for directions for performing an Agonist assay.

1. Prepare a stock solution of 0.5% DMSO in Assay Medium.
2. Prepare a 10X stock of Test Compounds in Assay Medium with 0.5% DMSO.
3. Prepare a 10X stock of agonist in Assay Medium with 0.5% DMSO. We recommend running a dose response curve to determine the optimal agonist concentration. For antagonist assays, we recommend stimulating cells initially with an agonist concentration in the EC_{50}-EC_{80} range.
4. Prepare a 10X stock of antagonist in Assay Medium with 0.5% DMSO. We recommend running a dose response curve to determine the optimal inhibition concentration for the Antagonist solution.
5. Add 4 µl of the 10X stock of Test Compounds to the Test Compound wells.
6. Add 4 µl of the stock solution of 0.5% DMSO to the Stimulated Control wells, the Unstimulated Control wells, and the Cell-free Control wells.
7. Add 4 µl of the 10X stock of antagonist in Assay Medium with 0.5% DMSO to the Antagonist Control wells.
8. If desired, incubate the Test Compounds with the cells humidified 37°C/5% CO_2 incubator before proceeding. Typically, a 30-minute incubation is sufficient.
9. Add 4 µl of the 10X stock solution of agonist to the Test Compound wells, the Stimulated Control wells, and the Antagonist Control wells.
10. Add 4 µl of Assay Medium with 0.5% DMSO to the Unstimulated Control and Cell-free Control wells.

11. Incubate the Antagonist assay plate in a humidified 37°C/5% CO_2 incubator for 16 hours. Then proceed to **Section 10.3** for Substrate Loading and Incubation.

10.3 Substrate Preparation, Loading, and Incubation

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

Prepare LiveBLAzer™-FRET B/G Substrate Mixture (CCF4-AM) Substrate Mixture and load cells in the absence of direct strong lighting. Turn off the light in the hood.

1. Prepare Solution A: 1 mM LiveBLAzer™-FRET B/G Substrate (CCF4-AM) Substrate Mixture in dry DMSO by adding 912 µl of DMSO per mg of dry substrate. Store the aliquots of the stock solution at –20ºC until use. The molecular weight of the LiveBLAzer™-FRET B/G Substrate (CCF4-AM) is 1096 g/mol.
2. Prepare 6X Loading Solution:
   a. Add 6 µl of Solution A to 60 µl of Solution B and vortex.
   b. Add 904 µl of Solution C to the above solution and vortex.
   c. Add 30 µl of Solution D to the above solution and vortex.
3. Remove assay plate from the humidified 37°C/5% CO_2 incubator.

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

4. Add 8 µl of the 6X Substrate Mixture to each well.
5. Cover the plate to protect it from light and evaporation.
6. Incubate at room temperature for 2 hours.

10.4 Detection

Make measurements 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.

10.4.1 Instrumentation, Filters, and Plates

- Fluorescence plate reader with bottom reading capabilities.
- Recommended filters for a filter based fluorescence plate reader:
  - Excitation filter: 409/20 nm
  - Emission filter: 460/40 nm
  - Emission filter: 530/30 nm
- Recommended settings for a monochrometer based instrument:
  - Excitation filter: 409 nM
  - Emission filter: 460 nM
  - Emission filter: 530 nM

Note: 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 (see section **11.2**).

10.4.2 Reading an Assay Plate

1. Set the fluorescence plate reader to bottom-read mode with optimal gain and 5 reads.
2. Allow the lamp in the fluorescence plate reader to warm up for at least 10 minutes 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)

11. Data Analysis

11.1 Background Subtraction and Ratio Calculation

We recommend that you subtract the background for both emission channels (460 nm and 530 nm).

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.

11.2 Visual Observation of Intracellular Beta-lactamase Activity Using 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 with either a xenon or mercury excitation lamp may be used to view the LiveBLAzer™-FRET B/G Substrate (CCF4-AM) signal in cells. To visually inspect the cells, you will need a long-pass filter passing blue and green fluorescence light, so that your eye can visually identify whether the cells are fluorescing green or blue.

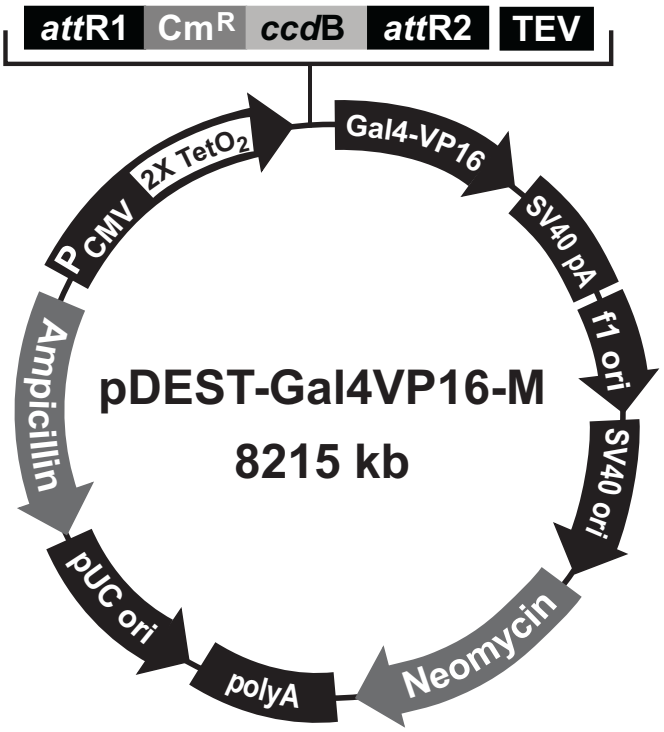
Recommended filter sets for observing beta-lactamase activity are described below and are available from Chroma Technologies (800-824-7662, www.chroma.com).

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. For epifluorescence microscopes, a long-pass dichroic mirror is needed to separate excitation and emission light and should be matched to the excitation filter (to maximally block the excitation light around 405 nm, yet allow good transmission of the emitted light).

12. Tango™ pDEST-Gal4VP16-M Vector Map



Comments for pDEST-Gal4VP16-M
8215 nucleotides

CMV promoter: bases 1-588
    TATA box: bases 502-508
    Tetracycline operator (2X $TetO_2$) sequence: bases 518-557
*att*R1 recombination site: bases 691-815
Chloramphenicol resistance gene: bases 924-1583
*ccd*B gene: bases 1925-2230
*att*R2 recombination site: bases 2271-2395
TEV cleavage site (ENLYFQM): bases 2404-2424
Gal4-VP16 transcriptional activator: bases 2428-3117
    Gal4 DNA binding domain: bases 2428-2865
    VP16: bases 2887-3117
SV40 polyadenylation region: bases 3378-3644
f1 origin: bases 3770-4225
SV40 early promoter and origin: bases 4385-4693
Neomycin resistance ORF: bases 4752-5546
Polyadenylation region: bases 5610-5658
pUC origin: bases 5900-6573 (complementary strand)
Ampicillin (*bla*) resistance ORF: bases 6718-7578 (complementary strand)

13. References

1. Zlokarnik, G., *et al,* **Quantitation of Transcription and Clonal Selection of Single Living Cells with β-Lactamase as Reporter,** (1998) *Science*; **279:** p84-88.
2. Kunapuli P, Ransom R, Murphy K, Pettibone D, Kerby J, Grimwood S, Zuck P, Hodder P, Lacson R, Hoffman I, Inglese J, Strulovici B, **Development of an Intact Cell Reporter Gene β-lactamase Assay for G Protein-coupled Receptors,** (2003) *Analytical Biochem.;* **314:** 16-29.
3. Xing, H., Pollok, B., *et al*, **A Fluorescent Reporter Assay For The Detection of Ligands Acting Through G1 Protein-coupled Receptors,** (2000) *J. Receptor & Signal Transduction Research*; **20**(4), 189-210.
4. Qureshi, S., *et al,* **A One-Arm Homologous Recombination Approach for Developing Nuclear Receptor Assays in Somatic Cells,** (2003) *Assay and Drug Dev. Tech;* **Vol 1,** No. 6:p755-66.
5. Peekhaus, N. et al, **A β-Lactamase-Dependent Gal4-Estrogen Receptor βTransactivation Assay for the Ultra-High Throughput Screening of Estrogen Receptor βAgonists in a 3,456-Well Format,** (2003) Assay and Drug Dev Tech, **Vol. 1**, No. 6: 789-800.
6. Chin, J., *et al*, **Miniaturization of Cell-Based β-Lactamase-Dependent FRET Assays to Ultra-High Throughput Formats to Identify Agonists of Human Liver X Receptors**, (2003) Assay and Drug Dev. Tech.; **Vol. 1**, **No. 6**: 777-787.
7. Whitney M, Rockenstein E, Cantin G, Knapp T, Zlokarnik G, Sanders P, Durick K, Craig FF, Negulescu PA., **A Genome-wide Functional Assay of Signal Transduction in Living Mammalian Cells,** (1998)*Nat. Biotechnol.*;**16(13)**:1329-33.
8. Andersson, S., Davis, D. L., Dahlbäck, H., Jörnvall, H., and Russell, D. W., **Cloning, Structure, and Expression of the Mitochondrial Cytochrome P-450 Sterol 26-Hydroxylase, a Bile Acid Biosynthetic Enzyme,** (1998*) J. Biol. Chem.*; **264**, 8222-8229.
9. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K, **Current Protocols in Molecular Biology** (1994) (New York: Greene Publishing Associates and Wiley-Interscience).
10. Boshart, M., Weber, F., Jahn, G., Dorsch-Häsler, K., Fleckenstein, B., and Schaffner, W. (1985). **A Very Strong Enhancer is Located Upstream of an Immediate Early Gene of Human Cytomegalovirus.** *Cell* **41**, 521-530.
11. Chen, C., and Okayama, H. (1987). **High-Efficiency Transformation of Mammalian Cells by Plasmid DNA.** *Mol.Cell. Biol*. **7**, 2745-2752.
12. Chu, G., Hayakawa, H., and Berg, P. (1987). **Electroporation for the Efficient Transfection of Mammalian Cells with DNA.** *Nuc. Acids Res.* **15**, 1311-1326.
13. Felgner, P. L., Holm, M., and Chan, H. (1989). Cationic **Liposome Mediated Transfection.** *Proc. West. Pharmacol. Soc.* **32**, 115-121.
14. Felgner, P. L., and Ringold, G. M. (1989). **Cationic Liposome-Mediated Transfection**. *Nature* **337**, 387-388.
15. Goodwin, E. C., and Rottman, F. M. (1992). **The 3´-Flanking Sequence of the Bovine Growth Hormone Gene Contains Novel Elements Required for Efficient and Accurate Polyadenylation.** *J. Biol. Chem.* **267**, 16330-16334.
16. Gossen, M., and Bujard, H. (1992). **Tight Control of Gene Expression in Mammalian Cells by Tetracycline-Responsive Promoters**. *Proc. Natl. Acad. Sci. USA* **89,** 5547-5551.
17. Hillen, W., and Berens, C. (1994). **Mechanisms Underlying Expression of Tn10 Encoded Tetracycline Resistance.** *Annu. Rev. Microbiol.* **48,** 345-369.
18. Hillen, W., Gatz, C., Altschmied, L., Schollmeier, K., and Meier, I. (1983). **Control of Expression of the Tn10-encoded Tetracycline Resistance Genes: Equilibrium and Kinetic Investigations of the Regulatory Reactions.** *J. Mol. Biol.* **169,** 707-721.
19. Kozak, M. (1987). **An Analysis of 5´-Noncoding Sequences from 699 Vertebrate Messenger RNAs.** *Nuc. Acids Res.* **15**, 8125-8148.
20. Kozak, M. (1991). **An Analysis of Vertebrate mRNA Sequences: Intimations of Translational Control.** *J. Cell Biol.* **115**, 887-903.
21. Kozak, M. (1990). **Downstream Secondary Structure Facilitates Recognition of Initiator Codons by Eukaryotic Ribosomes**. *Proc. Natl. Acad. Sci. USA* **87**, 8301-8305.
22. Nelson, J. A., Reynolds-Kohler, C., and Smith, B. A. (1987). **Negative and Positive Regulation by a Short Segment in the 5´-Flanking Region of the Human Cytomegalovirus Major Immediate-Early Gene.** *Mol. Cell. Biol.* **7**, 4125-4129.
23. Neumann, J. R., Morency, C. A., and Russian, K. O. (1987). **A Novel Rapid Assay for Chloramphenicol Acetyltransferase Gene Expression.** *BioTechniques* **5**, 444-447.

24. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). **Molecular Cloning: A Laboratory Manual, Second Edition** (Plainview, New York: Cold Spring Harbor Laboratory Press).

25. Shigekawa, K., and Dower, W. J. (1988). **Electroporation of Eukaryotes and Prokaryotes: A General Approach to the Introduction of Macromolecules into Cells.** *BioTechniques* **6**, 742-751.

26. Southern, P. J., and Berg, P. (1982). **Transformation of Mammalian Cells to Antibiotic Resistance with a Bacterial Gene Under Control of the SV40 Early Region Promoter.** *J. Molec. Appl. Gen.* **1,** 327-339.

27. Wigler, M., Silverstein, S., Lee, L.-S., Pellicer, A., Cheng, Y.-C., and Axel, R. (1977). **Transfer of Purified Herpes Virus Thymidine Kinase Gene to Cultured Mouse Cells.** *Cell* **11,** 223-232.

28. Kapust, R. B., Tozser, J., Copeland, T.D., Waugh, D.S.. (2002) **The P10 specificity of tobacco etch virus protease.** *Biochem. Biophys. Res. Com.* **294,** 949-955.

29. Dull, T., Zufferey, R., Kelly, M., Mandel, R. J., Nguyen, M., Trono, D., and Naldini, L. (1998) **A Third-Generation Lentivirus Vector with a Conditional Packaging System.** *J. Virol.* **72**, 8463-8471.

30. Luciw, P. A. (1996) in **Fields Virology** (Fields, B. N., Knipe, D. M., Howley, P. M., Chanock, R. M., Melnick, J. L., Monath, T. P., Roizman, B., and Straus, S. E., eds), 3rd Ed., pp. 1881-1975, Lippincott-Raven Publishers, Philadelphia, PA.

31. Kjems, J., Brown, M., Chang, D. D., and Sharp, P. A. (1991) **Structural Analysis of the Interaction Between the Human Immunodeficiency Virus Rev Protein and the Rev Response Element.** *Proc. Natl. Acad. Sci. USA* **88,** 683-687.

32. Yao, F., Svensjo, T., Winkler, T., Lu, M., Eriksson, C., and Eriksson, E. (1998). **Tetracycline Repressor, tetR, Rather than the tetR-Mammalian Cell Transcription Factor Fusion Derivatives, Regulates Inducible Gene Expression in Mammalian Cells**. *Hum. Gene Ther.* 9, 1939-1950.

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