



Promega

Technical Manual

HaloTag[®] Protein Purification System

INSTRUCTIONS FOR USE OF PRODUCTS G6270 AND G6280.



www.promega.com

HaloTag[®] Protein Purification System

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

1. Description	1
2. Product Components and Storage Conditions	3
3. Materials and Preparation	4
4. Protocols	6
A. General Guidelines for Using the HaloTag [®] Protein Purification System	7
B. HaloTag [®] Fusion Protein Expression With Single Step (KRX) Competent Cells.....	9
C. Preparation of Cell Lysate.....	10
D. Batch Method for HaloTag [®] Purification.....	11
E. Batch/Column Combination Method for HaloTag [®] Purification.....	13
F. Recommendations for FPLC.....	15
G. Optimizing HaloLink [™] Resin Usage and Purification Protocol (Optional)	16
5. Additional Protocols	18
A. Creating HaloTag [®] Fusion Protein Constructs	18
B. Detection of HaloTag [®] Fusion Proteins	19
C. Recommendations for Expression in Mammalian Cells	19
6. Reagent Compatibility	21
7. Troubleshooting	25
8. Appendix	33
A. HaloTag [®] Technology	33
B. Composition of Buffers.....	34
C. References.....	34
D. Related Products.....	35

1. Description

The HaloTag[®] Protein Purification System^(a-d) is based on a unique tag, the HaloTag[®] protein, which allows covalent, efficient and specific capture of the proteins expressed in *E. coli* as N-terminal HaloTag[®] fusion proteins. The HaloTag[®] protein is a mutated hydrolase (1-3) that covalently attaches to the

1. Description (continued)

HaloLink™ Resin via an immobilized chloroalkane. The HaloTag® Protein Purification System allows stringent washing because of the covalent attachment of the HaloTag® protein to the resin. TEV Protease cleaves the target protein from the HaloTag® protein which is bound to the HaloLink™ Resin^(a,b). Next, the TEV Protease, which has an N-terminal (HQ)₅ tag, is removed from the protein of interest using the HisLink™ Resin^(c,d), and the purified protein of interest is recovered. The general purification scheme is depicted in Figure 1.

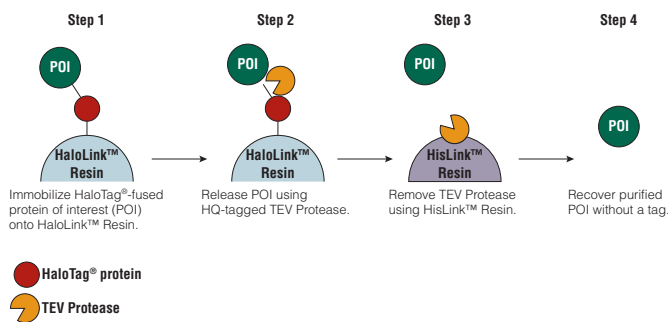


Figure 1. Streamlined purification process leads to higher yield of purified protein.

Benefits of the HaloTag® Protein Purification System:

- **Higher purity protein recovered at higher yields:** HaloTag® fusions are immobilized onto the HaloLink™ Resin through a covalent, stable linkage between the resin and the HaloTag® protein, making it possible to capture proteins expressed even at low levels.
- **Reduced background contaminants:** Extensive, stringent washes can be performed after covalent immobilization of HaloTag® fusion proteins, which is not possible with noncovalent, affinity interactions, and this may result in recovery of protein with higher purity.
- **Efficient release of target protein:** TEV Protease provided with this system supports site-specific and efficient on-resin cleavage, which results in recovery of the target protein without the HaloTag® protein. The TEV Protease contains an (HQ)₅ tag and is removed by binding to HisLink™ Resin.
- **Easy to use:** One purification buffer can be used throughout the protocol, significantly simplifying the process and reducing purification time.

This manual is designed to provide general guidelines for protein expression using either the pFN18A HaloTag® T7 Flexi® Vector (Cat.# G2751) or the pFN18K HaloTag® T7 Flexi® Vector (Cat.# G2681) and Single Step (KRX) Competent Cells (Cat.# L3001, L3002) with general cell lysis and purification protocols. Optimal expression and purification conditions may be dependent on the target of interest and may require additional optimization.

The HaloTag® purification protocol may also be applied to recombinant proteins expressed as C-terminal HaloTag® fusions or to HaloTag® fusions expressed in other systems, such as mammalian expression systems. When protein expression levels are low, expression conditions may need to be further optimized.

2. Product Components and Storage Conditions

Product	Size	Cat.#
HaloTag® Protein Purification System Sample Pack	each	G6270

Includes:

- 10ml HaloLink™ Resin, 10ml of 25% slurry
- 0.5ml HisLink™ Protein Purification Resin, 50% slurry
- 200µl TEV Protease, for use with HisLink™ Resin

Product	Size	Cat.#
HaloTag® Protein Purification System	each	G6280

Includes:

- 100ml HaloLink™ Resin, 100ml of 25% slurry
- 5ml HisLink™ Protein Purification Resin, 50% slurry
- 1.6ml TEV Protease, for use with HisLink™ Resin

TEV Protease Storage Buffer: 50mM HEPES, 1mM DTT, 0.5mM EDTA, 125mM NaCl, 50% Glycerol, 0.1% Triton.

Storage Conditions: Store the HaloLink™ Resin and HisLink™ Resin at 4°C. Do not freeze the resins. Store the TEV Protease at -20°C.

3. Materials and Preparation

Materials to Be Supplied by the User

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

Protein Expression

- appropriate HaloTag® Flexi® Vector (e.g., for *E. coli*: pFN18A/K HaloTag® T7 Flexi® Vectors; Cat.#s G2751 and G2681)
- Flexi® System Entry/Transfer (Cat.# C8640)
- Single Step (KRX) Competent Cells (Cat.# L3001, L3002)
- LB medium
- antibiotics
- glucose (20% w/v)
- rhamnose (20% w/v)

Cell Lysis

- HaloTag® Purification Buffer (Section 8.B)
- sonicator for cell breakage.
- RQ1 RNase-Free DNase (Cat.# M6101) or DNase I (0.5mg/ml)
- RNase A Solution (Cat.# A7973)
- lysozyme (10mg/ml)
- **Optional:** HaloTag® TMR Ligand (Cat.# G8252)

Purification

- 15ml conical tubes and other plasticware used in the purification process
- columns if using batch/column protocol or FPLC (see Sections 4.E. and 4.F.)
- tube rotator (e.g., Multipurpose Rotator Model 151 from Scientific Industries, Inc.)

Preparations

HaloTag® Protein Purification Buffer

Prepare HaloTag® Purification Buffer by combining 50mM HEPES (pH 7.5) and 150mM NaCl. This buffer can be used throughout the purification process.

Note: 1mM DTT, 0.5mM EDTA and 0.005% IGEPAL CA630 (final concentrations) can be added to the HaloTag® Purification Buffer during protein purification (See Optional Additives).

Cleavage Solution

The cleavage solution releases the protein of interest from the HaloLink™ Resin.

Prepare the solution on the day of protein purification. Dilute TEV Protease ~16-fold in HaloTag® Purification Buffer (Section 7.B).

Example: For purification from 50ml of cell culture, prepare cleavage solution by adding 66µl of TEV Protease into 1.1ml of HaloTag® Purification Buffer. Mix well and store on ice until use.

Note: Return TEV Protease to -20°C immediately after use.

HaloTag® TMR Ligand (optional)

Prepare 50µM HaloTag® TMR Ligand by mixing 2µl of 5mM stock with 198µl of Nuclease-Free Water or HaloTag® Purification Buffer. Store at 4°C, and use within a week.

Note: If the expression level is low (i.e., fusion protein cannot be detected on a Coomassie® blue-stained denaturing gel), you may label with the HaloTag® TMR Ligand, analyze by SDS PAGE and detect fluorescence using a fluorimaging instrument (555ex/585em)

Optional Additives

The following reagents can be added to basic HaloTag® Protein Purification Buffer during purification.

1mM DTT

0.5mM EDTA

0.005% IGEPAL® CA-630

Notes:

1. DTT can reduce background caused by nonspecific linkages to the fusion protein and is also beneficial for TEV Protease cleavage.
2. EDTA inhibits metalloprotease activity in the cell lysate and is also beneficial for TEV Protease cleavage, which is sensitive to divalent metals such as Zn²⁺ and Cu²⁺. DO NOT add during nuclease treatment in cell lysis because it will inhibit nuclease activity and may lead to viscous solution.
3. IGEPAL® CA-630 (Sigma Cat.# 18896) prevents the HaloLink™ Resin from sticking to plasticware and reduces nonspecific binding. IGEPAL® CA-630 is chemically indistinguishable from Nonidet® P-40.
4. IGEPAL® CA-630 is not stable in solution. Use all IGEPAL® CA-630-containing solutions within a week. We recommend first preparing a 10% stock solution of IGEPAL® CA-630 (in water) and using a final concentration of 0.005% v/v. The effect of IGEPAL® CA-630 is protein-dependent, and may need to be determined empirically. See Section 6 for general guidelines.

4. Protocols

This manual contains protocols for protein purification using batch and batch/column methods, as well as recommendations for FPLC systems. Figure 2 shows the general workflow for protein purification using batch and batch/column methods. Examples of purified proteins are shown in Figure 3. Protein purification is monitored by capture efficiency (comparing flowthrough with the starting material). The efficiency of TEV Protease cleavage/elution and TEV Protease removal by the HisLink™ Resin is measured by comparing starting material (tube S) and the protein recovered (E1) with the TEV Protease treated sample (tube S-TEV) and the elutions before (E1) and after (E2) treatment with HisLink™ Resin.

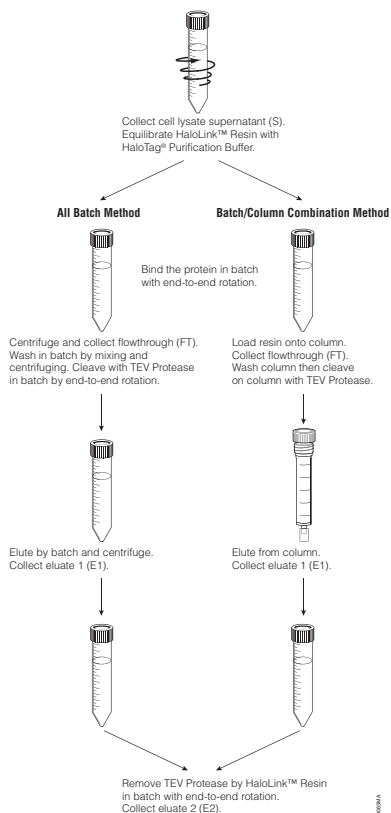


Figure 2. Workflow of HaloTag®-mediated protein purification using the HaloTag® Protein Purification System.

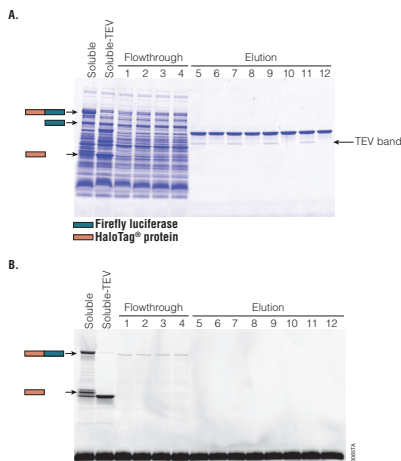


Figure 3. SDS-PAGE Analysis of proteins purified using the batch and batch/column protocols. Panel A. Coomassie[®] Blue-stained gel. **Panel B.** Proteins labeled with the HaloTag[®] TMR Ligand and detected using a fluoroimaging instrument (555_{Ex}/585_{Em}). **For Panel A and B.** Lanes 1, 2, 5, 6, 7, 8 are duplicate Batch purifications. Lanes 5 and 7 are Elution 1 (E1); lanes 6 and 8 are Elution 2 (E2). Lanes 3, 4, 9, 10, 11, 12 are duplicate Batch/Column Purifications. Lanes 9 and 11, Elution 1 (E1). Lanes 10 and 12, Elution 2 (E2). S = cell lysate supernatant. S-TEV = cell lysate supernatant treated with TEV protease.

4.A. General Guidelines for Using the HaloTag[®] Protein Purification System

Optimal purification is dependent on several factors, including protein expression level, the amount of soluble, active HaloTag[®] fusion in the lysate, the volume of HaloLink[™] Resin used for capture, washing conditions to remove nonspecific background, the efficiency of TEV Protease cleavage and the elution conditions.

We recommend resuspending the *E. coli* cell paste in 1/10th of the cell culture volume of HaloTag[®] Purification Buffer and using settled HaloLink[™] Resin at 1/50th of the original cell culture volume. The amount of cleavage solution, elution volume and HisLink[™] Resin are dependent on the volume of settled HaloLink[™] Resin. They can be scaled up/down proportionally. See Table 1 for reference.

**4.A. General Guidelines for Using the HaloTag® Protein Purification System
(continued)**

Table 1. Recommended Protein Purification Components.

LB media	50ml	500ml	1L
Cell pellet weight	0.2-0.5g	2-5g	4-10g
HaloTag® Protein Purification Buffer (for cell lysis)	5ml	50ml	100ml
25% HaloLink™ Resin	4ml (1ml settled)	40ml (10ml settled)	80ml (20ml settled)
Batch binding	15ml conicle tube	50ml conicle tube	Large containers
Columns for batch/column purification	Pierce Cat.# 89897	Bio-Rad Econo-Pac® #732-1010/732-1011	Bio-Rad Econo-Pac® #732-1010/732-1011
Columns for FPLC purification (e.g., ÄKTAexplorer™ column)	GE Tricorn™ 5/100 (flow rate 0.1-0.2ml/minute)	GE XK 16/100 (flow rate 0.5-1ml/minute)	GE XK 16/100 (flow rate 0.5-1ml/minute)
Cleavage solution	1.1ml (66µl of TEV Protease)	11ml (660µl of TEV Protease)	22ml (1,320µl of TEV Protease)
Protein Elution volume (2X settled HaloLink™ Resin volume)	2ml	20ml	40ml
50% HisLink™ Protein Purification Resin	50µl	500µl	1ml
Possible Protein Yield*	1-4mg	10-40mg	20-80mg

* Yield will depend upon the target protein.

The following protocols are for purification of proteins expressed in 50ml LB media. The volumes can be scaled up or down proportionally. We recommend screening the expression and solubility of your protein of interest before starting large-scale protein preparations.

- Batch binding is strongly recommended, since it promotes efficient capture of the HaloTag® fusion protein, especially when the HaloTag® protein active site is not fully accessible to the ligands due to steric hindrance or the HaloTag® fusion protein is in low abundance.
- We do not recommend binding HaloTag® fusions on a column with gravity loading since the flow rate, and thus, the contact time of HaloTag® fusion and its ligand on the resin, cannot be effectively controlled. In case gravity loading is applied, reload the lysate through the column multiple times, and check flowthrough to ensure efficient capture of fusion protein before washing and cleavage steps.
- If the HaloLink™ Resin is packed into a column for FPLC, we recommend using small diameter columns, such as GE Tricorn™ 5/100 for 1ml of settled HaloLink™ Resin with flow rate of 0.1–0.2ml/minute and GE XK 16/100 for 5ml of settled HaloLink™ Resin with flow rate of 0.5–1ml/minute for sample loading (see recommendations in Section 4.F).

4.B. HaloTag® Fusion Protein Expression With Single Step (KRX) Competent Cells

The following is recommended for auto-induction using KRX cells (4,5). Please refer to the *Single Step (KRX) Competent Cells Technical Manual #TB352* for more information.

Prepare Expression Culture

1. Inoculate a fresh colony into 5ml of LB media containing the proper antibiotic and 0.2–0.4% glucose, and grow at 37°C for 6–8 hours or overnight.
Note: Glucose is used to further reduce basal expression. For very toxic proteins, use higher glucose concentrations, such as 0.8% w/v.
2. Dilute the starter culture 1:100 in fresh LB media containing 0.05% glucose and 0.05% rhamnose.
Note: Glucose and rhamnose levels may be varied for maximum expression (5).
3. Grow cells at 25°C for another 17–20 hours.


Cell Harvest

4. Collect the cells by centrifugation at 4,000 × g for 20 minutes, 4°C.
5. Store the cell pellet at –20°C if not used immediately.


4.C. Preparation of Cell Lysate

Cell Lysis by Sonication

The following protocol is for a cell pellet from 50ml LB medium. This sonication method is optimized for the Misonix Sonicator 3000 using a Microtip Probe 419 with output power of 3.5. Sonication conditions for other sonicator must be determined empirically.

 **Caution:** Over sonication will inactivate the HaloTag® Protein and it will not bind to the HaloLink™ Resin.

1. Resuspend cell pellet completely in 5ml (1/10th culture volume) of HaloTag® Purification Buffer.
2. Add 50µl (1:100 dilution) each of the following, and incubate on ice for 5–10 minutes.
 - 10mg/ml lysozyme
 - RQ1 RNase-Free DNase or 0.5mg/ml DNase I
 - Optional: Protease inhibitors (see Section 6)
3. Sonicate on ice, using 5-second bursts with 5-second cooling time in between for a total of 2 minutes. **Note:** If it is not being used immediately, the cell lysate can be stored at -20°C. Thaw the frozen lysate in an ice/water bath immediately before use.

 Avoid frothing/overheating, as this will inactivate the HaloTag® Protein and it will not bind to the HaloLink™ Resin.

Optional: To dissociate chaperonins from HaloTag®-target fusion protein, add the following to the cell lysate:

- a. ATP to a final concentration of 2mM;
 - b. MgSO₄ or MgCl₂ to a final concentration of 10mM
- Incubate at 37°C for 10 minutes

4. **Immediately** before purification, spin cell lysate at 10,000 × g for 15–30 minutes at 4°C, and collect the supernatant.
5. Transfer the supernatant to another tube; store on ice. Save 100µl of the cell lysate supernatant on ice for further use in Sections 4.D–G.

Cell Lysis by Freeze-Thaw Treatment

1. Resuspend cell pellet completely in 5ml (1/10th culture volume) of HaloTag® Protein Purification Buffer.
2. Add 100µl (1:50 dilution) of each of the following. Mix well and incubate on ice for 5–10 minutes.
 - 10mg/ml lysozyme (0.2mg/ml final concentration)
 - RQ1 RNase-free DNase or 0.5mg/ml DNase I
 - Optional: Protease inhibitors (see Section 6).

3. Freeze the cell suspension in a dry ice/ethanol bath for 2-5 minutes or place at -20 to -70°C until completely frozen.
4. Thaw in an ice/water bath with occasionally mixing.
5. Repeat Steps 2 and 3 for a total of 3-5 times. Store at -20°C or collect supernatant as described for lysis by sonication.
6. The total cell lysate can be stored at -20°C . Thaw the lysate on ice immediately before use.

Optional: To dissociate chaperonins from HaloTag[®]-target fusion protein, add the following to the cell lysate:

- a. ATP to a final concentration of 2mM;
- b. MgSO_4 or MgCl_2 to a final concentration of 10mM

Incubate at 37°C for 10 minutes

7. **Immediately** before purification, spin cell lysates at $10,000 \times g$ for 15-30 minutes at 4°C .
8. Transfer the supernatant to another tube; store on ice. Save 100 μl of the cell lysate supernatant on ice for further use in Sections 4.D-G.

4.D. Batch Method for HaloTag[®] Purification

The following protocol is for cells prepared from a 50ml cell culture and using 1ml of settled HaloLink[™] Resin. See Figure 3 for example results.

HaloLink[™] Resin Equilibration

1. Thoroughly resuspend the HaloLink[™] Resin by inverting the bottle until completely mixed.
2. Add 4ml of HaloLink[™] Resin to a 15ml conical tube.
3. Spin at $1,000 \times g$ for 5 minutes at room temperature.
4. Discard the supernatant.
5. Add 10ml of HaloTag[®] Purification Buffer to the tube; invert the tube until thoroughly mixed.
6. Spin down resin as in Step 3, and remove supernatant.
7. Repeat two more times, for a total of 3 washes. After the last wash, do not remove the supernatant until immediately before use. This will prevent the resin from drying out.

4.D. Batch Method Protocol for HaloTag® Purification (continued)

Binding/Immobilization

8. Remove the supernatant from the tube, and add 5ml of cell lysate supernatant (prepared in Section 4.C).
9. Mix well by inverting, and place the tube onto a tube rotator for end-over-end mixing for 1 hour at room temperature.

Note: Mixing is critical. Periodically check the tubes, and if the resin starts to settle/clump, take the tube off the rotator, and mix well by pipetting several times or gentle vortexing; then place it back onto the rotator.

10. Spin at $1,000 \times g$ for 5 minutes.
11. Transfer the supernatant into another tube; this is sample flowthrough (FT).

Optional: To remove chaperonins that might be associating with HaloTag® fusion protein, add 1ml (1 column volume) of 4mM ATP and 20mM $MgSO_4$ or $MgCl_2$ in HaloTag® Protein Purification Buffer onto the settled resin (final concentration of ATP is around 2mM and Mg^{2+} is around 10mM); Bind by rotating end-to-end at room temperature for 30 minutes to 1 hour. Collect the resin by centrifugation as before and proceed to washing steps.

Wash

12. Wash the resin with 10ml of HaloTag® Protein Purification Buffer; mix well by inverting the tube until thoroughly mixed.

Note: After a sufficient amount of HaloTag® fusion protein has been captured, the HaloLink™ Resin may become difficult to resuspend. If necessary, the resin can be resuspended by briefly vortexing or by pipetting several times.

13. Spin at $1,000 \times g$ for 5 minutes. Discard the supernatant.
14. Repeat for a total of 3 washes. After the last wash, do not remove the supernatant until immediately before use. This will prevent the resin from drying out.

TEV Protease Cleavage/Elution

15. Add 1ml of cleavage solution (see Section 3) into the settled resin; mix well by pipetting 3–4 times.
16. Incubate on the rotator with end-over-end mixing at room temperature for 1 hour.

17. During the incubation, label two 1.5ml microcentrifuge tubes:
S (supernatant) and S-TEV (supernatant + TEV).
 - Add 50µl of cell lysate supernatant saved from Section 4.C and 10µl of HaloTag® Protein Purification Buffer to one tube (tube S).
 - Add 50µl of cell lysate supernatant and 10µl cleavage solution to the other tube (tube S-TEV); mix well. This tube will be used to evaluate cleavage and find the size of the cleaved protein following SDS-PAGE.
 - Incubate the two tubes at room temperature for 1 hour and then place on ice.
18. Spin at 2,000–3,000 × g for 5 minutes.
Note: After efficient TEV cleavage, the resin will become very loose and difficult to settle. Centrifuge at a higher speed (up to 10,000 × g) if necessary.
19. Transfer supernatant to a 15ml conical tube.
20. Add 1ml of HaloTag® Protein Purification Buffer to the resin, and mix well by inverting the tube.
21. Spin at 2,000–3,000 × g for 5 minutes. Add the supernatant to the tube from Step 19, and mix well. This will be Elution 1 (E1).
 - a. E1 has 2X column volume.
 - b. Save 100µl of E1 on ice for downstream analysis.

TEV Protease Removal

22. Thoroughly resuspend the HisLink™ Resin.
23. Add 50µl of 50% HisLink™ Resin into the tube from Step 21. Incubate the tube on a rotator at room temperature for 20 minutes.
Note: HisLink™ Resin settles quickly, and should be thoroughly mixed just before use. It may be difficult to pipet using a regular tip. Use a wide-bore tip.
24. Spin at 1,000 × g for 5 minutes, and transfer supernatant to another tube. This is your purified protein. Remove a small fraction for analysis, this is elution 2 (E2).

Gel Analysis

25. Transfer 10µl each of S, S-TEV, FT, E1 and E2 into another set of tubes.
26. Add 10µl of HaloTag® Protein Purification Buffer.
Optional: Instead of HaloTag® Protein Purification Buffer, add 10µl of 50µM HaloTag® TMR Ligand and incubate at room temperature for 10 minutes. Following electrophoresis, TMR fluorescence can be detected using a fluorimaging instrument (555_{Ex}/585_{Em}).

4.D. Batch Method Protocol for HaloTag® Purification (continued)

27. Add 20µl of 2X SDS loading buffer (with 50mM DTT, final concentration).
28. Heat at 95°C for 2 minutes.
29. Load 12µl of each sample per lane for SDS-PAGE analysis.

4.E. Batch/Column Combination Method for HaloTag® Protein Purification

The following protocol is for cells prepared from 50ml cell culture with 1ml settled HaloLink™ Resin and Pierce column (Pierce Cat.# 89897). See Figure 3 for a typical result.

HaloLink™ Resin Equilibration

Equilibrate the HaloLink™ Resin as described in Section 4.D.

Binding/Immobilization

1. Bind the protein to the resin as described in Section 4.D, steps 8-9.
2. Load all resin onto column (Pierce Cat.# 89897), drain by gravity, collect the flowthrough (FT) and store on ice for further analysis. Keep some solution on top of the resin to avoid drying the resin.

Optional: To remove chaperonins that might be associating with HaloTag® fusion protein, add 1ml (1 column volume) of 2mM ATP and 10mM MgSO₄ or MgCl₂ in HaloTag® Protein Purification Buffer onto the settled resin; let the solution completely enter the resin. Close the end of the column tightly, and cap the top of the column to avoid evaporation. Incubate at room temperature for 30 minutes to 1 hour. Proceed with column washing.

Wash

3. Add a total of 20ml HaloTag® Protein Purification Buffer (20X column volume) and drain by gravity.
4. After all the buffer has drained, close the end of the column tightly to avoid drying the resin.

TEV Protease Cleavage/Elution

5. Add 1ml (1 column volume) of cleavage solution onto the settled resin.
6. Open the end of the column, and let about 1ml of the solution run out (1 column volume) Close the end of the column tightly.

Note: There should be some buffer left on top of the resin. If not, add ~50µl of cleavage solution to the resin.

7. Cap the top of the column to avoid evaporation, and incubate at room temperature for 1 hour.

Note: Periodically check the column for any leaking; if leaking occurs, change to a new press-on cap and reseal the end of the column tightly.

8. During the incubation, label two 1.5ml microcentrifuge tubes:
S (supernatant) and S-TEV (supernatant + TEV).
 - Add 50µl of cell lysate supernatant saved from Section 4.C and 10µl of HaloTag® Protein Purification Buffer to one tube (tube S).
 - Add 50µl of cell lysate supernatant and 10µl cleavage solution to the other tube (tube S-TEV); mix well. This tube will be used to evaluate cleavage and find the size of the cleaved protein following SDS-PAGE.
 - Incubate the two tubes at room temperature for 1 hour and then place on ice.
9. Add 2ml of HaloTag® Protein Purification Buffer to the column, being careful not to disturb the resin.
10. Open the end of the column, and collect a total of ~2ml (2 column volumes) of elution into a 15ml conical tube. This will be Elution 1 (E1).

Note: A larger elution volume may be collected, if necessary (e.g., 5 column volumes), by adding more buffer to the resin.
11. Save 100µl of E1 in a microcentrifuge tube, and store on ice for analysis.

TEV Protease Removal

Follow the procedure TEV Protease Removal found in Section 4.D.

Gel Analysis

Perform gel analysis as described in Section 4.D.

4.F. Recommendations for FPLC

The following protocol is a general guideline. It has been tested on GE ÄKTAExplorer™ instrument with HaloTag® fusion proteins expressed in 50ml of Single Step (KRX) cells using 1ml of settled HaloLink™ Resin, and in 250ml of Single Step (KRX) cells using 5ml of settled HaloLink™ Resin. Please use this as a starting point to develop an FPLC-based purification protocol. The HaloTag® fusion protein may be immobilized onto HaloLink™ Resin as described in Section 4.D, and then loaded onto a column. The rest of the purification steps are performed by FPLC.

We recommend using long, narrow columns such as GE Tricorn™ 5/100 series packed with 1-2ml of HaloLink™ Resin with a flow rate of 0.1-0.2ml/minutes or the XK 16/100 series of columns packed with 5ml of HaloLink™ Resin with a flow rate of 0.5-1ml/minute. Follow the manufacturer's recommendations to assemble the column. The protocol described here is for 50ml of cell culture.

Column Packing

1. Completely resuspend the HaloLink™ Resin as described in Section 4.D.

2. Load 4ml of resuspended HaloLink™ Resin into the column, and pack the column according to manufacturer's recommendations.
3. Wash the packed column with distilled water at a flow rate of 1ml/minute. Use at least 3-5 column volumes.
4. Equilibrate the packed column with HaloTag® Protein Purification Buffer at a flow rate of 1ml/minute. Use at least 10 column volumes.

Purification

5. Load 5ml of cell lysate (prepared in Section 4.C.) on the column at a flow rate of 0.1-0.2ml/minute.
Note: Higher flow rates may reduce binding.
6. Wash the column with 10-20 column volumes of HaloTag® Protein Purification Buffer at a flow rate of 1ml/minute or until the absorption level reaches baseline.
7. Load 1ml of cleavage solution onto the column at a flow rate of 1ml/minute. Stop the flow and incubate at room temperature for 1 hour.
8. Elute protein of interest using HaloTag® Protein Purification Buffer at a flow rate of 1ml/minute, and collect the fractions. Usually a total of 1-2ml (1-2 column volumes) of the elution is collected.
9. Remove TEV Protease as described in Section 4.D.
10. Evaluate purification by SDS PAGE analysis.

4.G. Optimizing HaloLink™ Resin Usage and Purification Protocol (Optional)

The HaloLink™ Resin is based on Sepharose® beads with the HaloTag®-binding ligand covalently attached. The binding capacity of the resin is >7mg of HaloTag® protein per 1ml of settled resin. This is equivalent to 4ml of the 25% slurry provided. For more details, please see the *HaloLink™ Resin Technical Manual* #TM250. In general, we recommend using 1ml of settled HaloLink™ Resin for HaloTag® fusion protein expressed in 50ml of LB media. If needed, this protocol can be used to optimize the ratio of starting material to HaloLink™ Resin. Proteins with high-level expression will require more resin, while reducing the amount of HaloLink™ Resin will be optimal for proteins expressed at a low level.

For this analysis, we recommend starting with a cell lysate prepared from 50ml cell culture as in Section 4.C. Cell growth is optimal with better aeration conditions, which are harder to achieve with smaller culture volumes. Starting with a 50ml culture will ensure better cell growth and will also more closely reflect the final conditions. Expression may be very different in smaller cultures.

HaloLink™ Resin Equilibration

1. Thoroughly mix the HaloLink™ Resin by inverting the bottle until the resin is completely resuspended.
2. Transfer 400µl of the HaloLink™ Resin to microcentrifuge tubes labeled 1-4.
3. Centrifuge at 1,000 × g at room temperature for 5 minutes.
4. Remove supernatant. Add in 1ml HaloTag® Protein Purification Buffer, mix thoroughly by inverting the tube until the resin is completely resuspended, centrifuge as in step 3.
5. Repeat for a total of 3 washes.
6. After the final spin, do not remove the supernatant until you are ready to use the resin; this will prevent the resin from drying out.

Binding and Washing

7. Add 100, 250, 500µl and 1ml of cell lysate supernatant prepared in Section 4.C. to the four tubes with settled resin from Step 6, mix thoroughly by inverting the tubes until the resin is completely resuspended. The ratio between start culture to settled resin will be 10:1, 20:1, 50:1 and 100:1, respectively.
8. Continue mixing at room temperature for 1 hour with end-over-end rotation.
9. Spin down the resin as in Step 3, before transferring the supernatant to another set of collection tubes labeled FT 1-4. Store on ice. These will be the flowthrough (FT) samples.
10. Add 1ml of HaloTag® Protein Purification Buffer to the tubes containing the resin, and mix thoroughly by pipetting. Spin the resin down as in Step 3.
11. Repeat Step 10 for a total of 3 washes.
12. After the last wash, do not remove the supernatant from the resin until immediately before the next step to avoid drying the resin.

Note: After immobilizing a sufficient amount of the HaloTag® fusion protein, the resin may become difficult to resuspend.

TEV Protease Cleavage/Elution

13. Add 100µl of cleavage solution (prepared in Section 3) into each tube containing settled resin. Mix thoroughly by pipetting.
14. Incubate at room temperature for 1 hour with constant end-over-end rotation.

15. During the incubation, label two 1.5ml microcentrifuge tubes:
S (supernatant) and S-TEV (supernatant + TEV).
 - Mix 50 μ l of cell lysate supernatant saved from Section 4.C. and 10 μ l of HaloTag[®] Protein Purification Buffer (tube S).
 - In a separate tube mix 50 μ l of cell lysate supernatant and 10 μ l of cleavage solution (tube S-TEV).
 - Incubate both tubes at room temperature for 1 hour.

Note: TEV cleavage may also be performed at 4°C overnight, especially for target proteins not stable at room temperature.
16. Spin down resin at 2,000–3,000 $\times g$ for at least 5 minutes, and save supernatant for analysis. This is elution 1 (E1).

Gel Analysis

17. Prepare samples for SDS-PAGE analysis. Mix 10 μ l of each sample, 10 μ l of HaloTag[®] Protein Purification Buffer and 20 μ l of 2X SDS loading buffer.

Optional: Replace the 10 μ l of HaloTag[®] Protein Purification Buffer with 10 μ l of 50 μ M HaloTag[®] TMR Ligand (Section 3), and incubate at room temperature for 5 minutes. The HaloTag[®] Ligand can be detected by fluorescence (555_{Ex}/585_{Em}) after gel electrophoresis. This is especially helpful for proteins expressed at low levels that cannot be detected by Coomassie[®] blue staining.
18. Analyze 12 μ l of the sample using SDS-PAGE.
19. Stain the gel with Coomassie[®] blue according to manufacturer's protocol. Alternatively, if the HaloTag[®] TMR Ligand was added, the HaloTag[®] fusion protein can be detected by fluorescence (555_{Ex}/585_{Em}). The efficiency of TEV Protease cleavage can be determined by comparing lanes S and S-TEV. The binding efficiency can be determined by comparing lanes S and FTs.
20. Determine the optimal amount of HaloLink[™] Resin needed for purification with the least amount of HaloTag[®] fusion in flowthrough and most protein recovered after TEV cleavage.
21. Optionally, the amount of protein recovered can also be determined by a standard protein assay, such as A₂₈₀/extinction coefficient, Bradford or BCA assays.

5. Additional Protocols

5.A. Creating HaloTag[®] Fusion Protein Constructs

For *E. coli* expression, we strongly recommend using the pFN18A or pFN18K HaloTag[®] T7 Flexi[®] Vectors and expressing the protein in the Single Step (KRX) Competent Cells, which have tighter basal control and higher protein expression.

The linker between the HaloTag® tag and the protein of interest was optimized to minimize steric hindrance and for cleavage with TEV Protease. The linker sequence is EPTT EDLYFQ SDNAIA, which contains an optimized Tobacco Etch Virus (TEV) protease recognition site. This makes it possible to remove HaloTag® tag from the fusion protein. When using the pFN18A or pFN18K HaloTag® T7 Flexi® Vectors, the sequence SDNAIA will be left on the N-terminus of the target protein following cleavage. This provides increased stability with regard to both temperature and denaturants, increased solubility and faster labeling kinetics, and results in markedly improved expression. We do not recommend using the pHT2 HaloTag® Vector, which was discontinued August 31, 2009.

5.B. Detection of HaloTag® Fusion Proteins

HaloTag® fusion proteins expressed at high levels (>20–50mg/l) in *E. coli* can be detected visibly on Coomassie® blue-stained denaturing protein gel. If the expression level is low, using the HaloTag® TMR Ligand (Cat.# G8251) also offers a quick and convenient way to visualize protein expression by fluorescent detection. The fluorescent TMR-conjugated ligand can be added to the fractions collected during protein purification and analyzed by SDS-PAGE. The same protocol can be used to monitor binding of HaloTag® fusion proteins to the HaloLink™ Resin by analyzing the starting material and the supernatant recovered after protein binding, which may contain unbound protein.

For other expression systems, the level of expression might be too low to be detected on a Coomassie® blue-stained denaturing protein gel. The fluorescent TMR-conjugated ligand is needed for detecting expression as well as for monitoring efficiency of immobilization after binding. Recommendations for HaloTag® protein labeling and detection can be found in the *HaloCHIP™ System Technical Manual #TM075*), the *HaloLink™ Resin Technical Manual #TM250* as well as in the *HaloLink™ Protein Array Technical Manual #TM310*.

5.C. Recommendations for Expression in Mammalian Cells

Mammalian cells are commonly used to characterize potential cellular properties and functions of a protein of interest. Although expression levels are usually lower compared to *E. coli*, mammalian expression is advantageous, particularly since post-translational processing and signal recognitions are usually conserved for recombinant proteins of vertebrate origin, where these processes are lacking in *E. coli* based expression.

Protein expressed in mammalian cells can be purified using the HaloTag® Protein Purification System with low background, since the HaloTag® protein is not endogenous to mammalian cells. The HaloTag® capture is covalent and irreversible, which allows efficient capture of fusion targets at lower expression levels. The major considerations for protein purification from mammalian cells are the expression vector, the cell lysis conditions and the cell to lysate ratio.

Expression Vectors

For protein expression and purification from mammalian cells, we recommend the pFC14A, pFC14K, pFN21A and pFN21K HaloTag® CMV Flexi® Vectors.

Cell Lysis

Pellet and lyse mammalian cells in HaloTag® Purification Buffer supplemented with BaculoGold™ protease inhibitor cocktail (Becton Dickinson #554779) according to the manufacture's suggestions. In general, we recommend resuspending 1×10^7 – 1×10^8 cells per 1ml of lysis solution. Cell lysis may be achieved by one of the following methods:

1. Sonication on ice using 10-seconds burst with 10-second cooling time in between for a total of 1minute. With a sonicator such as Misonix 3000 equipped with a Microtip Probe 419; for cells resuspended in concentration of 1×10^8 cell/ml use power output of 3.5; if the concentration is 10^7 cell/ml reduce the power output to 2.5.

Note: Sonication conditions for other sonicators must be determined empirically.

Avoid over sonication as it will inactivate the HaloTag® protein

2. Add 1% Triton® X 100 and 0.1% sodium deoxycholate to the HaloTag® Protein Purification Buffer and incubate on ice for 20 minutes followed by one cycle of sonication as described in Step 1.

Store the lysate at -70°C until use. Immediately before purification, centrifuge the lysate at $10,000 \times g$ for 20–30 minutes at 4°C .

Protein Purification

Apply soluble cell lysate from 10^9 – 10^{10} cells to 1ml of settled HaloLink™ Resin. Follow binding, wash, TEV Protease cleavage and TEV removal steps as described in Section 4.

6. Reagent Compatibility

Many reagents have been tested in this purification system on HaloTag® fusion proteins expressed in *E. coli*. The effects are summarized in Table 2. For a particular protein of interest, the effect of these reagents may be different and should be determined empirically.

Table 2. Reagents Tested in the HaloTag® Protein Purification System.

Reagents Tested	Comments	Effect
DTT	Use 1mM in HaloTag® Protein Purification Buffer; can be applied in all steps. Use up to 50mM during protein binding.	Reduces background caused by intermolecular disulfide linkages and assists in TEV Protease cleavage.
EDTA	Use 0.5mM in all purification steps except cell lysis step. Up to 4mM may be used during protein binding.	Assists in TEV Protease cleavage; inhibits nuclease and metalloprotease.
glycerol	Use during binding, up to 20% may be applied.	May promote protein adsorption onto HaloLink™ Resin if used during the wash step.
protease inhibitor cocktails	BD Biosciences BaculoGold™ protease cocktail is recommended.	Prevents proteolysis. Do not use with TEV cleavage.
PMSF	Use up to 5mM in binding steps.	Prevents proteolysis.
AEBSF	Protease inhibitor cocktails containing AEBSF should be avoided, especially when working with proteins expressed at low levels or in mammalian cells.	Interferes with the binding of the HaloTag® protein to the HaloLink™ Resin at low protein expression levels, especially for mammalian expression.
arginine	Use up to 50mM in binding step. Avoid concentrations higher than 2mM in other steps.	May reduce nonspecific binding and protein aggregation. May increase protein recovery.

6. Reagent Compatibility (continued)

Table 2. Reagents Tested in the HaloTag® Protein Purification System (continued).

Reagents Tested	Comments	Effect	
urea/guanidine	Do not use.	Denaturants will interfere with the binding of HaloTag® fusion proteins, which requires native, folded proteins.	
imidazole	Up to 500mM may be used during the protein binding step.	Is a common eluant from histidine-tag affinity purification.	
GSH	Not recommended	Is a common eluants from GST-tag affinity purification.	
Non-ionic Detergents			
Reduce background from hydrophobic interaction			
Tween®-20	Use up to 0.1%.	OK for all steps.	
Tween®-8	Use up to 0.1%.	OK for all steps.	
OTG	Use up to 0.01%.	High concentration may promote nonspecific adsorption to HaloLink™ Resin and may reduce the amount of protein eluted. The effect may be protein-dependent and may need to be determined empirically.	
CHAPS	Use up to 0.01%.		
OG	Use up to 0.01%.		
Brij35	Use up to 0.05%.		
Triton®X-114	Use up to 0.05%.		
Triton® X-100	Use up to 0.05%.		
NP-40 (IGEPAL® CA-630)	Use up to 0.05%.		
Metals			
Mg ²⁺	Use up to 20mM for all steps.		
Ca ²⁺	Use up to 20mM for all steps.	Required for proteins containing calcium.	
Zn ²⁺	Use up to 20mM in cell lysate. Not recommended in wash step or in TEV cleavage step.	At 20mM will cause precipitation in cell lysate, but does not inhibit HaloTag® capture. Inhibits TEV cleavage.	

Table 2. Reagents Tested in the HaloTag® Protein Purification System (continued).

Reagents Tested	Comments	Effect
Metals (continued)		
NaCl	Recommend 150mM in HaloTag® Protein Purification Buffer.	Higher concentration (up to 500mM) <ul style="list-style-type: none"> • Can be used for protein elution after TEV cleavage and TEV removal by HisLink™ Resin. • May reduce specific capture of HaloTag® fusion by HaloLink™ Resin. • Can reduce TEV cleavage efficiency. Lower concentrations (0–150mM) may be used if target protein is not stable in the presence of salt.
Other Buffers		
50mM CAPS (pH 10) 150mM NaCl	Do not use.	Can precipitate protein during cell lysis and might damage HaloLink™ Resin.
50mM Na acetate (pH 4), 150mM NaCl	Do not use.	Can precipitate protein during cell lysis.
50mM Tris HCl (pH 8.5), 150mM NaCl	Can be used for binding and TEV cleavage step.	May not be optimal for TEV Protease removal by HisLink™ Resin.
PBS buffer (pH 7.3)	Acceptable for cell lysis, binding and TEV cleavage step.	HisLink™ Resin.
TBS buffer (pH 7.6)		
50mM MOPS (pH 7), 150mM NaCl		
50mM MES (pH 6), 150mM NaCl	Acceptable for binding; optional for TEV cleavage.	Not recommended for TEV Protease removal by HisLink™ Resin.
50mM Na citrate (pH 5), 150mM NaCl	Can be tested for TEV cleavage step.	

6. Reagent Compatibility (continued)

Table 2. Reagents Tested in the HaloTag® Protein Purification System (continued).

Reagents Tested	Comments	Effect
Detergent-Based Cell Lysis Reagents		
FastBreak™ Reagent	Some highly active HaloTag® fusions have been observed to precipitate under these lysis conditions, which can reduce final protein yield.	<ul style="list-style-type: none"> • Results may be protein-dependent. • Diluting the lysate 2-4X with HaloTag® Protein Purification Buffer after the cells are completely lysed may improve protein solubility and binding to HaloLink™ Resin. • Wash extensively after protein immobilization to remove any factors that may interfere with TEV Protease cleavage.
Novagen BugBuster® Master Mix		
Pierce B-PER		
Pierce B-PER (in phosphate buffer)		
Sigma CellLytic™ reagent	<ul style="list-style-type: none"> • Not recommended. May reduce binding of the HaloTag® fusion protein to the HaloLink™ Resin. • TEV protease will be inhibited in this solution. 	

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

Protein Expression Solubility

Symptoms	Causes and Comments
No or low protein expression	Clone is incorrect. Verify insert by DNA sequence.
	Use Single Step (KRX) cells rather than BL21(DE3) type of strains for expression with pFN18A and pFN18K HaloTag® T7 Flexi® Vectors
	Optimize culture and growth conditions such as medium composition, temperature and induction conditions (e.g., O.D. ₆₀₀ and induction time) to improve yield.
	Check both total and soluble cell lysate and compare to uninduced culture. The protein may be insoluble.
	Label the lysate with HaloTag® TMR Ligand to detect low expression levels.
	The glycerol stock may not be robust. To achieve reliable expression, we recommend using fresh transformants.
	Protein is toxic to the cell. Minimize basal expression by increasing glucose level in the starter medium and in expression medium for late induction at high cell densities. Alternatively, induce at O.D. ₆₀₀ 0.4–1.2 for short period of time.
	Protein is degraded. Add protease inhibitor during cell lysis (see Section 6). Keep all samples at 4°C. Perform purification at 4°C.
Use other expression system such as cell-free protein synthesis or mammalian cell expression.	

7. Troubleshooting (continued)

Protein Expression Solubility

Symptoms	Causes and Comments
Protein is insoluble	<p data-bbox="481 282 840 332">Fusion protein may be insoluble. Optimize expression conditions:</p> <ul data-bbox="481 335 884 674" style="list-style-type: none"> <li data-bbox="481 335 840 356">• Lower the growth temperature (15–30°C). <li data-bbox="481 360 884 434">• Change the rhamnose concentration for induction in KRX cells (normal range is 0.05–0.2% w/v). <li data-bbox="481 437 884 487">• Increase glucose for induction at higher cell density (normal concentration is 0.05% w/v; 6) <li data-bbox="481 490 884 540">• Collect samples at different time points after induction to determine best induction time. <li data-bbox="481 543 884 593">• Induce at a higher cell density for a short period of time. <li data-bbox="481 596 884 645">• Increase aeration, such as using different shake flasks (7). <li data-bbox="481 649 795 670">• Use fresh colony for starter culture. <hr/> <p data-bbox="481 690 817 740">The cell lysis is not complete or the lysis conditions are not optimized.</p> <ul data-bbox="481 743 884 1158" style="list-style-type: none"> <li data-bbox="481 743 864 764">• Treat cells with lysozyme before sonication. <li data-bbox="481 768 781 789">• Avoid frothing during sonication. <li data-bbox="481 792 884 897">• Oversonication or overheating during sonication. Decrease sonication time and output settings. Keep all samples on ice during sonication. <li data-bbox="481 900 884 1029">• The pH is not optimal. If the pH of the lysis buffer is less than 7 and greater than 8.5, most proteins in <i>E. coli</i> lysate will precipitate. Use HaloTag® Protein Purification Buffer for lysis. See Section 6 for buffer compatibility. <li data-bbox="481 1032 884 1082">• Include reducing reagents and detergents compatible with the system during cell lysis. <li data-bbox="481 1085 884 1158">• Detergent-based lysis reagents may render some HaloTag® fusion proteins insoluble. Use sonication or freeze-thaw method instead. <hr/> <p data-bbox="481 1177 884 1252">Use other expression systems such as mammalian cell expression or cell-free protein synthesis.</p>

Immobilization of HaloTag® Fusion onto HaloLink™ Resin

Symptoms	Causes and Comments
Protein does not bind to HaloLink™ Resin or binding is inefficient	<p data-bbox="495 237 831 285">HaloLink™ Resin is mishandled or used improperly.</p> <ul data-bbox="495 290 880 497" style="list-style-type: none"> <li data-bbox="495 290 880 365">• HaloLink™ Resin is not equilibrated. Equilibrate the resin thoroughly with HaloTag® Protein Purification buffer. <li data-bbox="495 370 880 391">• Avoid drying the Resin during purification. <li data-bbox="495 396 880 497">• Expression level is high so the amount of HaloLink™ Resin is not sufficient. Optimize the amount of HaloLink™ Resin for purification (Section 4.G). <hr/> <p data-bbox="495 517 860 564">Cell lysis conditions are inefficient. Increase lysis time or change sonication conditions.</p> <hr/> <p data-bbox="495 584 880 632">HaloTag® fusion is not active or the active site for binding is blocked.</p> <ul data-bbox="495 637 898 1443" style="list-style-type: none"> <li data-bbox="495 637 898 685">• Optimize expression conditions to achieve functional fusion protein expression. <li data-bbox="495 690 898 764">• Lysate is too viscous. Treat the lysate with nucleases. Do not add EDTA during cell lysis step. <li data-bbox="495 769 898 868">• Cell debris is not completely removed. Centrifuge at higher speed for more time or filter the lysate supernatant through a 0.22 or 0.45µM filter right before use. <li data-bbox="495 873 898 973">• Samples were oversonicated or overheated during sonication. Decrease sonication time and output settings. Keep all samples on ice during sonication. <li data-bbox="495 977 898 1052">• Binding conditions were not correct. The optimal pH range is between pH 6–8.5. Check pH of the lysate if necessary. <li data-bbox="495 1057 898 1179">• Some additives used in the cell lysate may reduce the specific binding of the HaloTag® Ligand to HaloLink™ Resin especially when HaloTag® protein is in low abundance. See Table 2 for details. <li data-bbox="495 1184 898 1232">• Target protein forms a multimer. Dilute the cell lysate prior to binding. <li data-bbox="495 1237 898 1285">• Target protein forms aggregates. Add arginine during binding step (see Section 6). <li data-bbox="495 1290 898 1443">• The N-terminal structure of the target protein might interfere with the reactive site of the HaloTag® fusion. Alter the N-terminal sequence of the target protein if possible or insert extra amino acids between the TEV cleavage site and the target protein.

7. Troubleshooting (continued)

Immobilization of HaloTag® Fusion onto HaloLink™ Resin

Symptoms	Causes and Comments
Protein does not bind to HaloLink™ Resin or binding is inefficient (continued)	<p>Binding time is not sufficient. Increase binding time, such as overnight at 4°C.</p> <p>Flow rate is too high when loading with FPLC. Reduce the flow rate as recommended in Section 4.F.</p> <p>During batch binding, mixing is crucial. Check periodically and mix well if the resin starts to form clumps.</p>

Protein Elution

Symptoms	Causes and Comments
Poor protein elution after TEV Protease cleavage	<p>Protein did not express or is insoluble. Verify the clone by DNA sequencing and check expression and solubility. Verify the HaloTag® fusion protein is present in the soluble fraction.</p> <p>Not enough fusion protein loaded onto HaloLink™ Resin. Optimize resin usage and the purification protocol (Section 4.G).</p> <p>Protein is degraded during the purification process. Include protease inhibitor during cell lysis (see Section 6).</p> <p>Elution volume is not sufficient. Increase elution volume.</p> <p>TEV Protease cleavage is not efficient</p> <ul style="list-style-type: none"> • Check the TEV cleavage site. The P1' position cannot be a proline, and other residues such as T,W,R,L,E,I might be cleaved less efficiently by TEV protease (8). Use the cleavage site in pFN18A and pFN18K HaloTag® T7 Flexi® Vectors, which is an optimized TEV recognition site. • Increase the reaction time of TEV Protease cleavage, such as 4°C overnight. • Increase the amount of TEV Protease in the cleavage reaction. • TEV Protease is a cysteine protease. Its activity can be enhanced by DTT and inhibited by divalent metals (e.g., Zn²⁺ and Cu²⁺).

Protein Elution

Symptoms	Causes and Comments
Poor protein elution after TEV Protease cleavage(continued)	<p>TEV Protease cleavage is not efficient.</p> <ul style="list-style-type: none"> • Additives used in the TEV cleavage reaction might inhibit the activity of TEV Protease; avoid these additives if possible (see Section 6). • Detergents might block the TEV recognition site (9,10) and the effect may be target-dependent. Omit detergent in cleavage reaction or replace with a compatible detergent. • TEV Protease was not stored properly and may have lost activity. Store TEV Protease provided in the system at -20°C. Do Not store or leave it at other temperatures. <p>Protein is retained by the HaloLink™ Resin.</p> <ul style="list-style-type: none"> • HaloTag® protein is negatively charged at pH 7.5. Positively charged protein at this pH can interact with the HaloTag® protein through ionic interaction. Increase the ionic strength of the elution buffer by adding NaCl (i.e., 500mM final concentration). • Very hydrophobic proteins may interact non-specifically with HaloLink™ Resin. They may also precipitate under high salt conditions. Using a nonionic detergent (i.e., IGEPAL® CA-630; 0.005–0.05%) during elution may improve results.
Multiple bands or contaminants (other than TEV Protease) co-elute with target of interest	<p>Protein degraded. Add protease inhibitor in the lysis and wash buffer and, if necessary, to the final eluted sample (see Section 6). Work at 4°C throughout the purification process.</p> <p>Check for premature stops in the sequence. Eliminate them if possible.</p> <p>The presence of cell debris and a lysate that is too viscous can lead to increased nonspecific binding. Optimize lysis conditions and remove insoluble fractions as much as possible.</p> <p>Additives used in the binding process, such as high salt and certain detergents, can promote non-specific adsorption/aggregation of proteins onto the HaloLink™ Resin. Do not use these additives if possible.</p>

7. Troubleshooting (continued)

Protein Elution

Symptoms

Multiple bands or contaminants (other than TEV Protease) co-elute with target of interest (continued)

Causes and Comments

Equilibrate the HaloLink™ Resin in the presence of IGEPAL® CA-630 (the effective range is 0.005%–0.1% v/v). Use 0.005% IGEPAL® CA-630 throughout purification.

Too much HaloLink™ Resin used for capture. Reduce the amount of HaloLink™ Resin used; optimize according to Section 4.G.

Increase wash stringency.

- Increase wash volume.
- Nonspecific binding of contaminants on the resin is usually low; however, certain conditions can increase background. Increase the wash stringency by raising the salt concentration or adding detergents to the wash buffer. Do not use high salt and high detergent at the same time.
- Wash with different conditions, such as high-salt followed by low-salt solutions.

Target protein co-elutes with contaminants.

- Oversonication can lead to nonspecific protein binding to target of interest. Use optimized sonication conditions or other mild cell breakage methods.
- Add reducing agent such as DTT to reduce disulfide bond-linked contaminants (Section 6).
- Protein cofactors that are required for protein function or proper folding might copurify.
- Presence of *E. coli* chaperonins. These chaperonins can facilitate nascent protein folding (11,12). They include but are not limited to DnaK (70kDa), DnaJ (37kDa), GrpE (40kDa), GroEL (57kDa), and GroES (10kDa).
 - Dissociate the complex: pre-incubate the cell lysate with 2mM ATP, 10mM MgSO₄/MgCl₂ in the HaloTag® Protein Purification Buffer (pH 7.5) for 10 minutes at 37°C prior to purification.
 - DnaK may be removed by ion exchange chromatography or by passage of the sample over ATP agarose.
 - During washing, incubate the HaloLink™ Resin with 2mM ATP and 10mM MgSO₄ or MgCl₂ in the HaloTag® Protein Purification Buffer for 30 minutes or longer at room temperature followed by washing with HaloTag® Protein Purification Buffer.

Protein Elution

Symptoms	Causes and Comments
Protein precipitated	<p>Oversonication or improper cell lysis conditions may lead to target protein denaturation or aggregation. Use proper cell lysis conditions for optimal protein recovery.</p> <hr/> <p>Temperature is too low. Perform purification at room temperature.</p> <hr/> <p>The pI of target protein is close to 7.5; use a different pH for TEV Protease cleavage and elution (pH 6-8.5). See Section 6.</p> <hr/> <p>High salt will precipitate hydrophobic protein. Reduce the concentration of salt or include detergent in elution. Other proteins might need high salt to stay in solution. Determine these conditions empirically for each target of interest if necessary.</p> <hr/> <p>Include a reducing agent such as DTT to reduce aggregation formed by intermolecular disulfide bonds.</p>

Removal of TEV Protease

Symptoms	Causes and Comments
TEV Protease cannot be removed	<p>Insufficient incubation time (<20 minutes) with HisLink™ Resin.</p> <hr/> <p>Not enough HisLink™ Resin was used. Increase the amount of HisLink™ Resin (e.g., double the amount of HisLink™ Resin used).</p> <hr/> <p>Fully resuspend the HisLink™ Resin before pipetting. Make sure that it is fully suspended, and pipet the resin immediately. The resin is heavy and settles quickly. If the resin cannot be pipetted within 10-15 seconds of mixing, significant settling will occur, and the resin will need to be resuspended again. Use a wide-bore pipette tip to transfer the resin if necessary.</p> <hr/> <p>The HisLink™ Resin and protein elution are not mixed well; monitor during incubation to ensure thorough mixing.</p>

7. Troubleshooting (continued)

Removal of TEV Protease

Symptoms	Causes and Comments
TEV Protease cannot be removed (continued)	<p>HQ-tag on TEV Protease is not accessible. If the target protein can tolerate high salt, add NaCl to 500mM during HisLink™ treatment of the elution sample.</p> <p>TEV Protease is precipitated due to improper handling and storage. Although the activity of TEV protease is not significantly affected by the presence of precipitation, the aggregates will be hard to remove. Always store TEV protease at -20°C and return to -20°C immediately after use. If precipitation does occur, give it a quick spin at 4°C in a desktop centrifuge (e.g., at full speed for 5 minutes). Use only cleared supernatant to prepare diluted cleavage solution.</p> <p>The HisLink™ Resin is provided in water; equilibration is not required. However, if TEV Protease removal is not complete, pre-equilibration of the HisLink™ Resin might be necessary. The resin can be equilibrated by washing 3X with 10 resin volumes of HaloTag® Protein Purification Buffer. Wait about 10-20 seconds during each wash, and let the resin settle by gravity; remove supernatant that may contain fine particles.</p> <p>TEV Protease provided has a very high pI. It is positively charged at pH 9 or lower. Use other ionic exchange methods to remove TEV Protease if needed.</p>
Loss of target protein after treatment with HisLink™ Resin	<p>Target protein is not stable or forms soluble aggregates due to suboptimal expression and improper cell lysis conditions. These conditions are target-dependent and may need to be determined empirically.</p> <p>HisLink™ Resin incubation is too long (>20 minutes) or too much HisLink™ Resin is used. Reduce the incubation time to 20 minutes or the amount of HisLink™ Resin or use both.</p>

Symptoms	Causes and Comments
Loss of target protein after treatment with HisLink™ Resin (continued)	Nonspecific interaction of the target protein to HisLink™ Resin. The nature of the potential interaction depends on the target of interest. <ul style="list-style-type: none">• If the target protein can tolerate high salt, add NaCl to 500mM during HisLink™ Resin treatment of the elution sample to prevent nonspecific interaction.• Hydrophobic proteins will precipitate in the presence of high salt. Reduce the salt concentration and add detergent (e.g., 0.005–0.05% IGEPAL® CA-630) to prevent nonspecific interaction of target protein to HisLink™ Resin.• Avoid using high salt and detergent at the same time; this may result in protein aggregation and nonspecific adsorption onto HisLink™ Resin.

8. Appendix

8.A. HaloTag® Technology

The HaloTag® Technology is a technology platform for understanding protein function in biochemical and cellular environments. Specific applications include live or fixed cell imaging, gel-based analyses including post-translational modification of labeled fusion proteins, and isolation of proteins or protein complexes, all using a single genetic construct. This technology is comprised of two essential parts: the HaloTag® protein, to which a protein of interest is fused at either the N- or C-terminus, and the HaloTag® ligands, designed to covalently bind the HaloTag® protein. For other HaloTag® related applications, please consult Technical Manuals #TM075, #TM250 and #TM310.

8.B. Composition of Buffers

HaloTag® Purification Buffer

50mM HEPES, pH 7.5

150mM NaCl

Optional Additives:

1mM DTT

0.5mM EDTA

0.005% IGEPAL CA-630

LB Medium

10g/l Bacto®-tryptone

5g/l Bacto®-yeast extract

5g/l NaCl

Adjust the pH to 7.5 with NaOH.

Autoclave to sterilize. Allow the autoclaved medium to cool to 55°C.

glucose, 20% (w/v)

20g D-glucose

Add distilled water to 100ml, sterilize through a 0.2µm filter and store in aliquots at -20°C.

rhamnose, 20% (w/v)

10g L-rhamnose monohydrate

Add distilled water to 45ml, sterilize through a 0.2µm filter and store in aliquots at -20°C.

2X SDS Loading Dye

100mM Tris-HCl (pH 6.8)

4% SDS

0.2% bromophenol blue

20% glycerol

8.C. References

1. Los, G.V. *et al.* (2005) HaloTag® Interchangeable Labeling Technology for cell imaging and protein capture. *Cell Notes*, **11**, 2-6.
2. Los, G.V. and Wood, K. (2007) The HaloTag: a novel technology for cell imaging and protein analysis. *Meth. Mol. Biol.*, **356**, 195-208.
3. Los, G.V. *et al.* (2008) HaloTag: a novel protein labeling technology for cell imaging and protein analysis. *ACS Chem. Biol.*, **3**, 373-82.
4. Hartnett, J., Gracyalny, J. and Slater, M.R. (2006) The Single Step (KRX) Competent Cells: Efficient cloning and high protein yields. *Promega Notes* **94**, 27-30.
5. Schagat, T. *et al.* (2008) KRX autoinduction protocol: A convenient method for protein expression. *Promega Notes* **98**, 16-8.
6. Golovanov, A. P *et al.* (2004) A simple method for improving protein solubility and long-term stability. *J. Am. Chem. Soc.* **126**, 8933-9.
7. Millard, C.S. *et al.* (2003) A less laborious approach to the high-throughput production of recombinant proteins in *Escherichia coli* using 2-liter plastic bottles. *Protein Expr. Purif.* **29**, 311-20.
8. Kapust, R.B. *et al.* (2002) The P1' specificity of tobacco etch virus protease. *Biochem. Biophys. Res. Commun.* **294**, 949-55.
9. Lundback, A.K. *et al.* (2008) Exploring the activity of tobacco etch virus protease in detergent solutions. *Anal. Biochem.* **382**, 69-71.
10. Mohanty, A.K., Simmons, C.R. and Wiener, M.C. (2003) Inhibition of tobacco etch virus protease activity by detergents. *Protein Expr. Purif.* **27**, 109-14.

11. Horwich, A.L., Farr, G.W. and Fenton, W.A. (2006) GroEL-GroES-mediated protein folding. *Chem. Rev.* **106**, 1917-30.
12. Sigler, P.B. *et al.* (1998) Structure and function in GroEL-mediated protein folding. *Annu. Rev. Biochem.* **67**, 581-608.

8.D. Related Products

Product	Size	Cat.#
Single Step (KRX) Competent Cells	5 × 200µl	L3001
	20 × 50µl	L3002

Flexi® Vectors

Product	Size	Cat.#
pFC14A HaloTag® CMV Flexi® Vector	20µg	G9651
pFC14K HaloTag® CMV Flexi® Vector	20µg	G9661
pFC15A HaloTag® CMVd1 Flexi® Vector	20µg	G1611
pFC15K HaloTag® CMVd1 Flexi® Vector	20µg	G1601
pFC16A HaloTag® CMVd2 Flexi® Vector	20µg	G1591
pFC16K HaloTag® CMVd2 Flexi® Vector	20µg	G1571
pFC17A HaloTag® CMVd3 Flexi® Vector	20µg	G1551
pFC17K HaloTag® CMVd3 Flexi® Vector	20µg	G1321
pFN18A HaloTag® T7 Flexi® Vector	20µg	G2751
pFN18K HaloTag® T7 Flexi® Vector	20µg	G2681
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
pFN21A HaloTag® CMV Flexi® Vector	20µg	G2821
pFN21K HaloTag® CMV Flexi® Vector	20µg	G2831
pFN22A HaloTag® CMVd1 Flexi® Vector	20µg	G2841
pFN22K HaloTag® CMVd1 Flexi® Vector	20µg	G2851
pFN23A HaloTag® CMVd2 Flexi® Vector	20µg	G2861
pFN23K HaloTag® CMVd2 Flexi® Vector	20µg	G2871
pFN24A HaloTag® CMVd3 Flexi® Vector	20µg	G2881
pFN24K HaloTag® CMVd3 Flexi® Vector	20µg	G2981
HaloTag® Flexi® Vectors - CMV Deletion Series Sample Pack	9 × 2µg	G3780

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	Each	G6140
HaloLink™ Array (TnT® SP6 Wheat Germ) Two Slide System	Each	G6180
HaloLink™ Array Six Slide System	Each	G6190

Ligands for Protein Immobilization

Product	Size	Cat.#
HaloLink™ Resin	2ml	G1911
	5ml	G1912
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

^(b)For use only in the purchaser's own, internal research. No other use or transfer of this product is authorized without the express, written consent of Promega including, without limitation, Commercial Use. Commercial Use means any and all uses of this product and derivatives by a party for monetary or other consideration and may include but is not limited to use in: (1) product manufacture; and (2) to provide a service, information or data; and/or resale of the product or its derivatives, whether or not such product or derivatives are resold for use in research. With respect to such Commercial Use, or any diagnostic, therapeutic or prophylactic uses, please contact Promega for supply and licensing information. If the purchaser is not willing to accept the conditions of this limited use statement, Promega is willing to accept return of the unopened product and provide purchaser with a full refund. However, in the event that the product is opened, then purchaser agrees to be bound by the conditions of this limited use statement.

^(b)U.S. Pat. No. 7,429,472 and other patents pending.

^(d)Certain applications of this product may be covered by patents issued to parties other than Promega and applicable in certain countries. Because purchase of this product does not include a license to perform any of these patented applications, users of this product may be required to obtain a patent license depending upon the particular application and country in which the product is used.

^(d)U.S. Pat. Nos. 7,112,552, 7,304,015, 7,304,016 and 7,354,750 and other patents pending.

^(d)Certain applications of this product may be covered by patents issued to parties other than Promega and applicable in certain countries. Because purchase of this product does not include a license to perform any of these patented applications, users of this product may be required to obtain a patent license depending upon the particular application and country in which the product is used.

© 2009 Promega Corporation. All Rights Reserved.

Flexi, HaloTag and TNT are registered trademarks of Promega Corporation. FastBreak, HaloChip, HaloLink and HisLink are trademarks of Promega Corporation.

ÅKTAexplorer and Tricorn are trademarks and FluoroImager is a registered trademarks of GE Healthcare Biosciences. BaculoGold is a trademark of Becton, Dickinson and Company. BugBuster is a registered trademark of Novagen, Inc. CellLytic is a trademark of Sigma-Aldrich, Inc. Coomassie and Tween are registered trademarks of Imperial Chemical Industries, Ltd. Econo-Pac is a registered trademark of Bio-Rad Laboratories, Inc. IGEPAL is a registered trademark of Rhone-Poulenc AG Co. Nonidet is a registered trademark of Shell International Petroleum Company, Ltd. Triton is a registered trademark of Union Carbide Chemical & Plastics Technology Corporation.

Products may be covered by pending or issued patents or may have certain limitations. Please visit our Web site for more information.

All prices and specifications are subject to change without prior notice.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.