

TECHNICAL MANUAL

HaloTag[®] Technology: Focus on Imaging

Instructions for Use of Products

**G2991, G3221, G1001, G1002, G2801, G2802, G8251, G8252, G8272, G8273,
G8581, G8582, G8471, G8472, G9281, G9211, GA1110, GA1111, GA1120,
GA1121, HT1010, HT1020, HT1030, HT1040, HT1050, HT1060, HT1070,
HT1100, HT1110, P1691, P6751, P6741 and P6711**

HaloTag[®] Technology: Focus on Imaging

All technical literature is available at: www.promega.com/protocols/
 Visit the web site to verify that you are using the most current version of this Technical Manual.
 E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

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1. Introduction to HaloTag® Technology and Cell Imaging

Understanding the functional role of proteins and how they interact within the cell is increasingly important. Often multiple recombinant protein fusions are needed to accomplish this research, which at times can be slow and cumbersome because of inefficiencies from moving between different applications. As a result, there is a need for a recombinant protein tag that affords flexibility between expression and localization, protein purification, protein interaction discovery, screening and further functional analyses.

The HaloTag® platform^(a-h) addresses the need for flexibility in functional protein analysis. **This modular technology is based on the formation of a covalent bond between a fusion tag and synthetic ligands, and is designed to enable complete characterization of protein function in cellular and biochemical environments.** There are a variety of HaloTag® Ligands^(a-g) and kits available for fluorescent cell imaging and protein capture/purification, as well as reactive customizable Ligand Building blocks^(e,f,g).

The HaloTag® reporter protein is an engineered, catalytically inactive derivative of a hydrolase, which forms a covalent bond with HaloTag® ligands (Figure 1). Under physiological conditions this covalent bond forms rapidly and is highly specific and essentially irreversible, yielding a complex that is stable even under stringent conditions (1–3). The HaloTag® protein is a 33kDa monomeric protein not endogenous to mammalian, plant or *E. coli* cells; therefore, there are no levels of nonspecific activity, only high labeling specificity. This technology has been used successfully in many systems, including: mammalian cells (4), bacteria (5), yeast (6), plants (7) and animal models (8).

Advantages of Cell Imaging Using HaloTag® Fluorescent Ligands

Gain Precision with Temporal and Spatial Control of Labeling: The modular nature of HaloTag® technology and the rapid binding kinetics with its ligands allow direct imaging of both protein trafficking and turnover. The user has spatial and temporal control of labeling because available ligands have both varied fluorescent spectra and also cell-permeant and impermeant properties. For example, a researcher can label only the surface pool of a membrane-bound protein with a cell-impermeant ligand and follow its biology, internalization or other fate. Or a protein can first be labeled with a ligand of one “color”, and after a period of time labeled again with a ligand containing a dye of a different color. Such pulse-chase labeling allows direct observation of protein trafficking and/or turnover over time.

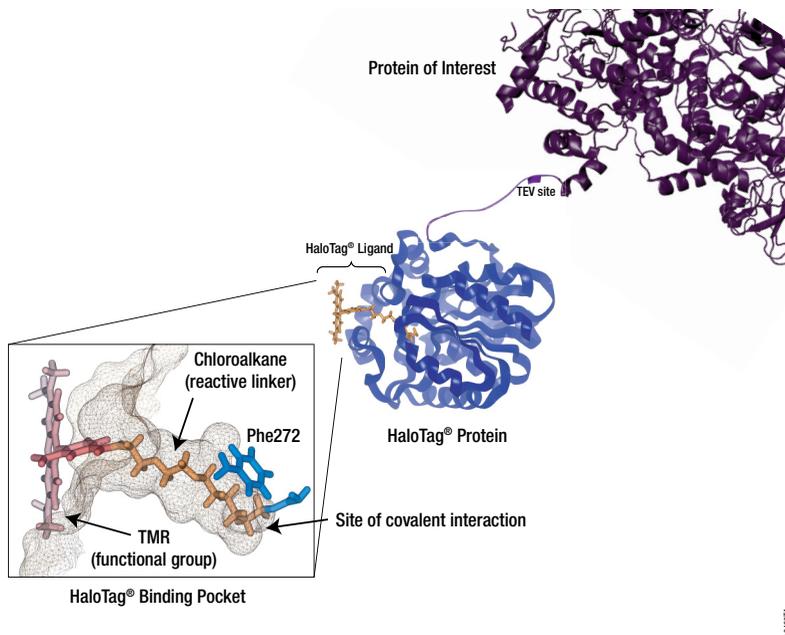


Figure 1. HaloTag® fusion protein displaying the linker to scale with TEV site and the binding pocket for the covalent interaction with HaloTag® ligands.

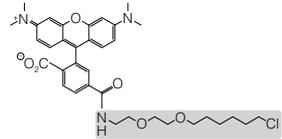
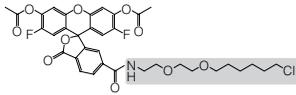
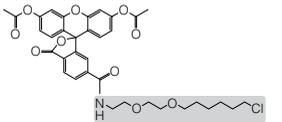
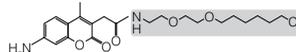
Label Live, then Image Live or Fixed Cells, and/or Directly Run a Gel for Quantification: All HaloTag® fluorescent ligands allow live-cell labeling and imaging. Labeling can be done rapidly with a wash step (within an hour) or overnight using a No-Wash protocol, depending on your workflow needs. Fixed cells manipulated by standard immunocytochemistry protocols can also be imaged because of the stability of the covalent bond between the HaloTag® fusion protein and ligands, and the stability of the dyes themselves. This stability also allows labeled cells to be analyzed directly by SDS-PAGE using a fluorescent scanner.

Design Only One Genetic Construct for Multiple Applications: Obtain new functionality without designing and cloning a new expression construct. Beyond cell imaging, the same fusion protein can be purified as a single pure protein, or pulled down with its partners to discover new protein interactions. Switch from HaloTag® fluorescent ligands to HaloTag® Purification and Pull-Down Systems using HaloLink™ Resin to efficiently capture and recover HaloTag® fusion proteins or complexes.

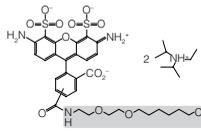
2. Product Components and Storage Conditions

2.A. Fluorescent Ligands

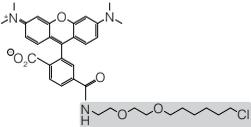
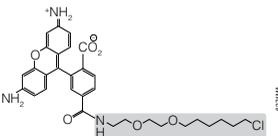
Cell-Permeant "Rapid" Ligands (for intracellular labeling)

Product	Size	Cat. #	Structure
HaloTag® TMR Ligand	15µl	G8252	
	30µl	G8251	
HaloTag® Oregon Green® Ligand	15µl	G2802	
	30µl	G2801	
HaloTag® diAcFAM Ligand	15µl	G8273	
	30µl	G8272	
HaloTag® Coumarin Ligand	15µl	G8582	
	30µl	G8581	

Cell-Impermeant "Rapid" Ligands (for cell-surface labeling)

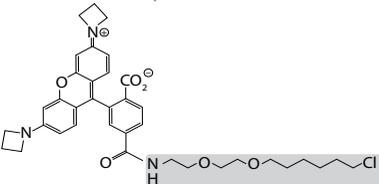
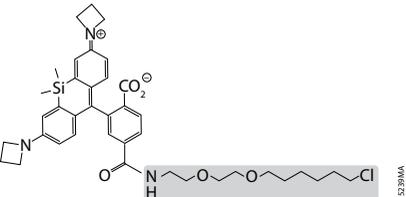
Product	Size	Cat. #	Structure
HaloTag® Alexa Fluor® 488 Ligand	15µl	G1002	
	30µl	G1001	
HaloTag® Alexa Fluor® 660 Ligand ^(d)	15µl	G8472	Structure not available
	30µl	G8471	

Cell-Permeant “No-Wash” Ligands (for intracellular labeling)

Product	Size	Cat.#	Structure
HaloTag® TMRDirect™ Ligand	30µl	G2991	
HaloTag® R110Direct™ Ligand	30µl	G3221	

Storage Conditions: Store all HaloTag® Ligands desiccated at –30°C to –10°C, protected from light. Avoid multiple freeze-thaw cycles and exposure to frequent temperature changes because these fluctuations can greatly alter product stability. We recommend dispensing ligands into aliquots prior to proper storage. See the expiration date on the product label.

Cell-Permeant, “Rapid, No-Wash” Ligands (for intracellular labeling; solid ligands that dissolve in DMSO)

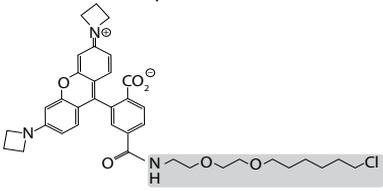
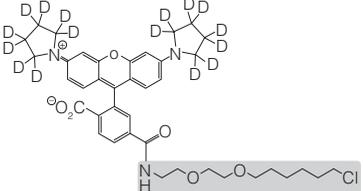
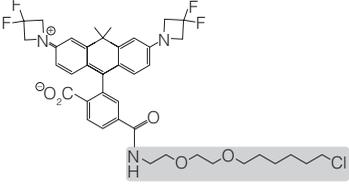
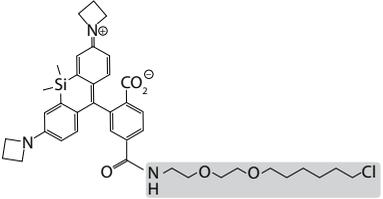
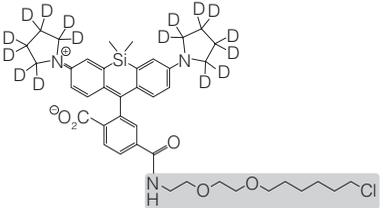
Product	Size	Cat.#	Structure
Janelia Fluor® 549 HaloTag® Ligand ^(a-c)	5µg	GA1110	
	3 × 5µg	GA1111	
Janelia Fluor® 646 HaloTag® Ligand ^(a-c)	5µg	GA1120	
	3 × 5µg	GA1121	

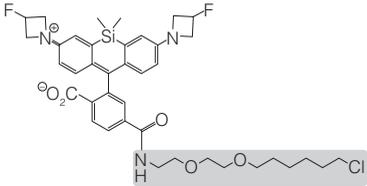
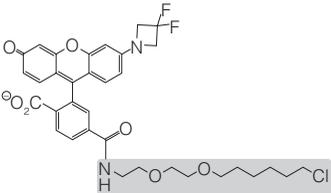
Storage Conditions: Store the Janelia Fluor® HaloTag® Ligands at –30°C to –10°C, protected from light.

Note: All Janelia Fluor® HaloTag® Ligands are recommended for single-use only. Do not store resuspended Janelia Fluor® HaloTag® Ligands.

2.A. Fluorescent Ligands (continued)

Cell-Permeant, "Rapid, No-Wash" Ligands (for intracellular labeling; 1nmol ligands that dissolve in aqueous buffer)

Product	Size	Cat.#	Structure
Janelia Fluor® 549 HaloTag® Ligand ^(a-c)	5 × 1 vial	HT1020	
Janelia Fluor® JFX554 HaloTag® Ligand	5 × 1 vial	HT1030	
Janelia Fluor® 585 HaloTag® Ligand	5 × 1 vial	HT1040	
Janelia Fluor® 646 HaloTag® Ligand ^(a-c)	5 × 1 vial	HT1060	
Janelia Fluor® JFX650 HaloTag® Ligand	5 × 1 vial	HT1070	

Product	Size	Cat.#	Structure
Janelia Fluor® 635 HaloTag® Ligand	5 × 1 vial	HT1050	
Janelia Fluor® 503 HaloTag® Ligand	5 × 1 vial	HT1010	

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PRODUCT	SIZE	CAT.#
Janelia Fluor® HaloTag® Red Sample Pack	3 vials	HT1100

Contains:

- 1 vial Janelia Fluor® 549 HaloTag® Ligand
- 1 vial Janelia Fluor® JFX554 HaloTag® Ligand
- 1 vial Janelia Fluor® 585 HaloTag® Ligand

PRODUCT	SIZE	CAT.#
Janelia Fluor® HaloTag® Far Red Sample Pack	3 vials	HT1110

Contains:

- 1 vial Janelia Fluor® 635 HaloTag® Ligand
- 1 vial Janelia Fluor® 646 HaloTag® Ligand
- 1 vial Janelia Fluor® JFX650 HaloTag® Ligand

Storage Conditions: Store the Janelia Fluor® and Janelia Fluor® JFX HaloTag® Ligands at –30°C to –10°C, protected from light.

Note: All Janelia Fluor® and Janelia Fluor® JFX HaloTag® Ligands are recommended for single-use only. Do not store resuspended Janelia Fluor® and Janelia Fluor® JFX HaloTag® Ligands.

2.A. Fluorescent Ligands (continued)

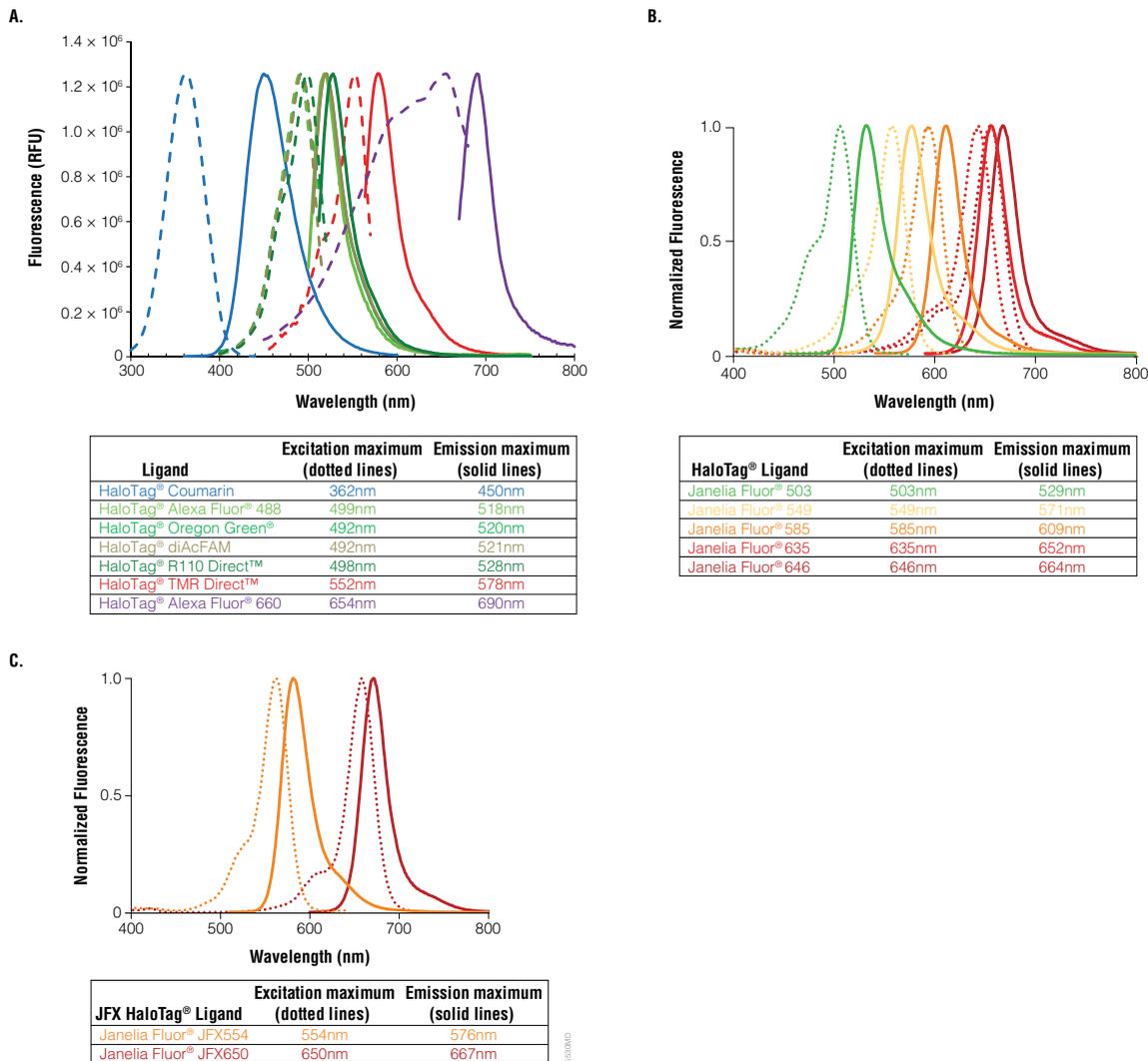
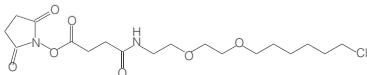
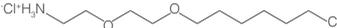
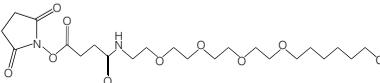
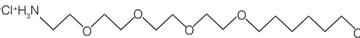


Figure 2. Excitation and emission spectra and table for the HaloTag® Ligands. Panel A. The wavelengths listed for the HaloTag® diAcFAM and Oregon Green® ligands were generated after hydrolysis of the diacetyl groups. Spectra for Alexa Fluor® 488 and Oregon Green® Ligands are identical and overlap each other. HaloTag® Alexa Fluor® 660 Ligand will have a reduced fluorescent emission at pH ≤4.0, concurrent with a shift in its absorbance peak at this low pH. **Panel B.** Excitation and emission spectra were generated for Janelia Fluor® HaloTag® Ligands and normalized to peak fluorescence. **Panel C.** Excitation and emission spectra were generated for Janelia Fluor® JFX HaloTag® Ligands and normalized to peak fluorescence.

2.B. Customizable Reactive Ligands

HaloTag® Ligand Building Blocks

Product	Size	Cat. #	Structure
HaloTag® Succinimidyl Ester (O2) Ligand	5mg	P1691	
HaloTag® Amine (O2) Ligand	5mg	P6711	
HaloTag® Succinimidyl Ester (O4) Ligand	5mg	P6751	
HaloTag® Amine (O4) Ligand	5mg	P6741	

Storage Conditions: Store Cat. # P1691 and P6751 at below -65°C under inert atmosphere. Store Cat. # P6711 and P6741 at -30°C to -10°C in an air-tight container away from light.

HaloTag® Antibodies

Product	Size	Conc.	Cat. #
Anti-HaloTag® pAb	200 μg	1mg/ml	G9281
Anti-HaloTag® Monoclonal Antibody (for Westerns)	200 μg	1mg/ml	G9211

Note: A complete listing of HaloTag® Purification and Pull-Down Systems and Reagents, including biotin ligands, is provided in Section 10, Related Products.



3. Generating a HaloTag® Fusion Protein

3.A. Beginning with an Expression-Validated HaloTag® ORF Clone

Promega offers expression-validated HaloTag® ORF clones in partnership with Kazusa DNA Research Institute. Cloning and validating the protein-coding region of a gene into a vector requires a significant amount of time, and often, there is a high failure rate prior to validating a clone. To circumvent this time sink for customers, we, in partnership with Kazusa, have produced premade, bench-validated ORF clones. This content is compatible with and optimized for HaloTag® protein analysis and TNT® T7 cell-free expression technologies. In addition, these validated ORF clones can be easily transferred into alternate vectors using the Flexi® Cloning System.

Find your protein precloned into a HaloTag® Vector and validated at: www.promega.com/FindMyGene

3.B. Creating Your Own HaloTag® Fusion Protein

Conventional Cloning into a Multiple Cloning Region

The pHTN HaloTag® CMV-neo Vector (Cat.# G7721, for N-terminal HaloTag® fusions) and the pHTC HaloTag® CMV-neo Vector (Cat.# G7711, for C-terminal HaloTag® fusions) are non-Flexi HaloTag® vectors with a common multiple cloning region designed to express an N-terminally or C-terminally tagged HaloTag® fusion protein in mammalian cells from a CMV promoter. In addition these vectors contain a neomycin (G418) selection cassette that allows antibiotic selection of stable cell lines expressing the fusion protein. The multiple cloning region allows the user to transfer the HaloTag® protein plus linker sequence into any vector of choice.

Flexi® Cloning Method

The Flexi® Vector System is a directional cloning method for protein-coding sequence. It is based on rare-cutting restriction enzymes, SgfI and PmeI, and provides a rapid, and high-fidelity way to transfer protein-coding regions without the need to resequence. All Flexi® Vectors carry the lethal barnase gene, which is replaced by the DNA fragment of interest, providing positive selection for the successful ligation of the insert. We recommend cloning into an N-terminal vector (first as pFN28A HaloTag® CMV-neo Flexi® Vector, Cat.# G8441) with later transfer into a C-terminal vector (such as pFC27K HaloTag® CMV-neo Flexi® Vector, Cat.# G8431) if needed.

4. Mammalian Live-Cell Labeling, Cell Fixation, ICC, SDS-PAGE, Flow Cytometry and Imaging

4.A. Rapid Labeling (15–60 minutes)

This protocol is intended for labeling live cells with HaloTag® TMR, diAcFAM, Oregon Green®, Coumarin, Alexa Fluor® 488 or Alexa Fluor® 660 Ligands.

Representative data are shown in Figures 3 and 4.

Materials to Be Supplied by the User

(Solution compositions are provided in Section 9.B.)

- chambered cover glass with cells expressing HaloTag® fusion protein
- complete culture medium appropriate for your cells at 37°C
- **optional:** culture medium, lacking phenol red at 37°C
- 1X PBS (pH 7.5, optional for washes)
- confocal microscope or widefield fluorescent microscope equipped with appropriate filter sets and/or lasers (see Section 9.A and Figure 2)
- 37°C + CO₂ cell culture incubator

1. Prepare a 1:200 dilution of HaloTag® Ligand in warm culture medium just prior to addition to cells. This is a 5X working stock solution.
2. Label cells by replacing one-fifth of the existing volume of medium with the 5X HaloTag® Ligand working stock solution, and mix gently.

This results in the recommended final labeling concentrations of 5µM TMR; 3.5µM Alexa Fluor® 660; 1µM diAcFAM, Oregon Green® or Alexa Fluor® 488; and 10µM Coumarin Ligand.

3. Incubate for 15 minutes in a 37°C + CO₂ cell culture incubator.
4. For cell-permeant ligands, gently replace the ligand-containing medium with an equal (or greater) volume of warm fresh medium (or 1X PBS [pH 7.5]). Repeat this two times, ending with warm complete medium, for a total of three complete rinses, and proceed to Step 5.

For cell-impermeant ligands (Alexa Fluor®-containing ligands) replace the ligand-containing medium with an equal (or greater) volume of warm fresh medium twice, and proceed to Step 7. Because they are cell-impermeant, these ligands do not require washing out of unbound ligand.

5. Incubate cells in complete culture medium at 37°C + CO₂ in a cell culture incubator for 30 minutes to wash unbound ligand.
6. Replace the medium with an equal volume of fresh warm culture medium. Use of medium lacking phenol red may improve imaging.
7. Transfer to a microscope, and capture images.

4.A. Rapid Labeling (15–60 minutes; continued)

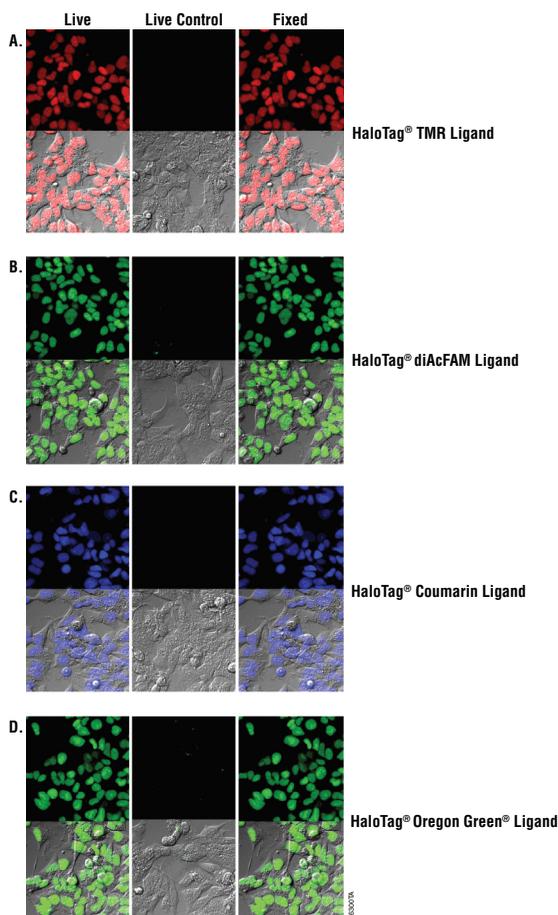


Figure 3. HaloTag[®] ligand labeling of live cells followed by fixation. HEK293 cells stably transfected with the HaloTag[®] gene fused to three copies of a nuclear localization sequence and nontransfected HEK293 controls labeled with HaloTag[®] TMR (**Panel A**), HaloTag[®] diAcFAM (**Panel B**), HaloTag[®] Coumarin (**Panel C**) or HaloTag[®] Oregon Green[®] Ligand (**Panel D**) as described in Section 4.A. In each panel showing stably transfected cells, labeling is restricted to the nucleus. Nontransfected (live control in figure) cells show no labeling. For HaloTag[®] TMR, diAcFAM and Oregon Green[®] Ligand labeling, images were collected on the Olympus FV500 confocal microscope with appropriate filter sets. For HaloTag[®] Coumarin Ligand labeling images were collected by epifluorescent illumination using DAPI filter cube and a CCD camera (Hamamatsu Orca). The top row of each panel is fluorescent signal only. The bottom row is differential interference contrast (DIC) with fluorescence overlay.

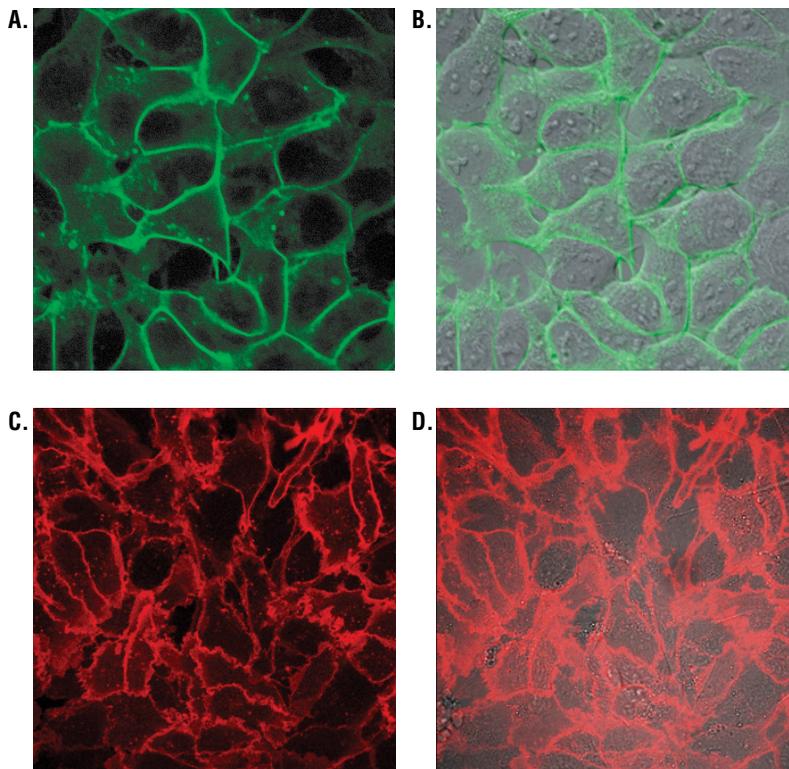


Figure 4. Live-cell labeling of cell surface HaloTag[®] fusion proteins. Panels A and B show HEK293 cells stably expressing HaloTag[®]-ECS (ExtraCellular Surface; comprised of a signal sequence and single transmembrane domain of β 1-integrin) fusion protein labeled with HaloTag[®] Alexa Fluor[®] 488 Ligand and then imaged. Panels C and D show U2OS cells expressing HaloTag[®]-EDG1 (GPCR receptor) fusion labeled with Alexa Fluor[®] 660 Ligand and then imaged. All labeling was done as described in Section 4.A. Confocal images show fluorescence (Panels A and C) is restricted to the cell surface, also seen clearly in the panels with the DIC/fluorescence overlay (Panel B and D). Images were generated on an Olympus FV500 confocal microscope using the appropriate filter sets.

4.B. No-Wash Labeling (overnight)

This protocol is intended for labeling of live cells with the cell-permeant HaloTag® TMRDirect™ or R110Direct™ Ligand. This protocol can be used to label cells that are adherent or nonadherent, and adherent cells can already be plated or still be in suspension at time of labeling. Representative data are shown in Figure 5.

Note: We do not recommend using the HaloTag® TMR Ligand (Cat.# G8251, G8252) for this protocol.

Materials to Be Supplied by the User

(Solution compositions are provided in Section 9.B.)

- cells expressing HaloTag® fusion protein (in suspension or plated)
- chambered cover glass or other cell culture device
- complete culture medium appropriate for your cells at 37°C
- **optional:** culture medium lacking phenol red at 37°C
- confocal microscope or widefield fluorescent microscope equipped with appropriate filter sets and/or lasers (Section 9.A and Figure 2)
- 37 °C + CO₂ cell culture incubator

1. Prepare a 1:200 dilution of HaloTag® TMRDirect™ or R110Direct™ Ligand in warm culture medium just prior to addition to cells. This is a 5X working stock solution.
2. For adherent cells: Replace one-fifth of the existing volume of medium with the 5X HaloTag® ligand working stock solution, and mix gently.

For cell suspensions: Add 5X ligand working stock to existing cell suspension, resulting in a 1X final concentration.

Step 2 results in the recommended final labeling concentration of 100nM HaloTag® TMRDirect™ or R110Direct™ Ligand.

3. Plate cells (if necessary), and incubate overnight in a 37°C + CO₂ cell culture incubator.
4. Gently replace the ligand-containing medium with an equal (or greater) volume of warm fresh medium, or fix cells (for endpoint assays; see Section 4.G).
5. Transfer to an imaging device, and capture images.

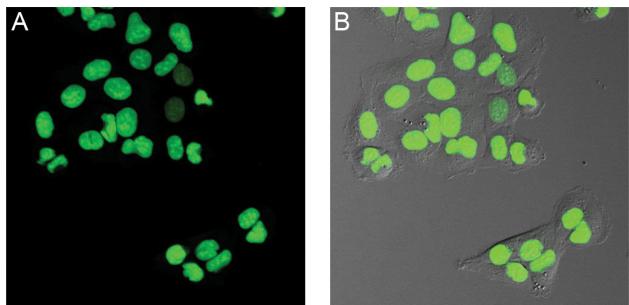


Figure 5. No-wash live-cell labeling results in high signal-to-noise ratio and specificity. U2OS cells stably expressing HaloTag® protein fused to three copies of a nuclear localization sequence were labeled with the HaloTag® R110Direct™ Ligand as described in Section 4.B. HaloTag® labeling shows a high signal-to-noise ratio and specificity: the label is restricted to the nucleus of all cells. **Panel A** shows fluorescence image of cells labeled with R110Direct™ Ligand; **Panel B** is an overlay of the fluorescent and DIC image. Images were generated on an Olympus FV500 confocal microscope using the appropriate filter sets.

4.C. Rapid, No-Wash Labeling with Janelia Fluor® HaloTag® Ligands (15 minutes to overnight)

This protocol is intended for labeling live cells with Janelia Fluor® 549 HaloTag® Ligand (Cat.# GA1110, GA1111) or Janelia Fluor® 646 HaloTag® Ligand (Cat.# GA1120, GA1121). These ligands have been demonstrated for use in super-resolution microscopy and single-molecule tracking approaches (10). Representative data are shown in Figure 6.

Materials to Be Supplied by the User

(Solution compositions are provided in Section 9.B.)

- chambered cover glass with cells expressing HaloTag® fusion protein
 - complete culture medium appropriate for your cells, at 37°C
 - **optional:** culture medium, lacking phenol red at 37°C
 - DMSO
 - confocal microscope or widefield fluorescent microscope equipped with appropriate filter sets and/or lasers (see Section 9.A and Figure 2)
 - 37°C + CO₂ cell culture incubator
1. Resuspend Janelia Fluor® 549 HaloTag® Ligand in 37.8µl of DMSO and Janelia Fluor® 646 HaloTag® Ligand in 35.5µl of DMSO. This will create a 200µM stock solution that is recommended for single-use only. Storage in DMSO is not recommended and will result in reduced ligand stability.
 2. Prepare a 1:200 (1µM final) dilution of each HaloTag® Ligand in warm culture medium just prior to addition to cells. This is a 5X working stock solution.
 3. Label cells by replacing one-fifth of the existing volume of medium with the 5X HaloTag® Ligand working stock solution, and mix gently. This results in the recommended final labeling concentration of 200nM.
 4. Incubate for 15 minutes or longer in a 37°C + CO₂ cell culture incubator.

4.C. Rapid, No-Wash Labeling with Janelia Fluor® HaloTag® Ligands (15 minutes to overnight; continued)

5. Replace the medium with an equal volume of fresh warm culture medium.

Optional: Replace the medium with an equal volume of fresh warm culture medium lacking phenol red. Use of medium lacking phenol red may improve imaging.

6. Transfer to a microscope and capture images.

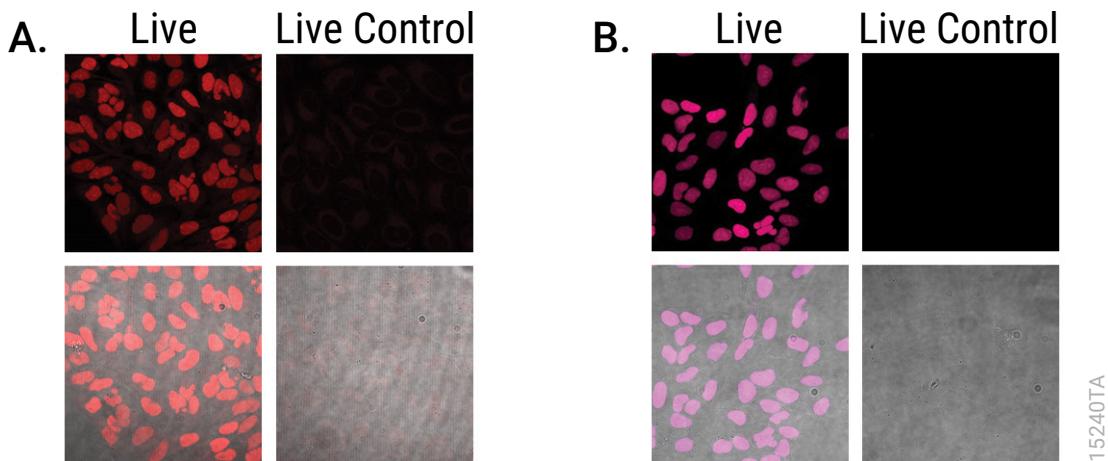


Figure 6. Live cell labeling of U2OS cells expressing nuclear HaloTag® protein. Parental U2OS cells and U2OS cells stably expressing HaloTag® protein fused to three copies of a nuclear localization sequence were adhered to glass-bottom chamber slides and labeled with 200nM Janelia Fluor® 549 HaloTag® Ligand or Janelia Fluor® 646 HaloTag® Ligand for 15 minutes. Cells were imaged with 561nm laser excitation for Janelia Fluor® 549 HaloTag® Ligand (**Panel A**) and 637nm laser excitation for Janelia Fluor® 646 HaloTag® Ligand (**Panel B**). In HaloTag®-expressing cells, labeling is restricted to the nucleus. Parental cells (live control in figure) show no labeling. The top row of each panel is fluorescent signal only. The bottom row shows transmitted images collected at the same respective laser excitation with the fluorescence images overlaid. Images were collected using a Nikon Eclipse Ti confocal microscope equipped with NIS Elements software and a 40X plan fluor oil immersion objective.

4.D. Rapid Labeling with 1nmol Janelia Fluor® and Janelia Fluor® JFX HaloTag® Ligands

This protocol is intended for labeling live cells with 1nmol Janelia Fluor® and Janelia Fluor® JFX HaloTag® Ligands (Cat.# HT1010, HT1020, HT1030, HT1040, HT1050, HT1060, HT1070, HT1100, HT1110). Representative data are shown in Figure 7.

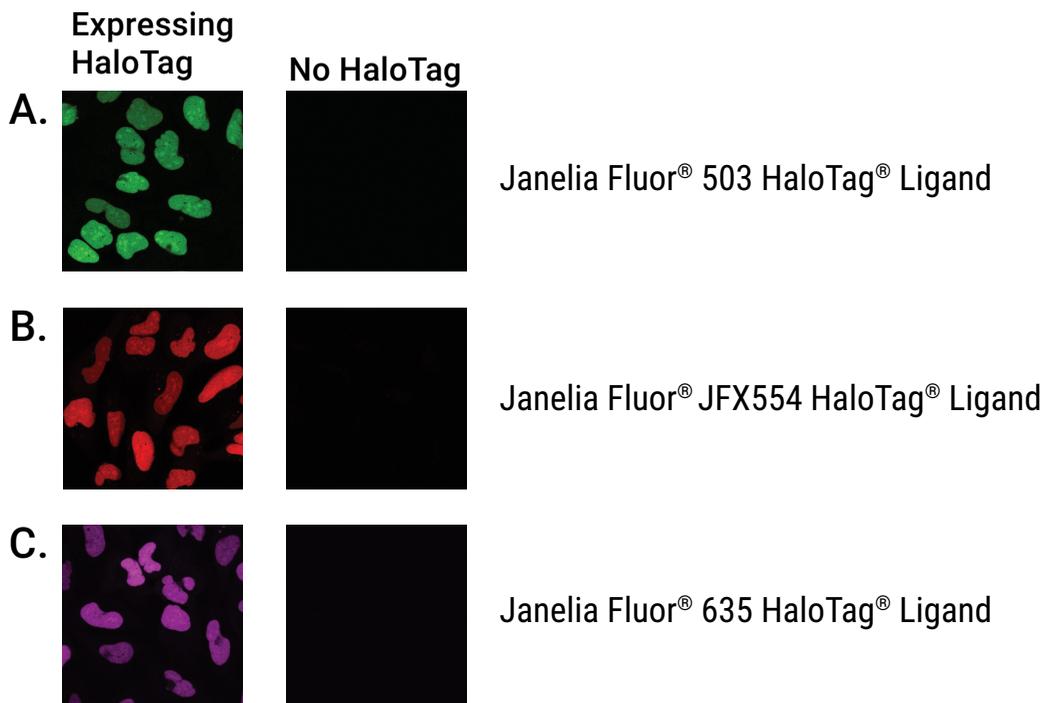
Materials to Be Supplied by the User

(Solution compositions are provided in Section 9.B.)

- optical bottom chamber with cells expressing HaloTag® fusion protein
 - complete culture medium appropriate for your cells, at 37°C
 - **optional:** culture medium, lacking phenol red at 37°C
 - confocal microscope or widefield fluorescent microscope equipped with appropriate filter sets and/or lasers (see Section 9.A and Figure 2)
 - 37°C + CO₂ cell culture incubator
1. Equilibrate a vial of 1nmol Janelia Fluor® or Janelia Fluor® JFX HaloTag® Ligand to room temperature.
 2. Add 1ml of cell medium or chosen aqueous buffer.
 3. Incubate the Janelia Fluor® or Janelia Fluor® JFX HaloTag® Ligand in medium for 2–3 minutes with intermittent agitation to dissolve the ligand. Do **not** pipet or vortex to resuspend the ligand. This will yield a 5X (1µM) working stock solution.
 4. Add the 5X (1µM) working stock solution to cells at a 1X final concentration of 200nM as a recommended starting point. Further concentration optimization may be necessary. Cells expressing lower levels of HaloTag® fusion protein may require lower concentrations that could enhance the signal-to-background ratio.
 5. Incubate the cells with the Janelia Fluor® or Janelia Fluor® JFX HaloTag® Ligand for 30 minutes (except for Janelia Fluor® 503 HaloTag® Ligand that requires a 1-hour incubation) at 37°C + CO₂ in a cell culture incubator. If using lower concentrations of ligand, longer incubation times may be required.
 6. Aspirate medium and replace with fresh cell medium. Alternatively, replace with phenol red-free medium to minimize background signal.

Note: For nonadherent cells and cells that strongly express HaloTag® protein, ligand-containing medium can remain on cells although the background signal will be higher compared to replacing with fresh medium. We recommend using the 1nmol Janelia Fluor® 585 and 635 HaloTag® Ligands for cells with lower HaloTag® expression when not replacing cell medium.

4.D. Rapid Labeling with 1nmol Janelia Fluor® and Janelia Fluor® JFX HaloTag® Ligands (continued)



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Figure 7. Live cell labeling of U2OS cells expressing nuclear HaloTag® protein using 1nmol Janelia Fluor® HaloTag® Ligands. Parental U2OS cells and U2OS cells stably expressing HaloTag® protein fused to three copies of a nuclear localization sequence were adhered to glass-bottom chamber slides and labeled with Janelia Fluor® 503, Janelia Fluor® JFX554 or Janelia Fluor® 635 HaloTag® Ligand for 30 minutes at 37°C + CO₂ in a cell culture incubator. Cell medium was replaced with a phenol-red free medium. Cells were imaged with 488nm laser excitation for Janelia Fluor® 503 HaloTag® Ligand (**Panel A**), 561nm laser excitation for Janelia Fluor® JFX554 HaloTag® Ligand (**Panel B**) and 637nm laser excitation for Janelia Fluor® 635 HaloTag® Ligand (**Panel C**). In HaloTag®-expressing cells, labeling is restricted to the nucleus. Parental cells (without HaloTag) show no labeling. Images were collected using a Nikon AX/AXR confocal microscope with a plan fluor 40X oil objective.

4.E. Pulse-Chase Labeling

This protocol is intended as a guide for pulse-chase labeling of cells expressing HaloTag® fusions to directly observe protein turnover or protein trafficking. This protocol is intended for labeling live cells with any of the HaloTag® ligands. Representative data are shown in Figure 8.

Materials to Be Supplied by the User

(Solution compositions are provided in Section 9.B.)

- optical bottom chamber with cells expressing HaloTag® fusion protein
 - complete culture medium appropriate for your cells at 37°C
 - **optional:** culture medium lacking phenol red at 37°C
 - 1X PBS (pH 7.5, optional for washes)
 - confocal microscope or widefield fluorescent microscope equipped with appropriate filter sets and lasers (Section 9.A and Figure 2)
 - 37°C + CO₂ cell culture incubator
1. Prepare a 5X working stock solution of pulse labeling ligand in warm culture medium just prior to adding to cells.
 2. Label cells by replacing one-fifth of the existing volume of medium with the 5X HaloTag® ligand working stock solution, and mix gently. This results in the recommended final labeling concentrations of 5µM TMR, 3.5µM Alexa Fluor® 660; 1µM diAcFAM, Oregon Green® or Alexa Fluor® 488; 10µM Coumarin; 100nM TMRDirect™ or R110Direct™; 200nM Janelia Fluor® 549, Janelia Fluor® 646, Janelia Fluor® JFX554, Janelia Fluor® 585, Janelia Fluor® JFX650, Janelia Fluor® 635 or Janelia Fluor® 503 HaloTag® Ligand.

Note: If protein fusion is membrane-bound with HaloTag® protein on the surface, pulse labeling should be done with an impermeable ligand (Alexa Fluor® 488 or Alexa Fluor® 660 Ligand) to label the surface (plasma membrane) pool first.

3. For rapid-labeling ligands (TMR, Oregon Green®, diAcFAM, Coumarin and Alexa Fluor® Ligands), incubate for 15 minutes in a 37°C + CO₂ cell culture incubator.

For no-wash-labeling ligands (TMRDirect™ and R110Direct™ Ligands), incubate overnight in a 37°C + CO₂ cell culture incubator.

For rapid, no-wash-labeling ligands, (Janelia Fluor® 549 HaloTag® Ligand and Janelia Fluor® 646 HaloTag® Ligand), incubate for 15 minutes in a 37°C + CO₂ cell culture incubator.

For rapid labeling with 1nmol Janelia Fluor® 549, Janelia Fluor® JFX554, Janelia Fluor® 585, Janelia Fluor® 646, Janelia Fluor® JFX650 or Janelia Fluor® 635 HaloTag® Ligand, incubate for 30 minutes in a 37°C + CO₂ cell culture incubator.

For rapid labeling with 1nmol Janelia Fluor® 503 HaloTag® Ligand, incubate for 60 minutes in a 37°C + CO₂ cell culture incubator.

4. If chase labeling will occur immediately following the pulse, then proceed immediately to Step 5.

If chase labeling will not occur immediately, simply replace the ligand-containing medium with an equal volume of warm fresh medium and either allow time to pass prior to chase and/or execute intended biology as per your specific needs prior to Step 6.

4.E. Pulse-Chase Labeling (continued)

5. Prepare a 1:1,000 dilution of chase ligand in warm culture medium just prior to addition to cells, and gently replace the medium currently on your cells with an equal volume of this 1X chase labeling ligand.

6. For rapid-labeling ligands (TMR, Oregon Green[®], diAcFAM, Coumarin and Alexa Fluor[®] Ligands), incubate for 15 minutes in a 37°C + CO₂ cell culture incubator.

For no-wash-labeling ligands (TMRDirect™ and R110Direct™ Ligands), incubate overnight in a 37°C + CO₂ cell culture incubator.

For rapid, no-wash-labeling ligands (Janelia Fluor[®] 549 and Janelia Fluor[®] 646 HaloTag[®] Ligands), incubate for 15 minutes in a 37°C + CO₂ cell culture incubator.

For rapid labeling with 1nmol Janelia Fluor[®] 549, Janelia Fluor[®] JFX554, Janelia Fluor[®] 585, Janelia Fluor[®] 646, Janelia Fluor[®] JFX650 or Janelia Fluor[®] 635 HaloTag[®] Ligand, incubate for 30 minutes in a 37°C + CO₂ cell culture incubator.

For rapid labeling with 1nmol Janelia Fluor[®] 503 HaloTag[®] Ligand, incubate for 60 minutes in a 37°C + CO₂ cell culture incubator.

7. For the cell-permeant rapid-labeling ligands (TMR, Oregon Green[®], diAcFAM and Coumarin Ligands) gently replace the ligand-containing medium with an equal (or greater) volume of warm fresh medium (or 1X PBS [pH 7.5]). Repeat this two times, ending with warm complete medium, for a total of three complete rinses and proceed to Step 8.

For the cell-impermeant rapid-labeling ligands (Alexa Fluor[®] 488 or 660 Ligand), gently replace the ligand-containing medium with an equal (or greater) volume of warm fresh medium twice and proceed to Step 10.

For no-wash-labeling ligands (TMRDirect™ and R110Direct™), gently replace the ligand-containing medium with an equal (or greater) volume of warm fresh medium, and proceed to Step 10.

For rapid, no-wash-labeling ligands (Janelia Fluor[®] 549 and Janelia Fluor[®] 646 HaloTag[®] Ligands), gently replace the ligand-containing medium with an equal (or greater) volume of warm fresh medium, and proceed to Step 10.

For rapid labeling with 1nmol Janelia Fluor[®] 549, Janelia Fluor[®] JFX554, Janelia Fluor[®] 585, Janelia Fluor[®] 646, Janelia Fluor[®] JFX650, Janelia Fluor[®] 635 or Janelia Fluor[®] 503 HaloTag[®] Ligand, gently replace the ligand-containing medium with an equal (or greater) volume of warm fresh medium, and proceed to Step 10.

8. Incubate cells in complete culture medium at 37°C + CO₂ in a cell culture incubator for 30 minutes to wash out unbound ligand.
9. Replace the medium with an equal volume of fresh warm culture medium (use of medium lacking phenol red may improve imaging).
10. Transfer to a microscope, and capture images.

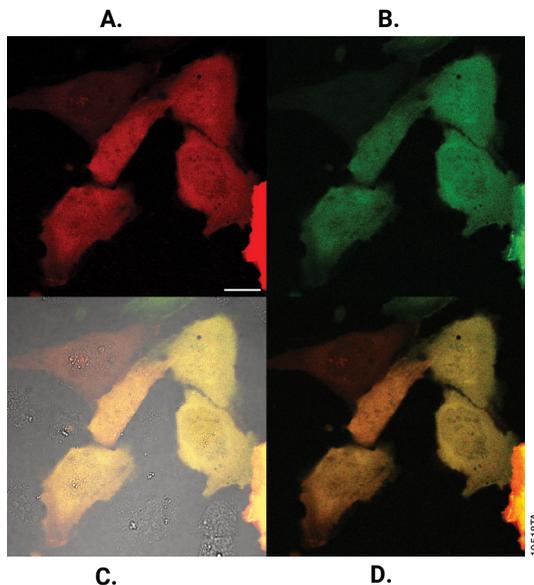


Figure 8. Pulse-chase labeling of cells expressing an intracellular HaloTag® protein using No-Wash labeling results in direct observation protein turnover. U2OS cells transiently expressing HaloTag® protein alone were labeled with HaloTag® TMRDirect™ Ligand for 20 hours followed by HaloTag® R110Direct™ Ligand for 20 hours and then imaged by confocal microscopy. The HaloTag® protein is small enough to be seen both in the cytoplasm and the nucleus. TMRDirect™ fluorescence is seen labeling the protein pool made prior to and during the first 20 hours of expression by the cells (**Panel A**), while R110Direct™ fluorescence is seen as labeling the protein pool made in the last 20 hours of expression by cells (**Panel B**). **Panel C** is an overlay of the two fluorescent channels and the DIC. **Panel D** is an overlay of the two fluorescent channels. Scale bar is 20µm.

4.F. Fixing Cells after Labeling

This protocol is intended to serve as a guide for fixation of cells expressing a HaloTag[®] fusion protein. The covalent bond that forms between the ligand and HaloTag[®] protein during live-cell labeling and the stability of the dyes allows you to subsequently fix, permeabilize and wash the cells using standard conditions without significant loss of the specific fluorescent signal. We recommend the use of paraformaldehyde (PFA) as a fixative because it crosslinks proteins in cells and at the membrane and has the added benefit of reducing cell loss from the growth surface.

Fixed cells can be treated with detergents, such as Triton[®] X-100, to permeabilize cells for downstream immunocytochemical applications. The conditions here are sufficient to permeabilize the plasma membrane. Alternative or additional detergents might be necessary to permeabilize other structures. Representative data are shown in Figures 3, 9 and 10.

Materials to Be Supplied by the User

(Solution compositions are provided in Section 9.B.)

All solutions should be at room temperature unless otherwise specified.

- 4% paraformaldehyde/0.2M sucrose/1X PBS (pH 7.5), warmed to 37°C
 - 1X PBS buffer (pH 7.5)
 - 1X PBS + 0.1% Triton[®] X-100
 - confocal microscope or widefield fluorescent microscope equipped with appropriate filter sets and lasers (Section 9.A and Figure 2).
1. Follow Steps 1–3 of Section 4.A (Rapid Labeling) or 4.B (No-Wash Labeling), Steps 1–4 of Section 4.C (Rapid, No-Wash Labeling) or Section 4.D (Rapid Labeling with 1nmol Janelia Fluor[®] and Janelia Fluor[®] JFX HaloTag[®] Ligands) or Steps 1–6 of Section 4.E (Pulse-Chase Labeling) to label cells with a HaloTag[®] ligand, if desired.
 2. Replace the medium with an equal volume of warm 4% paraformaldehyde/0.2M sucrose/1X PBS (pH 7.5), and incubate for 10 minutes at room temperature.
 3. Replace fixative with an equal volume of 1X PBS + 0.1% Triton[®] X-100, and incubate for 10 minutes at room temperature.
 4. Replace the detergent-containing solution with an equal volume of 1X PBS, and then either transfer to a microscope and capture images, store at 4°C, or proceed to Section 4.G for immunocytochemistry protocol.

4.G. Multiplexing with HaloTag® Using Immunocytochemistry (ICC)

This protocol is intended to serve as a guide for multiplexing with HaloTag® technology in fixed cells by ICC using any antibody of interest. The particular antibody used in this protocol is a purified rabbit polyclonal antibody raised against the HaloTag® protein (9). The antibody was purified using Protein G affinity resin and has been shown to detect HaloTag® fusion proteins in both immunocytochemistry and Western blot applications with high sensitivity and specificity. Further, this antibody labels HaloTag® fusion proteins independently of HaloTag® ligands. Because these labels do not interfere with one another, HaloTag® fusion proteins can be colabeled with a fluorescent ligand and the Anti-HaloTag® pAb in conjunction with an anti-rabbit secondary antibody bearing a spectrally distinct fluorescent tag. Representative data are shown in Figures 9 and 10.

Materials to Be Supplied by the User

(Solution compositions are provided in Section 9.B.)

All solutions should be at room temperature.

- cells fixed following the steps in Section 4.F
 - fluorophore-conjugated anti-rabbit secondary antibody (Ab) of choice
 - PBS + 2% serum from same host as secondary Ab + 0.01% Triton® X-100
 - PBS + 1% serum from same host as secondary Ab
 - PBS + 0.1% sodium azide (optional, for storage)
 - confocal microscope or widefield fluorescent microscope equipped with appropriate filter sets and lasers (Section 9.A and Figure 2)
1. Replace the 1X PBS with an equal volume of PBS + 2% serum from same host as secondary Ab + 0.01% Triton® X-100, and block for 1 hour at room temperature.
 2. Dilute the Anti-HaloTag® pAb 1:500 in PBS + 1% serum from same host as secondary Ab to a final labeling concentration of 2µg/ml.
 3. Replace the blocking solution with the antibody solution, and incubate for 1 hour at room temperature.
 4. Wash cells twice with PBS + 1% serum from same host as secondary Ab for 10 minutes at room temperature.
 5. Dilute the secondary antibody according to the manufacturer's recommendations in PBS + 1% serum from same host as secondary Ab.
 6. Replace the wash solution with the secondary antibody solution, and incubate for 30 minutes at room temperature.
 7. Wash cells twice with PBS + 1% serum from same host as secondary Ab for 10 minutes each wash at room temperature.
 8. Replace wash solution with 1X PBS.
 9. Transfer to a microscope, and capture images. Store cells at 4°C in 1X PBS or PBS + 0.1% sodium azide.

4.G. Multiplexing with HaloTag[®] Using Immunocytochemistry (ICC; continued)

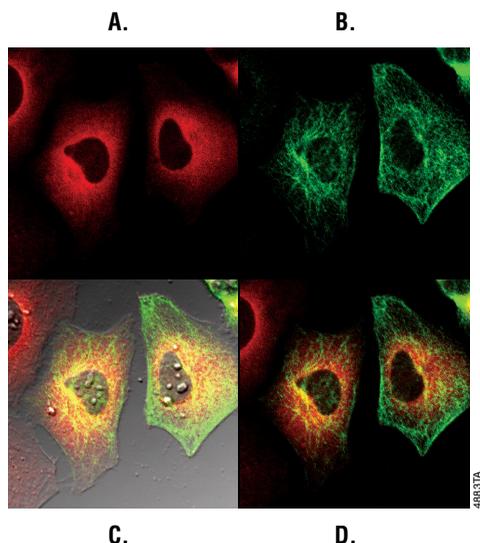
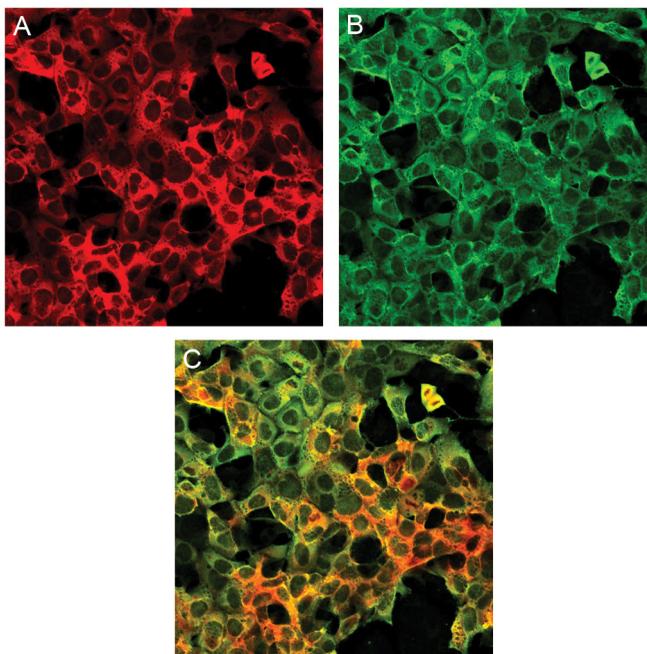


Figure 9. Multiplex HaloTag[®] labeling using TMR ligand with an antibody of interest. HeLa cells transiently transfected with p65-HaloTag[®] fusion (3) construct (p65 is a cytoplasmic signaling protein) were labeled with HaloTag[®] TMR Ligand as described in Section 4.A. Cells were then fixed as described in Section 4.F, stained with a mouse Anti- β III Tubulin mAb followed by Alexa Fluor[®] 488-conjugated goat anti-mouse IgG (Molecular Probes) as described in Section 4.G. TMR fluorescence is seen in the cytoplasm (**Panel A**), and Alexa Fluor[®] 488 fluorescence shows microtubules (**Panel B**). **Panel C** shows an overlay of both fluorescence channels with the DIC image, while **Panel D** shows an overlay of just the fluorescence channels. Images were generated on an Olympus FV500 confocal microscope in sequential mode using appropriate filter sets.



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Figure 10. Distinct labeling of a single HaloTag® fusion by a HaloTag® Ligand and Anti-HaloTag® pAb. HEK293 cells stably transfected with a p65-HaloTag® construct (p65 is primarily cytoplasmic in cells at rest) were labeled with HaloTag® TMR Ligand as described in Section 4.A, fixed as described in Section 4.E, then processed for immunocytochemistry using the Anti-HaloTag® pAb as described in Section 4.F. The secondary antibody used was Alexa Fluor® 488-conjugated anti-rabbit-IgG (Jackson ImmunoResearch). Distinct labeling is demonstrated by the fact that the red fluorescence associated with specific HaloTag® TMR Ligand labeling (**Panel A**) does not prevent green fluorescence associated with specific Anti-HaloTag® pAb labeling (**Panel B**). Merging the fluorescent images (**Panel C**) confirms that the HaloTag® fusion protein is labeled specifically (primarily cytoplasmic) as expected. Images were collected on the Olympus FV500 confocal microscope in sequential mode using appropriate filter sets.

4.H. Quantitation of HaloTag® Protein Fusions, SDS-PAGE Analysis

The following protocol is intended to serve as a guide for SDS-PAGE-based HaloTag® applications. Because the covalent bond between the HaloTag® protein and ligand withstands denaturation and the fluorescent dyes in the ligands are stable, you can directly quantify labeled fusion protein following SDS-PAGE using a fluorescent scanner (i.e., cell-to-gel analysis).

Such gels also can be subsequently used for Western blot analysis using the Anti-HaloTag® mAb at a final labeling concentration of 1 µg/ml (1:1,000 dilution; see Section 5 below for protocol).

Note: Although all of the HaloTag® ligands perform in this function, the HaloTag® TMR, TMRDirect™ or R110Direct™ Ligands are recommended for direct SDS-PAGE fluorescence scanning of an intracellularly expressed protein fusion. Either of the AlexaFluor®-containing ligands is recommended for extracellularly expressed proteins that place HaloTag® on the surface of cells. Representative data are shown in Figure 11.

Materials to be Supplied by the User

(Solution compositions are provided in Section 9.B.)

- cells expressing HaloTag® fusion protein
 - 1X PBS (pH 7.5)
 - 1X SDS sample buffer
 - heat block or water bath at 95°C
 - equipment and running buffer necessary for SDS-PAGE
 - fluorescent scanner
1. To label cells, use one of the following protocols: Steps 1–3 of Section 4.A or 4.B (Rapid or No-Wash Labeling); Steps 1–4 of Section 4.C (Rapid, No-Wash Labeling) or Section 4.D (Rapid Labeling with 1nmol Janelia Fluor® and Janelia Fluor® JFX HaloTag® Ligands); or Steps 1–6 of Section 4.E (Pulse-Chase Labeling).

Note: There is no need to go through the 30-minute wash step for an intracellularly expressed protein fusion labeled by Rapid Labeling protocol as the amount of unbound ligand is minimal and will migrate on the gel much faster than the labeled fusion protein (with sample dye front).
 2. Replace ligand-containing medium with 1X PBS (pH 7.5) in order to avoid a prominent band on the gel stemming from complete medium.
 3. Lyse cells by replacing the 1X PBS from the wells with approximately 100–150 µl of 1X SDS sample buffer per cm² of cell growth area.
 4. Collect cell lysate, and incubate for 5 minutes at 95°C.
 5. Perform SDS-PAGE by loading approximately 10 µl (5–10 µg total protein) of each sample per well of the gel or store samples at –20°C for later use.
 6. Analyze the gel on a fluorescence scanner.

Note: The dye front may contain fluorescent material that can complicate detection (unbound ligand and/or tracking dyes used in sample buffers). To eliminate these sources of nonspecific fluorescence, simply run the gel until the dye front migrates off of the gel or cut the dye front off of the bottom of the gel before scanning.

4.1. Flow Cytometry Analysis

Representative data are shown in Figure 12.

Materials to Be Supplied by the User

(Solution compositions are provided in Section 9.B.)

- large-well format plate of HaloTag[®]-expressing cells and negative control cells that do not express the HaloTag[®] fusion protein
 - complete culture medium appropriate for your cells at 37°C
 - for adherent cells: 1X PBS (pH 7.5) and 1X PBS containing 3mM EDTA (nonenzymatic) or trypsin solution (see Step 2 below)
 - flow cytometer with appropriate filter sets and lasers for ligand used (Section 9.A and Figure 2)
 - 37°C + CO₂ cell culture incubator
1. Label cells expressing HaloTag[®] protein with ligand of choice (Section 4.A, Rapid Labeling; 4.B, No-Wash Labeling; 4.C, Rapid, No-Wash Labeling; or 4.D, Rapid Labeling with 1nmol Janelia Fluor[®] and Janelia Fluor[®] JFX HaloTag[®] Ligands).

Recommended controls:

- a. Label cells that are not expressing HaloTag[®] protein to assess background with chosen ligand.
 - b. Prepare nonlabeled cells expressing HaloTag[®] protein to assess background from endogenous cell fluorescence or morphological changes due to expression.
2. For adherent cells with HaloTag[®] fusion protein expressed on the cell surface: Rinse cells twice with an equal volume of 1X PBS, incubate in 1X PBS with 3mM EDTA or use other nonenzymatic solution at 37°C for 5–15 minutes (depending on cell line) in order to gently get the cells off of the plate.

For adherent cells with HaloTag[®] fusion protein expressed inside the cell: Suspend cells using trypsin-containing solution in a manner appropriate for the cell line.

3. Collect, pellet, count and resuspend cells in medium at 37°C to a concentration of 0.5 to 1 × 10⁶ cells/ml.
4. Analyze cells by flow cytometry.

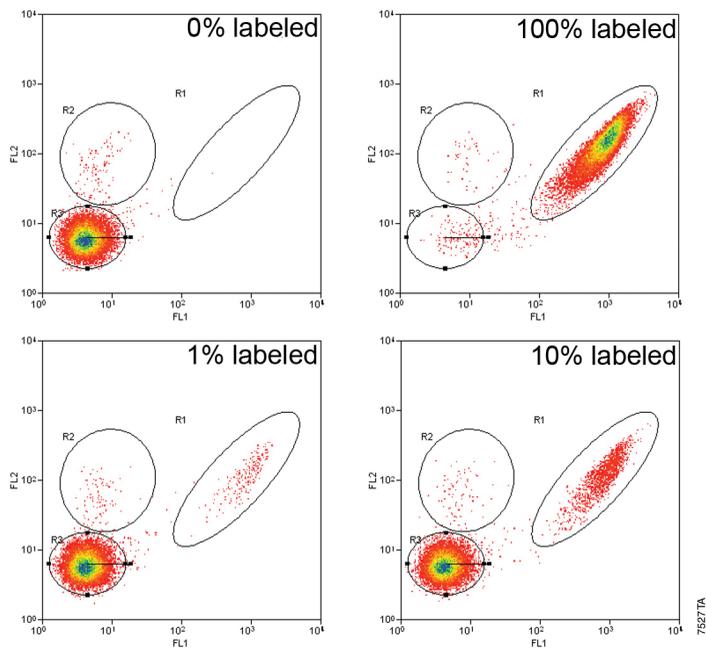


Figure 12. Successful flow cytometry analysis of labeled HaloTag® protein-expressing cells. U2OS cells stably expressing HaloTag® protein fused to three copies of a nuclear localization sequence were labeled with the HaloTag® R110Direct™ Ligand as described in Section 4.B, and known numbers (1%, 10% or 100% labeled) were gated from unlabeled cells. Flow cytometry of HaloTag® protein expressing cells was performed using a MoFlo® instrument with approximately 20,000 cells represented on each graph. R1 indicates cells labeled with R110, R2 are dead cells (by propidium iodide), and R3 indicates unlabeled cells. Channel overlap between propidium iodide and R110Direct™ fluorescence was not compensated for in analysis. Results show that labeled cells are successfully gated from nonlabeled cells.

5. Protein Detection Using HaloTag® Monoclonal Antibody (for Western blotting)

The following protocol is meant to serve as a general guide for the use of Anti-HaloTag® Monoclonal Antibody (Cat.# G9211) in Western blotting. The Anti-HaloTag® pAb (Cat.# G9281) is recommended for all other immunological detection methods. Representative data are shown in Figure 13.

Materials to Be Supplied by the User

- mammalian cells expressing HaloTag® fusion
 - appropriate mammalian lysis buffer (e.g., Mammalian Lysis Buffer, Cat.# G9381)
 - SDS-PAGE gel and gel running apparatus
 - nitrocellulose or PVDF membrane and Western blotting apparatus
 - TBST (1X TBS with 0.1% Tween®)
 - 5% BSA in TBST
 - anti-mouse IgG secondary antibody appropriately conjugated for Western blotting analysis and corresponding detection substrate (e.g., Anti-Mouse IgG (H+L), AP Conjugate Cat.# S3721) and Western Blue® Stabilized Substrate for Alkaline Phosphatase, Cat.# S3841)
 - rotating platform for gentle agitation
1. Lyse mammalian cells expressing HaloTag® fusion using an appropriate lysis buffer of choice.
Recommended control: Cells that are not expressing HaloTag® (nontransfected).
 2. Run lysates on SDS-PAGE, and transfer to nitrocellulose or PDVF membrane as per manufacturer protocol.
 3. Place resulting nitrocellulose or PVDF membranes in TBST containing 5% BSA to block for an hour at room temperature or overnight at 4°C, with gentle agitation.
 4. Replace blocking buffer with a solution of anti-HaloTag® mAb at 1:1,000 dilution in TBST, and leave at room temperature for 1 hour with gentle agitation.
 5. Replace primary antibody-containing solution with TBST. Wash 3 times for 15 minutes at room temperature with gentle agitation.
 6. Replace wash with anti-mouse IgG conjugated secondary antibody at manufacturer recommended concentration (e.g., for anti-mouse IgG-AP conjugated use at 1:7,500 dilution) in TBST and leave for 30 minutes at room temperature with gentle agitation.
 7. Replace secondary solution with TBST. Wash 3 times for 15 minutes at room temperature with gentle agitation.
 8. Detect HaloTag® protein bands using appropriate substrate (e.g., AP substrate).



Figure 13. Western blot detection of EZH2-HaloTag® (HT) fusion protein (~120kDa) using Anti-HaloTag® Monoclonal Antibody (mAb). Blot shows specific and robust detection of the HaloTag® fusion from HEK293T cell lysate. Western blotting using AP-based secondary antibody and substrate was used as described in protocol of Section 5. Lane 2 is the nontransfected control.

6. How to Generate HaloTag® Ligands from Building Blocks

The following protocols are meant to serve as a guide for the generation of HaloTag® ligands using HaloTag® Ligand Building Blocks. Specific example of generating a fluorophore-containing ligand is described in Figure 14.

6.A. General Protocol for Reporter Group Labeling (conjugation to 2-[(6-chloro-hexyloxy)-ethoxy]-ethylamine)

Materials to Be Supplied by the User

- succinimidyl ester reporter group
 - base (triethylamine, diisopropylethylamine, etc.)
 - DMF (dimethylformamide)
 - instrument and reagents for HPLC or silica gel chromatography
1. Add 1.5 to 3 equivalence of (02) or (04) amine to one equivalent of the succinimidyl ester of the reporter group in DMF and follow by base (triethylamine, diisopropylethylamine, etc.).
 2. Stir reaction for 8–16 hours at room temperature.
 3. Purify by preparative scale HPLC or silica gel chromatography, depending on lipophilicity of your construct.

6.B. Protein Labeling with HaloTag® Amine Ligand

Materials to Be Supplied by the User

- protein to be conjugated
 - water, 0.1M MES (pH 4.7–6.0) or 0.1M sodium phosphate (pH 7.3)
 - DMSO or DMF
 - EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride)
 - Sephadex® G-25 (for gel filtration separation of ligated protein)
1. Dissolve the protein to be modified at a concentration of 10mg/ml in either water, 0.1M MES (pH 4.7–6.0), or 0.1M sodium phosphate (pH 7.3).
 2. Prepare HaloTag® Amine (02 or 04) Ligand stock solution at 5mg/ml in DMSO or DMF.
 3. Take a tenfold-molar-excess-to-protein aliquot of the HaloTag® ligand stock solution and dilute it in buffer (same as what is used in Step 1).
 4. Add the aqueous HaloTag® ligand stock solution to the protein solution; the final protein concentration will be 5mg/ml or greater.
 5. Add EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) in tenfold molar excess to protein as a 0.1–0.5M aqueous solution and react for 2 hours at room temperature.
 6. Purify the ligated protein by gel filtration separation using Sephadex® G-25 in the buffer of choice.

6.C. Antibody Labeling with HaloTag® Succinimidyl Ester Ligand

Materials to Be Supplied by the User

- antibody to be conjugated
 - 0.1M sodium phosphate, 0.15M sodium chloride (pH 7.2; pH 7.0 to 7.6 can be used)
 - DMSO or DMF
 - Sephadex® G-25 (for gel filtration separation of ligated antibody) or means of dialysis against 0.1M sodium phosphate, 0.15M sodium chloride, 10mM EDTA (pH 7.2)
1. Dissolve antibody in 0.1M sodium phosphate, 0.15M sodium chloride (pH 7.2) at 1–5mg/ml.
 2. Prepare HaloTag® Succinimidyl Ester (02 or 04) Ligand stock solution in 5mg/ml DMSO or DMF.
 3. Add 10–40µl of HaloTag® Ligand stock solution per milliliter of 1mg/ml antibody solution and incubate at room temperature for 30 minutes.
Note: A 12-fold molar excess works well to ensure at least one label per antibody. Most protocols for general antibody labeling uses 20-fold excess, but higher concentrations of ligand to antibody will label more amines. Further, excess labels may or may not hinder the antibody-epitope interaction.
 4. Purify the ligated antibody by gel filtration using Sephadex® G-25 or by dialysis against 0.1M sodium phosphate, 0.15M sodium chloride, 10mM EDTA (pH 7.2).

6.D. Specific Example Protocol for Generating an Alexa Fluor® 488 HaloTag® Ligand

1. Begin with 5mg of Alexa Fluor® 488 NHS ester (7.8×10^{-6} mol) from Life Technologies.
2. In purchased vial, add a small magnetic stir bar and dissolve the dye in 0.5ml DMF (stored over molecular sieves to remove excess water).
3. With continuous stirring, add a threefold excess of amino ligand (2.3×10^{-5} mol) followed by one drop of N,N-diisopropylethylamine (excess).

Note: Add the amine ligand as a stock solution in either DMF or methylene chloride. It stores well at -78°C in solution.

If using a dye-succinimidyl ester (SE), you can use 1.5 to 3 equivalents of ligand to dye-SE.

4. Stir for 12 hours and monitor by analytical HPLC (C12 or C18) using 0.1% TFA, aqueous phase and acetonitrile, organic phase.
5. Once the reaction is determined to be complete, dilute the reaction mixture with 1ml of water and inject onto a preparative HPLC column (Varian Microsorb 60-8 C18; 250×21.4 mm). Purify the compound using the same mobile phase as in the analytical HPLC in Step 4.

7. Labeling Proteins in Mammalian Cell Lysates or Proteins Expressed in Cell-Free Systems

Fluorescent labeling of HaloTag[®] fusion protein with the HaloTag[®] TMRDirect[™] Ligand provides a rapid and convenient method to monitor protein expression and the purification efficiency. HaloTag[®] TMRDirect[™] Ligand (555_{Ex}/585_{Em}) is used in the example below because it is provided with the HaloTag[®] Mammalian Protein Detection and Purification System (Cat.# G6795). If desired, HaloTag[®] R110Direct[™] Ligand (502_{Ex}/527_{Em}) can easily be substituted for this ligand.

For further information regarding HaloTag[®] labeling after expression refer to Technical Manual TM348 *HaloTag[®] Mammalian Protein Detection and Purification Systems* at: www.promega.com/protocols

Materials to Be Supplied by the User

(Solution compositions are provided in Section 9.B.)

- lysate containing HaloTag[®] fusion protein
 - DMSO or 1X PBS (pH 7.5), optional
 - 4X SDS sample buffer
 - heat block or water bath at 70°C
 - equipment and running buffer necessary for SDS-PAGE
 - fluorescent scanner
1. Dilute the HaloTag[®] TMRDirect[™] Ligand stock solution (100µM) twofold in DMSO to make a 50µM working solution that can be stored, protected from light, at -20°C. Alternatively, the stock solution can be prepared in 1X PBS but cannot be stored.
 2. Combine 10µl of lysate containing the HaloTag[®] fusion protein or the equivalent amount of unbound fraction with 19µl of HaloTag[®] Protein Purification Buffer and 1µl of 50µM HaloTag[®] TMRDirect[™] Ligand.
 3. Incubate at room temperature for 15 minutes protected from light.
 4. Add 10µl of 4X SDS sample buffer (Section 9.B), and heat at 70°C for 3 minutes.
 5. Load 10µl onto an SDS-polyacrylamide gel.
 6. Scan the gel on a fluorescent scanner and quantitate band intensities.

Note: The dye front might contain fluorescent material that can complicate detection. Run the gel until the dye front migrates off of the gel or cut the dye front off of the bottom of the gel before scanning it.

8. Labeling *E. coli* Lysate to Analyze Protein Expression Level

These protocols are intended to serve as a guide for the labeling of *E. coli* lysates and intact bacteria.

8.A. Expressing HaloTag® Fusions in *E. coli*

Materials to Be Supplied by the User

(Solution compositions are provided in Section 9.B.)

- transformed *E. coli* KRX colonies expressing HaloTag® fusion protein (Single Step (KRX) Competent Cells [Cat. # L3002])
- LB containing appropriate antibiotics
- glucose
- L-Rhamnose Monohydrate (Cat. # L5701 or L5702)
- bacterial growth incubator at 25°C and 37°C

Note: These expression conditions are unique to *E. coli* KRX cells.

1. Inoculate a colony into 2ml of LB + appropriate antibiotics and grow overnight at 37°C.

Note: For toxic proteins add 0.4% glucose to the medium.

2. Dilute the overnight culture 1:100 into 2ml of auto induction medium.

8.B. Labeling *E. coli* Lysate to Analyze Protein Expression Level

Mode of Induction	Approximate O.D. at Induction	Growth Temperature	Growth Time
Late: 0.15% glucose + 0.2% rhamnose	$A_{600} = 1-1.2$	25°C	24 hours
Early: 0.05% glucose + 0.05% rhamnose	$A_{600} = 0.6-0.8$	25°C	16-18 hours

Materials to Be Supplied by the User

- 50mM HEPES (pH 7.5)
- 10X Fast Break™ Cell Lysis Reagent (Cat. # V8571, V8572 or V8573)
- lysozyme
- RQ1 RNase-Free DNase (Cat. # M6101)
- 4X SDS sample buffer
- freezer at -70°C
- centrifuge capable of 14,000 × g at 4°C
- heat block or water bath at 70°C
- equipment and running buffer necessary for SDS-PAGE
- fluorescent scanner

8.B. Labeling *E. coli* Lysate to Analyze Protein Expression Level (continued)

1. Pellet 1.5ml culture and resuspend in 0.5ml of 50mM HEPES (pH 7.5). Freeze for 20 minutes at -70°C .
2. Thaw the cells at room temperature and lyse by adding 0.5ml lysis buffer (1X FastBreak™ Cell Lysis Reagent + 2mg/ml + lysozyme + 0.02 units RQ1 RNase-free DNase) and incubate for 30 minutes with constant rotation.
3. Keep 0.5ml of lysed cells for crude lysate analysis and centrifuge the remaining 1ml soluble lysate at $14,000 \times g$ for 30 minutes at 4°C .
4. Label both crude and soluble lysates with the HaloTag® fluorescent ligand. Dilute the ligand into a $50\mu\text{M}$ working solution (below). Incubate for 20 minutes at room temperature.

Component	Volume
Lysate	20 μl
Ligand ($50\mu\text{M}$)	1 μl
1X 50mM HEPES (pH 7.5)	9 μl
Total Volume	30μl

5. Add 10 μl of 4X SDS loading buffer, and incubate for 2 minutes at 70°C .
6. Analyze 10–20 μl by SDS-PAGE.
7. First detect by a fluorescent scanner, and then stain using Coomassie® blue.

Note: The dye front might contain fluorescent material that can complicate detection. Run the gel until the dye front migrates off of the gel or cut the dye front off of the bottom of the gel prior to scan.

8.C. Labeling *E. coli* for Imaging Analysis

Representative data are shown in Figure 14.

Materials to Be Supplied by the User

- *E. coli* culture expressing HaloTag® fusion protein (Section 8.A)
 - bacterial growth incubator at 25°C
 - centrifuge capable of $2,000 \times g$
 - 1X PBS
 - chambered coverglass
 - 0.1% gelatin
 - confocal microscope equipped with appropriate filter sets and lasers (Section 9.A and Figure 2)
1. To label bacteria, dilute an overnight culture 1:10 in LB, add HaloTag® TMRDirect™ Ligand or HaloTag® TMR Ligand to a final concentration of $1\mu\text{M}$ (1:100 dilution for TMRDirect™ or 1:5,000 for TMR) and label at 25°C without mixing for 1 hour.
 2. Prepare the chambered cover glass by precoating the glass chambers with 0.1% gelatin (200 μl /chamber of 8-well coverglass) for 1 hour at room temperature.

3. To wash unbound ligand, follow Steps a–e below, 3 times using 1X PBS.
 - a. Pellet the cells at $2,000 \times g$ for 5 minutes.
 - b. Resuspend in 1ml of 1X PBS.
 - c. Incubate without mixing for 30 minutes at room temperature.
 - d. Pellet the cells at $2,000 \times g$ for 5 minutes.
 - e. Discard the 1X PBS.
4. For imaging, after the third wash, resuspend the cells in 200 μ l of 1X PBS and proceed to Step 5.
For SDS-PAGE analysis, you can collect the cells, lyse and analyze for labeling efficiency on a gel as described above in Section 8.B.
5. To plate the labeled *E. coli*, aspirate the gelatin from the chambered cover glass and then immediately add the cells; plating these at undiluted, 1:2.5, 1:5 and 1:10 dilutions. Allow 2–3 hours for the cells to adhere before imaging.
6. Transfer to a confocal microscope and capture images.

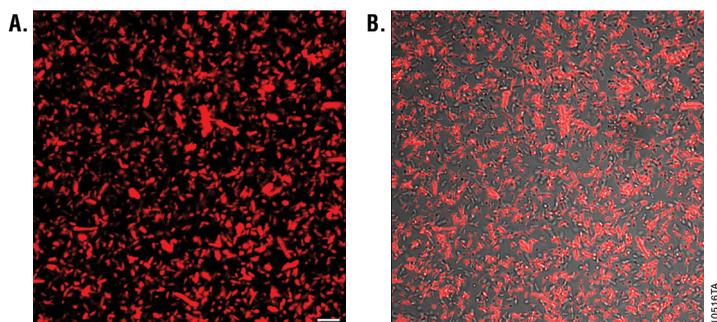


Figure 14. Distinct labeling of HaloTag[®] expressed in *E. coli*. *E. coli* expressing HaloTag[®] fusion proteins were labeled with the HaloTag[®] TMR Ligand as described in Section 8.C and then imaged using a confocal microscope. Bright TMR labeling with low background is shown in these images. Imaging using the fluorescent channel is shown in **Panel A**, and the fluorescent/DIC overlay is shown in **Panel B**. Images were generated on an Olympus FV500 confocal microscope using the appropriate filter sets. Scale bar = 20 μ m.



9. General Information, Tips and Troubleshooting

9.A. General Information

Recommended Standard Filter Sets for Imaging

Ligand	Recommended Filter Set
HaloTag® Alexa Fluor® 660, Janelia Fluor® 646, Janelia Fluor® JFX650 and Janelia Fluor® 635 HaloTag® Ligands	Cy® 5 filter set (~640nm _{Ex} /700nm _{Em})
HaloTag® TMR and TMRDirect™ Ligands, Janelia Fluor® 549, Janelia Fluor® JFX554 and Janelia Fluor® 585 HaloTag® Ligands	TRITC filter set (555nm _{Ex} /580nm _{Em})
HaloTag® Alexa Fluor® 488, Oregon Green® and R110Direct™ Ligands and Janelia Fluor® 503 HaloTag® Ligand	FITC filter set (488nm _{Ex} /520nm _{Em})
HaloTag® Coumarin Ligand	DAPI/AMCA filter set (345nm _{Ex} /445nm _{Em})

No Detectable Toxicity

The nonJanelia Fluor® HaloTag® Ligands have shown no detectable toxicity or morphological side effects at the recommended labeling conditions in all cell lines tested, including but not limited to U2OS, CHO, HEK293, HeLa, SW48 and Human Neural Progenitor cells. In addition, Janelia Fluor® 549 and Janelia Fluor® 646 HaloTag® Ligands were tested for toxicity at 200nM by CellTiter-Glo® Assay after overnight incubation in U2OS and HEK293 cells, and no loss in viability was observed. All other ligands that were not Janelia Fluor® HaloTag Ligands show no detectable toxicity by CellTiter-Glo® Assay after incubation for 24 hours in U2OS and CHO cells (the Direct ligands were tested at 10X recommended concentration for this).

Signal-to-Noise Ratio

The labeling recommendations provided here should result in a good signal-to-noise ratio.

However, the specific signal is a function of:

1. The level of HaloTag® fusion protein expression
2. The cell type and health of the cells because unbound ligand washing is an active cellular process
3. The quantum yield and stability of the fluorophore

As such, the recommended starting points for ligand concentration and wash time can be optimized. If you observe a significant amount of residual nonspecific fluorescence in your cells, we recommend lowering the ligand concentration and/or extending the wash times. This problem also can result from a high passage number cell line, which can cause signaling pathway changes. As an alternative, residual nonspecific fluorescence can be alleviated by fixing cells with paraformaldehyde and permeabilizing with 0.1–1.0% Triton® X-100 (Section 4.F).

Limitations of diAcFAM Ligand

The photo-instability of diAcFAM limits its use for quantitative fluorescent cell imaging but helps in labeling applications where the photo-instability may be advantageous, such as fluorescence recovery after photobleaching (FRAP).

Both the HaloTag® Oregon Green® and R110Direct™ Ligands exhibit significantly increased photostability compared with diAcFAM and are useful tools in cell-based imaging applications that require quantitation of fluorescence or the use of high excitation energies with a green ligand.

Use of HaloTag® Ligands in Serum-Containing Medium

All HaloTag® ligands may be directly diluted into serum-containing medium. However, the HaloTag® diAcFAM and Oregon Green® Ligands must be added to the cells immediately following this dilution because the diacetyl groups can be hydrolyzed by serum esterases. This deacetylation converts the HaloTag® diAcFAM and Oregon Green® Ligands into cell-impermeant fluorescent ligand derivatives. Once inside the cell, these ligands are modified, and, as for all of the other cell-permeant ligands, excess unbound ligand is eliminated from the cell in a time- and temperature-dependent manner by an active cellular mechanism.

While all cell types tested allow entry of the cell-permeant ligands and access to all major subcellular compartments, there are differences in rates of efflux. In general, efflux of the HaloTag® diAcFAM and Oregon Green® Ligands is significantly slower than that of other ligands, and the rate of efflux can vary significantly between cell types. Cells that have delayed efflux of unbound modified ligand might have limiting amounts of this active mechanism or suffer from poor health. Thus, while the use of serum results in a strict timing requirement for the addition of ligand to the cell culture, the presence of serum minimizes stress to cells and maintains their ability to actively eliminate unbound ligand, which results in lower background.

Streamlining the Protocol

The cell-impermeant Alexa Fluor® 488 and Alexa Fluor® 660 Ligands do not require a 30-minute wash step as unbound ligand does not enter cells. Among the cell-permeant ligand options, HaloTag® TMR and Coumarin Ligands are easily washed from cells. Therefore, you may be able to streamline the protocol in Section 4.A, Steps 4–6, without affecting the signal-to-noise ratio.



9.B. Composition of Buffers and Solutions

4% paraformaldehyde/0.2M sucrose/1X PBS (pH 7.5)

Prepare fresh for each use.

LB Broth (1X)

- 1% casein peptone Type M
- 0.5% NaCl
- 0.5% yeast extract

1X PBS buffer (pH 7.5)

- 137mM NaCl
- 2.68mM KCl
- 1.47mM KH_2PO_4
- 8.1mM Na_2HPO_4

4X SDS-sample buffer

- 0.24M Tris
- 2% SDS
- 50.4% glycerol
- 0.4M DTT
- 3mM bromophenol blue

Titrate to pH 6.8 using HCl.

1X SDS-sample buffer

1:4 dilution of 4X SDS Sample Buffer in filtered water.

Tris-buffered saline (TBS)

- 20mM Tris-HCl (pH 7.5)
- 150mM NaCl

9.C. 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

Fusion protein not expressed or expressed only at low levels

Causes and Comments

Poor transfection efficiency. Optimize transfection conditions and use high-quality endotoxin-free DNA.

Consider a construct in which the order of the HaloTag[®] protein and the protein of interest are reversed; N- or C-terminal fusions may perform differently.

Check reading frame of construct by sequencing or performing a rapid cell-free translation (such as TNT[®] T7 Quick Coupled Transcription Translation System, Cat.# L1170) followed by labeling, SDS-PAGE and fluorescence scanning of gel.

Weak or inappropriate regulatory elements; consider changing promoters and/or other regulatory elements.

Symptoms

Causes and Comments

Weak or absent fluorescent signal in labeled cells

Avoid proteolysis if lysing cells in PBS; add protease inhibitors during lysis, and perform lysis at 4°C.

Culture cells for a longer period of time before labeling to ensure that you have adequate protein expression and cell density.

Optimize cell health. Expose cells only to complete culture medium at 37°C under proper CO₂ conditions throughout labeling and imaging.

Optimize cell-labeling protocols. Increase labeling time.

Store the HaloTag® ligands desiccated at -30°C to -10°C, and protect them from light. Dispense the HaloTag® ligand into aliquots, avoiding multiple freeze-thaw cycles. Storing resuspended Janelia Fluor® HaloTag® Ligands is **not** recommended.

Use freshly diluted HaloTag® Ligand, and add immediately to label cells; avoid deacetylation. See Section 9.A.

Ensure that you are using the appropriate filter set for imaging. See Section 9.A and Figure 2.

Adjust the settings on your fluorescence detection instrument (e.g., laser power, PMT gain and aperture for a confocal microscope).

High background fluorescence in live cells

Decrease HaloTag® ligand concentration.

Increase time of wash and perform washes using complete medium (i.e., with serum).

Image cells in complete medium lacking phenol red.

Strictly follow temperature recommendations during labeling.

Adjust instrumentation settings (e.g., reduce laser power, reduce gain on PMT or aperture for a confocal microscope).

High background fluorescence in fixed cells

Increase time of wash and/or perform washes at a higher Triton® X-100 concentration or with a different detergent.

Decrease staining concentration

Use an alternative culture dish or glass because some HaloTag® ligands can bind to charged surfaces.

9.C. Troubleshooting (continued)

Symptoms	Causes and Comments
Cells detached from surface	<p>Handle cells gently to ensure that they remain attached to the surface and/or reduce the number of times you replace the medium (i.e., reduce the number of steps by using Direct™ ligands in the no-wash protocol instead of the ligands in the rapid labeling protocol).</p> <p>Perform all labeling, washes and imaging steps using complete culture medium, and keep cells in a 37°C + CO₂ incubator as much as possible.</p> <p>Increase seeding density or time in culture to allow cells to proliferate and adhere more tightly.</p> <p>Use an attachment matrix such as poly-L-lysine, fibronectin or collagen.</p>
Cell death or toxicity	<p>Modify transfection protocol or try less toxic transfection reagent. Ensure DNA is endotoxin-free.</p>

9.D. References

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Additional References

Learn more about the HaloTag® technology and imaging:

www.promega.com/resources/technologies/halotag-technology/

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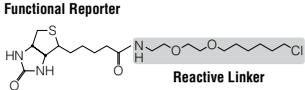
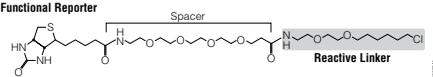
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10. Related Products

Biotin Ligands

Product	Size	Cat.#	Structure
HaloTag® Biotin Ligand	30µl	G8281	 <p>Functional Reporter</p> <p>Reactive Linker</p>
HaloTag® PEG-Biotin Ligand	30µl	G8591	 <p>Functional Reporter</p> <p>Spacer</p> <p>Reactive Linker</p>

HaloTag® Purification and Pull-Down Systems

Product	Size	Cat.#
HaloTag® Mammalian Pull-Down and Labeling System	24 reactions	G6500
HaloTag® Complete Pull-Down System	1 each	G6509
HaloTag® Mammalian Pull-Down System	24 reactions	G6504
HaloTag® Mammalian Protein Detection and Purification System	1 each	G6795
HaloTag® Mammalian Protein Purification System	1 each	G6790
HaloTag® Mammalian Protein Detection and Purification System Sample Pack	1 each	G6799
HaloTag® Protein Purification System	1 each	G6280
HaloTag® Protein Purification System Sample Pack	1 each	G6270
HaloCHIP™ System	20 reactions	G9410

HaloTag® Purification and Pull-Down Reagents

Product	Size	Cat.#
HaloLink™ Resin	1.25ml	G1912
	2.5ml	G1913
	10ml	G1914
	25ml	G1915
HaloLink™ Magnetic Beads	40 reactions	G9311

Cloning Vectors

Conventional

Product	Size	Cat.#
pHTN HaloTag® CMV-neo Vector	20µg	G7721
pHTC HaloTag® CMV-neo Vector	20µg	G7711

Flexi® Cloning Vectors

Product	Size	Cat.#
pFN28A HaloTag® CMV-neo Flexi® Vector	20µg	G8441
pFC27K HaloTag® CMV-neo Flexi® Vector	20µg	G8431

Many additional Flexi® vectors are available for your convenience. You can see all HaloTag® Flexi® Vectors at www.promega.com by searching for “halotag flexi vectors”. Additionally, information about the full line of reagents and tools available for the HaloTag® technology is available at: www.promega.com/resources/technologies/halotag/

11. Summary of Changes

The following changes were made to the 11/23 revision of this document:

1. Removed discontinued Cat.# P1681.
2. Added new 1nmol Janelia Fluor® and Janelia Fluor® JFX HaloTag® Ligands.
3. Inserted new Section 4.D and renumbered the subsequent subsections and figures.
4. Updated Step 1 for Sections 4.F, 4.H and 4.I.
5. Updated Sections 2.B, 9.A, 9.C, 9.D and 10.
6. Updated document font.



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