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Functional Protein Analysis using HaloTag® Technology

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Functional Protein Analysis using HaloTag[®] Technology

Understanding the functional role of proteins and their intracellular behavior is increasingly important. Often multiple protein fusion tags are required to fully characterize a specific protein of interest, such as use of one or more fusion tags for imaging and protein capture/purification applications. Recloning the same protein-coding DNA sequence of interest with multiple tags can be slow and cumbersome, requiring revalidation of the new construct in functional assays prior to use. What is needed is a single recombinant protein tag that provides application flexibility and superb performance for protein expression and localization, protein purification, protein interaction discovery, screening and further functional analyses. The HaloTag[®] Technology and the HaloTag[®] fusion proteins address this research need.

HaloTag® technology is based on an engineered catalytically inactive bacterial hydrolase of 34kDa that reacts, under physiological conditions, with chloroalkane ligands (HaloTag® ligands) to form a highly specific and irreversible covalent bond. By changing the ligands it is possible to control the function and application of the tagged protein of interest.

The HaloTag® system is designed to provide broad experimental flexibility in both cell-based and biochemical assays without switching fusion tags or recloning. HaloTag® ligands coupled to magnetic and nonmagnetic beads and glass slides are available. Cell-permeable and impermeable fluorescent ligands and biotin ligands are also available as well as reactive ligands to enable the creation of custom HaloTag®-linked constructs (**Figure 6.1**).

Features and Benefits

- **Multiple Applications:** Use a single protein fusion tag for all your needs.
- **Minimal Background:** HaloTag® protein has no homology to other mammalian cell proteins.
- **Irreversible Covalent Attachment:** Covalent binding to HaloTag® ligands and surfaces allows stringent washing, resulting in low background.

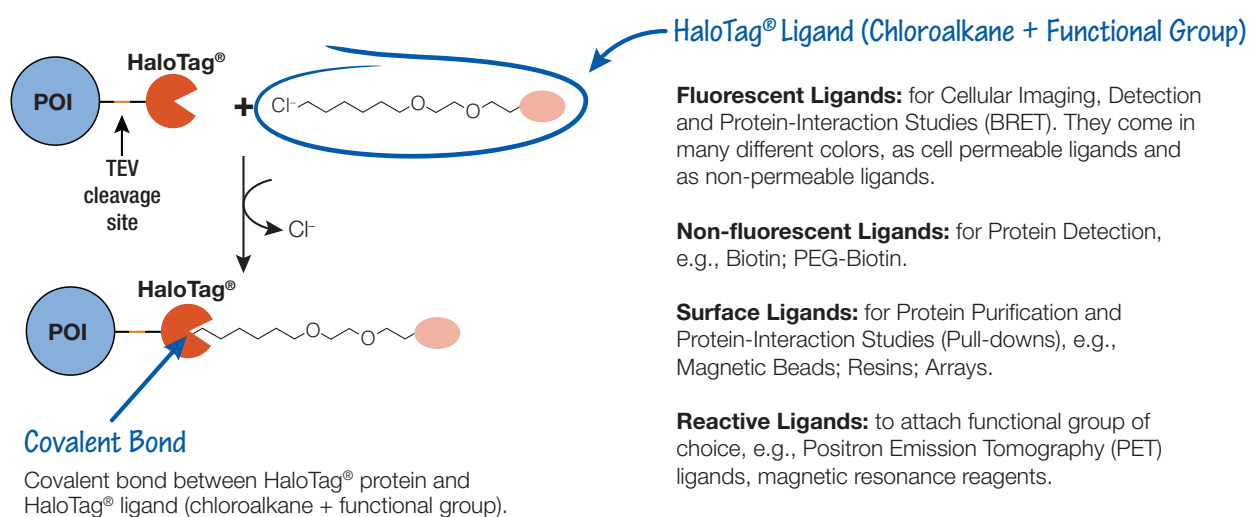


Figure 6.1. Schematic of HaloTag® technology consisting of the HaloTag® protein fused to a protein of interest (POI) and a selection of synthetic HaloTag® ligands carrying different functional groups. HaloTag® ligands specifically and covalently bind to the HaloTag® protein fusions.

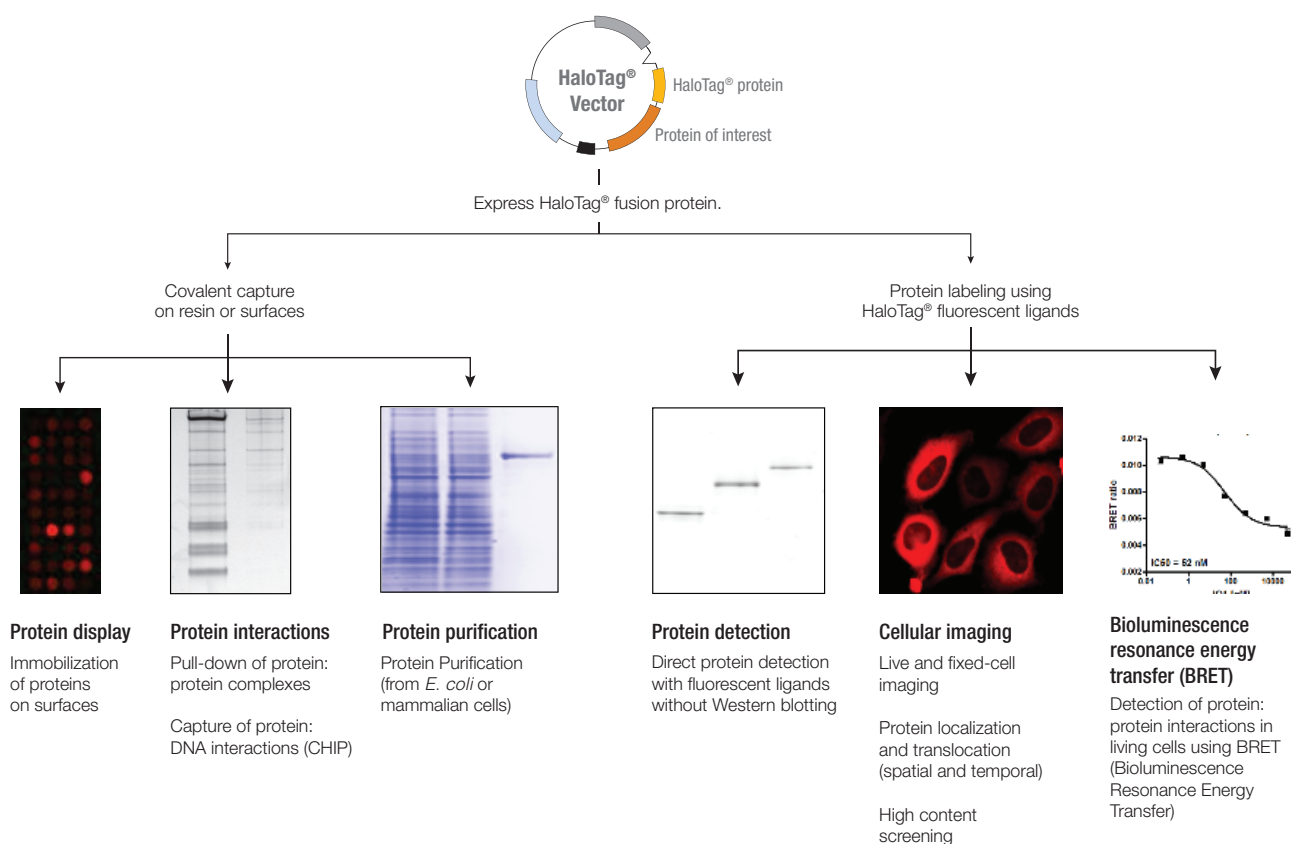


Figure 6.2. One genetic HaloTag® construct can be used for multiple applications.

References

- Urh, M. *et al.* (2012) HaloTag, a Platform Technology for Protein Analysis. *Curr Chem Genomics*. **6**,72-8.
- Los, G.V. *et al.* (2008) HaloTag: a novel protein labeling technology for cell imaging and protein analysis. *ACS Chem Biol*. **3**(6), 373-82.

Cellular Imaging

Fluorescent HaloTag® Ligands for Cellular Imaging

Cellular Imaging: Live and fixed cells; protein localization and trafficking; protein turnover and pulse-chase analysis; FACS analysis.

Description

Fluorescent HaloTag® ligands (see Figure 6.3) are available for the detection of HaloTag® protein fusions in live cells for direct imaging or for labeling in fixed cells. HaloTag® imaging can be multiplexed with fluorescent proteins (e.g., GFP) in live cells and fluorescent antibody staining in fixed cells and tissues. HaloTag® ligands are based on small organic dyes; they retain their fluorescent properties after fixation, enabling multiplexing of immunocytochemistry experiments.

Principle

Proteins of interest fused to HaloTag® protein can be labeled with various fluorescent ligands in live or fixed cells using a simple labeling protocol (Figure 6.4). HaloTag® ligands are noncytotoxic and allow permanent labeling due to covalent attachment to the HaloTag® protein. There are different types of fluorescent ligands for intracellular protein labeling (cell-permeant) as well as for cell surface protein labeling (cell-impermeant). Rapid ligands can label as quickly as 5–15 minutes after addition to cells expressing the HaloTag® protein. The direct label ligands can be added at the time of cell plating or transfection and will label the tagged protein of interest (POI) as it is expressed (without the need to wash away the unbound ligand). In addition to direct labeling, the dyes can be used in standard labeling protocols that include wash steps. Lastly, permeant and impermeant ligands of different colors can be used in tandem to track protein translocation to and from the plasma membrane (Figure 6.5). The range of differently colored HaloTag® ligands allows easy changes in POI color and easy integration with other fluorophores like GFP or other cellular labeling dyes.

Features and Benefits

- **Localization:** Robust protein imaging in live or fixed cells (e.g., 4% PFA-fixation).
- **Trafficking and Turnover:** Directly observe spatially- or temporally-separated protein populations with one or two colors in live cells. Label first with cell-impermeant and then with cell-permeable ligands.
- **Cell Sorting:** Simple non-antibody based cell labeling with multiple fluorophores.
- **Flexible:** Variety of HaloTag® ligands in different colors allows flexible combination with other labeling dyes (e.g., DNA staining dyes) as well as with immunofluorescence reagents.

Ligand	Excitation Maximum	Emission Maximum	Intended Use of Ligands	Ordering Information
HaloTag® Coumarin	362nm	450nm	Intracellular labeling	G8581, G8582
HaloTag® Alexa Fluor® 488	499nm	518nm	Cell-surface labeling	G1001, G1002
HaloTag® Oregon Green®	492nm	520nm	Intracellular labeling	G2801, G2802
HaloTag® DiAcFAM	492nm	521nm	Intracellular labeling	G8272, G8273
HaloTag® R110 Direct™ ("No Wash" ligands)	498nm	528nm	Intracellular labeling	G3221
HaloTag® TMR Direct™ ("No Wash" ligands)	552nm	578nm	Intracellular labeling	G2991
HaloTag® Alexa Fluor® 660	654nm	690nm	Cell-surface labeling	G8471, G8472

Figure 6.3. Maximum excitation and emission spectra for the HaloTag® Ligands.

Cellular Imaging

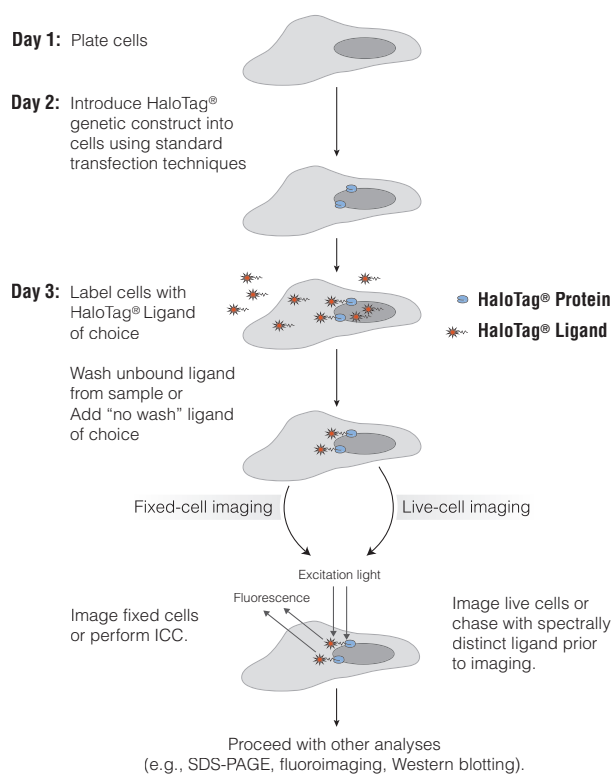


Figure 6.4. Overview of protocol for live-cell and fixed-cell imaging using HaloTag® Technology. No washing steps are required with the "No Wash" DIRECT® HaloTag® ligands.

References

- Yamaguchi, K. *et al.* (2009) Pulse-chase experiment for the analysis of protein stability in cultured mammalian cells by covalent fluorescent labeling of fusion proteins. In: *Reverse Chemical Genetics, Methods in Molecular Biology* **577**, H. Koga ed. Humana Press.
- Huybrechts, S.J. *et al.* (2009) Peroxisome dynamics in cultured mammalian cells. *Traffic* **10**(22),1722-33.
- Svendsen, S. *et al.* (2008) Spatial separation and bidirectional trafficking of proteins using a multi-functional reporter. *BMC Cell Biol.* **9**(17).

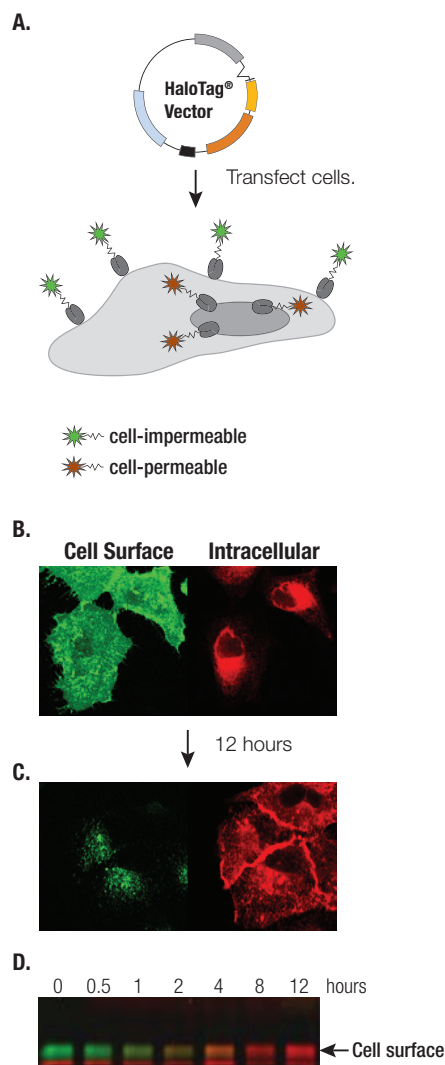


Figure 6.5. Membrane trafficking of β -integrin fragment fused to HaloTag® (N-terminal fusion). **(A)** Cells stably expressing β 1Int-HaloTag were labeled first with the cell-impermeable ligand HaloTag® Alexa Fluor® 488 and then with the cell-permeable HaloTag® TMR ligand. **(B)** Images were taken with a confocal microscope immediately after labeling to show cell-surface and intracellular localization of β 1Int-HaloTag. **(C)** Cells were re-imaged 12 hours after labeling to show receptor internalization and trafficking to the plasma membrane. **(D)** Fluorescan image of an SDS gel: Cell lysates taken at 0, 0.5, 1, 2, 4, 8 and 12 hours after labeling were run on SDS-PAGE. Fluorescan shows bidirectional trafficking of β 1Int-HaloTag as reflected in the upper band (cell surface β 1Int-HaloTag) and lower band (intracellular β 1Int-HaloTag). Image copyright BMC Cell Biology. Usage license can be found at: <http://www.biomedcentral.com/authors/license>. Image has been modified from original published version.

Protein Interaction Analysis

NanoBRET™: Live Cell Protein:Protein Interaction Assays

Study protein:protein interactions in living cells, even at physiological expression levels, using Bioluminescence Resonance Energy Transfer (BRET) real-time measurements.

Description

NanoBRET™ uses the very bright NanoLuc® Luciferase as a BRET donor and the HaloTag® Protein as an acceptor (**Figure 6.6**). NanoBRET™ Technology has many advantages compared to other BRET technologies, including higher signal-to-background ratios that enable BRET measurements in a broad dynamic assay window. NanoLuc® Luciferase is a small protein (19 kDa) with a very high light output (100X brighter than *Renilla* Luciferase). In combination with the HaloTag® protein and the optimized red-shifted NanoBRET™ Ligand, this new technology allows BRET measurements at physiological expression levels and may be suitable for BRET measurements in difficult-to-transfect cells and primary cells. The NanoBRET™ Nano-Glo® Detection System provides the NanoBRET™ Nano-Glo® Substrate used by NanoLuc® Luciferase to generate the donor signal and the HaloTag® NanoBRET™ 618 Ligand for the fluorescent energy acceptor. This ligand is added directly to cells during plating, and the NanoBRET™ Nano-Glo® Substrate is added to the sample just before measuring donor and acceptor emission.

Principle

NanoBRET™ technology measures the interaction between the energy donor fusion Protein A-NanoLuc® Luciferase and the energy acceptor fusion, Protein B-HaloTag® protein (**Figure 6.6**). As it can be difficult to predict the optimal orientation and placement of the energy donor and acceptor, we recommend evaluating all possible combinations of N- and C-terminal protein fusions to NanoLuc® and HaloTag® proteins on both protein interaction partners (A, B), since both orientation and composition of the fusion protein can affect protein

expression and/or activity. To select the best BRET pairs, eight different fusion constructs are used (four NanoLuc® protein fusions and four HaloTag® protein fusions; **Table 6.1**).

Features and Benefits

- **High Signal-to-Background Values:** Excellent signal-to-background and high dynamic range due to large spectral separation of donor emission and acceptor emission. Very low bleeding of donor emission into acceptor emission.
- **Bright Donor Signal:** Strong signal from donor enables use of weak promoters for physiological expression levels; lower cell number; difficult-to-transfect cells.
- **Simple Background Calculation:** A simple background calculation is possible by performing the assay in the absence of the NanoBRET™ fluorescent ligand. This feature is not possible in other BRET systems that use intrinsically fluorescent proteins.
- **Custom Assay Design Options:** NanoBRET™ Starter Systems are available in MCS or Flexi® options to enable simple generation of NanoLuc® and HaloTag® fusion proteins of your choice. Flexi® Vectors enable easy shuttling of ORFs from one vector to another (Chapter 1).
- **Ready to use ORF Clones Available:** Choose from >9,000 human HaloTag® ORF clones to design your assay with the Flexi Starter System.
- **Prebuilt NanoBRET™ Assays Available:** A large number of prebuilt, optimized NanoBRET™ assays are available to measure protein:protein interactions in key target areas such as epigenetics, transcription and signaling.

Protein Interaction Analysis

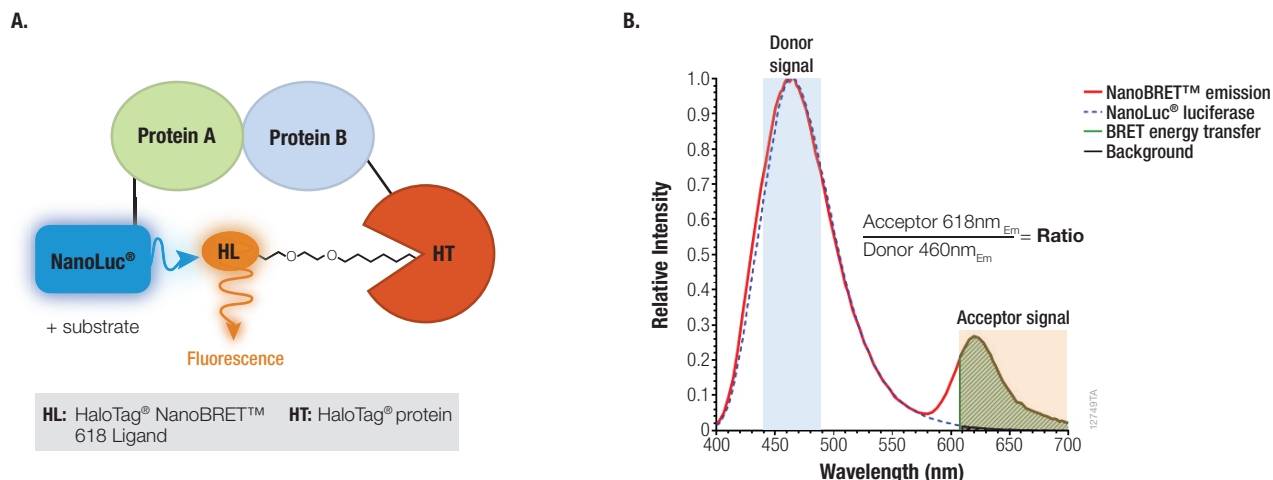


Figure 6.6. The NanoBRET™ Assay. **Panel A.** Depiction of energy transfer from a NanoLuc®-Protein A fusion (energy donor) to a fluorescently labeled HaloTag®-Protein B fusion (energy acceptor) upon interaction of Protein A and Protein B. **Panel B.** Spectral separation of the NanoLuc® emission (460nm) and the fluorescent HaloTag® NanoBRET™ ligand emission (618nm), and calculation of the NanoBRET™ ratio.

Table 6.1. HaloTag® and NanoLuc® Fusion Construct Combinations.

Test Pair	N-Terminal HaloTag® Fusion Protein	C-Terminal HaloTag® Fusion Protein	N-Terminal NanoLuc® Fusion Protein	C-Terminal NanoLuc® Fusion Protein
1	Protein X		Protein Y	
2	Protein X			Protein Y
3		Protein X	Protein Y	
4		Protein X		Protein Y
5	Protein Y		Protein X	
6	Protein Y			Protein X
7		Protein Y	Protein X	
8		Protein Y		Protein X

Ordering Information

NanoBRET™ Nano-Glo Detection Systems:
(Cat.# **N1661**, **N1662**, **N1663**)

NanoBRET™ MCS Starter System: (Cat.# **N1811**)

NanoBRET™ Flexi Starter System:
(Cat.# **N1821**)



Additional Information

For full list of pre-built NanoBRET™ protein:protein interaction (PPI) assays see www.promega.com/nanobret

References

Deplus, R. *et al.* (2013) TET2 and TET3 regulate GlcNAcylation and H3K4 methylation through OGT and SET1/COMPASS. *EMBO J.* **32**(5), 645-55.

Protein Interaction Analysis

HaloTag® Mammalian Pull-Down System

Capture and analysis of interacting proteins.

Description

HaloTag® Mammalian Pull-Down System is used to isolate and identify intracellular protein complexes from mammalian cells. HaloTag® fusion proteins form a highly specific covalent bond with the HaloLink™ Resin, allowing rapid and efficient capture of protein complexes even at very low, endogenous levels of bait protein expression. These benefits, coupled with the low nonspecific binding of the HaloLink™ Resin, improve the rate of successful complex capture and identification of physiologically relevant protein interactions in mammalian cells. The HaloTag® Mammalian Pull-Down and Labeling System also includes the HaloTag® TMRDirect™ Ligand, which allows optimization of protein expression levels, to study cellular localization, trafficking and protein turnover using the same HaloTag® genetic construct.

Principle

The basic HaloTag® pull-down experimental scheme is depicted in **Figure 6.7**. HaloTag® fusion proteins can be expressed in mammalian cells either transiently or stably, and used as bait to capture interacting proteins or protein complexes. After cell lysis, the HaloTag® fusion protein, bound to its interacting protein partners, is captured on the HaloLink™ Resin. The captured complexes are gently washed and eluted either using SDS elution buffer (or other denaturing conditions such as 8M urea), or cleaved from the resin using TEV protease*. The recovered complexes are suitable for analysis by a variety of methods including, Western blotting and mass spectrometry.

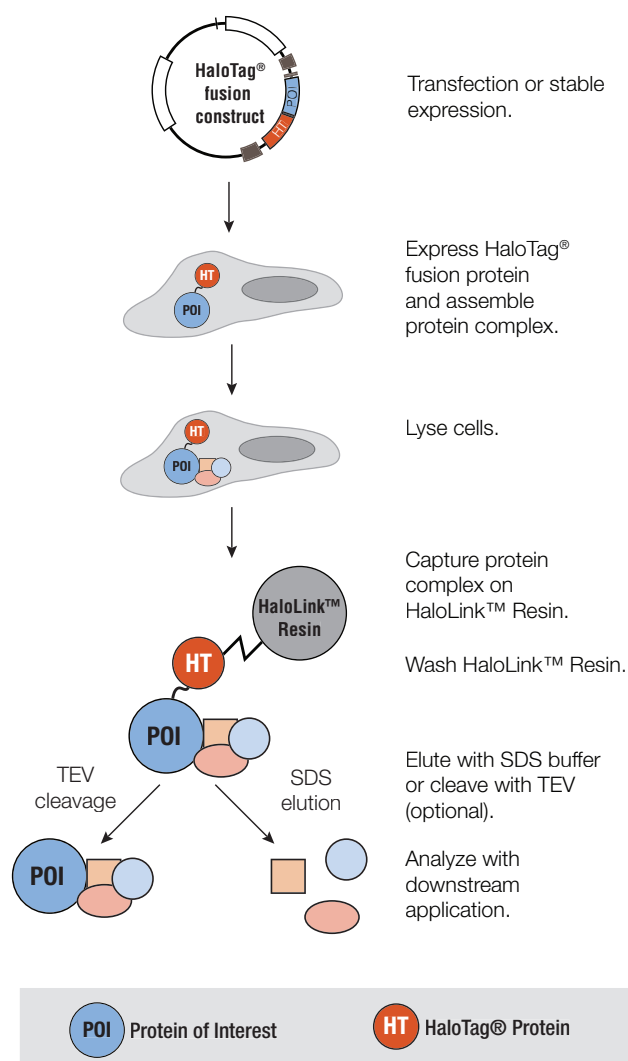


Figure 6.7. Schematic of the HaloTag® Mammalian Pull-Down System protocol.

Protein Interaction Analysis

Features and Benefits

- **Improved Capture:** Rapid and covalent attachment of HaloTag® protein to its resin enhances capture of protein partners, including transient interactions.
- **Compatibility:** Compatible with all downstream methods of analysis, including mass spectrometry.
- **Ability to Fluorescently Label HaloTag® Fusion Protein:** Optimize protein expression levels and determine cellular protein localization.

Additional Information

*TEV protease is also available separately.

References

- Galbraith, M.D. *et al.* (2013) HIF1A Employs CDK8-Mediator to Stimulate RNAPII Elongation in Response to Hypoxia. *Cell* **153**(6), 1327–39.
- Kalashnikova, A. *et al.* (2013) Linker histone H1.0 interacts with an extensive network of proteins found in the nucleolus. *Nucl. Acids Res.* **41**(7) 4026–35.

Ordering Information

HaloTag® Mammalian Pull-Down System
(Cat.# [G6504](#))

HaloTag® Mammalian Pull-Down and Labeling
System (Cat.# [G6500](#))

HaloTag® TMRDirect™ Ligand (Cat.# [G2991](#))



Protein Interaction Analysis

HaloCHIP™ System

Capture of Protein:DNA interactions.

Description

The HaloCHIP™ System is used for the covalent capture of intracellular protein:DNA complexes without the use of antibodies. This kit offers an efficient and robust alternative to the standard chromatin immunoprecipitation (ChIP) method and contains HaloLink™ Resin, Mammalian Lysis Buffers, Wash Buffers and HaloCHIP™ Blocking ligands.

Principle

Proteins of interest (e.g., transcription factors) are expressed in cells as HaloTag® fusion proteins, cross-linked to DNA with formaldehyde, and captured covalently on HaloLink™ Resin. Covalent capture allows the use of extensive and stringent wash conditions that are not possible when antibodies and other noncovalent tags are used for pull-down. The ability to use stringent wash conditions results in reduced background and an increased signal-to-background ratio of detected DNA fragments. After covalent capture, stringent washing removes nonspecifically bound nuclear proteins and DNA; heating reverses the crosslinking between the DNA and the HaloTag®-bound transcription factor, releasing the DNA for subsequent analysis (either RT-PCR or sequencing). See **Figure 6.8**.

Features and Benefits

- **No Requirement for Antibody:** No need for ChIP-qualified antibodies.
- **Obtain Results Faster:** Obtain data in 24–48 hours with fewer steps, minimizing potential experimental errors and reducing artifacts.
- **Improved Signal-to-Background Ratios:** Enables detection of small changes in protein binding patterns using a minimal number of cells.

References

Nagaki, K. *et al.* (2012) Isolation of centromeric-tandem repetitive DNA sequences by chromatin affinity purification using a HaloTag7-fused centromere-specific histone H3 in tobacco. *Plant Cell Rep.* **31**(4), 771–9.

Felder, T. *et al.* (2011) Characterization of novel proliferator-activated receptor gamma coactivator-1alpha (PCG-1alpha) in human liver. *J. Biol. Chem.* **286**, 42923–36.

Ordering Information

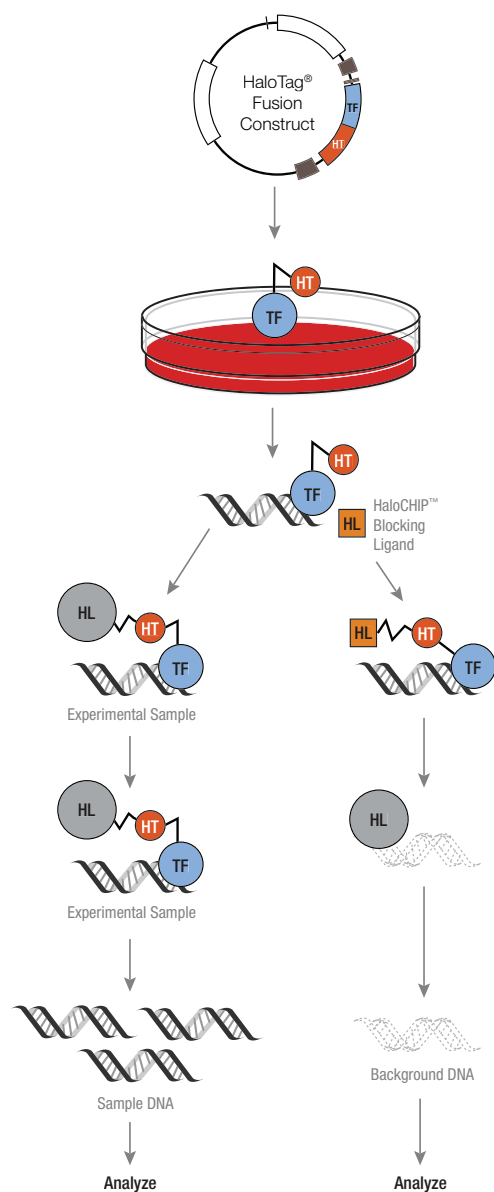
HaloCHIP™ System (Cat.# **G9410**)

Protein Interaction Analysis

Covalent Capture of Chromatin Complexes Using HaloTag® Technology

HT HaloTag® protein
 TF Transcription factor
 HL HaloLink™ Resin
 HL HaloCHIP™ Blocking Ligand

Illustration



Steps

Transfection

Expression of HaloTag®
Fusion Protein

Formaldehyde Crosslinking

Lysis, Sonication

Split Sample (For Blocking
Control Only)

Add HaloCHIP™ Blocking Ligand to the control sample to prevent binding to HaloLink™ Resin.

Capture using the
HaloLink™ Resin

Wash HaloLink™ Resin.

Covalent capture allows highly stringent washes to remove nonspecific proteins and DNA.

Release of DNA by Reversal
of Crosslinks

Protein remains bound to the HaloLink™ Resin.

1 to 1.5 Days

66533MB

Figure 6.8. Schematic diagram of the HaloCHIP™ System protocol.

Protein Interaction Analysis

HaloLink™ Protein Array System

Create your individual HaloTag® protein array to study protein:protein, protein:drug, protein:nucleic acid interactions; arrays for antibody screening and enzymatic functional analysis.

Description

The HaloLink™ Array Six Slide System provides an easy way to create custom protein arrays. HaloTag® fusion proteins are covalently attached to hydrogel-coated glass slides (optimized for low nonspecific binding) and can be used for many different binding and interaction studies.

Principle

To produce custom HaloTag® protein arrays, protein-coding sequences are cloned into appropriate HaloTag® Flexi® Vectors (see Chapter 1 for Flexi® Cloning System). HaloTag® fusion proteins are expressed either in cell-free (purchased separately) or cell-based expression systems (**Figure 6.9**). The HaloLink™ Array Gasket is applied to the HaloLink™ Slide, creating 50 leak-free wells. HaloTag® protein fusions are applied and captured on the HaloLink™ Slide, creating a custom array.

Features and Benefits

- **Irreversible Binding of Captured Protein:** HaloTag® fusion proteins bind to the HaloLink™ Slide via a covalent bond.
- **No Protein Pre-Purification Step:** The protein of interest is immobilized directly from crude cell-free or cell-based expression system lysates.
- **Reduced Nonspecific Binding:** Less nonspecific binding issues due to unique hydrogel coating of the HaloLink™ Slides.
- **Extensive, and Stringent Washing Allowed:** Covalent binding of HaloTag® fusion proteins to the HaloLink™ Slide, allows extensive washing, possibly resulting in lower background.

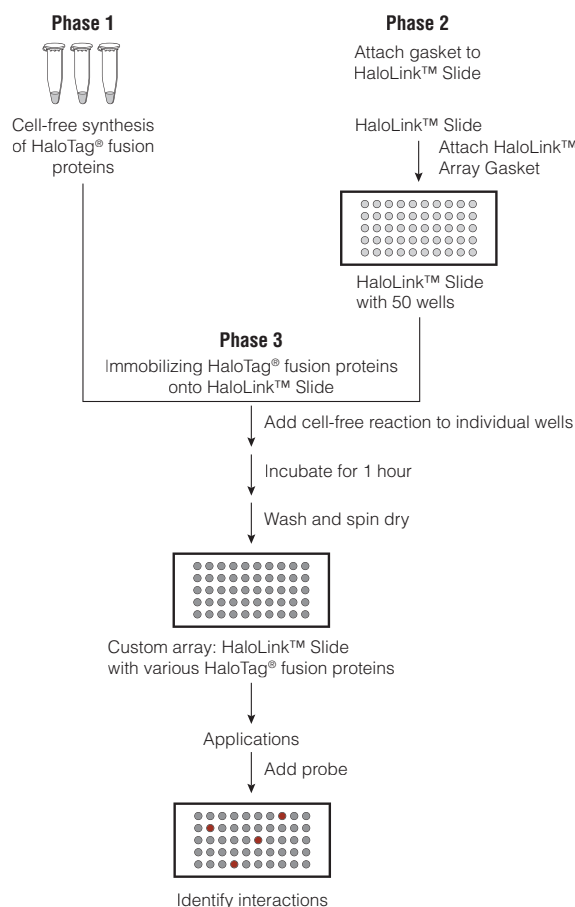


Figure 6.9. HaloLink™ Protein Array System overview.

Protein Interaction Analysis

References

Hurst, R. *et al.* (2009) Protein-protein interaction studies on protein arrays: Effect of detection strategies on signal-to-background ratios. *Analytical Biochem.* **392**, 45–53.

Hoppe, S. *et al.* (2012) Microarray-based method for screening of immunogenic proteins from bacteria. *J. Nanobiotechnol.* **10**, 12.

Wang, J. *et al.* (2013) A versatile protein microarray platform enabling antibody profiling against denatured proteins. *Proteomics Clin. App.* **7**, 378–8

Promega Products

HaloLink™ Array Six Slide System
(Cat.# **G6190**)

