HaloLink™ Resin: High Capacity, Specificity and Scalable Throughput for Protein Analysis

By Marjeta Urh, Ph.D.[1], Dan Simpson, Ph.D.[1], Jacqui Sankbeil, M.S.[1], Danette Hartzell, Ph.D.[1], Natasha Karassina, M.S.[1], Natalie Betz, Ph.D.[1], Nadine Nassif, M.S.[1], Jami English, M.S.[1], Ji Zhu, Ph.D.[2], Poncho Meisenheimer, Ph.D.[2], Dieter Klaubert, Ph.D.[2], Bob Bulleit, Ph.D.[1], Keith Wood Ph.D.[1], [1]Promega Corporation, [2]Promega Biosciences, Inc.

Abstract

HaloLink™ Resin provides a new method for specific, covalent and oriented immobilization of proteins onto surfaces. The strategy is based on the HaloTag™ Protein, which is derived from a catalytically inactive hydrolase engineered to form a covalent bond with a specific ligand. Here we demonstrate the high binding capacity and minimal nonspecific binding of the HaloLink™ Resin. We also show that the covalent HaloTag™ bond provides stability during dilution and stringent washing, minimizing the loss of the HaloTag™ fusion proteins from the surface. By analyzing protein:protein interactions and enzyme activity for several fusion proteins, we also demonstrate that fusion proteins bound to the HaloLink™ Resin maintain functionality. HaloLink™ Resin is not a purification resin; however, in conjunction with protease cleavage, it can be used to isolate a protein of interest.

The new HaloLink™ Resin enables covalent and oriented attachment of HaloTag™ fusion proteins to a solid surface.

Introduction

Proteomics is a rapidly growing field characterized by developing technologies for the study of proteins and their functions. The challenge that we face is to elucidate the function of all proteins and how they assemble into complex networks responsible for key cellular processes. Surface-based proteomics tools will play an important role in this effort. To advance, surface-based proteomics requires general and facile methods for immobilizing proteins on solid surfaces in known orientations without disrupting protein structure or function. Futhermore, this immobilization must exhibit high binding capacity and minimal nonspecific adsorption (1).

The HaloTag™ Technology[(a,b,c)] comprises the HaloTag™ Protein and a system of interchangeable synthetic ligands that specifically and covalently bind to the HaloTag™ Protein. These ligands impart multiple functions to a HaloTag™ fusion protein including imaging and immobilization. Thus one genetic construct can be used in various in vitro and in vivo (cell-based) assays. The HaloTag™ fusion proteins can be easily constructed using any of the HaloTag™ Vectors, the pFC8A and pFC8K (HaloTag™) CMV Flexi® Vectors (Cat.# C3631 and C3641) or the HaloTag™ pHT2 Vector (Cat.# G8241).

The HaloLink™ Resin[(b)] enables covalent and oriented attachment of HaloTag™ fusion proteins to a solid surface. The resin comprises HaloTag™ Ligand linked to a Sepharose® surface. The binding capacity is high, and nonspecific binding is low. The covalent nature of the HaloTag™ bond provides stability during dilution and stringent washing, minimizing the loss of the HaloTag™ fusion proteins from the surface. We demonstrate the utility of the HaloLink™ Resin in several different applications including protein:protein interactions in vivo and in vitro, analysis of enzymatic activity of proteins on the surface and purification of proteins of interest in conjunction with specific protease cleavage. Immobilizing proteins onto a solid support using the HaloLink™ Resin is a simple procedure that can be completed in 60 to 90 minutes (Figure 1). Currently the method is optimized for proteins expressed in cell-free expression systems (in vitro) or in mammalian cells.

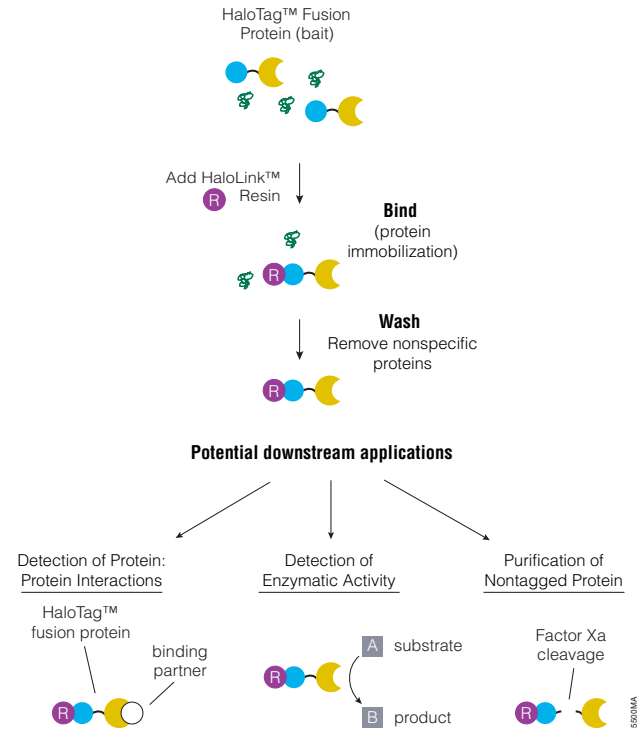

Figure 1. Overview of the HaloLink™ Resin immobilization protocol and potential downstream applications.

HaloLink™ Resin: High Binding Capacity, Specificity and Stability of Protein Binding

An important feature of any affinity binding resin is its binding capacity. Since HaloLink™ Resin is an immobilization resin and not a purification resin, we estimate binding capacity by the amount of the protein bound to the resin rather than the amount of eluted protein. Based on such experiments, we have determined the binding capacity to be at least 7mg of HaloTag™ fusion protein per milliliter of settled resin. This is comparable to the binding capacities of other affinity binding resins.

Another important feature of affinity resins is minimal nonspecific protein binding. This aspect is especially important when the resin is used for analysis of protein:protein interactions in pull-down assays. We evaluated nonspecific binding by treating HaloTag™ Resin with rabbit reticulocyte lysates used for in vitro protein expression according to the immobilization protocol described in *HaloLink™ Resin Technical Manual* #TM250. We boiled the resin in SDS loading buffer to remove all nonspecifically bound proteins and analzyed the supernatant by SDS-PAGE, along with dilutions of lysate used in the reaction. The lane containing the supernatant of resin boiled in SDS buffer contains almost undetectable amounts of protein, indicating that nonspecific binding of HaloLink™ Resin is low (Figure 2, Panel A).

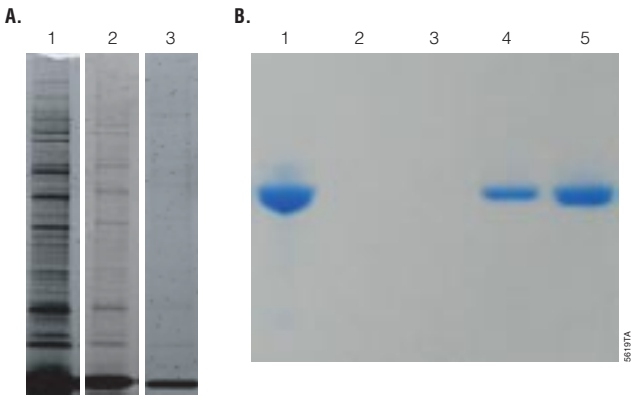

Figure 2. Specific and stable binding of HaloTag™ fusion proteins to HaloLink™ Resin. Panel A. Low nonspecific binding of proteins to HaloLink™ Resin. HaloLink™ Resin (50µl) was treated with rabbit reticulocyte lysate (400µg) according to the binding protocol in reference 7, boiled in SDS loading buffer, and the supernatant was analyzed by SDS-PAGE (lane 3). Fluorescent dye (Sypro® Ruby; Molecular Probes Cat.# 12000) was used to detect proteins. Lane 1, 500-fold diluted rabbit reticulocyte lysate; lane 2, 1,000-fold diluted rabbit reticulocyte lysate. **Panel B.** HaloTag™-GST fusion protein binds to HaloLink™ Resin stably. Equivalent amounts of settled HaloLink™ Resin and GST binding resin (25µl) were incubated with 160µg of HaloTag™-GST fusion protein; resins were washed and resuspended in PBS. After incubation at 4°C, supernatants were removed and analyzed by SDS-PAGE. Lane 1, 10% of the input HaloTag™-GST fusion protein; lane 2, supernatant from the HaloLink™ Resin removed at 10 minutes; lane 3, supernatant from the HaloLink™ Resin removed at 24 hours; lane 4, supernatant from the GST-binding resin removed at 10 minutes; lane 5, supernatant from the GST-binding resin removed at 24 hours.

Many applications used for protein analysis require extensive washing to remove nonspecifically bound proteins. A unique feature of HaloLink™ Resin is that proteins are bound to the resin covalently, which allows users to wash away nonspecifically bound proteins while retaining the fusion protein. To demonstrate this point, we bound HaloTag™-GST fusion protein to either HaloLink™ Resin or to a GST-binding resin. Following binding and washing, the resins carrying bound proteins were resuspended in phosphate-buffered saline (PBS). After an incubation period, we removed supernatant and analyzed it for the presence of the protein. The resin was resuspended in buffer once more, and after an additional 24-hour incubation, the second supernatant was analyzed. Results of the experiment demonstrate that HaloTag™-GST protein is stably attached to the HaloLink™ Resin, since protein was not detected in the supernatant; in contrast the HaloTag™-GST fusion protein leaches from the GST binding resin with each dilution (Figure 2, Panel B).

Detecting Protein:Protein Interactions

Pull-down assays probe interactions between a fusion protein consisting of an affinity tag and protein of interest (bait) and potential interacting partners (prey) in solution. Bait-prey complexes are isolated from solution by specific binding of the bait to the affinity resin. The most common pull-down technique is based on the GST affinity tag (2).

We performed pull-down experiments using HaloTag™ Protein in conjunction with HaloLink™ Resin. The properties of the HaloTag™ Protein provide some important advantages that increase chances of successful isolation of interacting partners. First the HaloTag™ Protein provides covalent attachment to the resin. This allows extensive washing to remove nonspecifically bound proteins. Second, HaloTag™ Protein binds the resin rapidly with high affinity. These features allow efficient immobilization of proteins present at low concentrations (e.g., proteins expressed in the in vitro expression systems). Thus HaloTag™ Protein in conjunction with HaloLink™ Resin uniquely enables detection of protein interactions when both proteins are expressed in vitro.

We used the well characterized interacting partners c-Jun and c-Fos (3) to demonstrate the pull-down capabilities of the HaloLink™ Resin. The c-Fos-HaloTag™ fusion was created by inserting the c-*fos* sequence into pFC8A Flexi® Vector; the c-*jun* sequence was inserted into pFV1A. Both proteins were expressed separately in TNT® T7 Quick Coupled Transcription/Translation System. The c-Jun (prey) was labeled with [^{35}S]methionine to facilitate detection. The HaloLink™ Resin was added to the in vitro reaction expressing the c-Fos-HaloTag™ fusion protein (bait), allowing immobilization of the bait

onto the resin. In parallel we set up a control reaction containing the HaloLink™ Resin and in vitro expression system without the bait. The in vitro expression reaction [^{35}S]-labeled c-Jun was added to both resins: the experimental sample (containing the bait) and the control (no bait). The bait and the prey were allowed to interact and then washed with buffer containing a low amount of nonionic detergent (7). To elute the captured prey, the resin was resuspended in SDS loading buffer, boiled at 95°C and analyzed by SDS-PAGE. The results in Figure 3 show that the prey protein was recovered only from the resin carrying the bait and not from the control sample. Thus the pull-down of c-Jun by the c-Fos-HaloTag™ fusion protein was efficient and specific as judged by absence of any prey protein in the control lane.

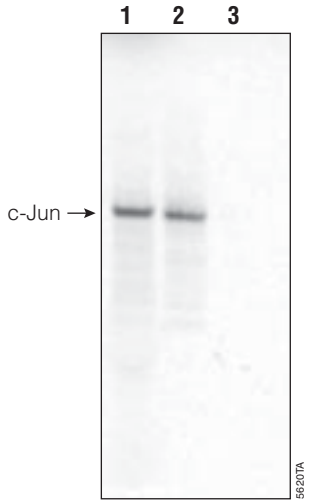

Figure 3. Detecting protein interactions using HaloLink™ Resin in a pull-down experiment. The c-Fos-HaloTag™ fusion protein was expressed in vitro; 20µl of the transcription/translation reaction containing c-Fos-HaloTag™ fusion was added to 50µl of the HaloLink™ Resin; immobilization was carried out according to the protocol (7). Prey protein (c-Jun) was synthesized and labeled with [^{35}S]-methionine in vitro, and 20µl of the reaction was added to the HaloLink™ Resin carrying the c-Fos-HaloTag™ fusion. In parallel, a control was carried out in which prey was added to the resin without the bait. After incubation and extensive washing [five 1ml wash steps using 0.05% IGEPAL® (Sigma Cat.# CA-630) and 1% BSA], proteins were eluted from the resin by boiling in SDS loading buffer and separated by SDS-PAGE, and the results were scanned on a Typhoon® Imager (Amersham Biosciences). Lane 1, in vitro reaction containing expressed ^{35}S-methionine-labeled c-Jun; lane 2, the pull-down reaction c-Fos-HaloTag™:c-Jun; Lane 3, control reaction (no c-Fos-HaloTag™ protein).

Detecting Regulated Protein:Protein Interactions

In the pull-down experiments described above, we precharged the HaloLink™ Resin with the bait protein and then added the prey. In the following experiment, we tested two additional parameters. First, could we efficiently detect protein:protein interactions when the protein binding partners are allowed to interact first and then the complexes are pulled down by adding the resin? Second, could we follow formation of protein:protein interactions that are modulated by a small molecule?

To test these parameters we analyzed the interaction between FK506 binding protein (FKBP) and the FKBP-rapamycin binding domain of mTOR (FRB) proteins (4–6). Rapamycin is needed for interaction between FKBP and FRB; FRB binds to a preformed FKBP-rapamycin complex (4). Formation of the ternary complex (FKBP-rapamycin-FRB) can be modulated by changing rapamycin concentration. An increase in rapamycin concentration results in increased FKBP-FRB interaction, predicting that we will recover increasing amounts of the prey protein (FRB) in the pull-down reaction with increasing concentration of rapamycin. Also, FRB should not be detected in the absence of rapamycin.

FKBP protein was inserted into the pFC8A Flexi® Vector to create a C-terminal HaloTag™-FKBP fusion protein. The FRB protein was expressed as a GST fusion from pFV2A plasmid. After incubation, protein complexes were subsequently pulled down by HaloLink™ Resin. FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System (Cat.# L5001) was used to fluorescently label the prey (GST-FRB) protein. Both proteins were expressed in vitro and mixed in the presence of various concentrations of rapamycin. Figure 4 shows that recovery of the prey protein is dependent on the concentration of rapamycin in the binding reaction and that no FRB was detected in the absence of rapamycin.

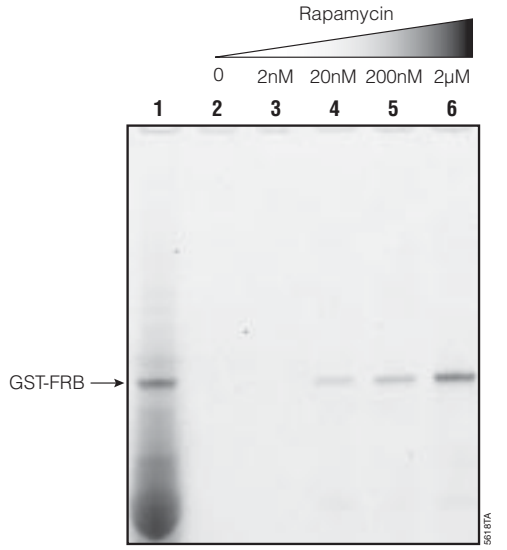

Figure 4. Detection of protein:protein interactions. GST-FRB and HaloTag™-FKBP fusion proteins were expressed using TNT® T7 Quick Coupled in vitro Transcription/Translation System (Cat.# L1170). FluoroTect™ Green$_{Lys}$ Labeling System (Cat.# L5001) was used to label the prey. The two reactions were mixed (20µl) in the absence of rapamycin or in the presence of indicated amounts of rapamycin. Samples were incubated 1 hour to allow proteins to bind, and then HaloLink™ Resin (50µl) was added to pull down the complexes. Reaction was incubated for 1 hour at room temperature with mixing; prey protein was eluted with SDS loading buffer and analyzed by SDS-PAGE. A Typhoon® scanner was used to detect fluorescent signal. Lane 1, input in vitro transcription/translation reaction containing GST-FRB; lane 2, control reaction in the absence of rapamycin; Lanes 3–6, reactions containing indicated amounts of rapamycin. Arrow indicates GST-FRB protein.

Detecting Protein:Protein Interactions in a 96-Well Plate Format

We have adapted HaloLink™ Resin pull-down to a 96-well format. We used the c-Jun-HaloTag™ fusion protein and GST-c-Fos protein pair to evaluate reproducibility of the assay across a 96-well plate. Both proteins were expressed in vitro; prey protein (c-Fos-GST) was fluorescently labeled using the FluoroTect™ $Green_{Lys}$ Labeling System. Proteins were mixed and allowed to interact. In control samples, prey protein was mixed with in vitro extract without the bait protein. HaloLink™ Resin was added to all experimental samples and to all controls. Samples were transferred into wells across the Wizard® SV 96 Lysate Clearing Plate (Cat.# A2241), and washed. Prey protein was eluted and analyzed. The data indicate that recovery of prey protein was consistent and that controls were consistently clean (Figure 5), demonstrating that HaloLink™ Resin can be successfully used in a 96-well format.

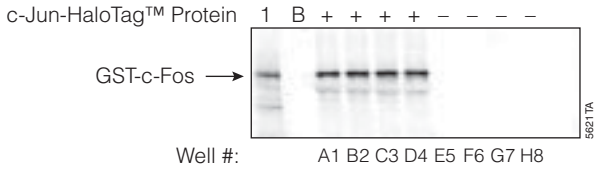

Figure 5. Consistent recovery of prey protein in a 96-well format pull-down experiment. Samples of c-Jun-HaloTag™:GST-c-Fos pull-down and controls were loaded into indicated wells across the 96-well plate. Lanes labeled "+", sample containing the bait (c-Jun-HaloTag™ fusion protein); lanes labeled "–", control sample without the bait. Both proteins were expressed using the TNT® T7 Quick Coupled in vitro Expression System; prey protein (c-Fos-GST) was synthesized in the presence of FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System (Cat.# L5001) to obtain fluorescently labeled protein. After the synthesis was completed, proteins were mixed and allowed to interact; four replicas of the experiment were set up. In control samples (n = 4) prey protein was mixed with TNT® extract without the bait protein. HaloLink™ Resin was added to all experimental samples and to all controls. After a 30-minute incubation to allow capture of complexes (7), samples were transferred into wells across the Wizard® SV 96 Lysate Clearing Plate (Cat.# A2241), which was assembled on the Manifold Base of the Vac-Man® 96 Vacuum Manifold (Cat.# A2291). Vacuum pressure was used to wash the resins. Prey protein was eluted with 50µl of 1X SDS loading dye and collected into a clean flat-bottom 96-well plate. An aliquot of each sample (12µl) was analyzed by SDS-PAGE. Lane 1, input FluoroTect™-labeled GST-cFos; lane B, blank.

Detecting Protein Interactions in vivo

While detecting protein interactions in vitro is important for understanding protein function, it is equally important to be able to analyze protein interactions in vivo. We used the interacting proteins p65 and IκB, to demonstrate that HaloLink™ Resin can be used to isolate protein complexes from mammalian cells. HeLa cells were transiently transfected with pFC8A Flexi® Vector encoding p65-HaloTag™ fusion protein. Twenty-four hours post transfection cells were collected and lysed using mechanical disruption. Resin was added to the lysed transfected cells and also to control reactions containing nontransfected cells. Proteins covalently attached to HaloLink™ Resin were analyzed by Western blot using antibodies directed against IκB. Results in Figure 6 demonstrate that IκB could be detected only in transfected cells as a result of IκB-p65-HaloTag™ fusion interactions and not in nontransfected cells that do not contain the p65-HaloTag™ fusion partner.

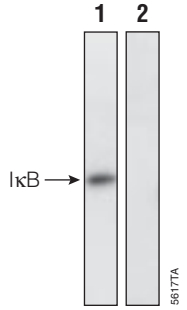

Figure 6. Detecting protein:protein interactions in vivo using HaloLink™ Resin by Western blot. HeLa cells were grown in 10cm Petri dishes and transfected with pFC8A carrying p65-HaloTag™ fusion protein. Twenty-four hours post transfection cells were collected in 1ml of buffer and lysed using a glass homogenizer. HaloLink™ Resin (100µl) was added to 100µl of the total cell lysate. In parallel, nontransfected cells were also processed to serve as a negative control. Resin was incubated with the cell lysate for 1 hour at room temperature, washed, and prey protein was eluted with SDS loading buffer. Proteins were resolved by SDS-PAGE and analyzed by Western blot using antibodies directed against IκB. Lane 1, protein from transfected cells eluted from resin; lane 2, control sample (nontransfected cells).

Immobilizing Enzymes onto HaloLink™ Resin

HaloLink™ Resin can also be used to capture and covalently immobilize enzymes from complex protein mixtures. We tested whether enzymes maintain their enzymatic activity once attached onto the surface by measuring enzymatic activity of luciferase-HaloTag™ fusions. The luciferase-HaloTag™ fusion and luciferase without the tag were expressed in vitro. HaloLink™ Resin was added to both extracts, and proteins were immobilized according to the protocol described in the Technical Manual (7). Over 60% of the total enzymatic activity of the luciferase-HaloTag™ fusion was detected on the HaloLink™ Resin (Figure 7), indicating that the bound fusion protein retained enzymatic activity. The rest of the activity was detected in the unbound fraction. These results demonstrate that proteins immobilized onto the HaloLink™ Resin maintain their native conformation and enzymatic activity. As expected, no activity is associated with resin that was incubated with luciferase not fused to the HaloTag™ Protein; thus binding to resin is specific and HaloTag™ Protein-dependent.

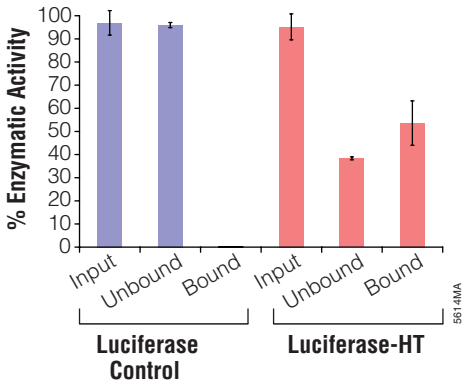

Figure 7. Detecting enzymatic activity of proteins immobilized onto the HaloLink™ Resin. Luciferase-HaloTag™ fusion protein was synthesized in vitro, and 20µl of the reaction was added to 50µl of the HaloLink™ Resin. To assess the specificity of attachment, nontagged luciferase was expressed and added to the resin as a negative control. The graph shows the percent of enzymatic activity in different fractions. The input material (in vitro transcription/translation reaction) has 100% activity; the enzymatic activity in the unbound fraction and the activity on the resin are 40% and 60%, respectively.

Purifying Proteins Using HaloLink™ Resin

HaloLink™ Resin allows permanent attachment of proteins onto the resin. Such permanent attachment does not allow purification of the HaloTag™-fusion proteins by simple elution. However, the pFC8A and pFC8K Flexi® Vectors contain a protease cleavage site (Factor Xa) situated in the linker sequence between the HaloTag™ Protein and the protein of interest. This allows release of the protein of interest from the HaloLink™ Resin by Factor Xa protease cleavage. Because the cleavage is performed on the protein bound to the resin, any contaminating proteins can be removed by extensive washing before the cleavage reaction, and the released target protein is pure and tag-free. We demonstrated this using a luciferase-HaloTag™ fusion protei expressed in vitro and immobilized onto the HaloLink™ Resin. Following immobilization, the fusion protein was cleaved with Factor Xa. The supernatant was collected and analyzed by SDS-PAGE. Release of pure luciferase without the tag is demonstrated in Figure 8.

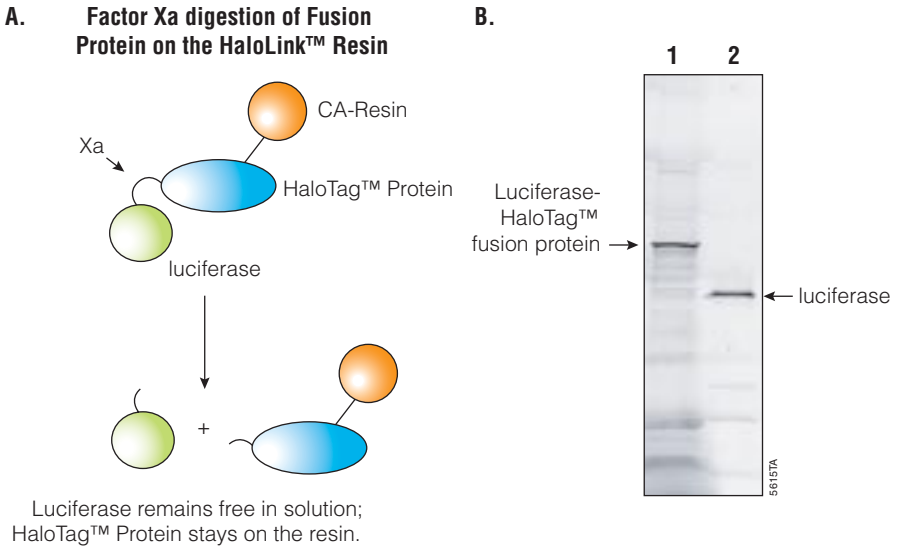

Figure 8. Release of fusion protein off the resin by proteolytic cleavage. Panel A. Schematic showing use of Factor Xa cleavage to release fusion protein from the HaloLink™ Resin. **Panel B.** Luciferase-HaloTag™ fusion was expressed in vitro and labeled with FluoroTect™ $Green_{Lys}$ System (Cat.# L5001). A 20µl aliquot of the reaction was added to 50µl of HaloLink™ Resin and incubated 30 minutes at room temperature followed by washing (7). Resin carrying luciferase-HaloTag™ fusion on the surface was treated with Factor Xa for 1 hour at room temperature; supernatant was collected and analyzed for the presence of nontagged luciferase by SDS-PAGE and scanned on a Typhoon® imager. Lane 1, 4µl of in vitro transcription/translation reaction containing luciferase-HaloTag™ fusion protein (50µl of this reaction was added to HaloLink™ Resin). Lane 2, supernatant collected post Factor Xa cleavage; 4µl of 50µl cleavage reaction was loaded.

Conclusions

HaloLink™ Resin provides a convenient way to covalently immobilize HaloTag™ fusion proteins onto a solid support in an oriented fashion. HaloTag™ fusion proteins can be easily synthesized using any of the HaloTag™ Vectors. These vectors are optimized for in vitro or in vivo expression in mammalian cells. We show that HaloLink™ Resin enables studies of protein function in a variety of assays. Immobilized proteins can be used in protein pull-down assays to isolate protein binding partners in vivo (mammalian cells) or in vitro, or they can be evaluated for their enzymatic activity. Target proteins can be released from the resin by proteolytic cleavage, resulting in pure protein. Immobilization of proteins onto HaloLink™ Resin is covalent, rapid and selective, allowing efficient binding of proteins without purification or protein modification prior to immobilization. These characteristics make the HaloTag™ Protein and HaloLink™ Resin well suited for studies of protein functions in vivo and in vitro.

References

1. Luy, Y.Y. *et al.* (2004) *J. Am. Chem. Soc.* **126**, 9024–32.
2. Kaelin, W.G. *et al.* (1991) *Cell* **64**, 521–32.
3. Vogt, P. N. (2002) *Nat. Rev. Cancer* **2**, 465–9.
4. Banaszynski L.A. *et al.* (2005) *J. Am. Chem. Soc.* **127**, 4715–21.
5. Thurl E.H. and Lawrence C.J. (2003) *Sci. STKE* (http://stke.sciencemag.org/) **212**, re15.
6. Chen J. et al. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 4947–51.
7. *HaloLink™ Resin Technical Manual* #TM250, Promega Corporation.

Protocol

- *HaloLink™ Resin Technical Manual* #TM250, Promega Corporation. (www.promega.com/tbs/tm250/tm250.html)
- *HaloTag™ Interchangeable Protein Labeling Technology Technical Manual* #TM260, Promega Corporation. (www.promega.com/tbs/tm260/tm260.html)

Acknowledgments

The authors thank the following HaloLink™ team members for their contributions: Elaine Schenborn, Brian McNamara, Gary Kobs, Linda Link, Michael Ma, Eric Gulliksen, Brett Schumacher, Tanya Quint, Promega Corporation; Michael Scurria, Promega Biosciences, Inc.

Ordering Information

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

Related Products

Product	Size	Cat.#
HaloTag™ pHT2 Vector	20µg	G8241
pFC8A (HaloTag™) CMV Flexi® Vector	20µg	C3631
pFC8K (HaloTag™) CMV Flexi® Vector	20µg	C3641
HaloTag™ TMR Ligand	30µl	G8251
HaloTag™ diAcFAM Ligand	30µl	G8271
HaloTag™ Coumarin Ligand	30µl	G8581
HaloTag™ Biotin Ligand	30µl	G8281
HaloTag™ PEG-Biotin Ligand	30µl	G8591
TNT® T7 Quick Coupled Transcription/ Translation System*	40 reactions	L1170
FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System*	40 reactions	L5001
Wheat Germ Extract Plus	40 × 50 reactions	L3250
	10 × 50 reactions	L3251

*For Laboratory Use.

[a] Patent Pending. For research use only. Researchers may use this product in their own research and they may transfer this product and its derivatives to others for research use provided that at the time of transfer a copy of this label license is given to the recipients and recipients agree to be bound by the terms of this label license. Research performed on behalf of another party, whether for monetary consideration or free of charge, is permitted. In addition, researchers must do one of the following: (1) use HaloTag™ ligands (including but not limited to a ligand linked to dyes, ligands that can be linked to other moieties, and ligands chemically linked onto surfaces) purchased exclusively from Promega or Promega's authorized distributors or (2) contact Promega to obtain a license. With respect to manufacture or sale of research products, or any diagnostic, therapeutic or prophylactic uses, please contact Promega for supply and licensing information. No reach-through payments shall be owed to Promega relating to an organization's commercialization of products that are the discoveries resulting from research use of this product or its derivatives, provided that such products of the organization do not fall within the scope of the valid claims of any issued patents assigned or licensed to Promega, or that such commercialization would not be a violation of the terms of this label license. No other use or transfer of this product or its derivatives is authorized without the express, written consent of Promega. 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] Patent Pending. For research use only. Researchers may use this product in their own research. Research performed on behalf of another party, whether for monetary consideration or free of charge, is permitted. No reach-through payments shall be owed to Promega relating to an organization's commercialization of products that are the discoveries resulting from research use of this product or its derivatives, provided that such products of the organization do not fall within the scope of the valid claims of any issued patents assigned or licensed to Promega, or that such commercialization would not be a violation of the terms of this label license. No other use or transfer of this product is authorized without the express, written consent of Promega. With respect to manufacture or sale of research products, 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.

[c] The CMV promoter and its use are covered under U.S. Pat. Nos. 5,168,062 and 5,385,839 owned by the University of Iowa Research Foundation, Iowa City, Iowa, and licensed FOR RESEARCH USE ONLY. Research Use includes contract research for which monetary or other consideration may be received. Other commercial users must obtain a license to these patents directly from the University of Iowa Research Foundation.

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