TECHNICAL MANUAL

HaloTag® Mammalian Pull-Down and Labeling Systems

Instructions for Use of Products
G6500 and G6504

Revised 8/15
TM342

HaloTag® Mammalian Pull-Down and Labeling Systems

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

1. Description

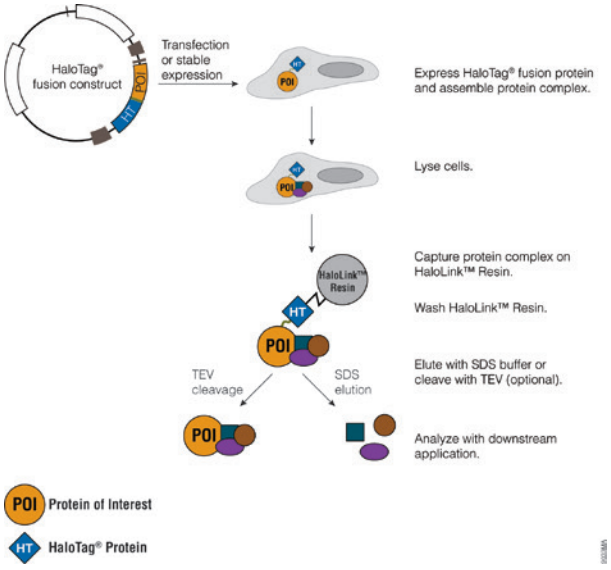
Protein function is highly regulated through a complex network of protein interactions and modifications. Deconvolution of protein interactions is necessary to fully understand these processes.

The HaloTag® Mammalian Pull-Down System[a] (Cat.# G6504), was developed to isolate and identify intracellular protein complexes from mammalian cells. HaloTag® fusion proteins form a highly specific and covalent bond with HaloLink™ Resin, allowing rapid and efficient capture of dilute protein complexes. These benefits, coupled with the low nonspecific protein binding features of the HaloLink™ Resin, improve the rate of successful complex capture and identification from mammalian cells.

The HaloTag® Mammalian Pull-Down and Labeling System (Cat.# G6500) also includes the HaloTag® TMR Direct™ Ligand, which allows the study of cellular localization, trafficking and protein turnover using the same HaloTag® genetic construct.

The general HaloTag® pull-down experimental scheme is depicted in Figure 1. HaloTag® fusion proteins can be expressed in mammalian cells either transiently or stably and used as bait to capture interacting protein partners.

After cellular lysis, the HaloTag® fusion protein, bound to its interacting protein partners, is captured on the HaloLink™ Resin. The captured complexes are gently washed and eluted using SDS elution buffer or cleaved from the resin using TEV (Tobacco Etch Virus) protease. The recovered complexes are suitable for analysis by a variety of methods including SDS-PAGE, Western blotting or mass spectrometry. As a negative control, we recommend using cells expressing the HaloTag® Control Vector (Cat.# G6591); alternatively, untransfected cells can be used. HaloTag® vectors are available as N- or C-terminus constructs and with promoters supporting various levels of expression (Table 1).



In addition to identification of intracellular protein interactions and protein cellular localization, HaloTag® technology is also applicable to the study of intracellular protein:DNA interactions (HaloCHIP™ System; Cat.# G9410). The ability to perform several different experiments with the same fusion protein eliminates the need to make multiple constructs for each desired study and allows flexibility to easily expand to other areas of research.

To perform in vitro pull-downs, where the HaloTag® fusion bait protein is bound to the HaloLink™ Resin and introduced to a pool of proteins containing potential binding partners, see the protocols in the *HaloLink™ Resin Technical Manual* #TM250.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
HaloTag® Mammalian Pull-Down and Labeling System	**24 reactions**	**G6500**

Each system contains sufficient reagents for 24 reactions or 12 pull-down experiments. The standard pull-down experiment consists of 1 experimental reaction and 1 negative-control reaction. Includes:

- 1.25ml HaloLink™ Resin
- 10ml Mammalian Lysis Buffer
- 25ml 10X TBS Buffer
- 1 vial Protease Inhibitor Cocktail, 50X
- 1.3ml SDS Elution Buffer
- 30µl HaloTag® TMRDirect™ Ligand

PRODUCT	SIZE	CAT.#
HaloTag® Mammalian Pull-Down System	**24 reactions**	**G6504**

Each system contains sufficient reagents for 24 reactions or 12 pull-down experiments. The standard pull-down experiment consists of 1 experimental reaction and 1 negative-control reaction. Includes:

- 1.25ml HaloLink™ Resin
- 10ml Mammalian Lysis Buffer
- 25ml 10X TBS Buffer
- 1 vial Protease Inhibitor Cocktail, 50X
- 1.3ml SDS Elution Buffer

Storage Conditions: Store the 10X TBS Buffer and SDS Elution Buffer at room temperature. Store the HaloLink™ Resin and Mammalian Lysis Buffer at 4°C. Store the Protease Inhibitor Cocktail and HaloTag® TMRDirect™ Ligand at –20°C.

2. Product Components and Storage Conditions (continued)

Available Separately

PRODUCT	SIZE	CAT.#
HaloTag® TMRDirect™ Ligand	**30µl**	**G2991**

The HaloTag® TMRDirect™ Ligand is NOT interchangeable with the HaloTag® TMR Ligand and is recommended ONLY for use with the "no-wash" labeling protocol.

PRODUCT	SIZE	CAT.#
HaloTag® Control Vector	**20µg**	**G6591**

The HaloTag® Control Vector provides protein expression of the HaloTag® protein in mammalian cells, *E. coli* or in vitro expression systems dependent on human cytomegalovirus (CMV) intermediate early enhancer, T7 or SP6 RNA polymerase promoters. It can be used as a control for any HaloTag® experimental system and can be used for both stable and transient HaloTag® expression in mammalian cells; for stable expression, co-transfection with a vector containing a selectable marker is required.

Storage Conditions: Store at –20°C.

PRODUCT	SIZE	CAT.#
Protease Inhibitor Cocktail, 50X	**1ml**	**G6521**

The Protease Inhibitor Cocktail, 50X, is a mixture of six different protease inhibitors with different target protease specificities. This product is provided in a freeze-dried format and can be reconstituted using either 100% ethanol or DMSO.

Storage Conditions: Store at –20°C.

PRODUCT	SIZE	CAT.#
Mammalian Lysis Buffer	**40ml**	**G9381**

The Mammalian Lysis Buffer is designed for use with HaloTag® Mammalian-based expression systems such as the HaloTag® Mammalian Pull-Down and Labeling Systems (Cat.# G6500 and G6504) as well as the HaloCHIP™ System (Cat.# G9410).

Storage Conditions: Store at 4°C.

3. Preparation of Materials and Reagents

Materials and Equipment to Be Supplied by the User

(Solution compositions are provided in Section 8.A.)

- cells for transfection or a stable cell line expressing the desired HaloTag® fusion protein
- vector encoding HaloTag® fusion protein (Cat.#s G9651, G9661, G1611, G1601, G1591, G1571, G1551, G1321, G2821, G2831, G2841, G2851, G2861, G2871, G2881 or G2981) in the form of transfection-grade DNA
- HaloTag® Control Vector (Cat.# G6591) in the form of transfection-grade DNA
- cellular growth media
- transfection reagents
- PBS - tissue culture certified
- ethanol
- IGEPAL® CA-630 (Sigma Cat.# 18896)
- rotating or shaking platform (i.e., tube rotator from Scientific Equipment Products; or other mixing devices such as the IKA-SCHÜTTLER MTS2)
- microcentrifuge
- cell culture incubator
- glass homogenizer (e.g., 2ml Kontes Dounce Tissue Grinder; Thermo Fisher Scientific Cat.# K885300-0002) or 25- to 27-gauge needle
- disposable cell lifter (e.g., Thermo Fisher Scientific Cat.# 08-773-1)

Additional Materials and Equipment to Be Supplied by the User for Cell Labeling System (Cat.# G6500; Section 6).

- chambered cover glass (i.e., Thermo Fisher Scientific Cat.# 155409) or similar cell culture device
- 37°C + 5% CO_2 cell culture incubator
- 4% paraformaldehyde/0.2M sucrose/1X PBS (pH 7.5)
- 1X PBS buffer (pH 7.5)
- 1X PBS + 0.1% Triton® X-100
- imaging device equipped with appropriate filter sets and lasers ($555nm_{Ex}/580nm_{Em}$)

Note: IGEPAL® CA-630 prevents the HaloLink™ Resin from sticking to plasticware and reduces nonspecific binding. IGEPAL® CA-630 is chemically indistinguishable from Nonidet® P-40. IGEPAL® CA-630 is not stable in solution. Use all solutions containing IGEPAL® CA-630 within a week. We recommend first preparing a 10% stock solution of IGEPAL® CA-630 (in water) and using a final concentration of 0.05% v/v. The effect of IGEPAL® CA-630 is protein-dependent and may need to be determined empirically.

3.A. Before You Begin

Reconstitute the Protease Inhibitor Cocktail. Prepare a 50X stock solution of the Protease Inhibitor Cocktail by resuspending it in 1ml of 100% ethanol. Store the reconstituted stock at 4°C.

Note: The Protease Inhibitor Cocktail only needs to be reconstituted the first time the system is used.

3.B. Prepare Reagents

Prepare the following reagents fresh for each experiment. The quantities given are for a standard protein pull-down experiment consisting of one experimental reaction and one control reaction. If you are performing a different number of reactions, adjust the volumes accordingly.

1. Prepare 1X TBS solution by adding 2ml of 10X TBS to 18ml of sterile water.
2. Set aside 2ml of the 1X TBS solution from Step 1 to use as the diluting buffer.
3. Prepare the resin equilibration/wash buffer by adding 90µl of 10% IGEPAL® CA-630 to the remaining 18ml of 1X TBS from Step 1.

3.C. Creating HaloTag® Fusion Protein Constructs

Instructions for creating HaloTag® fusion protein constructs can be found in the *Flexi® Vector Systems Technical Manual*, #TM254, and the *HaloTag® Interchangeable Labeling Technology Technical Manual*, #TM260. We strongly recommend verifying the initial clone by DNA sequencing and examining expression in an appropriate cell line. The HaloTag® mammalian vectors are available for N-terminus fusions or C-terminus fusions and for different levels of expression ranging from high, medium, low or ultra-low (Table 1).

Note: A library of prepared HaloTag® clones are available. This collection has been validated extensively and is ready for use: **www.promega.com/FindMyGene**

Table 1. Relative Mammalian Protein Expression Levels for HaloTag® Flexi® Vectors.

C-Terminal Vectors	Cat.#	Expression Level
pFC14A HaloTag® CMV Flexi® Vector	G9651	High
pFC14K HaloTag® CMV Flexi® Vector	G9661	High
pFC15A HaloTag® CMV*d1* Flexi® Vector	G1611	Medium
pFC15K HaloTag® CMV*d1* Flexi® Vector	G1601	Medium
pFC16A HaloTag® CMV*d2* Flexi® Vector	G1591	Low
pFC16K HaloTag® CMV*d2* Flexi® Vector	G1571	Low
pFC17A HaloTag® CMV*d3* Flexi® Vector	G1551	Ultra-Low
pFC17K HaloTag® CMV*d3* Flexi® Vector	G1321	Ultra-Low

N-Terminal Vectors	Cat.#	Expression Level
pFN21A HaloTag® CMV Flexi® Vector	G2821	High
pFN21K HaloTag® CMV Flexi® Vector	G2831	High
pFN22A HaloTag® CMV*d1* Flexi® Vector	G2841	Medium
pFN22K HaloTag® CMV*d1* Flexi® Vector	G2851	Medium
pFN23A HaloTag® CMV*d2* Flexi® Vector	G2861	Low
pFN23K HaloTag® CMV*d2* Flexi® Vector	G2871	Low
pFN24A HaloTag® CMV*d3* Flexi® Vector	G2881	Ultra-Low
pFN24K HaloTag® CMV*d3* Flexi® Vector	G2981	Ultra-Low

3.D. Collecting Mammalian Cells Expressing HaloTag® Fusion Proteins

1. For transient expression of fusion proteins, plate one 15cm dish with 30ml of cells at 3–4 × 10^5 cells/ml (or 1–1.2 × 10^7 cells total) for each sample to be processed. Grow cells overnight in the appropriate medium, temperature and CO_2 content for the cell line being used. Cells should be grown to 70–80% confluency.

   Notes:

   1. These cell densities have been used with HeLa and HEK-293 cells. Other cell lines may require optimization to determine the best cell density to use. It is also possible to grow cells in suspension.
   2. If you are using cells that are stably expressing a HaloTag® protein, plate the cells at similar densities, but skip Step 2 and grow until the cells have reached 80–90% confluency.

3.D. Collecting Mammalian Cells Expressing HaloTag® Fusion Proteins (continued)

2. Transfect cells with the DNA construct encoding the HaloTag® fusion protein following the transfection reagent manufacturer recommendations.

   Notes:

   If using HeLa or HEK-293 cells, we recommend the following conditions for transfection, using FuGENE® HD Transfection Reagent (Cat.# E2311). (For other transfection reagents, follow the manufacturer's recommendations.) See the general recommendations in the *FuGENE® HD Transfection Reagent Technical Manual*, #TM328, using the following conditions for a 15cm dish containing 1–1.2 x 10^7 cells total:

   1. Add 30µg of endotoxin-free transfection-grade DNA to 1ml of minimal medium (such as OPTI-MEM® (Invitrogen, 51985-091) and mix.
   2. Add 90µl of room temperature FuGENE® HD Reagent directly to the medium; mix immediately.
   3. Incubate the medium/DNA/FuGENE® HD mixture for 15 minutes at room temperature.
   4. Add the mixture to a plate in a drop-wise manner and gently rock to distribute the transfection mixture.

3. Harvest cells typically 24–48 hours after transfection (or at 80–90% confluency for stable cell lines). Remove the medium and gently wash the cell layer with 20–25ml of ice-cold PBS. Aspirate to remove the PBS wash.

4. Add 25–30ml of ice-cold PBS to the cells, and gently scrape to collect cells into conical tubes. Centrifuge the cells at 4°C for 5–10 minutes at 2,000 × *g*, and discard the PBS. Store the cell pellets at –80°C **for at least 30 minutes** prior to lysing them as described in Section 4. We have successfully kept cell pellets expressing HaloTag® fusions frozen at –80°C for up to 6 months.

4. Protein Pull-Down Protocol

The protocol provided in this manual is optimized for mammalian cells. If you are using other biological systems, you will need to optimize the lysis and binding steps.

The Mammalian Lysis Buffer is optimized for this system (see composition in Section 8.A). Other lysis buffers might be compatible with this system as well. In addition, nondetergent buffers may work when used in combination with a freeze-thaw. To prevent the disruption of the protein complexes, sonication is not recommended. Additives and cofactors known to be necessary to preserve the protein interaction complexes may be added at this point. We do not recommend using SDS, IGEPAL® CA-630 or Tween® 20 detergents in the lysis buffer because these inhibit capture of the protein onto the HaloLink™ Resin.

Note: The Protease Inhibitor Cocktail is optimized to work with this system (see composition in Section 8.A). Other protease inhibitor cocktails can reduce binding efficiency. Avoid using any cocktails that are known to contain 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) or that are of unknown composition.

Phase 1. Equilibrate Resin

1. Mix the HaloLink™ Resin by inverting the bottle until you have obtained a uniform suspension.
2. For each pull-down experiment, dispense 200µl of HaloLink™ Resin into two 1.5ml microcentrifuge tubes (one tube for the experimental sample and one tube for the negative control sample). Centrifuge for 1 minute at 800 × *g*. Carefully remove and discard the supernatant, leaving the resin at the bottom of the tube.
3. Add 800µl of Resin Equilibration/Wash Buffer (Section 3). Mix thoroughly by inverting the tube. Centrifuge for 2 minutes at 800 × *g*. Carefully remove and discard the supernatant, leaving the resin at the bottom of the tube. Repeat this wash step 2 additional times for a total of 3 washes. Do not remove the final wash supernatant until you are ready to bind lysates (Phase 3). This will prevent the resin from drying out.

Phase 2. Prepare HaloTag® Fusion Protein and Control Lysates

The following protocol should yield enough material for mass spectroscopy analysis. It is possible to scale down the amount of cells and reagents used for initial optimization experiments or to detect protein interactions by Western blot analysis. In this case, we recommend using half the amount of cells and half the volume of resin and other reagents described below. The incubation times and temperatures should remain unchanged.

1. For each sample, grow approximately 1–1.2 × 10^7 cells as described in Section 3.D.

   Note: If your protein shows extremely low or high expression, you may need to adjust the amount of starting cells up or down by a factor of two- to fivefold.

2. Thaw the frozen cell pellet prepared in Section 3, and resuspend the cells in 300µl of Mammalian Lysis Buffer; pipette or briefly vortex to mix.

   Notes:

   1. Mammalian Lysis Buffer is optimized for total cellular lysis, including the nucleus. If cytoplasmic and nuclear fractions need to remain as separate pools, perform an initial cytoplasmic lysis as described in Section 5.B. If you are processing the cytosolic fraction only, discard the nuclear pellets.
   2. If you will be processing both the cytosolic and nuclear fractions, the nuclear pellets can be lysed subsequently with the Mammalian Lysis Buffer as described here. If you know your complex requires certain cofactors or small molecules to maintain complex integrity, please add these to the Mammalian Lysis Buffer and the wash buffer.

4. Protein Pull-Down Protocol (continued)

3. Add 6µl of the 50X Protease Inhibitor Cocktail and incubate on ice for 5 minutes.
4. To reduce lysate viscosity following the incubation, homogenize with a Dounce glass homogenizer (2ml size) on ice using 25–30 strokes using the large pestle (B). Alternatively, pass the cells through a 25- or 27-gauge needle 5–10 times.

   Note: We do not recommend sonication because protein complexes may fall apart, and overheating may reduce the HaloTag® protein activity.
5. Centrifuge the sample at 14,000 × *g* for 5 minutes at 4°C to clear the lysate.

   Note: If processing nuclear fractions these may be optionally treated with RQ1 RNase-Free DNase (Cat.# M6101) to reduce DNA content in the nuclear lysate. After the standard lysis protocol, add 30µl of 10X RQ1 DNase 10X Reaction Buffer and 3µl of RQ1 RNase-Free DNase. Incubate at room temperature for 10 minutes with gentle shaking. Continue with the standard lysis protocol.
6. Transfer the clear lysate to new tube, and place the tube on ice until Phase 3.

Phase 3. Bind Protein Complexes

1. Immediately before binding, dilute the 300µl of clear lysate prepared in Phase 2 with 700µl of 1X TBS (Section 6).

   Note: We recommend you determine the binding efficiency (Section 5.A), and to do so you will need to set aside 10µl of the diluted lysate as the prebinding fraction. Store this fraction on ice.
2. Remove the Resin Equilibration/Wash Buffer supernatant from the equilibrated resin, and add the remainder of the diluted lysate.
3. Incubate with mixing on a tube rotator (or equivalent device) for 15 minutes at room temperature. Make certain that the resin does not settle to the bottom of the tube; settling will reduce binding efficiency.

   Notes:

   1. In most cases 15 minutes binding time is sufficient to capture abundant protein complexes. For low abundance or larger protein complexes, this incubation time can be extended to 30-60 minutes at room temperature. Longer incubation times may increase non-specific binding.
   2. To capture membrane associated protein complexes extend the binding incubation time to 60 minutes at room temperature.
   3. For unstable or temperature sensitive protein complexes the binding can be performed at 4°C for 2 hours to overnight.
4. Centrifuge the tubes for 2 minutes at 800 × *g*, and discard the supernatant.

   Note: To determine the binding efficiency (Section 5.A) set aside 10µl of the supernatant as the unbound fraction. Store this fraction on ice.

Phase 4. Washing

1. Add 1ml of Resin Equilibration/Wash Buffer (Section 3) to each tube, and mix thoroughly by gently inverting the tube several times. Centrifuge for 2 minutes at 800 × *g*. Discard the wash. Repeat three additional times, for a total of four washes.
2. Add 1ml of Resin Equilibration/Wash Buffer (Section 3), and mix thoroughly by inverting the tube several times. Incubate at room temperature for 5 minutes with mixing. Centrifuge for 2 minutes at 800 × *g*. Discard the wash.

   Note: The stability of different protein complexes will depend on the binding affinities between the proteins in the complex, and the washing conditions may need to be optimized.

Phase 5. Protein Elution

1. For each sample, resuspend the resin with 50µl of SDS Elution Buffer. Incubate the tubes for 30 minutes with shaking at room temperature.

   Note: In some instances, it is possible to substitute the SDS Elution Buffer for the optional urea elution buffer as described in Section 5.D (composition in Section 8.A). Samples eluted in urea may be directly digested with Lys-C prior to mass spectroscopy analysis. See Section 5.D for more details.
2. Centrifuge for 2 minutes at 800 × *g*, and carefully transfer the eluate to a fresh tube leaving the resin at the bottom.

   Notes:

   1. Resin particles in the eluted fraction could be problematic if the sample is to be analyzed directly in solution by mass spectroscopy (see Section 5.D). If resin particles are present in the eluate, we recommend transferring the eluted fraction to a spin column with a 1.5 ml collection tube and spinning for 15 seconds at 10,000 × *g*.
   2. This elution method releases the interacting protein partners and leaves behind the HaloTag® fusion protein, which is covalently bound to the resin. Alternatively, TEV Protease cleavage can be used to isolate the entire complex including the bait protein originally fused to the HaloTag® protein (Section 5.C).

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5. Additional Protocols

5.A. Detecting HaloTag® Fusion Proteins

HaloTag® fusion protein expression and binding efficiency can be tested quickly and conveniently by fluorescent detection using the HaloTag® TMRDirect™ Ligand provided with the Cat.# G6500 System (also available separately; Cat. # G2991) or the HaloTag® TMR Ligand (Cat. # G8251, G8252).

1. For the HaloTag® TMRDirect™ Ligand (Cat.# G2991), dilute the stock solution (100µM) 1:2 in DMSO to make a 50µM working solution. For the HaloTag® TMR Ligand (Cat.# G8251, G8252), dilute the stock solution (5mM) 1:100 in DMSO for a final concentration of 50µM.
2. Mix 10µl of diluted lysate containing the HaloTag® fusion protein or the equivalent amount of unbound fraction (saved in Phase 3, Steps 1 and 4) with 19µl of water and 1µl of 50µM HaloTag® TMR Ligand.
3. Incubate at room temperature for 15 minutes protected from light.
4. Add 10µl of 4X SDS gel loading buffer (Section 8.A) and heat at 70°C for 5 minutes or 95°C for 2 minutes.
5. Load 5–10µl onto an SDS-polyacrylamide gel.
6. Quantitate band intensities for the before- and after-binding fractions on a fluorescent detection scanner such as the Typhoon®, GE Healthcare Bio-sciences (excitation 532nm; emission 580nm).
7. To determine the binding efficiency, divide the band intensity of the sample after binding by the band intensity of the sample prior to binding. This value represents the percentage of unbound protein. Subtract the unbound percentage from 100% to calculate the percentage bound.

5.B. Cytoplasmic Lysis

1. Thaw the cell pellets. Add 300µl of Cytoplasmic Lysis Buffer (composition in Section 8.A) and pipette or vortex briefly.
2. Add 6µl of 50X Protease Inhibitor Cocktail and incubate on ice for 30 minutes.
3. Homogenize with a Dounce glass homogenizer (2ml size) using 25–30 strokes on ice using the large pestle (B). Alternatively, pass cells through a 25- or 27-gauge needle 5–10 times to complete lysis.
4. Centrifuge at 800 × *g* for 20 minutes at 4°C to separate the cytosol from the nuclear pellet.
5. To process the cytosolic fractions, transfer the clear lysate to a new tube, and place the tubes on ice until protein dilution and binding (Phase 3; Section 4). If you do not need the cytosolic fraction, discard the supernatant without disturbing the nuclear pellets at the bottom of the tube.
6. To process the nuclear fractions, use the standard lysis protocol (Phase 2; Section 4).

5.C. TEV Protease Cleavage of Fusion Proteins

An optimized TEV (Tobacco Etch Virus) cleavage site has been engineered between the HaloTag® protein and the target of interest in the HaloTag® Flexi® Vectors.

TEV cleavage should release all the protein partners including the untagged bait protein. Below is a protocol recommended for the use of ProTEV Plus (Cat.# V6101 and V6102) and optional removal of the TEV protease using MagneHis™ Ni-Particles (Cat.# V8560). If a different TEV enzyme is used, follow the manufacturer's recommendations.

1. Following the last wash (Phase 4, Section 2), resuspend the resin in 50µl 1X ProTEV Buffer and 30 units of ProTEV Plus Protease.
2. To improve cleavage, incubate at 25°C with shaking for 1 hour, making certain that the resin does not settle.
3. Centrifuge for 2 minutes at 800 × *g*, and carefully transfer the eluate to fresh tube leaving the resin at the bottom of the centrifuged tube.

   Notes:

   1. Resin particles in the eluted fraction could be problematic if the sample is to be analyzed directly in solution by mass spectroscopy (see Section 5.D). If resin particles are present in the eluate, we recommend transferring the eluted fraction to a spin column with a 1.5 ml collection tube and spinning for 15 seconds at 10,000 × *g*.
   2. This elution method releases the entire complex including the bait protein originally fused to the HaloTag® protein and interacting protein partners.

4. **Optional:** To remove the ProTEV Plus enzyme, add 5µl of MagneHis™ Ni-Particles to the supernatant and incubate for 20 minutes with shaking or rotation at room temperature. Place the tube on a magnetic separation stand, and allow the particles to collect. Transfer the supernatant containing the cleaved protein complex to a new tube.

   Note: Incubation with the MagneHis™ Ni-Particles may result in the loss of some material as a result of nonspecific binding. Consider the benefits of enzyme removal versus the possibility of reduced protein yield.

5.D. Downstream Analysis and Applications

Samples can be analyzed by SDS electrophoresis followed by silver stain detection (Figure 2), Western blotting or mass spectroscopy (MS). In addition, samples eluted via TEV cleavage, have the potential to be used in functional assays provided the TEV cleavage buffer used is compatible with the downstream assay. Use the following volumes as starting points for the various applications.

Western blot analysis: 2–5µl
Silver staining gel analysis: 10–15µl
Mass spectroscopy analysis: 35–40µl

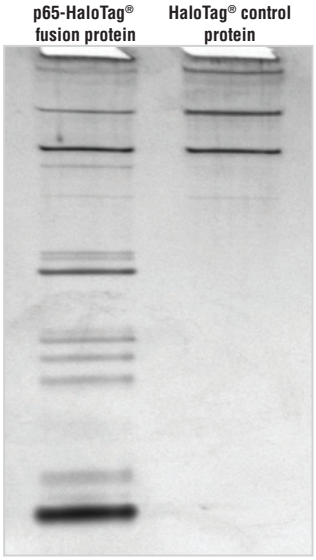

Figure 2. Example of a pull-down experiment detected by silver staining after SDS-PAGE separation.

Mass Spectroscopy Analysis

Mass spectroscopy analysis to identify recovered proteins can be performed in a variety of fashions.

For samples eluted in SDS one possibility is to resolve the samples by gel electrophoresis and perform in-gel digestion of the protein bands present primarily or uniquely in the experimental lane. Alternatively, bands present in both the experimental and control lanes can be analyzed. Following in-gel digestion with trypsin and extraction of peptides (Trypsin Gold; Cat.# V5280), the samples can be analyzed by MALDI or LC/MS/MS. In some cases, a large number of proteins are recovered, and resolving individual protein bands is not practical. In these situations, it is possible to partially resolve the samples by performing gel electrophoresis just long enough for the samples to enter the gel and then perform in-gel digestion on gel pieces representing the entire sample. The individually digested and extracted fractions can be pooled together as complex protein mixtures or analyzed separately by LC/MS/MS or other sensitive means of identification.

When the protein complexes are recovered by TEV cleavage (Section 5.C) or urea elution (Section 4, Phase 5), it is possible to perform the tryptic digestion directly in-solution without the need to resolve the samples using gel electrophoresis. Samples eluted in urea may be directly digested with Lys-C prior to mass spectroscopy analysis. For samples that will be directly injected into mass spectroscopy apparatus, it is important that no resin particles have been carried over in the eluted fraction. If resin particles are present in the eluate, we recommend transferring the eluted fraction to a spin column with a 1.5 ml collection tube and spinning for 15 seconds at 10,000 × *g*.

Note: For information about mass spectrometry services by our partner MS Bioworks please visit our web site: **www.promega.com/msbioworks/**

5.E. SDS-PAGE Analysis of HaloTag® Protein Fusions

The following protocol is intended to serve as a guide for SDS-PAGE-based HaloTag® applications. The covalent bond between the HaloTag® fusion protein and HaloTag® ligand withstands denaturation and thus allows rapid and direct analysis following SDS-PAGE application, such as fluorimaging (i.e., cell-to-gel analysis). The gels also can be used for Western blot analysis using the Anti-HaloTag® pAb at a final labeling concentration of 1µg/ml (1:1,000 dilution).

Although all of the HaloTag® ligands perform in this function, the HaloTag® TMR Ligand is recommended for direct SDS-PAGE fluorescence scanning applications. Representative data are shown in Figure 3.

5.E. SDS-PAGE Analysis of HaloTag® Protein Fusions (continued)

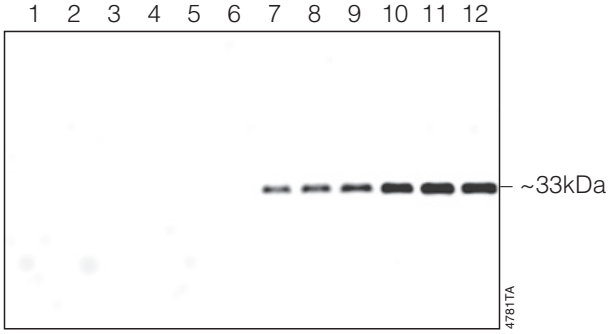

Figure 3. SDS-PAGE analysis shows fast, efficient and highly specific labeling of the HaloTag® protein in live cells. CHO-K1 untransfected control cells (lanes 1–6) or cells transiently transfected with HaloTag® pHT2 Vector (lanes 7–12) were labeled with 5µM HaloTag® TMR Ligand for different periods of time at 37°C (0.5, 1, 2, 5, 15 or 30 minutes) and treated as described in Section 4. Following SDS-PAGE, the gel was analyzed on a Hitachi FMBIO® fluorescence imaging system.

Materials to be Supplied by the User
(Solution compositions are provided in Section 8.A.)

- 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, follow Steps 1–4 of Section 6.A or 6.B, ending with cells in 1X PBS.
2. Lyse cells by replacing the 1X PBS from the wells with ~75–100µl of 1X SDS sample buffer per cm^2 of cell growth area (e.g., 150–200µl of 1X SDS sample buffer per well of a 24-well plate that has a 1.9cm^2 growth area).
3. Collect cell lysate, and incubate for 5 minutes at 95°C.
4. Perform SDS-PAGE by loading 10µl (5–10µg total protein) of each sample per well of the gel, or store samples at –20°C for later use.
5. Analyze the gel on a fluorescence scanner.

   Note: The dye front might 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.
6. After scanning, the proteins can be transferred to nitrocellulose for Western blot analysis if used promptly (i.e., gel is not fixed, remains moist and is not left in buffer or deionized water).

6. Cell Imaging Protocols

The same HaloTag® construct used for protein complex isolation can be used for cellular localization, protein trafficking and protein turnover studies. The following protocols use the HaloTag® TMRDirect™ Ligand provided in the HaloTag® Mammalian Pull-Down and Labeling System (Cat.# G6500). The HaloTag® TMRDirect™ Ligand and other HaloTag® Ligands for cellular imaging are also available separately and are listed in Section 8.B. Additional information is available at: **www.promega.com/protocols/**

These protocols are intended to serve as a guide for fluorescent ligand labeling of live cells expressing a HaloTag® protein that are grown on chambered cover glass slides (our recommended format for imaging purposes). You may need to optimize conditions to better fit your individual needs. For example, if cells attach poorly to the growth substrate, we recommend growing cells on a treated surface, such as one coated with collagen or poly-L-lysine.

Note: Fluorescent dyes are light-sensitive. Avoid exposing the cells to strong light during the ligand labeling and washing procedure.

Note: Use these protocols only for complementary cellular imaging studies. Once the HaloTag® protein is labeled with the HaloTag® TMRDirect™ Ligand it cannot be used for protein complex isolation. Use the protocols described in Sections 3 and 4 to perform the pull-down experiments.

6.A. Live-Cell Imaging

This protocol can be used to label adherent or nonadherent cells. Adherent cells that have been plated or are still in suspension can be used. Labeling of the HaloTag® protein can be accomplished concurrently with expression by adding the HaloTag® TMRDirect™ Ligand at the time of transfection or at the time of plating for stable cell lines. Alternatively, the HaloTag® TMRDirect™ Ligand can be added at a later time up to 15–18 hours prior to imaging. Representative data are shown in Figure 4.

1. Plate cells in chambered cover glass or other cell culture device, and allow them to reach appropriate confluency for desired transfection method.
2. Transfect cells with the DNA construct encoding the HaloTag® fusion protein following the manufacturer's recommendations (skip this step if the cells are stably expressing the HaloTag® protein).
3. Labeling can be performed at this time or at a later time but no later than 15–18 hours prior to imaging as follows.

6.A. Live-Cell Imaging (continued)

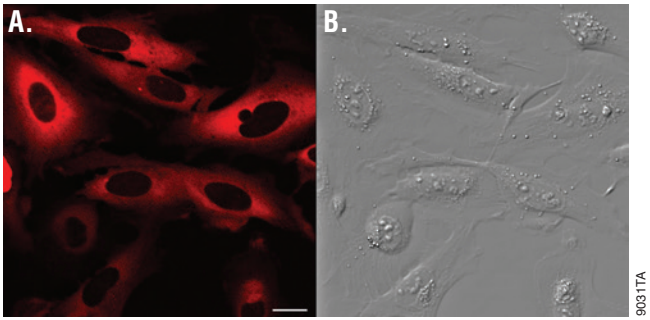

Figure 4. U2OS cells stably expressing p65-HaloTag® protein and labeled with the HaloTag® TMRDirect™ Ligand. The cells were imaged using a confocal microscope using the appropriate filter sets. Scale bar represents 20µm.

4. Prepare a 1:200 dilution of HaloTag® TMRDirect™ Ligand in warm culture medium just prior to adding it to the cells. This is a 5X working stock solution at 500nM. The final labeling concentration will be 100nM of HaloTag® TMRDirect™ Ligand.

   For adherent cells: Replace one-fifth of the existing volume of medium with the 5X HaloTag® ligand working stock solution, and mix gently.

   For cells in suspension: Add 5X ligand working stock to existing cell suspension so that the resulting final concentration of HaloTag® Ligand Working Stock Solution is 1X.

5. After adding the ligand, incubate overnight in a 37 °C + 5% CO_2 cell culture incubator.

6. Gently replace the ligand-containing medium with an equal (or greater) volume of warm fresh medium, or fix solution (for endpoint assays; see Section 6.B).

7. Transfer the cell chamber to an imaging device equipped with appropriate filter sets and lasers ($555nm_{Ex}/580nm_{Em}$), and capture images.

6.B. Fixed-Cell Imaging

This protocol is intended to serve as a guide to fixing cells that are expressing a HaloTag® fusion protein. The covalent bond that forms between the ligand and HaloTag® protein during live-cell labeling allows you to subsequently fix, permeabilize and wash the cells under stringent conditions without significant loss of the specific fluorescent signal. We recommend using 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 further eliminate nonspecific labeling and 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.

1. Label the cells following Steps 1–5 of Section 6.A.
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.
5. Transfer to a microscope, and capture images using a confocal microscope or wide-field fluorescent microscope equipped with appropriate filter sets and lasers ($555nm_{Ex}/580nm_{Em}$) or proceed to immunocytochemistry (ICC).

7. 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	Possible Causes and Comments
Cannot detect HaloTag® fusion proteins	HaloTag® fusion protein was not expressed in the cell lysate. Confirm protein expression using Western blot or HaloTag® fluorescent ligands. • Modify transfection conditions.
	If using HaloTag® fluorescent ligands for detection, verify that the fluorescent scanner has the appropriate filters and/or sensitivity. • Use a fluorescent scanner with the appropriate sensitivity (e.g., Typhoon®, GE Healthcare Bio-sciences). For scanners with lower sensitivity, sample volume may need to be increased
Inefficient binding of HaloTag® fusion protein onto HaloLink™ Resin	Inefficient lysis: • Do not skip freeze-thaw steps (Sections 3.D and 5.B). Freeze cells at –80°C for at least 30 minutes prior to thawing and lysing. • Increase the number of strokes if homogenizing with glass Dounce homogenizer or increase the number of passes through a 25- to 27-gauge needle. • If cells are difficult to lyse, increase the volume of Mammalian Lysis Buffer to 500µl or incubation time in Mammalian Lysis Buffer to 15 minutes prior to mechanical homogenization.

7. Troubleshooting (continued)

Symptoms	Possible Causes and Comments
Inefficient binding of HaloTag® fusion protein onto HaloLink™ Resin efficiency; Section 5.A). (continued)	Suboptimal binding conditions (check binding efficiency; Section 5.A). • Do not use protease inhibitor cocktails containing 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) because it is known to significantly reduce the binding of HaloTag® fusion proteins to HaloLink™ Resin. **Use the Protease Inhibitor Cocktail provided with the kit.** • Increase the binding time to 30 minutes to 1 hour at room temperature (may increase overall background binding). • For 4°C binding, increase binding time to 2 hours to overnight. • If altering lysis buffer, confirm the formulation is compatible with HaloTag® protein binding by testing with the HaloTag Control Vector (Cat.# G6591).
HaloTag® fusion protein is inactivated.	• Do not sonicate the cells. • Possible steric hindrance. • Positioning of HaloTag® fusion in the complex precludes binding to the resin.
Unable to detect interacting protein partners	Inefficient protein capture (see above).
	Low expression levels. You may need to increase the number of cells used.
	Unstable protein complex. • Minimize handling time or lower binding temperature. • Reduce the number or volume of washes (may increase overall background). • Add cofactors to lysis and wash buffers to stabilize complexes. • If no interacting partners are identified, consider moving the HaloTag® protein from N- or C-terminus to the opposite terminus.
	Inefficient elution: • Increase the volume of the SDS elution buffer or increase elution time and/or temperature. • If cleaving by TEV protease, increase the amount of enzyme or the cleavage reaction time. Cleavage of larger protein complexes can be improved by using lower molecular weight versions of TEV protease. • Perform a second elution to increase yield.

Symptoms	Possible Causes and Comments
High background	Excessive nonspecific protein bands in experimental and/or control samples. • Equilibrate HaloLink™ Resin in recommended buffer containing 0.05% IGEPAL® CA-630. • Ensure lysate is diluted with 1X TBS buffer just prior to binding to the HaloLink™ Resin. • Increase the number and/or volume of washes or extend the time for one or more washes. Include salt, and/or detergents in the wash buffer. • Reduce the volume of HaloLink™ Resin. • If protein is overexpressed reduce the amount of cells. • Perform TEV cleavage instead of SDS elution. • If nonspecific proteins are chaperons, heat-shock proteins, or other stress-related proteins, reduce expression level or consider a stable cell line.
Weak or absent fluorescent signal in labeled cells	Poor transfection efficiency. Optimize the transfection conditions, and use high-quality, endotoxin-free DNA.
	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 CO_2 conditions throughout labeling and imaging.
	Optimize cell-labeling protocols (e.g., increase labeling time and/or ligand concentration.)
	Use freshly diluted HaloTag® Ligand, and add immediately to label cells.
	Ensure that you are using the appropriate filter set for imaging ($555nm_{Ex}/580nm_{Em}$).
	Adjust the settings on your fluorescence detection instrument (e.g., laser power, PMT gain and aperture for a confocal microscope).
	Prevent photobleaching of HaloTag® Ligand by analyzing fluorescent signal for only a short period of time using the lowest laser or lamp power possible.

8. Appendix

8.A. Composition of Buffers and Solutions

Supplied with System

HaloLink™ Resin
25% slurry in 25% ethanol

Mammalian Lysis Buffer

50mM	Tris-HCl (pH 7.5)
150mM	NaCl
1%	Triton® X-100
0.1%	Na deoxycholate

10X TBS Buffer

1M	Tris-HCl (pH 7.5)
1.5M	NaCl

Protease Inhibitor Cocktail
Reconstituted 50X stock will contain:

5mM	benzamidine HCl
2.75mM	phenanthroline
500µM	bestatin
1mM	leupeptin
250µM	pepstatin A
50mM	PMSF

SDS Elution Buffer

1%	SDS
50mM	Tris-HCl (pH 7.5)

To Be Supplied by User

cytoplasmic lysis buffer

20mM	Tris (pH 7.5)
5mM	$MgCl_2$
10mM	$NaCl_2$
1mM	DTT
1mM	EDTA

4X SDS-PAGE gel loading buffer

240mM	Tris-HCl (pH 6.8)
3mM	bromophenol blue
50%	glycerol
400mM	dithiothreitol
2%	SDS

urea elution buffer

8M	urea
100mM	Tris, pH 8.5

4% paraformaldehyde/0.2M sucrose/1X PBS (pH 7.5)
Prepare fresh for each use.

1X PBS Buffer (pH 7.5)

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

1X SDS sample buffer

60mM	Tris-HCL (pH 6.8)
0.75mM	bromophenol blue
12.6%	glycerol
100mM	dithiothreitol
0.5%	SDS

8.B. Related Products

HaloTag® Flexi® Vectors for Protein Expression in Mammalian Systems

Product	Size	Cat.#
pFC14A HaloTag® CMV Flexi® Vector	20µg	G9651
pFC14K HaloTag® CMV Flexi® Vector	20µg	G9661
pFC15A HaloTag® CMV*d1* Flexi® Vector	20µg	G1611
pFC15K HaloTag® CMV*d1* Flexi® Vector	20µg	G1601
pFC16A HaloTag® CMV*d2* Flexi® Vector	20µg	G1591
pFC16K HaloTag® CMV*d2* Flexi® Vector	20µg	G1571
pFC17A HaloTag® CMV*d3* Flexi® Vector	20µg	G1551
pFC17K HaloTag® CMV*d3* Flexi® Vector	20µg	G1321
pFN21A HaloTag® CMV Flexi® Vector	20µg	G2821
pFN21K HaloTag® CMV Flexi® Vector	20µg	G2831
pFN22A HaloTag® CMV*d1* Flexi® Vector	20µg	G2841
pFN22K HaloTag® CMV*d1* Flexi® Vector	20µg	G2851
pFN23A HaloTag® CMV*d2* Flexi® Vector	20µg	G2861
pFN23K HaloTag® CMV*d2* Flexi® Vector	20µg	G2871
pFN24A HaloTag® CMV*d3* Flexi® Vector	20µg	G2881
pFN24K HaloTag® CMV*d3* Flexi® Vector	20µg	G2981
HaloTag® Flexi® Vectors—CMV Deletion Series Sample Pack	9 × 2µg	G3780
HaloTag Control Vector	20µg	G6591

HaloTag® Flexi® Vectors for Protein Expression in E. coli

Product	Size	Cat.#
pFN18A HaloTag® T7 Flexi® Vector	20µg	G2751
pFN18K HaloTag® T7 Flexi® Vector	20µg	G2681

HaloTag® Flexi® Vectors for Protein Expression in Cell-Free Systems

Product	Size	Cat.#
pFN19A HaloTag® T7 SP6 Flexi® Vector	20µg	G1891
pFN19K HaloTag® T7 SP6 Flexi® Vector	20µg	G1841
pFC20A HaloTag® T7 SP6 Flexi® Vector	20µg	G1681
pFC20K HaloTag® T7 SP6 Flexi® Vector	20µg	G1691

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8.B. Related Products (continued)

Flexi® Cloning System Products

Product	Size	Cat.#
Flexi® System, Entry/Transfer	5 entry and 20 transfer reactions	C8640
Flexi® System, Transfer	100 transfer reactions	C8820
Carboxy Flexi® System, Transfer	50 transfer reactions	C9320

HaloTag® Products

Product	Size	Cat.#
HaloCHIP™ System	20 reactions	G9410
HaloLink™ Array (TnT® T7 Quick) Two Slide System	two 50-well arrays	G6140
HaloLink™ Array (TnT® SP6 Wheat Germ) Two Slide System	two 50-well arrays	G6180
HaloLink™ Array Six Slide System	6 slides	G6190
HaloTag® Protein Purification System	25ml	G6280
HaloTag® Protein Purification System, Sample Pack	2.5ml	G6270

Ligands for Protein Immobilization

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

Fluorescent Ligands for Cellular Imaging

Product	Size	Cat.#
HaloTag® TMR Ligand	15µl	G8252
HaloTag® TMRDirect™ Ligand	30µl	G2991
HaloTag® diAcFAM Ligand	15µl	G8273
HaloTag® Coumarin Ligand	15µl	G8582
HaloTag® Oregon Green® Ligand	15µl	G2802
HaloTag® Alexi Fluor® 488 Ligand	15µl	G1002
HaloTag® R110Direct™ Ligand	30µl	G3221

9. Summary of Changes

The following changes were made to the 8/15 revision of this document:

1. The document design was updated.