Achieve the Protein Expression Level You Need with the Mammalian HaloTag® 7 Flexi® Vectors

ABSTRACT A series of vectors containing deletions of human cytomegalovirus (CMV) immediate-early enhancer/promoter were created to provide different constitutive expression levels of HaloTag® 7 fusion proteins, providing greater control over the level of protein expression. This allows expression of enough fusion protein to image or capture but not so much to disturb normal function of the protein partner fused to the HaloTag® 7 protein.

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Sample Pack (Cat.# G3780)
The HaloTag® 7 CMV promoter deletion series and the full-length CMV promoter are available as a sample pack. This pack allows the cloning of a protein coding region into a donor vector and then transfer of the protein coding region to any or all of the complete set of HaloTag® 7 vectors.

INTRODUCTION

For protein overproduction with the purpose of overexpressing proteins, the human cytomegalovirus (CMV) immediate-early enhancer/promoter is often chosen for its robust expression levels. However, this high level of production can potentially disrupt the normal behavior of the protein or the physiology of the cell and may need to be adjusted. While protein overexpression studies yield important clues to the normal functioning of a gene product in the cell, the results must be interpreted with caution. When expressing protein fused to a tag such as the improved HaloTag® 7 reporter protein (1), these same concerns apply.

The HaloTag® 7 protein is an engineered, catalytically inactive derivative of a hydrolase that forms a covalent bond with HaloTag® Ligands. HaloTag® protein fusion can be used either for capture of the fusion and its molecular partners in the cell or for labeling with a variety of fluors. We developed the HaloTag® 7 Flexi® Vector CMV deletion series [a–d], a series of human CMV promoter deletions that offer different constitutive expression levels for HaloTag® fusion proteins. This allows researchers to express just enough protein for their application.

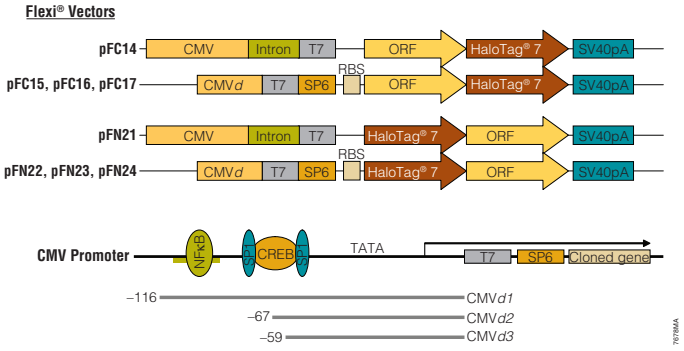

Figure 1. Expression elements of the mammalian HaloTag® 7 Flexi® Vectors. pFC14 and pFN21 contain the full-length (742 bp) human cytomegalovirus (CMV) immediate-early enhancer/promoter, which contains both the proximal and distal regulatory elements for strong expression. They also contain a chimeric intron, composed of the 5′-splice site from the β-globin intron and the 3′-splice site from an IgG intron, allowing increased expression levels. pFC15 and pFN22 contain CMV*d1*, a CMV promoter deletion retaining 116 bp upstream from the transcription start site. CMV*d1* contains a consensus NFκB and a partial-consensus CREB binding site (2), and two functional SP1/SP3 binding sites (3,4). pFC16 and pFN23 contain CMV*d2*, a deletion retaining 67 bp, while pFC17 and pFN24 contain CMV*d3*, a deletion retaining 59 bp. Both of these deletions have the weaker of the two SP1/SP3 binding sites (3,4). T7 and SP6 bacteriophage RNA polymerase promoters provide expression in cell-free systems and *E. coli* strains harboring T7 RNA polymerase (e.g., KRX or BL21(DE3)pLysS). The SV40 late polyadenylation signal provides efficient RNA processing in mammalian cells. CMV deletion vectors also contain an *E. coli* ribosome binding site in the 5′ UTR for expression in *E. coli* and S30 cell-free systems. "pF" indicates that these are Flexi® Vectors. The letter after "pF" indicates the position of HaloTag® 7: "N" for N-terminal and "C" for C-terminal. The number associated with the vector is a unique identifier of the type of expression and application. Vectors are available in ampicillin- and kanamycin-resistant versions, indicated by the addition of an "A" or "K" to the vector designation (e.g., pFN21A provides ampicillin resistance).

DIFFERENT EXPRESSION LEVELS: HALOTAG® 7 FLEXI® VECTOR CMV DELETION SERIES

A series of 5′ deletions of the human CMV promoter were synthesized and incorporated into a Flexi® Vector format such that either firefly or *Renilla* luciferase-HaloTag® 7 fusion proteins were expressed. The resulting activity levels were screened to create a series of successive deletions that provided a five- to tenfold drop in expression from one deletion to another. Deletions to –116 (CMV*d1*), –67 (CMV*d2*) and –59 (CMV*d3*), were chosen to make up the series (Figure 1). Expression levels depend on the cell type and the protein being expressed, and with the exception of CHO cells, the CMV deletions provide a consistent drop in expression with each successive deletion (Figure 2). HaloTag® activity mirrored luciferase activity (data not shown).

To complement the C-terminal HaloTag® 7 Vectors, the same promoters were used to build fusion Flexi® Vectors appending HaloTag® 7 on the amino-terminus of the protein of interest. *Luc2*, an engineered brighter version of firefly luciferase, was cloned into all vectors. Activity levels for luciferase (Figure 3) and HaloTag® (data not shown) were similar, with HaloTag® activity mirroring luciferase activity. Ampicillin- and kanamycin-resistant versions of each vector were made and provide similar expression levels. In addition, both C-terminal and N-terminal HaloTag® 7 versions of each promoter provide similar expression levels.

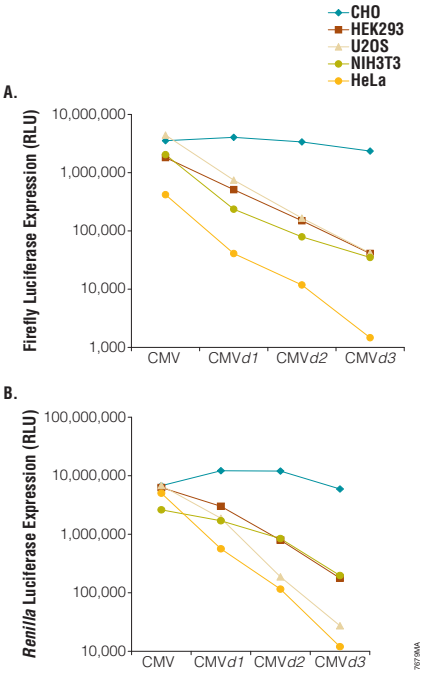

Figure 2. The CMV deletion series provides expression levels spaced about 0.5 to 1 log apart. Cell lines were maintained in complete medium containing 10% serum at 37 °C and 5–10% CO_2. Cells at 85% confluency were transiently transfected with firefly luciferase HaloTag® 7 constructs (**Panel A**) or *Renilla* luciferase HaloTag® 7 constructs (**Panel B**) using LT1 reagent (Mirus) in 6-well plates. After 48 hours, the activity of the HaloTag® protein and the luciferases were determined. The HaloTag® protein activity was assessed by taking a portion of the cells, incubating with HaloTag® TMR Ligand for 15 minutes, lysing cells in SDS gel loading buffer, incubating at 95 °C for 5 minutes, and analyzing by SDS-PAGE and fluoroimaging ($532_{EX}/580_{EM}$) using a Typhoon® 9400 and ImageQuant software (GE Healthcare Bio-sciences). Luciferase activity was measured using Dual-Glo® Luciferase Assay System.

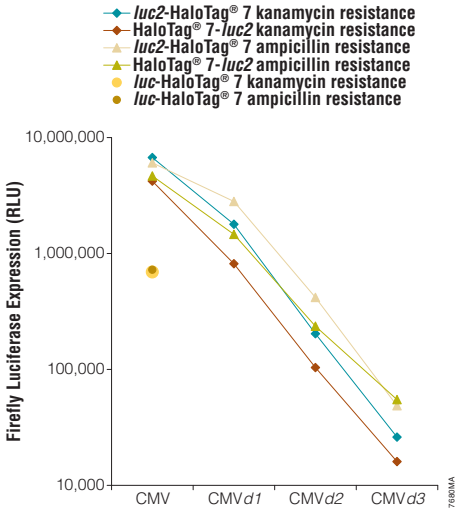

Figure 3. Amino- and carboxy-terminal HaloTag® 7 fusion provide similar expression levels. HeLa cells were transiently transfected with *luc* and *luc*2 HaloTag® 7 fusion constructs. Transient transfections and HaloTag® and luciferase assays were performed as described for Figure 2. Ampicillin- and kanamycin-resistant versions of each vector were made to facilitate shuttling protein coding regions between compatible Flexi® Vectors; ampicillin- and kanamycin-resistant versions provided similar expression levels.

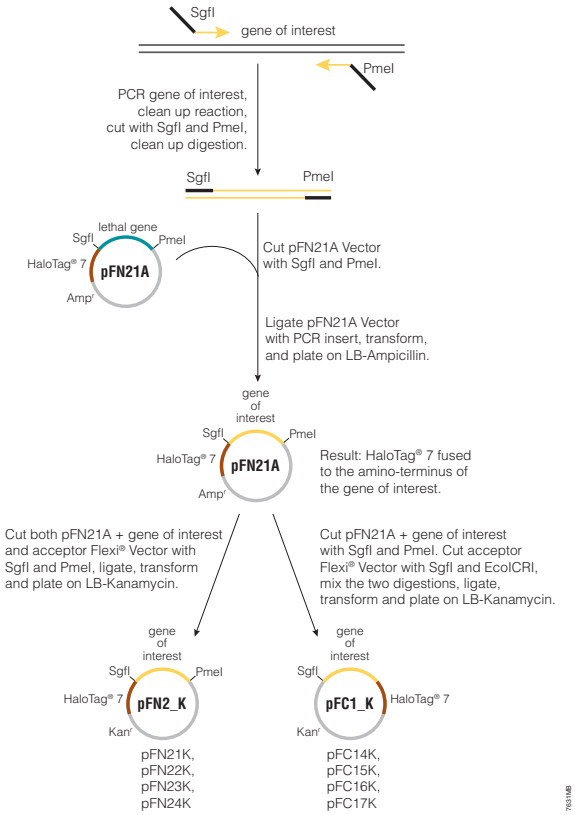

Figure 4. Using the CMV Deletion Series Sample Pack. Protein-coding regions can be cloned into the vectors by PCR using primers that append SgfI and PmeI sites to the amplimer, where PmeI now encodes the stop codon for the protein-coding region (see *Flexi® Vector Systems Technical Manual*, #TM254 for details). After restriction digestion, the protein coding region can be captured in pFN21A (HaloTag® 7) CMV Flexi® Vector. Because PCR is used in the process, sequence validation of the protein-coding region is advised. This sequence-validated clone now can be a donor of the protein-coding region for other HaloTag® 7 vectors included in the kit, as well as being used for mammalian expression of the HaloTag® 7 protein fused to your gene of interest. The shuttling of protein-coding regions between compatible Flexi® Vectors is a rapid, easy, efficient and high-fidelity process; it is not necessary to resequence the protein coding region after transfer to another vector. The kit provides every combination of orientation (N-terminal, C-terminal) and CMV promoter strength to provide appropriate expression levels of HaloTag® 7 fusion proteins in mammalian cells.

CMV DELETION SERIES SAMPLE PACK

The HaloTag® 7 CMV promoter deletion series and the full-length CMV promoter are available as a sample pack (Cat.# G3780). This pack allows the cloning of a protein coding region into a donor vector, pFN21A, and then transfer of the protein coding region to any or all of the complete set of mammalian HaloTag® 7 vectors (Figure 4).

p65/NFκB FUNCTIONAL ASSESSMENT AT DIFFERENT EXPRESSION LEVELS

The model system of p65/NFκB was used to examine proper function and cellular localization with HaloTag® 7 under different expression levels. In the absence of stimulation, the p65 protein resides primarily in the cytoplasm in most cells. Upon stimulation with the agonist TNF-α, p65 translocates to the nucleus, where it has been shown to bind directly and activate transcription of several targets, including the IκB promoter. The HaloCHIP™

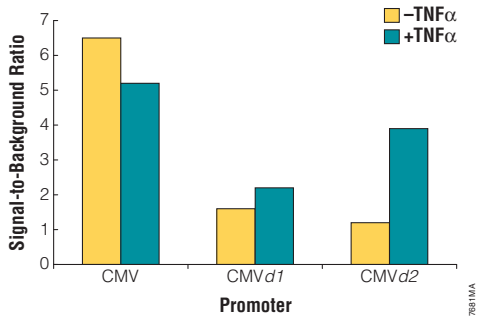

Figure 5. Agonist-dependent DNA binding of p65-HaloTag® 7 at varying expression levels using the HaloCHIP™ method. HeLa cells (4 × 10^5) were transfected with various p65-HaloTag® 7 constructs (pFC14, pFC15, and pFC16), then treated without or with TNF-α for 30 minutes. Sample crosslinking and processing was performed as described in the *HaloCHIP™ System Technical Manual*, #TM075. DNA fragments obtained from the HaloCHIP™ procedure were further purified, and the p65-specific promoter IκB was amplified using standard PCR (33 cycles). The signal-to-background ratios for each construct (+/– TNF-α) were determined and plotted.

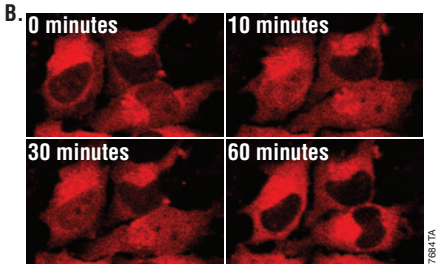

Figure 6. Agonist-dependent translocation of p65-HaloTag® 7 protein. HeLa cells (4 × 10^4) were plated in CG chamber (Nunc) and transiently transfected with the p65-HaloTag® 7 constructs using LT1 (Mirus). 24-hour-post-transfection cells were labeled with HaloTag® TMR Ligand as described in reference 3. Unbound ligand was washed out, and cells were imaged on an Olympus FV500 confocal microscope using the appropriate filter sets. For functional assay, cells were challenged with TNF-α (10 ng/ml), and images were collected every 10 minutes. p65-HaloTag® 7 moves into the nucleus within 10 minutes and moves back to the cytoplasm by 60 minutes.

method (5), which was designed to study the intracellular binding of HaloTag® fusion proteins to DNA without the use of antibodies, was employed to study the DNA binding response to TNF-α of the various p65-HaloTag® 7 fusion constructs. Analysis of the IκB promoter isolated from the HaloCHIP™ process (Figure 5) demonstrates that only CMV*d2* shows the proper increased DNA binding response to the TNF-α stimulation. Given the sensitivity of the p65/NFκB system to absolute protein levels and regulatory feedback loops, this demonstrates an example where low levels of expression are better suited to study proper physiology.

p65-HaloTag® 7 fusion protein cellular localization was difficult to determine when using the full-length CMV protein, as most of the cells were saturated with labeled fusion protein. However, the CMV deletion strains did provide appropriately labeled cells suitable for imaging with CMV*d2*, providing an optimal expression level. In cells expression p65 HaloTag® 7 fusion with the CMV*d2* promoter, it is easy to observe cytoplasmic-nucleus migration during a time-course treatment with TNF-α (Figure 6). These results are consistent with those observed using the HaloCHIP™ method (5).

SUMMARY

Overexpression of proteins in a cellular environment can potentially perturb protein function. To address these concerns, the HaloTag® 7 CMV promoter deletion series was created. This series allows fine tuning of protein expression levels as well as easy shuffling of the gene of interest between vectors. Proteins can be expressed at this level to study proper physiology combined with an easy, sensitive means of detection and purification.

REFERENCES

1. Encell, L. *et al.* (2008) (manuscript submitted)
2. Isomura, H., Tsurumi, T. and Stinski, M. F. (2004) *J.Virol.* **78**, 12788–99.
3. *HaloTag® Technology: Focus on Imaging Technical Manual* #TM260, Promega Corporation.
4. Isomura, H. *et al.* (2005) *J. Virol.* **79**, 9597–607.
5. Hartzell, D.D. *et al.* (2007) *Promega Notes* **97**, 18–21.

ORDERING INFORMATION

Product	Cat.#
pFC14A/K (HaloTag® 7) CMV Flexi® Vectors*	G9651/G9661
pFC15A/K (HaloTag® 7) CMV*d1* Flexi® Vectors*	G1611/G1601
pFC16A/K (HaloTag® 7) CMV*d2* Flexi® Vectors*	G1591/G1571
pFC17A/K (HaloTag® 7) CMV*d3* Flexi® Vectors*	G1551/G1321
pFN21A/K (HaloTag® 7) CMV Flexi® Vectors*	G2821/G2831
pFN22A/K (HaloTag® 7) CMV*d1* Flexi® Vectors*	G2841/G2851
pFN23A/K (HaloTag® 7) CMV*d2* Flexi® Vectors*	G2861/G2871
pFN24A/K (HaloTag® 7) CMV*d3* Flexi® Vectors*	G2881/G2981

Product	Size	Cat.#
HaloTag® 7 Flexi® Vectors — CMV Deletion Series Sample Pack	9 × 2 µg	G3780

* All Vectors are supplied in 20 µg aliquots.

[a]Patent Pending.

[b]For research use only. With respect to manufacture or sale of research products or services, or any diagnostic, therapeutic or prophylactic uses, please contact Promega for supply and licensing information.

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