Promega

Technical Manual

Protease-Glo™ Assay

INSTRUCTIONS FOR USE OF PRODUCTS G9451 AND G9461.

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PRINTED IN USA.
Revised 2/11

Part# TM303

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Protease-Glo™ Assay

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1. Description

The Protease-Glo™ Assay[a–e] is a novel method to detect and measure protease activities using a genetically engineered firefly (*Photinus pyralis*) luciferase and represents one example of the GloSensor™ platform technology (1). The assay uses a circularly permuted firefly luciferase, the GloSensor™-10F protein, with a protease recognition site as the protease substrate (1; Figure 1). This assay system allows rapid generation of protease substrates through molecular cloning and coupled transcription/translation cell-free expression, thus enabling the facile evaluation of protease function (2).

Oligonucleotides encoding a protease recognition sequence are designed and cloned into the GloSensor™-10F gene located on a linearized vector. The GloSensor™ protein containing the protease site of interest is then synthesized in a cell-free protein expression system and subsequently used as a protease substrate. Cleavage of the protease recognition sequence leads to activation of the GloSensor™ protein and light emission (Figure 1). The level of luminescence correlates to protease activity (1,2). The Protease-Glo™ Assay has the advantage of a bioluminescent readout, which provides easy quantitation, high sensitivity and wide dynamic range (1–3).

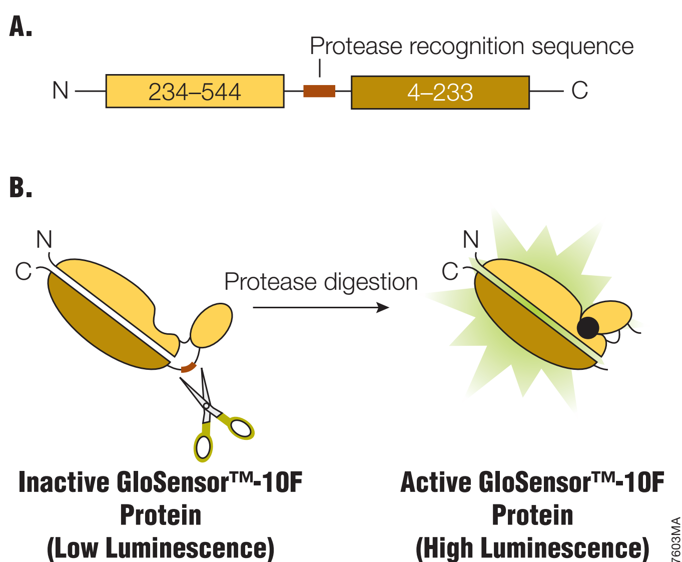
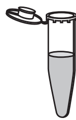
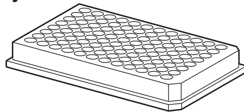
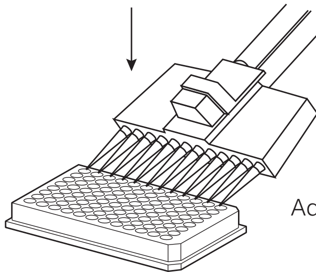


Figure 1. Modulation of firefly luciferase with a polypeptide linker between the N- and C-termini. Panel A. To generate the GloSensor™ protein, new N- and C-termini were created at amino acids 234 and 233, respectively. The protein-coding region of this circularly permuted firefly luciferase is carried on the pGloSensor™-10F Linear Vector. **Panel B.** Insertion of a short polypeptide linker containing a protease recognition sequence between the native N- and C-termini of firefly luciferase greatly reduces luciferase activity. Proteolytic cleavage of the polypeptide linker by the cognate protease (represented by the scissors) activates the luciferase enzyme, resulting in an increase in luminescent signal in the presence of a firefly luciferase substrate (represented by the black circle).







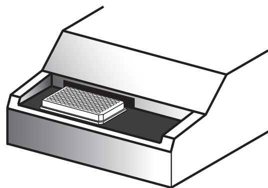


Figure 2. Overview of the Protease-Glo™ Assay. Oligonucleotides encoding the protease recognition sequence of interest are designed and cloned into the pGloSensor™-10F Linear Vector. The resulting GloSensor™[protease site] protein is expressed in a cell-free protein expression system and interrogated with the cognate protease. The Bright-Glo™ Assay Reagent is then added to an aliquot of the protease digestion reaction, and luminescence is measured.

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2. Product Components and Storage Conditions

Product	Size	Cat.#
Protease-Glo™ Assay	1 kit	G9451

Includes:

- 200µl Rapid Ligation Buffer, 2X
- 100 units T4 DNA Ligase
- 1.0µg pGloSensor™-10F Linear Vector
- 1ml Oligo Annealing Buffer
- 1.25ml Nuclease-Free Water
- 300µl TNT® SP6 High-Yield Wheat Germ Master Mix
- 10ml Bright-Glo™ Assay Buffer
- 1 vial Bright-Glo™ Luciferase Assay Substrate
- 15µg pGloSensor™-10F[TEV] Control Plasmid (150ng/µl)
- 1,000 U ProTEV Plus (5U/µl)
- 1ml ProTEV Buffer, 20X
- 250µl DTT, 100mM
- 1 Protocol

Storage Conditions: Store all components at –20°C, except the TNT® SP6 High-Yield Wheat Germ Master Mix, which must be stored at –70°C.

Available Separately

Product	Size	Cat.#
pGloSensor™-10F Linear Vector	1.0µg	G9461

3. Oligonucleotide Design

We strongly recommend that you use the Protease-Glo™ Oligonucleotide Designer tool available at: **www.promega.com/techserv/tools/** to design oligonucleotides. Simply input the single-amino acid code of the recognition sequence for your protease of interest, and the tool will design the oligonucleotide sequences.

If you choose to design your own oligonucleotides, use this section as a guide. Section 3.A provides guidelines to design the protease recognition portion of the oligonucleotides. Section 3.B provides guidelines to design the complete oligonucleotide sequences.

3.A. Selecting a Suitable Protease Recognition Sequence

Choose a protease target sequence. Recognize that a shorter amino acid sequence increases the probability that the GloSensor™ protein itself will be cleaved, while a longer sequence may result in a higher basal luminescence and, therefore, a reduced dynamic range. We have tested and recommend target sequences 4–14 amino acids long. General guidelines for target sequence selection are being developed continuously as more is learned about each protease. Published literature may serve as a reliable guide for protease recognition sequence selection; however, the optimal target sequence may have to be determined experimentally (4).

Use the pGloSensor™-10F Linear Vector containing a nonspecific target sequence or scrambled sequence or the pGloSensor™-10F[TEV] Control Plasmid as a negative control for your protease of interest recognition sequence.

3.B. Assay Oligonucleotide Design

Two complementary DNA oligonucleotides (oligonucleotides A and B, supplied by the user) are annealed to form a double-stranded linker for ligation into the pGloSensor™-10F Linear Vector (Figures 3 and 4).

For target sequences 4–14 amino acids long, the oligonucleotides should be 18–48bp in length. Since detection of a 48bp insert is difficult using an agarose gel, we have devised a method to detect inserts by SgfI/PmeI digestion (Section 4.E). The pGloSensor™-10F Linear Vector contains single sites for both SgfI and PmeI. Insertion of the oligonucleotides results in a 1.6kb fragment upon SgfI/PmeI digestion. A second confirmatory screen may be performed by expressing the GloSensor™[protease site] protein using the TNT® SP6 High-Yield Wheat Germ Master Mix (Section 4.F). The correct size of the GloSensor™ protein is 61kDa.

Note: Two oligonucleotides must be synthesized for each amino acid sequence tested. Standard desalting of the oligonucleotides is required prior to use; 5´ phosphorylation is not required. Gel purification is not required unless recommended by the oligonucleotide manufacturer.

Oligonucleotide A	5´ CTAGC—N_{12-42}—G 3´
Oligonucleotide B	3´ G—N_{12-42}—CCTAG 5´

Figure 3. Annealed oligonucleotides A and B. Note that the sequence for oligonucleotide B is represented in the 3´ to 5´ orientation.

3.B. Assay Oligonucleotide Design (continued)

Oligonucleotide A

Oligonucleotide A should contain the following elements:

- **5´ Overhang Sequence.** This five-nucleotide sequence (CTAGC) is complementary to the pGloSensor™-10F Linear Vector overhang and oligonucleotide B. Ligation of the annealed oligonucleotides A and B will create the flexible amino acid linker: Ala-Ser.
- **Protease Recognition Sequence.** A nucleic acid sequence encoding the protease recognition sequence (designated by $N_{12–42}$ in Figure 3) begins immediately after the 5´ overhang sequence to ensure the protease recognition sequence is in the correct reading frame. We recommend that this nucleotide sequence code for a total of 4–14 amino acids.
- **3´ Overhang sequence.** This single nucleotide (G) is complementary to the overhang of oligonucleotide B. Ligation of the annealed oligonucleotides A and B will create a flexible amino acid linker: Gly-Ser.

  Note: To create the flexible linker, the BglII restriction site will be destroyed during ligation of the annealed oligonucleotides (Figure 4 and Section 11.B).

Oligonucleotide B

Oligonucleotide B has regions that are complementary to oligonucleotide A with additional sequences for ligation into pGloSensor™-10F Linear Vector. Oligonucleotide B should contain the following elements:

- **5´ Overhang Sequence.** This five-nucleotide sequence (GATCC) is complementary to the pGloSensor™-10F Linear Vector overhang and oligonucleotide A. Ligation of the annealed oligonucleotides A and B will create the flexible amino acid linker: Gly-Ser.

  Note: To create the flexible linker, the BglII restriction site will be destroyed during ligation of the annealed oligonucleotides (Figure 4 and Section 11.B).
- **Reverse Complement of the Protease Recognition Sequence.** This nucleotide sequence is the reverse complement of the protease recognition sequence of oligonucleotide A.
- **3′ Overhang sequence.** This single nucleotide (G) is complementary to the overhang of oligonucleotide A. Ligation of the annealed oligonucleotides A and B will create a flexible amino acid linker: Ala-Ser.

An example of oligonucleotide sequences encoding the 7-amino-acid Tobacco Etch Virus (TEV) protease recognition sequence, Glu Asn Leu Tyr Phe Gln Ser, is shown in Figure 4. Each oligonucleotide (shown in the shaded boxes) in this example is 27 nucleotides long.

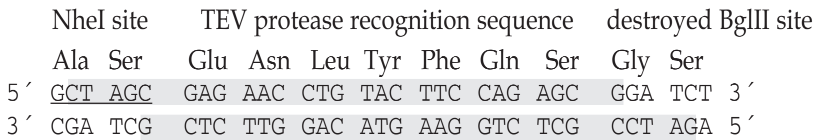

Figure 4. Example protease recognition sequence. The NheI site (underlined) is retained during the cloning process, but the BglII site is destroyed.

4. Cloning a Protease Recognition Sequence Insert into the pGloSensor™-10F Linear Vector

Materials to Be Supplied by the User
(Solution compositions are provided in Section 11.C.)

- oligonucleotide A (Section 3)
- oligonucleotide B (Section 3)
- competent cells with a transformation efficiency >1 × 10^8cfu/µg (e.g., JM109 Competent Cells, >10^8cfu/µg, Cat.# L2001)
- 1.5ml polypropylene microcentrifuge tubes or 17 × 100mm polypropylene tubes (e.g., Falcon™ tubes; Becton, Dickinson and Company Cat.# 2059)
- LB plates with ampicillin
- SOC medium

4.A. Oligonucleotide Dilution and Annealing

1. Resuspend each oligonucleotide (A and B) in TE buffer or Nuclease-Free Water to a final concentration of 100µM.
2. Assemble the annealing reaction as described below.

oligonucleotide A (100µM)	2µl
oligonucleotide B (100µM)	2µl
Oligo Annealing Buffer	46µl
Total volume	**50µl**

   Note: The final concentration of each oligonucleotide is 4µM.

3. Heat the annealing reaction at 90°C for 3 minutes.
4. Incubate for 15 minutes at room temperature.
5. The annealed oligonucleotides can be used immediately or stored at –20°C for up to 1 month. If the annealed oligonucleotides are stored at –20°C, thaw at room temperature prior to use. Avoid thawing the annealed oligonucleotides at temperatures above room temperature.

4.B. Ligation of an Insert into the pGloSensor™-10F Linear Vector

No manipulation of the pGloSensor™-10F Linear Vector is required prior to use in the ligation reaction.

1. Serially dilute the annealed oligonucleotides to a final concentration of 10nM just prior to assembling the ligation reaction.

annealed oligonucleotides	2µl
Nuclease-Free Water	38µl
Total volume	**40µl**

   a. Dilution 1: Dilute the annealed oligonucleotides from Section 4.A, Step 5, as described below.

   The final concentration of the annealed oligonucleotides is now 200nM.

   Note: Do not store the diluted oligonucleotides.

annealed oligonucleotides (200nM)	2µl
Nuclease-Free Water	38µl
Total volume	**40µl**

   b. Dilution 2: Dilute the annealed oligonucleotides from Step 1.a as described below.

Component	Negative Control Reaction (Minus Insert)	Standard Reaction
Rapid Ligation Buffer, 2X	5µl	5µl
pGloSensor™-10F Linear Vector (50ng)	1µl	1µl
annealed oligonucleotides A and B (10nM)	–	1µl
Nuclease-Free Water	3µl	2µl
T4 DNA Ligase	1µl	1µl
Total volume	**10µl**	**10µl**

   The final concentration of the annealed oligonucleotides is now 10nM.

2. Assemble the ligation reactions as described below. See Notes 1 and 2.
3. Mix the reactions by pipetting, and incubate at room temperature for 5–60 minutes. Alternatively, the reactions can be incubated overnight at 4°C.

Notes:

1. The 2X Rapid Ligation Buffer contains ATP, which degrades during temperature fluctuations. Avoid multiple freeze-thaw cycles and exposure to frequent temperature changes by making single-use aliquots of the buffer after it is thawed for the first time. Store the aliquots at –20°C.
2. Vortex the 2X Rapid Ligation Buffer before each use.

4.C. Transformation of *E. coli* with pGloSensor™[Protease Site] Plasmid

Be sure to use competent cells with a transformation efficiency of >1 × 10^8cfu/µg DNA to obtain the optimal number of colonies. Transformation efficiency can be determined by performing a control transformation reaction using a known quantity of supercoiled plasmid DNA and calculating the number of colony forming units (cfu) per microgram of DNA. See Note 2.

We recommend using JM109 Competent Cells, >10^8cfu/µg (Cat.# L2001). Other host strains, such as DH5α™, may be used. If you are using competent cells other than Promega JM109 Competent Cells, be sure to follow the appropriate transformation protocol. Select transformants on LB/ampicillin plates. For best results, do not use plates that are more than 1 month old.

1. Prepare two LB/ampicillin plates for each ligation reaction and control transformation. Equilibrate plates to room temperature prior to plating cells.
2. Remove the frozen, high-efficiency competent cells from –70°C storage, and place in an ice bath until just thawed (about 5 minutes). Mix cells by gently flicking the tube.
3. For each ligation reaction and transformation control, carefully transfer 50µl of cells into a sterile 17 × 100mm polypropylene tube on ice.

   Note: In our experience, using 17 × 100mm polypropylene tubes increases transformation efficiency. Tubes from some manufacturers bind DNA, thereby decreasing the colony number, and should be avoided.
4. Briefly centrifuge tubes containing the ligation reactions to collect contents at the bottom of the tube. Add 2µl of each ligation reaction to a tube of cells prepared in Step 3.

   To perform a transformation control, add 20pg of supercoiled plasmid DNA to one of the tubes prepared in Step 3.

   Note: The pGloSensor™-10F Linear Vector is not appropriate as a transformation control.
5. Gently flick the tubes to mix, and place them on ice for 20 minutes.
6. Heat-shock the cells for 45–50 seconds in a water bath at 42°C. Do not shake.
7. Immediately return the tubes to ice for 2 minutes.
8. Add 950µl of room-temperature SOC medium to each tube. LB broth may be substituted, but the number of colonies may be lower.
9. Incubate the tubes at 37°C with shaking (approximately 150rpm) for 1.5 hours.

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4.C. Transformation of *E. coli* with pGloSensor™[Protease Site] Plasmid (continued)

10. Plate 50µl of each transformation onto duplicate LB/ampicillin plates. If a higher number of colonies is desired, pellet the cells by centrifugation at 1,000 × *g* for 10 minutes, resuspend the cells in 200µl of SOC medium and plate 50µl of cells on each of two plates.
11. Incubate the plates overnight (16–24 hours) at 37°C. In our experience, at least 100 colonies per plate are routinely seen when using competent cells that are 1×10^8cfu/µg DNA if 50µl of unpelleted cells is plated.

Notes:

1. The negative control ligation reaction allows determination of the number of background colonies resulting from the pGloSensor™-10F Vector alone. A successful ligation reaction with insert typically yields at least 10 times more colonies than the negative control ligation reaction. The efficiency of ligation will depend on the length and sequence of the oligonucleotides.
2. The transformation efficiency of the competent cells is calculated as cfu/µg:

$$\frac{\text{colonies on control plate}}{\text{ng of supercoiled plasmid DNA plated}} \times \frac{1 \times 10^3\text{ng}}{\mu\text{g}}$$

4.D. Recombinant Plasmid DNA Purification

A standard plasmid miniprep procedure can be used to isolate plasmid DNA to screen for inserts. The miniprep process can be both laborious and time-consuming, particularly when large numbers of minipreps are required.

To generate a sufficient quantity of plasmid DNA for the subsequent cell-free transcription/translation and protease-detection reactions, we recommend performing a standard plasmid midiprep procedure. Although miniprep DNA is generally of sufficient purity, miniprep protocols may only yield enough plasmid DNA for one or two cell-free transcription/translation reactions. See Section 11.D for a list of appropriate plasmid DNA purification products.

4.E. Screening for Inserts Using SgfI/PmeI Digestion

We recommend screening 6–8 colonies. The pGloSensor™-10F Linear Vector contains a single SgfI site and a single PmeI site. Digestion with 10X Flexi Enzyme Blend (SgfI and PmeI) (Cat.# R1851) of the GloSensor™ plasmid containing an insert will yield two DNA fragments (approximately 3.4kb and 1.6kb). A typical digest reaction is performed as follows: 1µl of 10X Flexi Enzyme Blend (SgfI and PmeI) + 5µl (200–500ng) of plasmid DNA + 4µl of 5X Flexi Digest Buffer + 10µl of Nuclease-Free water. After mixing the reaction thoroughly by pipetting, incubate the reaction at 37°C for 2 hours. Add 5µl of loading dye (e.g., Blue/Orange Loading Dye, 6X, Cat.# G1881). Load 20µl of the reaction onto a 1% agarose gel, and separate DNA fragments by electrophoresis. Visualize the fragments by ethidium bromide staining. In our experience, pGloSensor™-10F plasmid that does not contain insert will yield the 1.6kb DNA fragment <1% of the time.

4.F. Screening for Inserts Using the TNT® SP6 High-Yield Wheat Germ Master Mix

A second confirmatory screen may be performed by expressing the GloSensor™[protease site] protein using the TNT® SP6 High-Yield Wheat Germ Master Mix. Protein can be expressed as recommended in the TNT® SP6 High-Yield Protein Expression System protocol (Section 5 or the *TNT® SP6 High-Yield Protein Expression System Technical Manual* #TM282) in the presence of label [e.g., [^{35}S]methionine or non-radioactive alternatives such as Transcend™ tRNA (Cat.# L5061) or FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System (Cat.# L5001)]. The appearance of a 61kDa protein, visualized after size-fractionating 2.5µl of the expressed protein by SDS polyacrylamide gel electrophoresis (SDS-PAGE), confirms the presence of an in-frame GloSensor™[protease site] protein.

This does not, however, confirm the presence of the correct oligonucleotide sequence. Absolute confirmation can be obtained by sequencing the construct. In our experience, 99% of constructs encoding the correctly sized 61kDa GloSensor™[protease site] protein were found to contain the correct oligonucleotides by sequencing.

4.G. Propagation of the pGloSensor™-10F[TEV] Control Plasmid

The pGloSensor™-10F[TEV] Control Plasmid is provided for use as a positive control in the protease assay. The plasmid can be propagated by transforming *E. coli* with 0.1ng DNA and selecting a positive clone on LB/ampicillin plates (Sections 4.C, 4.D and 4.E). We recommend retaining one positive clone as a glycerol stock stored at –70°C for future use.

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4.H. Flexi® Transfer of the GloSensor™[Protease Site] Protein-Coding Region

We have designed the pGloSensor™-10F Linear Vector to be compatible with our Flexi® Vector System. The GloSensor™[protease site]-coding region is flanked by recognition sequences for two rare-cutting enzymes, SgfI and PmeI. The Flexi® Vector System provides a rapid, efficient and high-fidelity way to transfer coding regions between a variety of Flexi® Vectors. The Flexi® Vectors contain various expression or peptide tag options to enable expression of native or fusion proteins to study protein structure and function as well as protein:protein interactions. For more information, see the *Flexi® Vector System Technical Manual* #TM254.

5. Production of the GloSensor™[Protease Site] Protein by Cell-Free Transcription/Translation

Materials to Be Supplied by the User

- [^{35}S]methionine, >1,000Ci/mmol (e.g., EasyTag™ L-[^{35}S]methionine, Perkin Elmer Cat.# NEG709A), Transcend™ tRNA (Cat.# L5061) or FluoroTect™ $\text{Green}_{\text{Lys}}$ in vitro Translation Labeling System (Cat.# L5001)

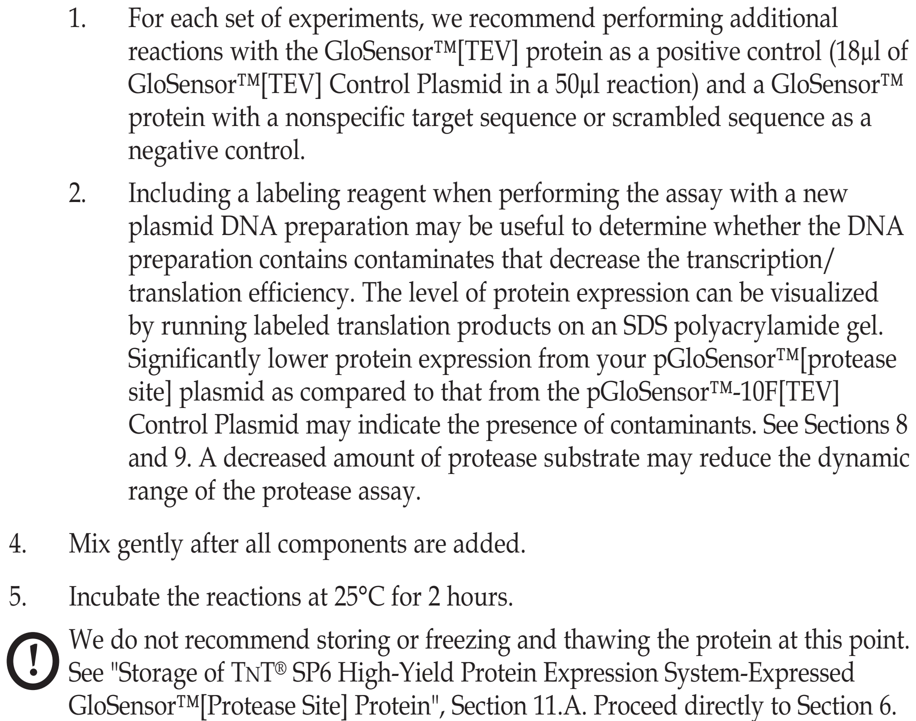
1. Remove the TNT® SP6 High-Yield Wheat Germ Master Mix from storage at –70°C. Rapidly thaw the master mix on ice or by hand warming, and immediately place on ice.

   Note: Except for reaction assembly and translation incubation, the TNT® SP6 High-Yield Wheat Germ Master Mix should be kept at 4°C or on ice. Assemble the TNT® reaction within 10 minutes of the lysate thawing. Any unused Master Mix should be refrozen in an ethanol/dry ice bath as soon as possible after thawing to minimize loss of translational activity. Do not expose the Master Mix to more than three freeze-thaw cycles.

2. After the master mix has thawed, gently mix several times with a pipette tip or by pipetting.

3. Following the example below, assemble a TNT® reaction for each of your pGloSensor™[protease site] plasmids and one reaction with no DNA. Add the reaction components to RNase-free and DNase-free 0.5ml or 1.5ml microcentrifuge tubes. See Notes 1 and 2.

Component	Reactions with Plasmid	No-DNA Control Reaction
TNT® SP6 High-Yield Wheat Germ Master Mix	30µl	30µl
pGloSensor™[protease site] plasmid	2–3µg	–
[^{35}S]methionine or Transcend™ tRNA or FluoroTect™ $\text{Green}_{\text{Lys}}$ tRNA (optional)	2–4µl	2–4µl
Nuclease-Free Water to a final volume	50µl	50µl


Notes:

1. For each set of experiments, we recommend performing additional reactions with the GloSensor™[TEV] protein as a positive control (18µl of GloSensor™[TEV] Control Plasmid in a 50µl reaction) and a GloSensor™ protein with a nonspecific target sequence or scrambled sequence as a negative control.
2. Including a labeling reagent when performing the assay with a new plasmid DNA preparation may be useful to determine whether the DNA preparation contains contaminates that decrease the transcription/translation efficiency. The level of protein expression can be visualized by running labeled translation products on an SDS polyacrylamide gel. Significantly lower protein expression from your pGloSensor™[protease site] plasmid as compared to that from the pGloSensor™-10F[TEV] Control Plasmid may indicate the presence of contaminants. See Sections 8 and 9. A decreased amount of protease substrate may reduce the dynamic range of the protease assay.

4. Mix gently after all components are added.
5. Incubate the reactions at 25°C for 2 hours.

We do not recommend storing or freezing and thawing the protein at this point. See "Storage of TNT® SP6 High-Yield Protein Expression System-Expressed GloSensor™[Protease Site] Protein", Section 11.A. Proceed directly to Section 6.

6. Protease Assay

Proteases constitute a large and varied class of proteins with different properties, activities and biological functions. Because this assay is based on the production of a GloSensor™[protease site] protein in a cell-free coupled transcription/translation system to serve as a substrate for a protease, one cannot anticipate how all combinations of substrates and proteases will perform in this assay.

The optimal protease digestion conditions must be empirically determined for each unique protease. See Section 11.A. Below we show an example of ProTEV Protease digestion of the translation products from the pGloSensor™-10F[TEV] Control Plasmid. We recommend assembling two sets of digests, one using the translation products of the pGloSensor™[protease site] plasmid (plus-DNA TNT® reaction) and the other using translation products of the no-DNA control reaction (no-DNA TNT® reaction). One reaction in each set will be digested with the protease, and the other will be the undigested negative control reaction. See Table 1.

Note: For each set of experiments, we recommend performing reactions with the GloSensor™[TEV] protein as a positive control and a GloSensor™ protein with a nonspecific target sequence or scrambled sequence as a negative control.

In experiments performed at Promega, the fold activation of the GloSensor™[TEV] protein digested with ProTEV Protease is generally >200 (Figure 5).

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6. Protease Assay (continued)

1. Assemble your reactions as described in Table 1. Add 15µl of the plus-DNA TNT® reaction from Section 5 to each of two RNase- and DNase-free 0.5ml tubes. Add 15µl of the no-DNA TNT® reaction from Section 5 to each of two RNase- and DNase-free 0.5ml tubes.

Table 1. Recommended Protease Digestions.

Tube	Plus-DNA TNT® Reaction	No-DNA TNT® Reaction	Protease
A	+	–	+
B	+	–	–
C	–	+	+
D	–	+	–

2. Prepare the 2X ProTEV buffer: Dilute the ProTEV Buffer, 20X, to 2X with water (1:10 dilution). Add 100mM DTT to a final concentration of 2mM (1:50 dilution). Add 15µl of 2X ProTEV buffer to tubes A, B, C and D.
3. Add 10U (2.0µl) of ProTEV Plus (5U/µl) to tubes A and C. Add 2.0µl of Nuclease-Free Water instead of protease to the negative control reactions (tubes B and D).
4. Incubate reactions at 30°C for 30 minutes.

We do not recommend storing or freezing and thawing the protein at this point. See "Storage of TNT® SP6 High-Yield Protein Expression System-Expressed GloSensor™[Protease Site] Protein", Section 11.A. Proceed directly to Section 7.

Plasmid DNAs encoding seven different GloSensor™[protease site] proteins, a GloSensor™[42AA] protein containing a glycine/serine-rich 42 amino acid sequence, and the full-length firefly luciferase protein have been transcribed and translated. When the GloSensor™[protease site] proteins were digested with the cognate protease, the fold activation ranged from 160 to 1,250 (Figure 6). We have demonstrated the general applicability of the Protease-Glo™ Assay for two additional proteases: enterokinase and PreScission, with fold activations of 185 and 2,600, respectively (1). Importantly, proteases with and without P´ requirements (nomenclature of Schechter and Berger; 5,6) were able to activate the GloSensor™[protease site] proteins.

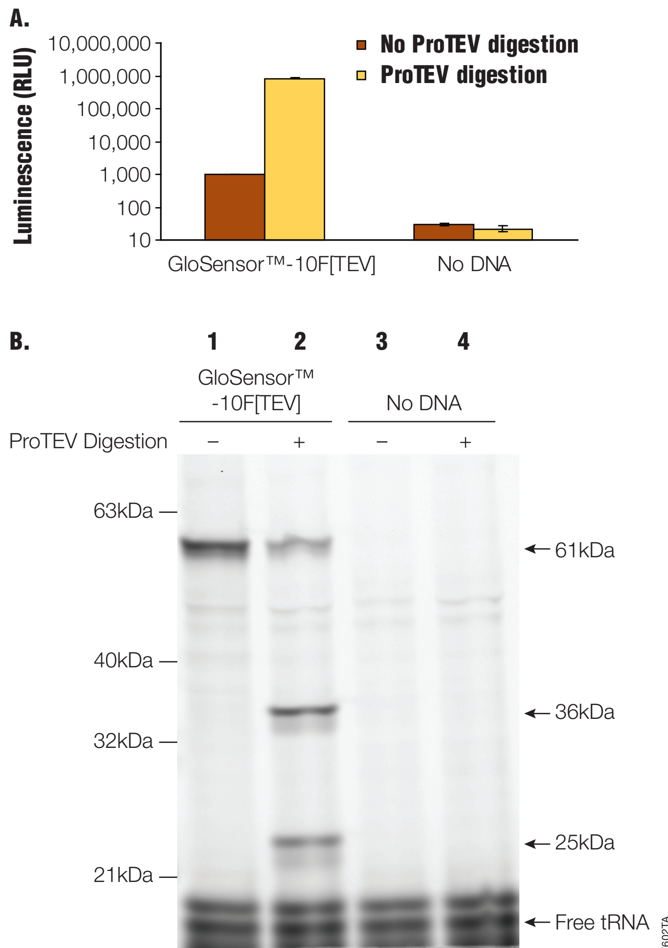

Figure 5. GloSensor™[TEV] protein activation by ProTEV Protease digestion. Plasmid DNA encoding the GloSensor™[TEV] protein was transcribed and translated using the TNT® SP6 High-Yield Wheat Germ Master Mix; a no-DNA control was performed in parallel. Following expression, 15µl of the TNT® reaction was combined with 15µl of 2X ProTEV Buffer (100mM HEPES [pH 7.5], 1mM EDTA, 2mM DTT) with or without ProTEV Protease (10 units). **Panel A.** Luminescent signal was measured by mixing an aliquot of each TNT® reaction with Bright-Glo™ Assay Reagent in triplicate and incubating for 5 minutes at room temperature. Luminescence was measured using a GloMax® 96 Microplate Luminometer and 1-second integration time. Error bars are the standard deviation of the mean. **Panel B.** The GloSensor™[TEV] protein and no-DNA control reaction were labeled using FluoroTect™ Green$_{Lys}$ tRNA, size fractionated on a 4–12% Bis-Tris NuPAGE® gel (Invitrogen), then visualized. In the reaction containing the GloSensor™[TEV] protein without ProTEV Protease digestion (lane 1), only the uncut 61kDa protein is visible. However, after ProTEV digestion, the smaller 36kDa and 25kDa protein fragments also are visible (lane 2). Note that the protease digestion was incomplete, and thus, the uncut 61kDa protein is visible. No labeled proteins were observed in the no-DNA control reactions, without or with ProTEV Protease digestion (lanes 3 and 4, respectively). The similar amount of free labeled tRNA visible in each lane indicates that a similar amount of lysate was added to each lane.

6. Protease Assay (continued)

Because the length of the protease site inserts differ (and for other, less well understood reasons), the levels of luminescence without protease digestion from the different GloSensor™[protease site] proteins vary. We typically observe that luminescence from the GloSensor™[protease site] protein after protease digestion is approximately 0.1–10% of the luminescence from full-length firefly luciferase. Therefore, although we are activating the proteins by protease digestion, luciferase activity is not fully restored. The GloSensor™[42AA] protein contains a long flexible 42-amino-acid flexible linker. This linker does not hinder luciferase activity to the same extent as protease site inserts. This is demonstrated in Figure 6, which shows that the GloSensor™[42AA] protein luminescence was approximately equal to luminescence of the GloSensor™[protease site] proteins after protease digestion and approximately 2% of the full-length firefly luciferase luminescence.

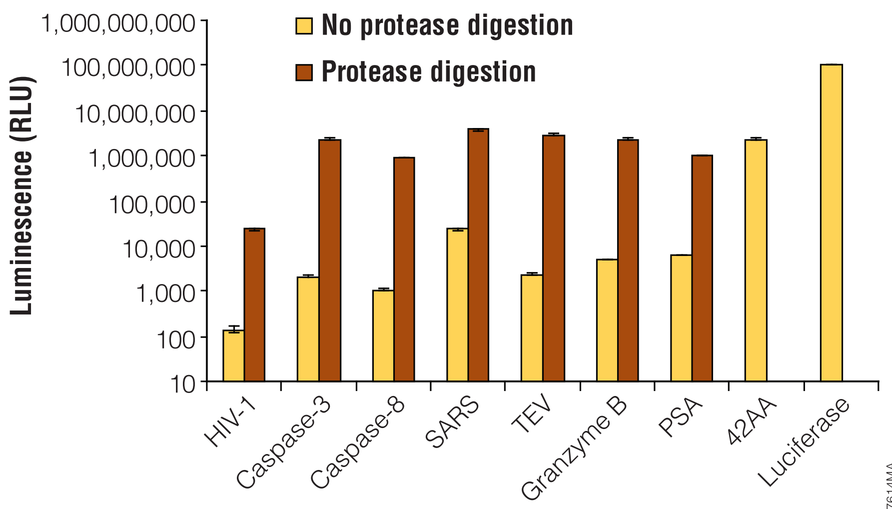

Figure 6. Examples of GloSensor™[protease site] activation by protease digestion. Plasmid DNA encoding seven different GloSensor™[protease site] proteins with protease recognition sequences for the indicated proteases were isolated. Plasmids encoding a GloSensor™[42AA] protein containing a 42-amino-acid sequence or the full length firefly luciferase protein were created for use as controls. Plasmid DNAs were transcribed and translated using TNT® SP6 High Yield Protein Expression System for 2 hours at 25ºC; a no-DNA control reaction was performed in parallel. Following expression, 25µl of the TNT® reaction was combined with 25µl of 2X protease buffer and the following cognate proteases: HIV-1 protease (30 units, AnaSpec), caspase-3 (1.7 units, UpState Biotechnology), caspase-8 (2.5 units, Chemicon), GST-SARS-CoV 3CL (6µg), ProTEV (3 units, Promega), granzyme B (112 units, BioMol), PSA (3.2µg, Sigma-Aldrich). For the GloSensor™[42AA] protein and full-length firefly luciferase protein, 25µl of the TNT® reaction was combined with 25µl of 100mM HEPES (pH 7.5). Following incubation at 30ºC for 1 or 2 hours, aliquots of each reaction were added to Bright-Glo™ Assay Reagent in triplicate and incubated for 5 minutes at room temperature. Luminescence was measured using a GloMax® 96 Microplate Luminometer and 1-second integration time. Error bars are the standard deviation of the mean.

7. Luminescence Detection Using the Bright-Glo™ Assay Reagent

1. Approximately 30 minutes prior to luminescence detection, place the Bright-Glo™ Assay Buffer on the bench top or in a 22°C water bath to allow the buffer to equilibrate to room temperature.
2. Immediately before luminescence detection, reconstitute the Bright-Glo™ Assay Reagent by transferring the contents of one bottle of Bright-Glo™ Assay Buffer to one vial of Bright-Glo™ Substrate. Mix by inversion until the substrate is thoroughly dissolved. See Notes 1–3.
3. If a labeling reagent was added to the TNT® reactions and if desired, reserve 5µl of each protease digestion and negative control reaction for SDS-PAGE analysis (Section 8).
4. Dilute the remaining volume of each protease digestion and negative control reaction 1:20 in nuclease-free water (e.g., 20µl reaction in 380µl water).
5. Add 100µl of the diluted protease digestion or negative control reaction per well of a white, flat-bottom 96-well plate. Analyze each reaction in triplicate.
6. Add 100µl of Bright-Glo™ Assay Reagent to each well.
7. Incubate for 2–5 minutes at room temperature.
8. Measure luminescence using a luminometer.

   Note: We perform our luciferase assays using a GloMax® 96 Microplate Luminometer with a 1-second integration.
9. Calculate the fold activation, referring to the tube designations in Table 1 if necessary. See Note 4.

$$\frac{\text{(luminescence from tube A)} - \text{(luminescence from tube C)}}{\text{(luminescence from tube B)} - \text{(luminescence from tube D)}}$$

Notes:

1. After reconstitution, unused Bright-Glo™ Assay Reagent can be stored at –70°C for up to 1 month. Avoid multiple freeze-thaw cycles. Store reagent protected from light.
2. To use frozen Bright-Glo™ Assay Reagent, thaw the reagent at temperatures below 25°C to ensure optimal performance. Mix well after thawing. The most convenient and effective method to thaw or equilibrate cold reagent is placing it in a water bath at room temperature.
3. Since luciferase activity is temperature-dependent, the temperature of the Bright-Glo™ Assay Reagent should be held constant while quantitating luminescence. This is achieved most easily by using reagent equilibrated to room temperature, which is near the temperature optimum of luciferase. Equilibration of the reagent prior to use is unnecessary when the Bright-Glo™ Assay Buffer is stored at room temperature.
4. We recommend calculating fold activation only if the luminescence of the tube B reaction is greater than three standard deviations above the luminescence of the tube D reaction.

8. Gel Analysis of Translation or Protease Digestion Products

It may be desirable to visualize the expressed GloSensor™[protease site] protein by adding a labeled reagent (Section 5). For example:

- Perform a secondary screen for inserts in the cloning process to confirm the presence of a 61kDa protein as described in Section 4.F.
- Confirm that the pGloSensor™-10F[protease site] plasmid DNA preparation results in a similar amount of expressed 61kDa protein as the pGloSensor™-10F[TEV] Control Plasmid. If there is significantly less expressed protein visible by SDS-PAGE as compared to protein expressed using the pGloSensor™-10F[TEV] Control Plasmid, the DNA preparation may contain contaminants that result in decreased transcription/translation efficiency. See Section 9.
- Confirm the specific digestion of the GloSensor™[protease site] protein by the protease. After protease digestion, the resultant protein fragments will be 36kDa and 25kDa in size (Figure 5). Note that the protease digestion may not be complete, and therefore, the undigested 61kDa protein also may be present.
- We strongly recommend performing gel analysis using the positive control and experimental proteins the first time the assay is performed.
- Troubleshoot unexpected luminescent results as recommended in Section 9.

A protocol for SDS-polyacrylamide gel analysis of radioactively labeled and fluorescently labeled proteins is given below. For more information about detecting proteins labeled with FluoroTect™ $\text{Green}_{\text{Lys}}$ tRNA, refer to the *FluoroTect™* $\textit{Green}_{\textit{Lys}}$ *in vitro Translation Labeling System Technical Bulletin* #TB285. For colorimetric or chemiluminescent detection of proteins labeled with Transcend™ tRNA, refer to the *Transcend™ Systems Technical Bulletin* #TB182. These Technical Bulletins are provided with the FluoroTect™ and Transcend™ products and are also available on our Web site at: **www.promega.com/tbs/**

1. To visualize the pGloSensor™[protease site] translation products: Once the 50µl translation reaction is complete or at any desired timepoint, remove a 2.5µl aliquot, and add 17.5µl of 1X SDS sample buffer.

   To visualize the protease digestion products of the protein: Add 5µl of protease digestion to 15µl of 1X SDS sample buffer.

   Note: When using precast SDS polyacrylamide gels, be sure to use the loading buffer recommended by the manufacturer.

2. Load samples onto an SDS-polyacrylamide gel, or store at –20°C. You do not need to separate labeled polypeptides from free amino acids by acetone precipitation.

3. Perform electrophoresis according to the gel manufacturer's instructions. Electrophoresis is usually performed until the bromophenol blue dye has run off the bottom of the gel. Disposal of unincorporated label may be easier if the gel is stopped while the dye front remains in the gel, because the dye front also contains unincorporated labeled amino acids.

4. To visualize fluorescently labeled protein products, scan the gel with a fluorescence imaging instrument (i.e., FluorImager® SI or FluorImager® 595, both with a 488 argon laser [Molecular Dynamics]; the Typhoon® [GE Healthcare], with a 532nm excitation, or the FMBIO® II [Hitachi], with a 505 channel).

   To visualize radioactively labeled products, fix the gel by soaking in 50% methanol, 7% acetic acid for 15 minutes followed by soaking in 7% glycerol, 7% methanol, 7% acetic acid for 5–10 minutes. Dry the gel before exposure to film as follows: Soak the gel in 10% glycerol for 5 minutes to prevent cracking during drying. Place the gel on a sheet of Whatman® 3MM filter paper, cover the top of the gel with plastic wrap and dry at 80°C for 30–90 minutes under vacuum using a conventional gel dryer. Dry completely. The gel may also be dried overnight using the Gel Drying Kit (Cat.# V7120). To decrease the likelihood of cracking gradient gels, dry them with the wells pointing down. Expose the gel on Kodak X-OMAT® AR film for 6–15 hours at room temperature. Alternatively, the fixed gel can be exposed to a phosphorimaging screen. Phosphorimaging systems provide greater sensitivity, greater speed and the ability to quantitate radioactive bands.

9. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: www.promega.com. E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
Low number or no colonies after ligation and transformation	Ligation reaction failed. Ligation reactions with insert typically yield at least 10 times more colonies than negative control reactions. If the ligation with insert yields the same number of colonies as the negative control ligation, this indicates a problem with the insert or ligation.
	The 2X Rapid Ligation Buffer contains ATP, which degrades during temperature fluctuations. Avoid multiple freeze-thaw cycles by making single-use aliquots of the buffer. Use a fresh vial of buffer.
	Improper dilution of the 2X Rapid Ligation Buffer. The Rapid Ligation Buffer is provided at a 2X concentration. Use 5µl in a 10µl reaction.
	Incorrect oligonucleotide sequences. Oligonucleotide A must have a 5´ overhang sequence of CTAGC and a 3´ overhang sequence of G, and oligonucleotide B must have a 5´ overhang sequence of GATCC and a 3´ overhang sequence of G. See Section 3.B.
	Oligonucleotides failed to anneal. Confirm oligonucleotide sequences. Repeat annealing and dilution steps, and use oligonucleotides immediately.
	Poor-quality oligonucleotides. Repeat ligations using HPLC-purified or gel-purified oligonucleotides for oligonucleotides that are greater than 45 nucleotides in length.
	Amount of annealed oligonucleotides in the ligation was not optimal. Titrate the amount of DNA to determine the optimal concentration.
	Ineffective transformation or poor-quality competent cells. Perform a control transformation with supercoiled plasmid DNA to ensure that the efficiency of the competent cells is >1 × 10^8cfu/µg DNA (Section 4.C). The pGloSensor™-10F Linear Vector is not an appropriate transformation control DNA.

Symptoms	Causes and Comments
Low translation efficiency or translation product smaller than the expected size (61kDa)	pGloSensor™[protease site] plasmid DNA contained ethanol or salt. Perform an ethanol precipitation to remove contaminants.
	Possible cloning error. Verify that the pGloSensor™[protease site] plasmid used in the transcription/translation reaction encodes a full-length protein (61kDa). A nucleotide change, insertion or deletion within the plasmid sequence can introduce a premature transcription or translation termination signal or disrupt an element necessary for efficient transcription or translation. Premature termination results in the synthesis of protein products that are smaller than expected (61kDa). Check the GloSensor™[protease site] protein-coding region for the presence of alternative stop codons.
	Incorrect oligonucleotide sequences. Reorder oligonucleotides from manufacturer, and repeat the ligation.
	Poor-quality DNA. Perform control transcription/translation reactions with a constant amount of Luciferase SP6 Control DNA (Cat.# L4741), adding in decreasing amounts of the pGloSensor™[protease site] plasmid. The luminescence from the pGloSensor™[protease site] protein will constitute an insignificant fraction of the total luminescence. Compare the level of luminescence for the control reactions. If a significant decrease in luciferase activity is observed with increasing amounts of pGloSensor™[protease site] plasmid, your DNA preparation is likely reducing protein synthesis efficiency.

9. Troubleshooting (continued)

Symptoms	Causes and Comments
High luminescence without protease digestion/no increase in luminescence after protease digestion	Protease recognition sequence was too long. Decrease the protease recognition sequence to ≤14 amino acids. Compare luminescence for undigested and protease-digested translation products encoded by the pGloSensor™-10F[TEV] Control Plasmid. Luminescence with your experimental constructs should be similar to or less than that with the pGloSensor™-10F[TEV] Control Plasmid.
	TNT® reaction component, protease digestion buffer or Bright-Glo™ Assay Reagent was contaminated with protease. Repeat the transcription/translation of your pGloSensor™[protease site] plasmid, and prepare fresh protease digestion buffer, using new tubes and other plasticware.
	The TNT® SP6 High-Yield Wheat Germ Master Mix contains the protease of interest. Express the pGloSensor™[protease site] plasmid in rabbit reticulocyte lysate-based TNT® SP6 Quick Coupled Transcription/Translation System (Cat.# L2080), or transfer the GloSensor™[protease site] protein-coding sequence to the pFN6A (HQ) Flexi® Vector (Cat.# C8511) and express the protein using the *E. coli* T7 S30 Extract System for Circular DNA (Cat.# L1130). Express the protein in all three systems, perform the protease digestion and compare luminescence from the undigested and protease-digested samples.
	Protease digestion conditions were incompatible with luciferase activity. Perform digestion at 22–30°C. Check compatibility of the protease digestion components and Bright-Glo™ assay.
	Decrease amount of GloSensor™[protease site] protein in each luciferase assay. Use as little as 100µl of 1:100 dilution of the protease digestion per 100µl of Bright-Glo™ Assay Reagent in the luciferase assay.
	Perform a ProTEV Plus digestion of the pGloSensor™-10F[TEV] Control Plasmid translation products as a positive control. Digestion of the pGloSensor™-10F[TEV] Control Plasmid translation products should yield fold activation >200 (Figure 5).

Symptoms	Causes and Comments
Low luminescence/no increase in luminescence after protease digestion	Perform a ProTEV Plus digestion of the pGloSensor™-10F[TEV] Control Plasmid translation products as a positive control. Digestion of the pGloSensor™-10F[TEV] Control Plasmid translation products should yield fold activation >200 (Figure 5).
	No expression or poor expression of the GloSensor™[protease site] protein. Label your TNT®-expressed protein using FluoroTect™ $Green_{Lys}$ tRNA, Transcend™ tRNA or [^{35}S]methionine. Check the expressed protein by SDS-PAGE. Compare the amount and size of protein expressed from your pGloSensor™[protease site] plasmid to that from the pGloSensor™-10F[TEV] Control Plasmid. Repeat the transcription/translation reaction if necessary. See comments for "Low translation efficiency or translation product smaller than the expected size (61kDa)".
	TNT® SP6 High-Yield Protein Expression System-expressed GloSensor™[protease site] protein was frozen and thawed after expression or protease digestions or before Bright-Glo™ Assay Reagent detection. We do not recommend storing or freezing and thawing the protein. Experimental data suggest that storage may be possible; however, stabilities of the GloSensor™[protease site] proteins tested varied. The stability of each specific protein must be empirically determined.
	No protease digestion of GloSensor™[protease site] protein. Label your TNT®-expressed protein using FluoroTect™ $Green_{Lys}$ tRNA, Transcend™ tRNA or [^{35}S]methionine, and perform the protease digestion. Check for digested protein fragments by SDS-PAGE. The digested protein fragments are 35 and 26kDa (Figure 5). Compare to digested pGloSensor™-10F[TEV] Control Plasmid translation products.

9. Troubleshooting (continued)

Symptoms	Causes and Comments
Low luminescence/no increase in luminescence after protease digestion (continued)	Protease nonspecifically degraded the GloSensor™[protease site] protein. • Digest translation products from the pGloSensor™-10F[TEV] Control Plasmid with protease of interest. Luminescence levels should be similar in the protease-digested and undigested samples.
	• Label TnT®-expressed protein using [^{35}S]methionine or non-radioactive alternatives such as Transcend™ tRNA (Cat.# L5061) or FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System (Cat.# L5001). Digest translation products with protease, and check the size of the protein by SDS-PAGE. The presence of bands other than the expected 25, 36 and 61kDa fragments or the presence of a smear of protein fragments indicates that the GloSensor™[protease site] protein is being nonspecifically degraded.
	The amino acid sequence of the protease recognition site is also present in the GloSensor™ amino acid sequence. We strongly recommend using the Protease-Glo™ Oligonucleotide Designer tool to design oligonucleotides A and B. This tool will search the GloSensor™ amino acid sequence to identify any sequences that match your input protease recognition sequence.
	Protease digestion conditions were incompatible with luciferase activity. Check compatibility of protease digestion buffer with the Bright-Glo™ Assay, paying particular attention to the NaCl concentration (Section 11.A). Perform protease digestions at 22–30°C and at a pH between 5.2 and 9.1 (Figure 7). Incubate translation products of a pGloSensor™ positive control plasmid (e.g., the pGloSensor™[42AA]; Section 11.A) with the protease digestion buffer of interest or PBS. The level of luminescence in the presence of the digestion buffer should not be significantly different than that in the presence of PBS.

Symptoms	Causes and Comments
Low luminescence/no increase in luminescence after protease digestion (continued)	Poor digestion of GloSensor™[protease site] protein. Perform digestion time course, temperature optimization and protease titration. Incubate the protease digestion at temperatures between 22°C and 30°C.
	When using a plate format to measure luminescence, use a white, flat-bottom 96-well plate (e.g., Cat.# Z3291), not a black or clear plate.
	Increase the amount of GloSensor™[protease site] protein per digestion. Prepare a 10X protease digestion buffer, and add 18µl of the translation reaction containing the GloSensor™ [protease site] protein and 2µl of 10X protease digest buffer. Add 1µl of protease to the digested samples and 1µl of water to the negative control reactions.
	Increase amount of GloSensor™[protease site] protein per luciferase assay. Add 100µl of a 1:4 dilution of protease digestion per 100µl of Bright-Glo™ Assay Reagent.
	The luminometer was not sufficiently sensitive. Perform a translation using the Luciferase SP6 Control DNA (Cat.# L4741), then perform luciferase assays with decreasing amounts of translated products. The dynamic range of the luminometer should be at least seven orders of magnitude (e.g., 10^1 to 10^8 relative light units). Luminometers that we have tested at Promega include the GloMax® 96 Microplate Luminometer (Cat.# E6501, E6511, E6521), GloMax® 20/20 Luminometer (Cat.# E5311), Molecular Devices SpectraMAX® L Microplate Luminometer and Turner Biosystems Veritas™ Microplate Luminometer and 20/20n Single Tube Luminometer.

10. References

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3. Fan, F. and Wood, K.V. (2007) Bioluminescent assays for high-throughput screening. *Assay Drug. Dev. Technol.* **5**, 127–136.
4. Harris, J.L. *et al.* (2001) Definition of the extended substrate specificity determinants for beta-tryptases I and II. *J. Biol. Chem.* **276**, 34941–7.
5. Schechter, I. and Berger, A. (1967) On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Comm.* **27**, 157–62.
6. Schechter, I. and Berger, A. (1968) On the active site of proteases. 3. Mapping the active site of papain; specific peptide inhibitors of papain. *Biochem. Biophys. Res. Comm.* **32**, 898–902.
7. *ProTEV Plus Promega Product Information* #9PIV610, Promega Corporation.
8. *Bright-Glo™ Luciferase Assay System Technical Manual* #TM052, Promega Corporation.

11. Appendix

11.A. General Considerations

Reagents

The GloSensor™[protease site] protein is expressed in vitro using the TNT® SP6 High-Yield Wheat Germ Master Mix. We strongly recommend including a positive control with each set of experiments to ensure protease activity is specific to the protease of interest. The Protease-Glo™ Assay is provided with the pGloSensor™-10F[TEV] Control Plasmid and ProTEV Plus to perform positive control reactions.

Storage of TNT® SP6 High-Yield Protein Expression System-Expressed GloSensor™[Protease Site] Protein

We recommend performing the protease digestion and luminescence measurement on the same day that the GloSensor™[protease site] protein is expressed. We do not recommend storing or freezing and thawing the protein. Experimental data suggest that storage may be possible; however, the stabilities of the GloSensor™[protease site] proteins tested varied. The stability of each specific protein must be empirically determined.

Protease Assays

Protease reactions should be performed under conditions recommended in the literature. Typically a 2X protease reaction buffer is made and mixed at a 1:1 ratio with the TNT® SP6 High-Yield Protein Expression System-expressed GloSensor™[protease site] protein. The protease of interest is added to the translation products and incubated at the desired temperature (≤30°C) for the desired time.

Some protease buffer conditions may be incompatible with the TNT® SP6 High-Yield Wheat Germ Master Mix. For example, use of buffers with an extreme pH may result in protein precipitation from the TNT® SP6 High-Yield Wheat Germ Master Mix. If this occurs, the protease digestions or Bright-Glo™ assays will fail. Successful digestion of the GloSensor™[TEV] protein by the ProTEV Protease, which is active over a wide pH range (7), demonstrates that the TNT® SP6 High-Yield Wheat Germ Master Mix and GloSensor™[TEV] protein are compatible within a pH range of 5.2 to 9.1 (Figure 7).

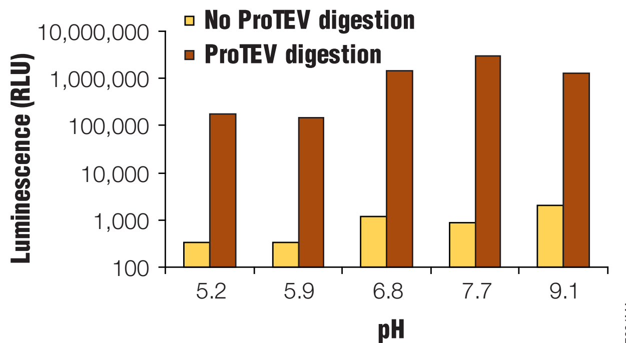

Figure 7. pH range for the GloSensor™[TEV] protein digestion. Plasmid DNA encoding the GloSensor™[TEV] protein was transcribed and translated using TNT® SP6 High-Yield Wheat Germ Master Mix. Following expression, 15µl of the TNT® reaction was combined with 15µl of 2X cleavage buffer at various pH values with or without ProTEV Protease (10 units). Following incubation, aliquots were mixed with the Bright-Glo™ Assay Reagent in triplicate and incubated for 5 minutes at room temperature. Luminescence was measured using a GloMax® 96 Microplate Luminometer and 1-second integration time. Error bars are the standard deviation of the mean. The pH values of the protease digestions were determined after mixing the 2X buffer with translated products and are indicated on the graph. Final buffer composition and protease digestion reaction pH values were 0.5mM EDTA, 1mM DTT plus: 50mM sodium acetate (pH 5.2), 50mM MES (pH 5.9), 50mM HEPES (pH 6.8), 50mM HEPES (pH 7.7) or 50mM CHES (pH 9.1).

11.A. General Considerations (continued)

TNT® SP6 High-Yield Protein Expression System-Expressed GloSensor™[Protease Site] Protein Positive Control

To determine if the experimental protease buffer conditions are compatible with the luminescent reaction, we recommend creating a GloSensor™ protein containing a long, flexible sequence to use as a positive control. For example, we have developed a GloSensor™[42AA] protein containing a 42 amino acid, glycine/serine-rich sequence (42AA). This 42 amino acid, glycine/serine-rich sequence is not recognized by native proteases in the TNT® SP6 High-Yield Wheat Germ Master Mix. Because of the length of this polypeptide, the basal luminescence of the GloSensor™[42AA] protein is approximately equal to the luminescence of the GloSensor™[TEV] protein after ProTEV Protease digestion (Figure 8).

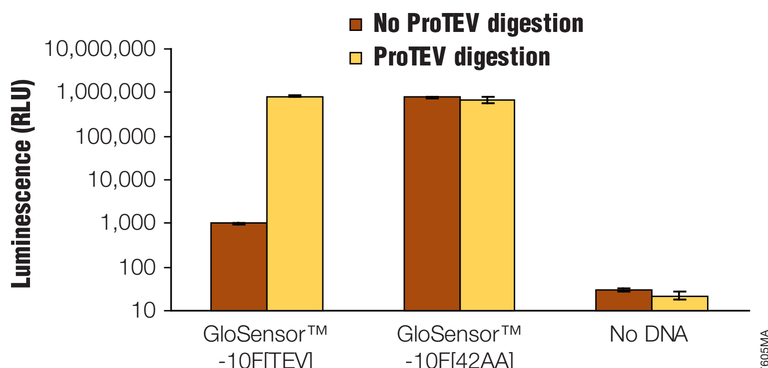

Figure 8. Comparison of luminescent signals from the GloSensor™[TEV] and GloSensor™[42AA] proteins. Plasmid DNAs encoding the GloSensor™[TEV] and GloSensor™[42AA] proteins were transcribed and translated using the TNT® SP6 High-Yield Wheat Germ Master Mix. Following expression, 15µl of the TNT® reaction was combined with 15µl of 2X ProTEV Buffer (100mM HEPES [pH 7.5], 1mM EDTA, 2mM DTT) with or without ProTEV Protease (3 units). Following incubation, aliquots were mixed with the Bright-Glo™ Assay Reagent in triplicate and incubated for 5 minutes at room temperature. Luminescence was measured using a GloMax® 96 Microplate Luminometer and 1-second integration time. Error bars are the standard deviation of the mean.

Bright-Glo™ Assay Reagent

We recommend the Bright-Glo™ Assay Reagent (Cat.# E2620) to measure luminescence. The Bright-Glo™ Assay Reagent is a robust, homogeneous luciferase assay chemistry that achieves high sensitivity. No noticeable effect on relative luminescence or signal stability is observed when up to 1.0mM $MgSO_4$, 3mM $CaCl_2$, 10mM KCl, 10mM PO_4, 30nM $CuSO_4$, 10µM $FeSO_4$ or 10µM $ZnSO_4$ is added to Bright-Glo™ assays (8). However, NaCl can affect relative luminescence. We determined the effects of NaCl concentration on the GloSensor™[42AA] protein luminescent signal (Figure 9). The luminescent signal from the GloSensor™[42AA] protein decreased with increasing NaCl concentration. When the GloSensor™[42AA] protein was incubated in a buffer with a final concentration of 2.5M NaCl, the luminescent signal decreased by 82% compared to a PBS-only control (140mM NaCl). For more information, see the *Bright-Glo™ Luciferase Assay System Technical Manual* #TM052.

Luciferase activity is temperature-dependent, so temperature is an important factor in experimental precision. Equilibrate the Bright-Glo™ Assay Reagent to room temperature before beginning measurements. Lower temperatures result in increased signal stability, but luminescent intensity is lowered. If cold reagent is used, luminescence will slowly increase during the experiment as the reagent warms. Higher temperatures cause an increase in luminescence, but the signal becomes less stable. This can occur if the samples are too warm or if the luminometer produces excess heat within the reading chamber.

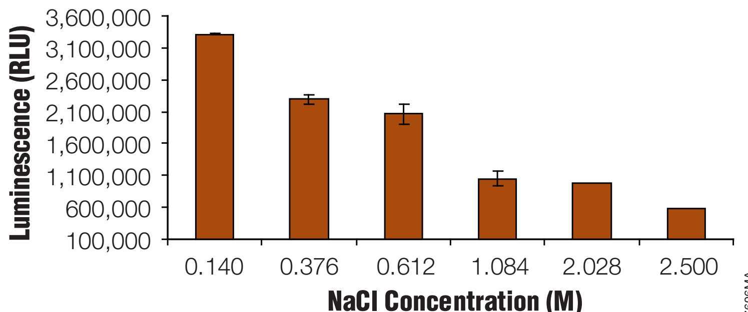

Figure 9. Effect of NaCl concentration on the GloSensor™[42AA] protein luminescent signal. Plasmid DNA encoding the GloSensor™[42AA] protein was transcribed and translated using TNT® SP6 High-Yield Wheat Germ Master Mix. Following expression, 15µl of the TNT® reaction was combined with 15µl of 2X PBS (pH 7.3) with increasing NaCl concentrations. The reactions were incubated for 15 minutes at room temperature in the presence of the indicated NaCl concentration. Aliquots were mixed with the Bright-Glo™ Assay Reagent in triplicate and incubated for 5 minutes at room temperature. Luminescence was measured using a GloMax® 96 Microplate Luminometer and 1-second integration time. Error bars are the standard deviation of the mean.

11.A. General Considerations (continued)

Luminometers

The detection instrument should be configured to measure light emission over a defined period, as opposed to measuring flash intensity or peak height. For luciferase assays using the Bright-Glo™ Assay Reagent, we recommend programming luminometers with a 1-second measurement. The dynamic range of the luminometer should be at least seven orders of magnitude (e.g., 10^1 to 10^8 relative light units).

The most convenient method to perform large numbers of Bright-Glo™ assays is to use a luminometer capable of processing multiple sample tubes or configure assays in a 96-well array and use a plate-reading luminometer. For high-throughput applications, we recommend first dispensing the desired volume of each sample into the assay tubes or wells of a plate, then manually adding 100µl of Bright-Glo™ Assay Reagent to each tube or well and measuring luminescence. The Bright-Glo™ Reagent is not designed for use with the automated reagent injectors that are integrated into some luminometers.

11.B. Vector Sequence Reference Points and Sequence Accession Numbers

Vector sequences for the pGloSensor™-10F Linear Vector (GenBank®/EMBL Accession Number EU693492) and pGloSensor™-10F[TEV] Control Plasmid (GenBank®/EMBL Accession Number EU693493) are available in the GenBank® database and on the Internet at: **www.promega.com/vectors/**. The pGloSensor™ Linear Vector is created by restriction enzyme digestion with NheI and BglII and, as a result, has overhangs of GATC. The listed locations of the vector features and restriction enzyme sites are in relation to base 1 and are numbered as though the NheI and BglII recognition sites were uncut and positioned side by side.

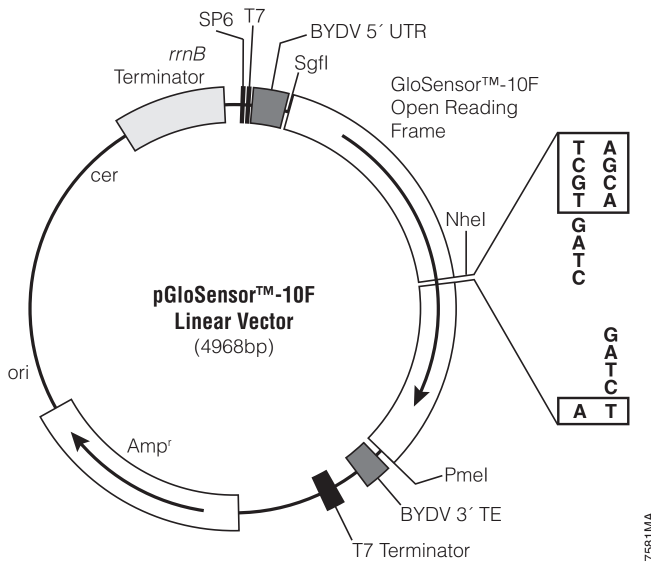

Figure 10. pGloSensor™-10F Linear Vector circle map and sequence reference points.

pGloSensor™-10F Linear Vector Sequence Reference Points:

SP6 RNA polymerase promoter (–17 to +1)	23–42
T7 RNA polymerase promoter (–17 to +1)*	46–63
Barley yellow dwarf virus (BYDV) 5´ untranslated region	65–201
SgfI recognition site	202–209
GloSensor™ protein-coding region	211–1848
NheI recognition site	1147–1152
BglII recognition site (destroyed during cloning process)	1153–1158
PmeI recognition site	1849–1856
BYDV 3´ translation enhancer	1875–1981
T7 terminator	2086–2133
β-lactamase (Amp^r) coding region	2467–3327
Origin of replication	3482–3518
cer site (site for *E. coli* XerCD recombinase)	4189–4474
rrnB transcription terminator	4525–4926

*The consensus T7 RNA polymerase promoter sequence extends only to +1 and does not match many standard T7 promoter primers available for sequencing applications.

11.B. Vector Sequence Reference Points and Sequence Accession Numbers (continued)

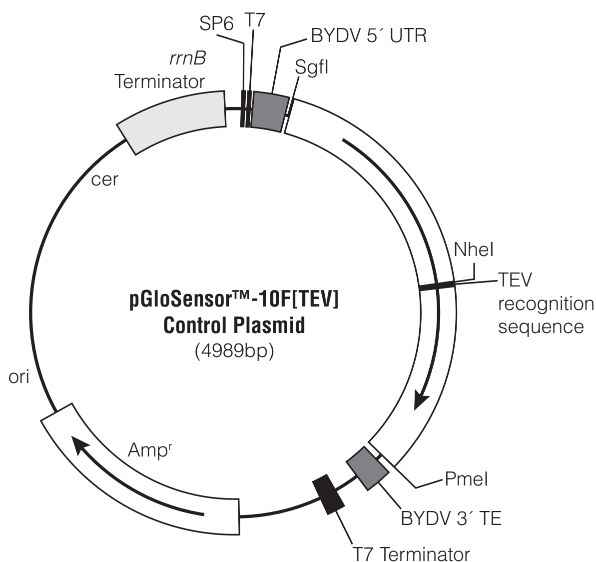

Figure 11. pGloSensor™-10F[TEV] Control Plasmid circle map and sequence reference points.

pGloSensor™-10F[TEV] Control Plasmid Sequence Reference Points:

SP6 RNA polymerase promoter (–17 to +1)	23–42
T7 RNA polymerase promoter (–17 to +1)*	46–63
Barley yellow dwarf virus (BYDV) 5´ untranslated region	65–201
SgfI recognition site	202–209
GloSensor™[TEV] protein-coding region	211–1875
NheI recognition site	1147–1152
PmeI recognition site	1870–1877
BYDV 3´ translation enhancer	1896–2002
T7 terminator	2107–2154
β-lactamase (Amp^r) coding region	2488–3348
Origin of replication	3503–3539
cer site (site for *E. coli* XerCD recombinase)	4210–4495
rrnB transcription terminator	4546–4947

*The consensus T7 RNA polymerase promoter sequence extends only to +1 and does not match many standard T7 promoter primers available for sequencing applications.

11.C. Composition of Buffers and Solutions

LB plates with ampicillin

Add 15g agar to 1 liter of LB medium. Autoclave. Allow the medium to cool to 50°C before adding ampicillin to a final concentration of 100µg/ml. Pour 30–35ml of medium into 85mm petri dishes. Let the agar harden. Store at 4°C for up to 1 month or at room temperature for up to 1 week.

Oligo Annealing Buffer (provided)

60mM	Tris-HCl (pH 7.9)
1.5M	NaCl
60mM	$MgCl_2$
10mM	DTT

2X Rapid Ligation Buffer (provided)

60mM	Tris-HCl (pH 7.8)
20mM	$MgCl_2$
20mM	DTT
2mM	ATP
10%	polyethylene glycol (MW8000, A CS Grade)

Store in single-use aliquots at –20°C. Avoid multiple freeze-thaw cycles.

1X SDS sample buffer

50mM	Tris-HCl (pH 6.8)
2%	SDS
0.1%	bromophenol blue
10%	glycerol
100mM	dithiothreitol

1X SDS sample buffer lacking dithiothreitol can be stored at room temperature. Dithiothreitol should be added from a 1M stock just before use.

SOC medium (100ml)

2.0g	Bacto®-tryptone
0.5g	Bacto®-yeast extract
1ml	1M NaCl
0.25ml	1M KCl
1ml	2M Mg^{2+} stock, filter-sterilized
1ml	2M glucose, filter-sterilized

Add Bacto®-tryptone, Bacto®-yeast extract, NaCl and KCl to 97ml of distilled water. Stir to dissolve. Autoclave and cool to room temperature. Add 2M Mg^{2+} stock and 2M glucose, each to a final concentration of 20mM. Bring the volume to 100ml with sterile, distilled water. The final pH should be 7.0.

TE buffer, 1X

10mM	Tris-HCl (pH 8.0)
1mM	EDTA

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11.D. Related Products

Product	Size	Cat.#
10X Flexi® Enzyme Blend (SgfI/PmeI)	25µl	R1851
	100µl	R1852
TNT® SP6 High-Yield Protein Expression System	10 reactions	L3261
	40 reactions	L3260
ProTEV Plus	1,000U	V6101
	8,000U	V6102
JM109 Competent Cells, >10^8cfu/µg*	5 × 200µl	L2001
LigaFast™ Rapid DNA Ligation System*	30 reactions	M8221
	150 reactions	M8225
FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System*	40 reactions	L5001
Transcend™ Colorimetric Non-Radioactive Translation Detection System*	30 reactions	L5070
Transcend™ Chemiluminescent Non-Radioactive Translation Detection System*	30 reactions	L5080
Transcend™ tRNA*	30µl	L5061

*For Laboratory Use.

Reporter Assay Systems

Product	Size	Cat.#
Bright-Glo™ Luciferase Assay System	10ml	E2610
	100ml	E2620
	10 × 100ml	E2650

For Laboratory Use.

Luminometers

Product	Size	Cat.#
GloMax® 96 Microplate Luminometer	each	E6501
GloMax® 96 Microplate Luminometer w/Single Injector	each	E6511
GloMax® 96 Microplate Luminometer w/Dual Injectors	each	E6521
GloMax®-Multi Base Instrument	each	E7031
GloMax®-Multi Luminescence Module	each	E7041
GloMax®-Multi Fluorescence Module	each	E7051
GloMax®-Multi Absorbance Module	each	E7061
GloMax® 20/20 Luminometer	each	E5311
GloMax® 20/20 Luminometer w/Single Auto-Injector	each	E5321
GloMax® 20/20 Luminometer w/Dual Auto-Injector	each	E5331
Luminometer Plates	50 plates	Z3291

Plasmid DNA Purification

Product	Size	Cat.#
Wizard® *Plus* SV Minipreps DNA Purification System*	50 preps	A1330
	250 preps	A1460
Wizard® *Plus* SV Minipreps DNA Purification System* + Vacuum Adapters	50 preps	A1340
	250 preps	A1470
PureYield™ Plasmid Miniprep System	10 preps	A1220
	50 preps	A1221
	250 preps	A1222
PureYield™ Plasmid Midiprep System	25 preps	A2492
	100 preps	A2495

*For Laboratory Use.

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