HaloTag™ Interchangeable Labeling Technology

Promega

Technical Manual No. 260

INSTRUCTIONS FOR USE OF PRODUCTS G8241, G8251, G8271, AND G8281.
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I. Description

The HaloTag™ Interchangeable Labeling Technology[a,b,c,d] is a novel tool for imaging live or fixed mammalian cells that express the HaloTag™ Protein or protein fusions, analyzing post-translational modification of labeled fusion proteins, and isolating proteins and protein complexes. The technology is based on efficient formation of a covalent bond between a specially designed reporter protein encoded by the HaloTag™ pHT2 Vector and a specific ligand in living cells, in solution or on a solid support. The HaloTag™ pHT2 Vector contains the open reading frame for a genetically engineered derivative of a hydrolase gene. This protein is not endogenous to mammalian cells.

The HaloTag™ Ligand can carry a variety of functionalities, including fluorescent labels, affinity handles and attachments to a solid phase. The covalent bond forms rapidly under general physiological conditions, is highly specific and essentially irreversible, yielding a complex that is stable even under stringent conditions. The open architecture of the technology enables use of different ligands. We currently offer ligands that can readily cross the cell membrane with either red or green fluorophores or biotin. Additional ligands will be offered to expand the range of applications, as well as solid supports for direct capture of the HaloTag™ Protein or protein fusions.

Note: Certain uses of this product may require a special license. Please see page 24 of this Technical Manual for more information.

Advantages of HaloTag™ Interchangeable Labeling Technology:

- **Label in Solution or on a Solid Support:** The HaloTag™ Ligands bind to the HaloTag™ Protein or protein fusions with high specificity and affinity.
- **Label Your HaloTag™ Protein in Live Cells:** The HaloTag™ Ligands readily cross the cell membrane.
- **Image Fixed Cells:** The covalent bond is stable, allowing imaging of fixed cells and analysis of the labeled protein under stringent conditions.
- **Introduce Novel Functionalities or Perform Sequential Labeling:** The open architecture of the technology enables the use of different ligands for multiple applications.
- **Design Only One Genetic Construct for Multiple Experiments:** Obtain new functionality by using a different HaloTag™ Ligand without having to design and clone a new expression construct.
- **Analyze Labeled Fusion Proteins Using SDS-PAGE, Mass Spectrometry, etc.:** The bound ligand is stable under denaturing conditions.

A. The HaloTag™ Protein and Ligands

The HaloTag™ Protein, a genetically engineered derivative of a hydrolase gene (Figure 1), is designed to efficiently form a covalent bond with the HaloTag™ Ligands. The HaloTag™ Protein is a monomer and can be fused to your protein of interest at either the N- or C-terminus.

The HaloTag™ Ligands are small chemical tags that readily cross the cell membrane and comprise the HaloTag™ Reactive Linker and the HaloTag™ Functional Reporter (Figure 2).

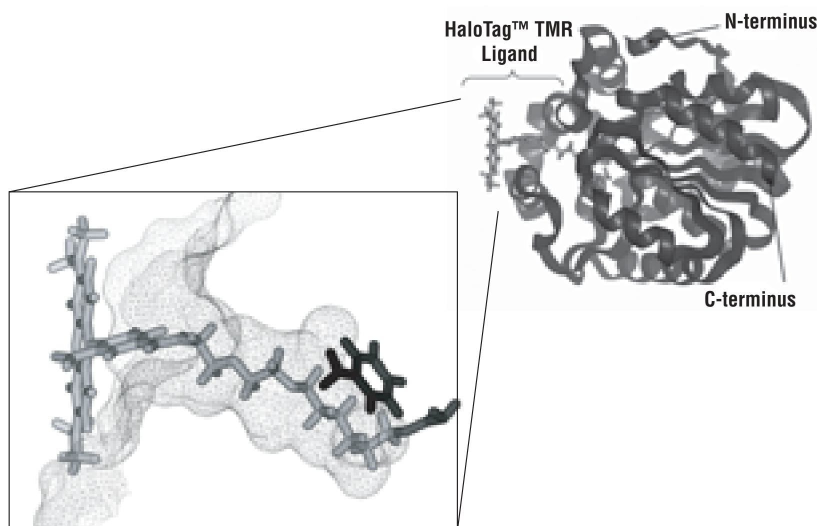

Figure 1. Structure of the HaloTag™ Protein with a HaloTag™ TMR Ligand modeled into the active site. The HaloTag™ Protein was created by mutating a wildtype hydrolase. Instead of forming an intermediate enzyme-substrate complex like the wildtype protein does, HaloTag™ Protein covalently traps the Reactive Linker. By coupling a fluorescent reporter to the Reactive Linker, a HaloTag™ Ligand is created for the HaloTag™ Protein that efficiently forms an irreversible bond to the fusion protein. Other reporter groups or solid supports may be attached to the Reactive Linker.

- The HaloTag™ TMR Ligand contains tetramethyl rhodamine (TMR) and is brightly fluorescent ($555_{Ex}/585_{Em}$). This ligand may be used for fluorescent labeling of the HaloTag™ Protein.
- The HaloTag™ diAcFAM Ligand contains a nonfluorescent diacetyl derivative of fluorescein. Upon cleavage of the diacetyl groups by cellular esterases diAcFAM is converted to brightly fluorescent FAM ($494_{Ex}/526_{Em}$). This Ligand may be used for fluorescent labeling of the HaloTag™ Protein.
- The HaloTag™ Biotin Ligand contains biotin and may be used as an affinity tag to capture a protein of interest using the strong biotin-streptavidin interaction.

B. The HaloTag™ pHT2 Vector

The HaloTag™ pHT2 Vector contains the open reading frame for the modified hydrolase gene cloned into a mammalian expression vector with the following features:

- Kozak sequence for translation initiation was added to the beginning of the gene.
- The following restriction sites were added for convenience: N-terminal (*Nhe* I, *Pvu* II, *Eco*R V, nonunique *Nco* I), C-terminal (*Pac* I, *Not* I).
  - *Bam*H I and *Nae* I restriction sites were added within the HaloTag™ gene ORF to allow convenient creation of protein fusions.
  - The *Eco*R V site was added to the vector before the ATG start codon of the HaloTag™ gene and cuts the pHT2 Vector in frame with the HaloTag™ ORF.
  - The *Bam*H I site was added immediately after the ATG start codon.
  - The *Nae* I site was added to the open reading frame just before the stop codon.

When designing HaloTag™ Protein-based fusions, preserving the functional activity of both fusion partners is one of the critical requirements to fusion protein design. To reduce potential three-dimensional structural hindrance effects of the fusion partners, we recommend inserting a polypeptide linker between fusion partners. The size and the sequence of the polypeptide linker should be determined empirically. A good place to start is with a 17-amino acid linker comprising Gly and Ser repeats.

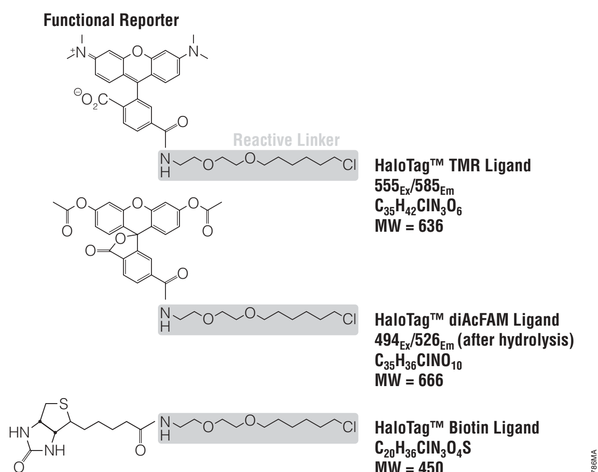

Figure 2. Structure of the ligands showing the Functional Reporters and the Reactive Linkers: HaloTag™ TMR Ligand, HaloTag™ diAcFAM Ligand and HaloTag™ Biotin Ligand. The HaloTag™ Ligands readily cross the cell membrane and can be used for live-cell imaging.

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To reduce potential three-dimensional structural hindrance effects of the fusion partners, we recommend inserting a polypeptide linker between fusion partners. The size and the sequence of the polypeptide linker should be determined empirically. A good place to start is with a 17-amino acid linker comprising Gly and Ser repeats.

Note: Using this vector requires generation of transfection-quality DNA.

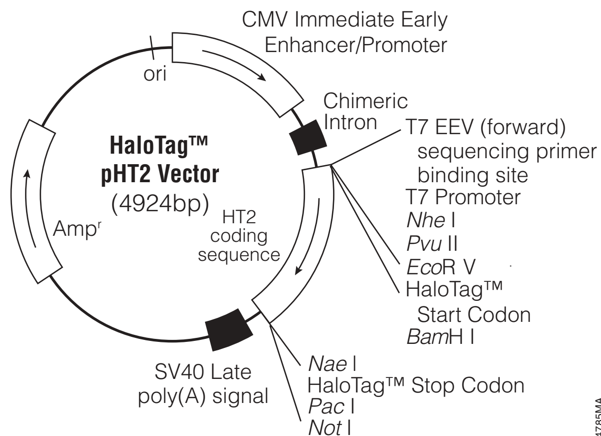

Figure 3. HaloTag™ pHT2 Vector circle map and sequence reference points.

HaloTag™ pHT2 Vector Sequence Reference Points:

CMV enhancer/promoter	1–742
Chimeric intron	857–989
T7 EEV (forward) sequencing primer binding site	1020–1041
T7 promoter	1034–1052
T3 (reverse) sequencing primer binding site	1996–2015
HaloTag™ open reading frame	1081–1971
SV40 late polyadenylation signal	2024–2245
β-lactamase (Amp^r) coding region	3232–4089

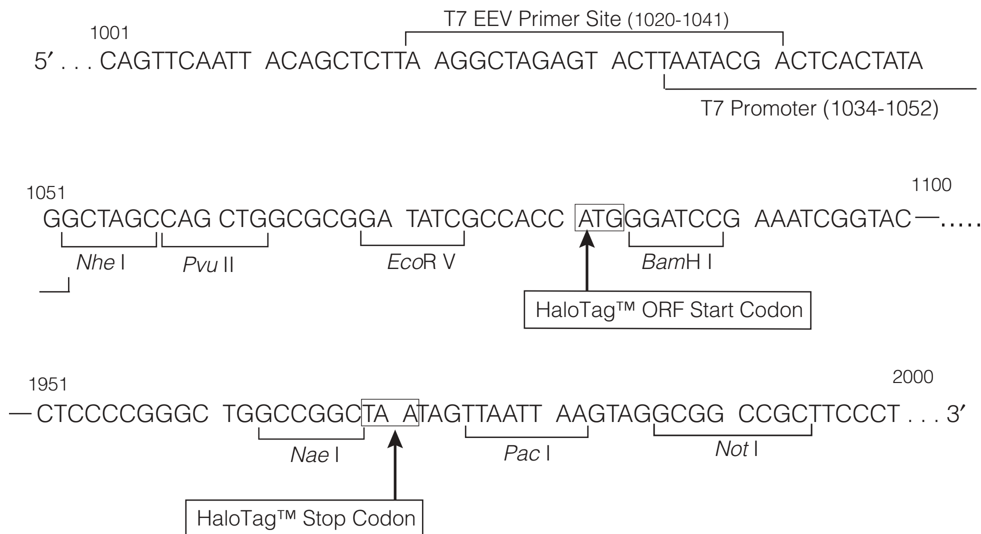

Figure 4. The sequence of the HaloTag™ pHT2 Vector showing potential cloning sites and primer hybridization.

II. Product Components

Product	Size	Conc.	Cat.#
HaloTag™ pHT2 Vector[a,b,c]	20µg		G8241
HaloTag™ TMR Ligand[a,d]	30µl	5mM	G8251
HaloTag™ diAcFAM Ligand[a,d]	30µl	10mM	G8271
HaloTag™ Biotin Ligand[a,d]	30µl	5mM	G8281

The HaloTag™ TMR and Biotin Ligands are supplied at 5mM in cell-culture-quality DMSO. The HaloTag™ diAcFAM Ligand is supplied at 10mM in cell-culture-quality DMSO.

Storage Conditions: Store the pHT2 Vector and ligands desiccated at –20°C. Avoid multiple freeze-thaw cycles and exposure to frequent temperature changes. These fluctuations can greatly alter product stability. We recommend dispensing ligands into aliquots and storing them at –20°C, desiccated and protected from light. When stored and handled properly, the performance of these products is guaranteed for six months from date of purchase.

Usage Note: The final recommended labeling concentration for the HaloTag™ TMR Ligand is 5µM, for the HaloTag™ diAcFAM Ligand is 10µM, and for the HaloTag™ Biotin Ligand is 5–25µM.

Table 1. Volume of Medium Plus Label Recommended per Well

Culture Vessel	Volume per well
8-well chamber slide	200µl
96-well plate	50µl
48-well plate	100µl
24-well plate	250µl
12-well plate	500µl
6-well plate	1,000µl

III. HaloTag™ Interchangeable Labeling Technology Protocols

A. Overview of the HaloTag™ Labeling Protocol

The figure below provides an overview of the HaloTag™ Labeling Protocol. The protocol should be empirically optimized for your experimental system.

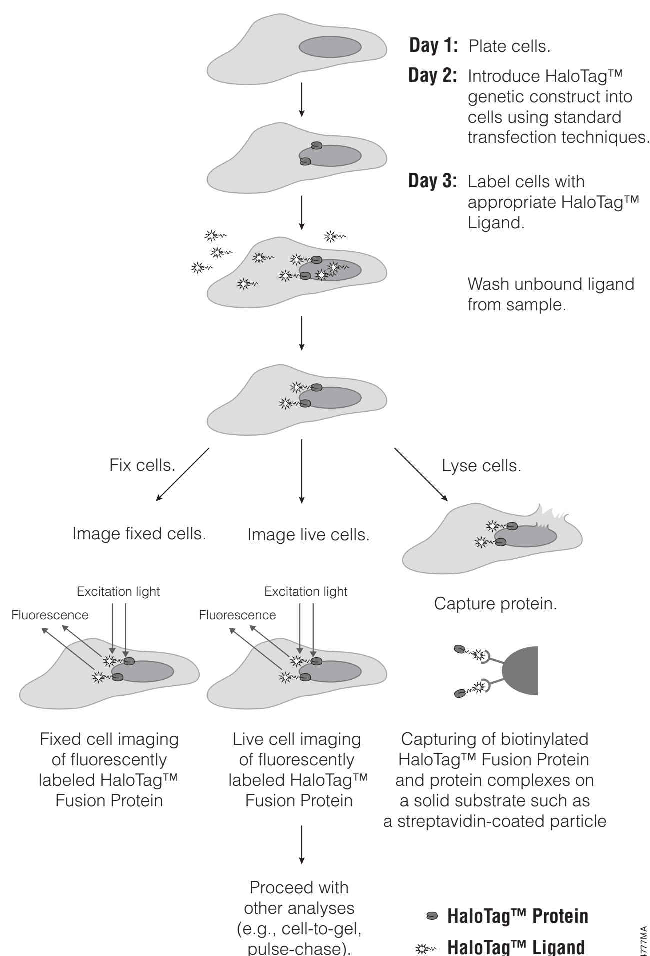

Figure 5. Overview of the HaloTag™ Interchangeable Labeling Technology applications.

B. Example Protocol for Labeling HaloTag™ Protein for Imaging Live or Fixed Cells

This example protocol is intended to serve as a guide. You should empirically optimize the cell culture protocol, transfection conditions, ligand concentration and labeling protocol for your experimental system.

Materials to Be Supplied by the User

- chambered cover glass
- transfection reagent
- endotoxin-free (transfection grade) plasmid DNA
- fetal bovine serum (FBS)
- serum-free cell culture medium
- PBS (37°C)
- fluorescent or confocal microscope
- 37°C cell culture incubator

Additional Materials for Imaging Fixed Cells

- 4% paraformaldehyde containing 0.5mM sucrose
- Triton® X-100
- 0.1% sodium azide/PBS solution

The following protocol was used for HeLa cells (ATCC #CCL-2) cultured in DMEM/F12 containing 10% FBS and no antibiotic (growth medium) on 8-well Lab-Tek® II chambered cover glass (Nalge Nunc Cat.# 155409) at 37°C, 5% CO_2. The cells were transfected with the HaloTag™ pHT2 Vector using a lipid transfection reagent according to the manufacturer's directions. The cells were imaged using a Olympus FV500 confocal microscope. Typical images of live and fixed cells expressing the HaloTag™ Protein labeled with the HaloTag™ TMR Ligand or the HaloTag™ diAcFAM Ligand are shown in Figure 6, Panels A and B. Figure 7 shows fixed cells expressing a p65-HaloTag™ fusion protein (1) labeled with the HaloTag™ TMR Ligand and counter labeled with Anti-βIII Tubulin Antibody.

Day 1: Plating Cells

1. Plate cells at a seeding density of 7.5–10 × 10^3 cells/cm^2 (9–12 × 10^3 cells/well) in 400µl growth medium.
2. Allow cells to grow using standard conditions (37°C, 5% CO_2) to ~85% confluence (~24–48 hours).

Day 2: Transfecting Cells

1. Transfect cells according to the manufacturer's instructions for the transfection reagent that you are using. See Section IV for more information about standard transfection methods.
2. Twenty four hours after adding transfection reagent, proceed with labeling protocol.

Day 3: Labeling Cells with HaloTag™ TMR or HaloTag™ diAcFAM Ligand

HaloTag™ TMR Ligand:

1. Prepare a 500-fold dilution of HaloTag™ TMR Ligand stock solution in 37°C growth medium (10µM).
2. Remove all but 100µl of growth medium. Cells should still be covered by medium. Add 100µl medium containing the HaloTag™ TMR Ligand to each well. The final recommended working concentration is 5µM.

HaloTag™ diAcFAM Ligand:

1. Prepare a 500-fold dilution of HaloTag™ diAcFAM Ligand stock solution in 37°C growth medium (20µM).
2. Remove all but 100µl of growth medium. Cells should still be covered by medium. *Immediately* add 100µl of medium containing the HaloTag™ diAcFAM Ligand to each well. The final recommended working concentration is 10µM.

Note: To avoid serum-induced hydrolysis of the HaloTag™ diAcFAM Ligand, add the Ligand to the cells **immediately** after diluting it.

Imaging Live Cells (Day 3 continued)

1. Incubate the cells with the desired HaloTag™ Ligand for 15 minutes at 37°C, 5% CO_2 in the dark.
2. Remove the ligand-containing medium.
3. Rinse the cells with 0.5ml/well PBS (37°C). Repeat two times for a total of three washes.
4. Replace the PBS with fresh growth medium (37°C), and return the cells to the incubator for 30 minutes (37°C, 5% CO_2).
5. Replace the growth medium with 400µl of PBS (37°C) or growth medium without phenol red (37°C).
6. Transfer the chambered cover glass to a microscope and capture images.

Imaging Fixed Cells (Day 3 continued)

1. Incubate the cells with the desired HaloTag™ Ligand for 15 minutes at 37°C, 5% CO_2 in the dark.
2. Remove the ligand-containing medium and rinse the cells with 400µl/well PBS (37°C).
3. Replace the PBS with 400µl freshly prepared 4% paraformaldehyde containing 0.5mM sucrose.
4. Incubate for 10 minutes at 37°C, 5% CO_2 in the dark.
5. Replace the fixative with PBS containing 0.1% Triton® X-100.
6. Incubate for 30 minutes at 37°C, 5% CO_2 in the dark.
7. Replace Triton® X-100 solution with 400µl PBS.
8. Transfer the chambered cover glass to a microscope stage and capture images. (Labeled cells may be stored in a 0.1% sodium azide/PBS solution protected from light. Mounted slides may be stored at room temperature, protected from light.)

Note: The HaloTag™ TMR Ligand is the dye of choice for cell-to-gel applications.

C. Example Protocol for Cell-To-Gel Analysis of HaloTag™ TMR-Labeled Cells

This protocol is intended to serve as a guide. You should empirically optimize the cell culture protocol, transfection conditions, ligand concentration and labeling protocol for your experimental system.

Materials to be Supplied by the User

- fetal bovine serum
- serum-free medium
- endotoxin-free (transfection grade) plasmid DNA
- transfection reagent
- SDS-PAGE running buffer
- SDS-PAGE sample buffer
- PBS (37°C)
- fluorescent imager
- 37°C incubator
- electrophoresis equipment

The following protocol was used for CHO-K1 cells (ATCC #CCL-61) transfected with the HaloTag™ pHT2 Vector using a lipid transfection reagent according to the manufacturer's protocol. Data generated using this protocol are presented in Figure 9.

Day 1: Plating Cells

1. Plate CHO-K1 cells in F12 medium containing 10% FBS (no antibiotic) in 24-well plates at a density of 1.2–1.4 × 10^5 cells/well.
2. Adjust the volume to 1,000µl/well and culture cells in standard conditions (37°C, 5% CO_2) to ~85% confluence (~18–24 hours).

Day 2: Transfecting Cells

1. Transfect cells following the manufacturer's instructions for the transfection reagent that you are using. See Section IV for more information about standard transfection methods.
2. Twenty-four hours after adding the transfection reagent, proceed with the labeling protocol.

Day 3: Labeling with HaloTag™ TMR Ligand

1. Prepare the HaloTag™ TMR Ligand solution for labeling.
   a. Prepare a 5µM solution of HaloTag™ TMR Ligand solution in F12 + 10% FBS.
   b. Remove the medium from the 24-well plate and replace it with 200µl of the 5µM HaloTag™ TMR Ligand solution.
2. Incubate the cells for 10 minutes at 37°C, 5% CO_2.
3. Remove the HaloTag™ TMR Ligand solution from the well.
4. Rinse the cells two times with 37°C PBS (1ml per well).

Day 3: Cell-To-Gel Analysis

1. Remove the PBS from the wells and add 200µl of 1X SDS-PAGE sample buffer to each well of the 24-well plate. Collect the lysate in a 1.5ml tube. Incubate for 5 minutes at 95°C.

   Note: Samples may be frozen at –20°C and analyzed later.
2. Load 10µl of each sample for SDS-PAGE analysis.
3. Analyze the gel on a fluorescent scanner.

D. Example Protocol for Expressing, Capturing and Detecting HaloTag™ Protein Fusion Expressed in Mammalian Cells

This protocol is intended to serve as a guide. You should empirically optimize the cell culture protocol, transfection conditions, and ligand concentration and labeling protocol for your experimental system.

Materials to Be Supplied By the User

- fetal bovine serum
- serum-free medium
- transfection reagent
- endotoxin-free (transfection grade) plasmid DNA
- streptavidin-coated particles
- 24-well culture plates
- protease inhibitors
- Tween® 20
- PBS (37°C)
- 37°C incubator
- magnetic stand

The following protocol was used with CHO-K1 (ATCC #CCL-61) cells cultured in 24-well plates (Fisher Cat.# 353047) transfected with the vector coding for a *Renilla* Luciferase-HaloTag™ Protein-FLAG fusion. This protocol used a lipid transfection reagent according to the manufacturer's instructions. Streptavidin MagneSphere® Paramagnetic Particles (Cat.# Z5481) were used to capture the proteins. Data generated using this protocol are presented in Figure 8.

Day 1: Plating Cells

1. Plate CHO-K1 cells in F12 medium containing 10% FBS, no antibiotic (growth medium) in 24-well plates at a density of 1.2–1.4 × 10^5 cells/well.
2. Adjust the volume to 1000µl/well and culture cells under standard conditions (37°C, 5% CO_2) to ~85% confluence (~18–24 hours).

Day 2: Transfecting Cells

1. Transfect cells following the manufacturer's instructions for the transfection reagent that you are using. See Section IV for more information about standard transfection methods.
2. Twenty-four hours after adding transfection reagent, proceed with labeling protocol.

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Day 3: Labeling the *Renilla* Luciferase-HaloTag™ Protein-FLAG Fusion with HaloTag™ Biotin Ligand

1. Prepare a 1,000- to 200-fold dilution of HaloTag™ Biotin Ligand stock solution in 37°C growth medium (5–25µM).
2. Replace growth medium with 200µl of diluted HaloTag™ Biotin Ligand solution.
3. Incubate cells with HaloTag™ Biotin Ligand for 15 minutes at 37°C, 5% CO_2.

   Note: You may need to optimize amount of Biotin Ligand and length of incubation.
4. Remove HaloTag™ Biotin Ligand-containing medium and quickly rinse the cells with 1ml/well warm PBS (37°C). Repeat two times for a total of 3 rinses.
5. Replace the PBS with fresh growth medium (37°C), and return the cells to the incubator for 60 minutes at 37°C, 5% CO_2.
6. Quickly wash the cells 2 times with 1.0ml/well warm PBS (37°C).
7. Add 200µl PBS containing protease inhibitors (Sigma #P8340) to each well as recommended by the manufacturer.
8. Lyse the cells by mechanical disruption.
9. Use cell lysates immediately or store them at –20°C for 1 month.

Day 3: Capturing the Biotin-Labeled HaloTag™ Protein

This protocol used MagneSphere® Paramagnetic Particles. The particles must be thoroughly resuspended and washed three times with PBS containing 0.05% Tween® 20. **Do not** allow particles to dry.

1. Mix 15–20µl paramagnetic streptavidin-coated particles and 50–60µl of cell lysate in 1.5ml tube. Incubate at room temperature on a shaker 15–60 minutes.

   Note: You may need to optimize the amount of cell lysate and amount of streptavidin particles for your experimental system.
2. Place the tube in the magnetic stand, and capture the magnetic particles.
3. Carefully remove the supernatant by aspiration (containing unbound protein) and save it for immunodetection.
4. Remove the tube from the magnetic stand, add PBS containing 0.05% Tween® 20 to the particles, and vortex quickly to break up magnetic particle aggregates.
5. Repeat the last three steps for a total of 3 washes.
6. To collect the proteins bound to the particles add ~50–60µl of SDS-PAGE Sample Buffer to the particles and heat the suspension 5 minutes at 95°C.
7. Place the tube in the magnetic stand and capture the magnetic particles.
8. Carefully collect the supernatant (bound proteins).
9. Analyze the samples immediately or store them at –20°C. Proteins can be resolved on SDS-PAGE and analyzed by Western blot.

Note: If using streptavidin-coated particles from another source, consult the manufacturer's instructions.

Note: Capture of some enzymes can be detected directly on the particles with an appropriate enzyme activity assay (see Figure 7). Resuspend the particles in a buffer that is appropriate for your particular assay.

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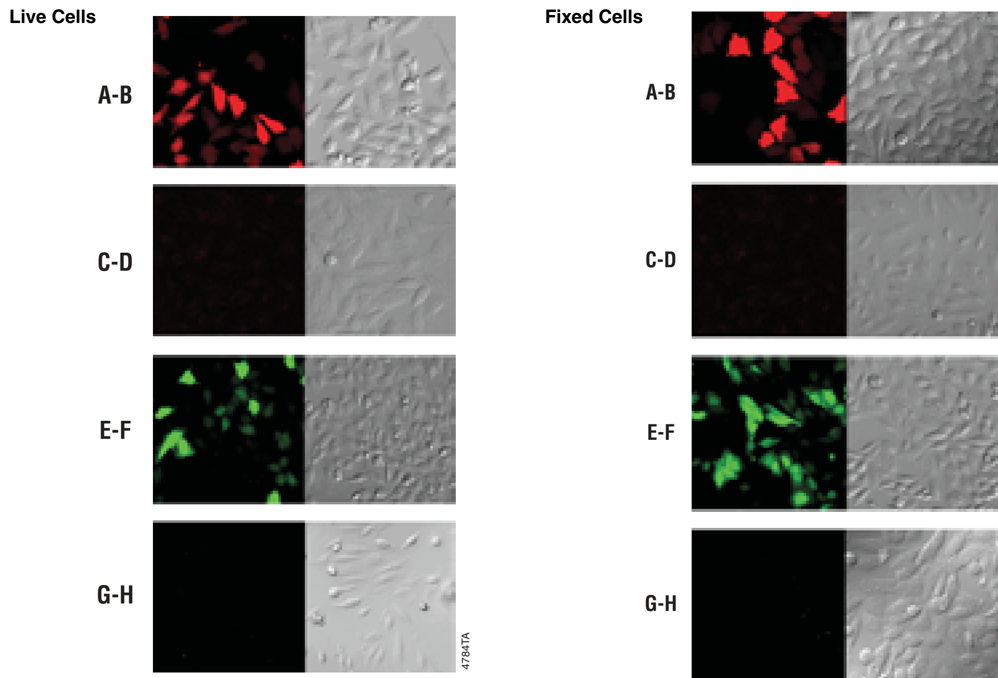

Figure 6. Images of live cells (Left Panel) and fixed cells (Right Panel) expressing HaloTag™ Protein and labeled with HaloTag™ TMR Ligand or HaloTag™ diAcFAM Ligand. HeLa cells were transiently transfected with the HaloTag™ pHT2 Vector and then labeled with 5μM HaloTag™ TMR Ligand (**Panels A and B**) or 10μM HaloTag™ diAcFAM Ligand (**Panels E and F**) according to the protocol described in Section III.B. Images were generated on the Olympus FV500 confocal microscope equipped with filters for fluorescence scanning and an environmental chamber to maintain physiologic conditions. HaloTag™ TMR Ligand was excited with a 545nm green HeNe laser (6.0% transmittance, PMT gain = 600), and HaloTag™ FAM Ligand was excited with a 488nm Argon laser (1.5% transmittance, PMT gain = 700). Transmitted light images (**Panels B, D, F and H**) were captured in parallel with live cell fluorescence measurements. Nontransfected cells were also treated with 5μM HaloTag™ TMR Ligand (**Panels C and D**) or 10μM HaloTag™ diAcFAM Ligand (**Panels G and H**), and images were captured using the same microscope settings. **Panels A and C** represent light passing through the emission filter in the red channel (lp >565nm); **Panels E and G** represent light passing through the emission filter in the green channel (bp, 510–535nm).

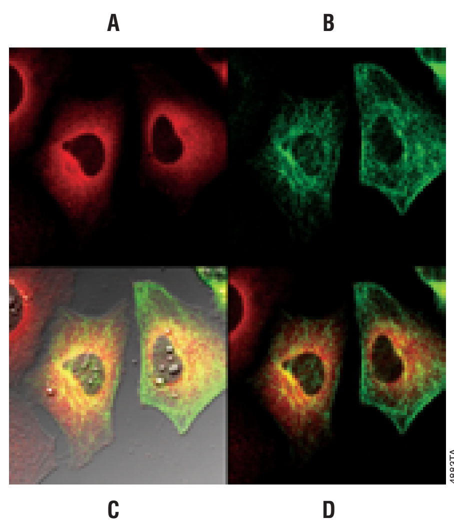

Figure 7. Fixed cells expressing p65-HaloTag™ Protein labeled with HaloTag™ TMR Ligand and counterstained for βIII-tubulin. HeLa Cells transiently transfected with plasmid encoding a p65-HaloTag™ fusion protein (1) were labeled with 5μM HaloTag™ TMR Ligand according to the protocol described in Section III.B. Cells were fixed with 3.7% paraformaldehyde, labeled with mouse Anti-βIII Tubulin Antibody (Cat.# G7121) at 1μg/ml followed by incubation with AlexaFluor™-488-conjugated goat-antimouse IgG (Molecular Probes). Images were generated on an Olympus FV500 confocal microscope in sequential mode using appropriate filter sets for TMR, Alexa Fluor™-488 or transmitted light. **Panel A.** TMR fluorescence. **Panel B.** Alexa Fluor™-488 fluorescence. **Panel C.** Overlaid Alexa Fluor™-488, TMR fluorescence and transmitted light. **Panel D.** Overlaid Alexa Fluor™-488 and TMR fluorescence.

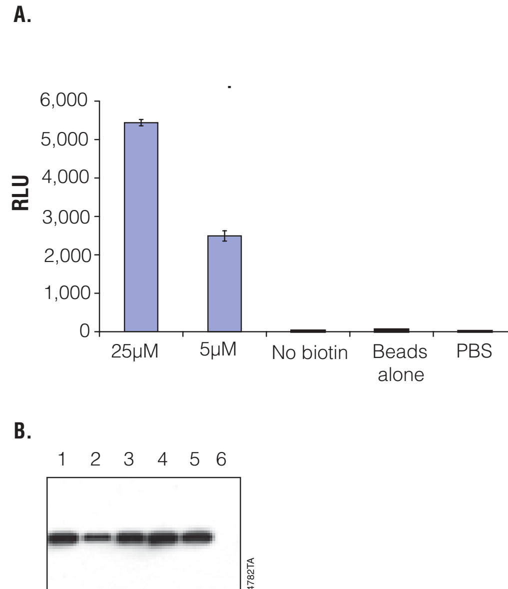

Figure 8. Efficient capture of a HaloTag™ Protein-based fusion on paramagnetic streptavidin (SA)-coated particles. CHO-K1 cells were transiently transfected with a vector encoding a *Renilla* luciferase-HaloTag™ Protein-FLAG fusion (RLUC-HT2-FLAG). Twenty-four hours later cells were treated with or without 5µM or 25µM of HaloTag™ Biotin Ligand for 15 minutes at 37°C. Unbound ligand was washed out, and biotin-labeled protein was captured on SA-coated paramagnetic particles (Cat.# Z5481). All procedures were done according to the protocol described in Section III.D. **Panel A.** To measure activity of the luciferase, particles on which RLUC-HT2-FLAG had been captured were transferred into a standard non-coated 96-well plate. An equal volume of 2X *Renilla* Luciferase Assay Substrate (Promega Part# E289B) was injected to the particles, and luminescent signal was detected on an Orion Microplate Luminometer (Berthold Detection Systems) over 10 seconds with a 2-second delay. **Panel B.** Cells transiently transfected with a vector coding for RLUC-HT2-FLAG fusion protein were treated with 25µM of HaloTag™ Biotin Ligand in DMSO (lanes 1–3) or DMSO alone (lanes 4–6) for 60 minutes at 37°C. Unbound ligand was washed out and biotin-labeled protein was captured on SA-coated paramagnetic particles. Cell lysates (lanes 1 and 4), non-bound (lanes 2 and 5) and bound protein (lanes 3 and 6) were incubated with SDS-PAGE Sample Buffer, resolved on SDS-PAGE, and analyzed by Western blot with anti-FLAG antibody M2 (Sigma).

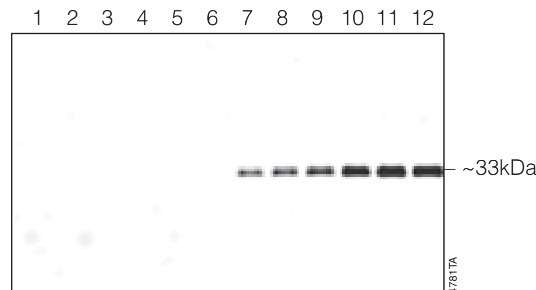

Figure 9. Fast, efficient and highly specific labeling of the HaloTag™ Protein expressed in CHO-K1 cells. CHO-K1 control cells (lanes 1–6) or cells transiently transfected with HaloTag™ pHT2 Vector (lanes 7–12) were treated with 5µM HaloTag™ TMR Ligand for different periods of time at 37°C (0.5, 1, 2, 5, 15, or 30 minutes). At the end of the ligand treatment, ligand-containing medium was removed, and the cells were quickly rinsed with PBS and lysed with SDS-PAGE sample buffer. Cell proteins were resolved by SDS-PAGE and analyzed on an Hitachi FMBIO® fluorescent scanner. All procedures were done as described in Section III.

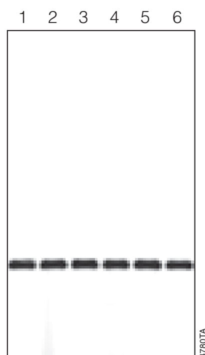

Figure 10. Fast, efficient, and highly specific labeling of the HaloTag™ Protein in vitro. Lysates (prepared in PBS plus protease inhibitors using mechanical disruption) of CHO-K1 cells that were transiently transfected with HaloTag™ pHT2 Vector were treated with 1.0µM HaloTag™ TMR Ligand for different periods of time at room temperature (0.5. 1, 2, 5, 15 or 30 minutes; lanes 1–6, respectively). The reaction was stopped by adding SDS-PAGE sample buffer. Cell lysate proteins were resolved by SDS-PAGE and analyzed on an Hitachi FMBIO® fluorescent scanner.

IV. General Considerations

The HaloTag™ TMR, HaloTag™ diAcFAM, and HaloTag™ Biotin Ligands readily cross the cell membrane, allowing labeling and detection of the HaloTag™ Protein in live mammalian cells or capture of proteins from cell lysates.

Ligand Concentration: Concentration of the HaloTag™ Ligands, transfection conditions, and cell labeling and washing protocols should be optimized for different cell lines and different applications. HaloTag™ TMR, HaloTag™ diAcFAM and HaloTag™ Biotin Ligands have shown no detectable toxicity or morphological side effects at recommended labeling conditions in the cell lines tested (HeLa, CHO-K1).

Serum: HaloTag™ Ligands can be added to serum-containing medium directly. The HaloTag™ TMR and HaloTag™ Biotin Ligands can be premixed with medium. The HaloTag™ diAcFAM Ligand must be mixed with media immediately before adding it to the cells. The diacetyl groups can be hydrolyzed by serum esterases converting the HaloTag™ diAcFAM Ligand, which can cross the membrane, into the FAM derivative, which cannot enter the cells.

Method of Detection: Fluorescence microscopy is a technique of choice for detection, subcellular localization and translocation of fluorescently labeled proteins. The ability to resolve cells, subcellular structures or fluorescently labeled proteins within specific cell compartments varies with instrument capabilities. We recommend using a confocal microscope with high numerical aperture objectives (2,3).

Fluorescence signal also can be detected and quantified using fluorescence scanners, flow cytometry or fluorescence plate readers. Accurate quantification of fluorescent signal could be complicated by a number of factors including low protein expression, instrument sensitivity and quality of cell growth surfaces.

Light Exposure: Fluorescent dyes are light sensitive. Avoid light exposure of the cells during ligand loading and washing procedures.

Transfection of Mammalian Cells: Maintaining healthy cell culture is essential for all mammalian cell-based applications. For established cell lines (e.g., CHO-K1 or HeLa), follow recommendations of cell supplier (e.g., ATCC). For additional information, consult references 4 and 5.

For transient transfection use your method of choice [e.g., lipofection (6,7), calcium phosphate (8,9), electroporation (10,11) or viral (12,13)]. Stable cell lines expressing HaloTag™ Protein or HaloTag™ Protein-based fusions also can be generated.

Note: Balance between sufficient protein expression, transfection efficiency and low toxicity is essential for generating reliable data.

The level of the HaloTag™ Protein expression depends on many factors including cell type, efficiency of transfection, type of promoter and protein coding sequence. Transfection can be toxic to cells. Transfection efficiency frequently correlates with toxicity of the transfection protocols. Balance between sufficient protein expression, transfection efficiency and low toxicity is essential for generating reliable data. Cells should be actively proliferating. The recommended cell density for most cell lines at transfection time is approximately 80–90% confluence. Preliminary experiments should be done to optimize cell density, amount of DNA and transfection reagent for transient transfection. Efficiency of transfection may be affected by the specific cell line, cell culture and transfection conditions, and specific DNA constructs.

For additional information, please contact your local Promega Branch Office or distributor. Contact information available at: **www.promega.com**.

E-mail:
techserv@promega.com

V. Troubleshooting

A. Fluorescence Labeling and Detection

Symptoms	Comments
Fusion protein not expressed or expressed only at low level	Check the reading frame of your construct by sequence analysis.
	Optimize transfection conditions, and use high-quality, endotoxin-free DNA.
	To stabilize expressed protein, add protease inhibitors during the cell lysis step.
	Consider changing promoters and/or other regulatory elements.
	Try construct with the order of the pro-. tein of interest and the HaloTag™ Protein reversed. Fusions in which the dehalogenase is at the C-terminus of the protein of interest may work better.
Expression too low for immunodetection	Use high-quality antibody for detection.
	Check transfection conditions to optimize expression.
Weak or no fluorescent signal	Make sure that transfection efficiency is optimized.
	Culture cells for a longer period of time before labeling to ensure that you have adequate protein expression.
	Protein expression can be improved by optimizing the health of the cells. Increase the seeding density or time in culture to allow cells to proliferate and adhere more tightly.
	Optimize cell-labeling protocols. Increase cell-labeling time. Increase the concentration of the HaloTag™ Ligand up to 25μM.
	Store the HaloTag™ Ligands at –20°C and protect them from light so that they do not lose labeling activity. Dispense the HaloTag™ Ligands into aliquots and avoid multiple freeze-thaw cycles. Use freshly prepared labeling solution to label your cells.

A. Troubleshooting (Fluorescence Labeling and Detection), continued

Symptoms	Comments
Weak or no fluorescent signal (continued)	Ensure that you are using the correct filter set for viewing. Use a standard FITC filter set to detect proteins labeled with the HaloTag™ diAcFAM Ligand. Use a standard TRITC filter set to detect proteins labeled with HaloTag™ TMR Ligand. Adjust the settings on your fluorescence detection instruments (laser power and PMT gain for a confocal microscope).
	To avoid hydrolysis of the diacetyl groups of the HaloTag™ diAcFAM Ligand, be sure to mix the HaloTag™ diAcFAM Ligand with cell culture medium immediately before adding it to the cells. You may also increase the concentration of the HaloTag™ diAcFAM Ligand up to 25µM.
	To prevent HaloTag™ Ligands from photobleaching, analyze fluorescent signal for only a short period of time. Use lower laser power.
	For cell-to-gel applications, we recommend using the HaloTag™ TMR Ligand for labeling.
High background fluorescence	Increase time for washing unbound ligand.
	Replace the medium with 37°C PBS or phenol red-free culture medium (37°C) immediately before imaging live cells.
	Adjust instrumentation settings (e.g., reduce laser power, reduce gain on PMT).
	The HaloTag™ Ligands can bind to charged surfaces. Use an alternative culture dish or glass.
Cells detached from surface	When imaging live cells, handle cells carefully to ensure they remain attached to surface.
Altered cell morphology	Label cells in the dark so that they are not exposed to intense light during labeling.
Poor transfection efficiency	Optimize transfection conditions, and use high-quality, endotoxin-free DNA.
Cell death or toxicity	Use less toxic transfection reagents. The DNA used for transfection must be endotoxin free.
	Increase seeding density or time in culture to allow cells to proliferate and adhere more tightly. Use an attachment matrix such as poly-L-lysine, fibronectin or collagen.

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V. Troubleshooting (continued)

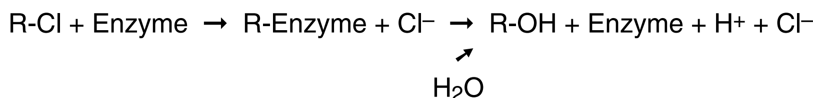
B. Biotin Labeling and Detection or Capture

Symptoms	Comments
Fusion protein not expressed or expressed only at low level	Check the reading frame of your construct by sequence analysis.
	Optimize transfection conditions, and use high-quality, endotoxin-free DNA.
	To stabilize expressed protein, add protease inhibitors during the cell lysis step.
	Consider changing promoters and/or other regulatory elements.
	Try construct with the order of the pro-. tein of interest and the HaloTag™ Protein reversed. Fusions in which the dehalogenase is at the C-terminus of the protein of interest may work better.
Expression too low for immunodetection	Use high-quality antibody for detection. Check transfection conditions to optimize expression.
Fusion protein not binding to streptavidin (SA) particles	Optimize labeling procedure to ensure ligand binding (time of labeling, concentration of HaloTag™ Biotin Ligand, time of washing unbound ligand).
	Use the cell-to-gel assay to test the ability of the protein to bind to the HaloTag™ Ligand.
	Change the design of the fusion if the ligand cannot bind (e.g., insert a polypeptide linker between the fusion partners).
	Be careful not to aspirate the particles during the washing steps. Save unbound material for immunodetection.
	Add protease inhibitors to the lysis step prevent degradation.
	Optimize the amount of SA particles added to the cell lysis sample. Handle SA particles as directed by the manufacturer.
Fusion protein not active	Some fusion constructs may produce fusion proteins that interfere with HaloTag™ Protein-Ligand binding.

VI. Appendix

A. HaloTag™ Technology and Chemistry

The native hydrolase is a monomer with MW ~33kDa. The activity of enzyme cleaves carbon-halogen bonds in aliphatic halogenated compounds involving a hydrolytic triad at the active site.



$$\text{R-Cl} + \text{Enzyme} \rightarrow \text{R-Enzyme} + \text{Cl}^- \xrightarrow{H_2O} \text{R-OH} + \text{Enzyme} + \text{H}^+ + \text{Cl}^-$$

In the reaction catalyzed by the native enzyme, an enzyme-substrate complex is formed by a nucleophilic attack involving Asp106 and the formation of an ester intermediate; His272 activates H_2O that hydrolyzes this intermediate, releasing product from the catalytic center (Figure 11; 14–16). A point mutation in the gene resulting in a His272Phe substitution impairs the hydrolysis step, leading to formation of a covalent bond between protein and ligand containing the functional reporters (Figure 12). The amino acid sequence of the protein was further optimized to provide better access to the active site by the ligand. These changes result in a dramatic increase in the kinetics of ligand binding rate by several thousand-fold, leading to almost immediate binding for HaloTag™ TMR Ligand binding to GST-HaloTag™ Protein fusion in vitro.

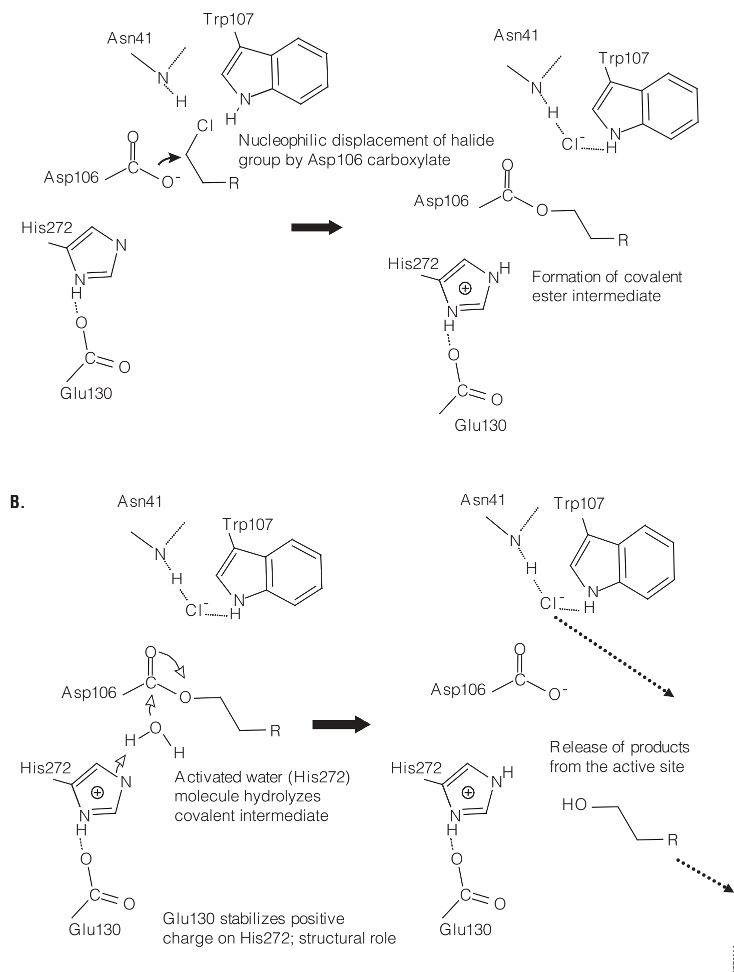


Figure 11. The catalytic mechanism of wildtype hydrolase.

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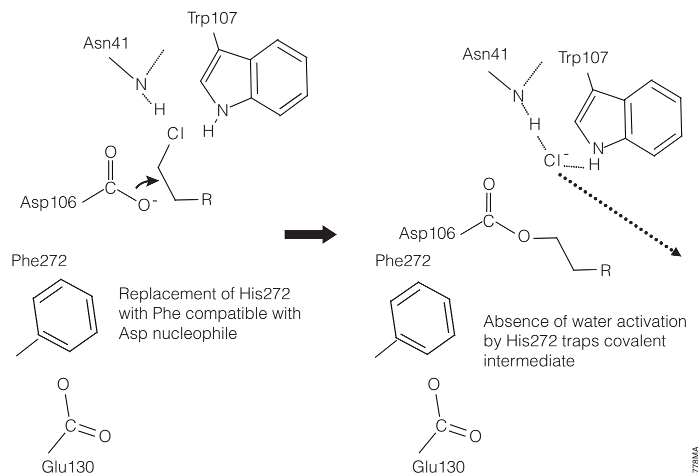
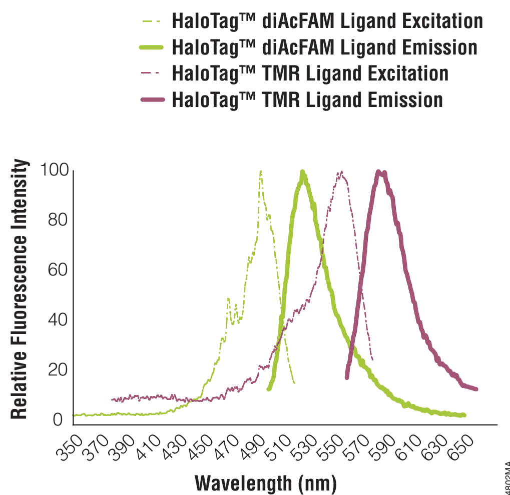


Figure 12. The engineered hydrolase encoded by the HaloTag™ pHT2 Vector includes the His272Phe substitution, which is critical for covalent bond formation.



Ligand	Excitation Maximum	Emission Maximum
HaloTag™ diAcFAM (upon hydrolysis)	494nm	526nm
HaloTag™ TMR	555nm	585nm

Figure 13. Excitation and emission spectra for the HaloTag™ TMR Functional Reporter and the HaloTag™ diAcFAM Functional Reporter (after hydrolysis of the diacetyl groups and conversion to the FAM derivative).

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B. HaloTag™ pHT2 Vector Features and Restriction Enzyme Sites

CMV Enhancer/Promoter: The CMV enhancer/promoter region present in the HaloTag™ pHT2 Vector allows strong, constitutive expression in many cell types. The promiscuous nature of the CMV promoter/enhancer has been demonstrated in transgenic mice, where its transcriptional activity was observed in 24 of the 28 murine tissues examined (17).

Chimeric Intron: Downstream of the enhancer/promoter region is a chimeric intron composed of the 5´-donor site from the first intron of the human β-globin and the branch and 3´-acceptor site from the intron between the leader and body of an immunoglobin gene heavy chain variable region (18). The sequences of the donor and acceptor sites, along with the branch point site, have been changed to match the consensus sequences for splicing (19). Transfection studies have demonstrated that the presence of an intron flanking the cDNA insert frequently increases the level of gene expression (20–22). The intron is located upstream of the HaloTag™ gene sequence to minimize the use of cryptic 5´-donor splice sites that may reside within the gene (23,24).

T7 Promoter: A T7 RNA polymerase promoter is located downstream of the chimeric intron and immediately precedes the HaloTag™ gene. This promoter can be used to synthesize RNA transcripts in vitro using T7 RNA Polymerase (Cat.# P2075) or the TNT® T7 Quick Coupled Transcription/Translation System (Cat.# L1170) or the TNT® T7 Coupled Reticulocyte Lysate System (Cat.# L4610).

SV40 Late Polyadenylation Signal: Polyadenylation signals cause the termination of transcription by RNA polymerase II and signal the addition of approximately 200–250 adenosine residues to the 3´-end of the RNA transcript (25). Polyadenylation has been shown to enhance RNA stability and translation efficiency (26,27). The late SV40 polyadenylation signal is extremely efficient and has been shown to increase the steady-state level of RNA approximately five-fold over levels achieved with the early SV40 polyadenylation signal (28).

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HaloTag™ pHT2 Vector Restriction Enzyme Sites

The following restriction enzyme tables were constructed using DNASTAR® sequence analysis software. Please note that we have not verified this information by restriction digestion with each enzyme listed. The location given specifies the 3´-end of the cut DNA (the base to the left of the cut site). For more information on the cut sites of these enzymes, or if you identify a discrepancy, please contact your local Promega Branch or Distributor. In the U.S., contact Promega Technical Services at 800-356-9526. Vector sequences are available in the GenBank® database (GenBank® Accession Number AY773970) and online at: www.promega.com/vectors/

Table 1. Restriction Enzymes That Cut the HaloTag™ pHT2 Vector Between 1 and 5 Times.

Enzyme	# of Sites	Location
***Acc* B7 I**	1	1125
***Acc* III**	2	1440, 1538
Afl II	2	820, 1017
***Alw*44 I**	3	2850, 3347, 4593
*Alw*N I	1	4498
*Asp*H I	5	721, 2854, 3351, 3436, 4597
***Ava* I**	2	1887, 1954
***Bal* I**	3	10, 64, 1506
***Bam*H I**	1	1084
***Ban* I**	4	618, 943, 2529, 4066
***Ban* II**	5	721, 1408, 1658, 1727, 2499
Bbs I	2	928, 1916
***Bcl* I**	1	1565
***Bgl* II**	1	4919
Blp I	1	1407
Bpu 1102 I	1	1407
Bsa I	3	882, 1516, 3953
*Bsa*A I	2	493, 2570
*Bsa*B I	1	2256
***Bsa*M I**	2	2075, 2168
Bsm I	2	2075, 2168
*Bsp*H I	3	3074, 3179, 4187
*Bsp*M I	1	844
*Bsr*G I	1	96
***Bss*H II**	1	1942
*Bss*S I	3	3043, 3350, 4734
***Bst*98 I**	2	820, 1017
***Bst*E II**	1	1201
***Bst*Z I**	3	1554, 1829, 1988
*Cfr*10 I	3	1717, 1963, 3934
***Cla* I**	2	1571, 2260
***Csp* I**	1	1541
***Dra* I**	4	2215, 3441, 4133, 4152
Dra II	1	3039
Dra III	1	2573
Drd I	5	809, 1333, 2617, 2936, 4805
Dsa I	3	513, 1079, 1168
Eag I	3	1554, 1829, 1988
Ear I	3	1370, 2278, 3220
***Ecl*HK I**	1	4019
***Eco*52 I**	3	1554, 1829, 1988
***Eco*ICR I**	1	719
***Eco*R I**	2	1471, 1510
***Eco*R V**	1	1071
Fsp I	2	2319, 3796
***Hae* II**	3	2415, 2423, 4667
***Hinc* II**	3	669, 1674, 2154
*Hin*d II	3	669, 1674, 2154
***Hin*d III**	1	748
***Hpa* I**	1	2154
***Nae* I**	1	1965
***Nco* I**	2	513, 1079
***Nde* I**	2	387, 2845
***Ngo*M IV**	1	1963
***Nhe* I**	1	1052
***Not* I**	1	1988
***Nru* I**	1	1652
Nsp I	3	1246, 1466, 2994
Pac I	1	1979
*Pfl*M I	1	1125
*Psp*A I	2	1887, 1954
***Pst* I**	1	830

Table 1. Restriction Enzymes That Cut the HaloTag™ pHT2 Vector Between 1 and 5 Times (continued).

Enzyme	# of Sites	Location	Enzymes	# of Sites	Location
***Pvu* I**	2	2300, 3650	***Ssp* I**	4	5, 52, 2778, 3214
***Pvu* II**	1	1060	***Stu* I**	1	1534
Rsr II	1	1541	***Sty* I**	3	513, 1079, 1364
***Sac* I**	1	721	*Tfi* I	2	1418, 1694
***Sac* II**	1	1171	***Tth*111 I**	2	1212, 1333
***Sca* I**	2	1030, 3538	***Vsp* I**	2	160, 3844
*Sgr*A I	1	1717	***Xma* I**	2	1887, 1954
***Sma* I**	2	1889, 1956	***Xmn* I**	2	1582, 3419
***Sna*B I**	1	493			
***Spe* I**	1	152			

Table 2. Restriction Enzymes That Do Not Cut the HaloTag™ pHT2 Vector.

***Acc* I**	*Bbr*P I	*Eco*72 I	***Mlu* I**	*Ppu*M I	*Srf* I
***Acc*65 I**	***Bbu* I**	*Eco*81 I	***Nar* I**	*Psh*A I	*Sse*8387 I
Afl III	*Bsp*120 I	*Eco*N I	***Nsi* I**	*Psp*5 II	*Swa* I
***Age* I**	*Bst*1107 I	*Ehe* I	*Pae*R7 I	***Sal* I**	***Xba* I**
***Apa* I**	***Bst*X I**	*Fse* I	*Pin*A I	***Sfi* I**	*Xcm* I
Asc I	***Bsu*36 I**	**I-*Ppo* I**	*Pme* I	***Sgf* I**	***Xho* I**
Avr II	***Csp*45 I**	*Kas* I	*Pml* I	***Sph* I**	
Bbe I	***Eco*47 III**	***Kpn* I**	*Ppu*10 I	*Spl* I	

Table 3. Restriction Enzymes That Cut the HaloTag™ pHT2 Vector 6 or More Times.

***Aat* II**	*Bsa*J I	*Dpn* II	***Hsp*92 I**	***Msp*A1 I**	*Sfa*N I
Aci I	***Bsp*1286 I**	*Eae* I	***Hsp*92 II**	***Nci* I**	***Sin* I**
Acy I	*Bsr* I	*Fnu*4H I	*Mae* I	***Nde* II**	***Taq* I**
***Alu* I**	***Bsr* S I**	***Fok* I**	*Mae* II	*Nla* III	***Tru*9 I**
***Alw*26 I**	*Bst*71 I	***Hae* III**	*Mae* III	*Nla* IV	***Xho* II**
***Ava* II**	***Bst*O I**	*Hga* I	*Mbo* I	*Ple* I	
Bbv I	*Bst*U I	***Hha* I**	***Mbo* II**	***Rsa* I**	
***Bgl* I**	***Cfo* I**	***Hinf* I**	*Mnl* I	***Sau*3A I**	
*Bsa*O I	***Dde* I**	***Hpa* II**	*Mse* I	*Sau*96 I	
*Bsa*H I	***Dpn* I**	*Hph* I	***Msp* I**	*Scr*F I	

Note: The enzymes listed in boldface type are available from Promega.

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D. Related Products

Transfection Reagents

Product	Size	Cat.#
TransFast™ Transfection Reagent	1.2mg	E2431
Tfx™-10 Reagent	9.3mg	E2381
Tfx™-20 Reagent	4.8mg	E2391
Tfx™-50 Reagent	2.1mg	E1811
Tfx™ Reagent Transfection Trio	5.4mg	E2400
Transfectam® Reagent	1mg	E1231
	0.5mg	E1232
ProFection® Mammalian Transfection System—Calcium Phosphate	40 reactions	E1200
ProFection® Mammalian Transfection System—DEAE Dextran	40 reactions	E1210

Transfection-Quality DNA Purification

Product	Size	Cat.#
PureYield™ Plasmid Midiprep System	25 preps	A2492
	100 preps	A2495
Wizard® Purefection Plasmid DNA Purification System[1*]	2 maxipreps/ 10 midipreps	A2150
Wizard MagneSil Tfx™ System*	4 × 96 preps	A2381
Endotoxin Removal Resin	100ml	A2191
4/40 Wash Solution	115ml	A2221

[1]For Laboratory Use. *Available in additional sizes.

Protein Fusion Vectors

Product	Size	Cat.#
Monster Green® Fluorescent Protein phMGFP Vector	20µg	E6421

Magnetic Particles

Product	Size	Cat.#
Streptavidin MagneSphere® Paramagnetic Particles	9ml (15 × 0.6ml)	Z5481

For Laboratory Use. Available in additional sizes.

Cell Viability Analysis

Product	Size	Cat.#
CellTiter-Glo® Luminescent Cell Viability Assay*	10ml	G7570
CellTiter-Blue® Cell Viability Assay*	20ml	G8080
CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay[1*]	200 assays	G3582

[1]For Laboratory Use. *Available in additional sizes.

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