TECHNICAL MANUAL

HaloTag® Complete Pull-Down System

Instructions for Use of Product
G6509

Revised 8/17
TM360

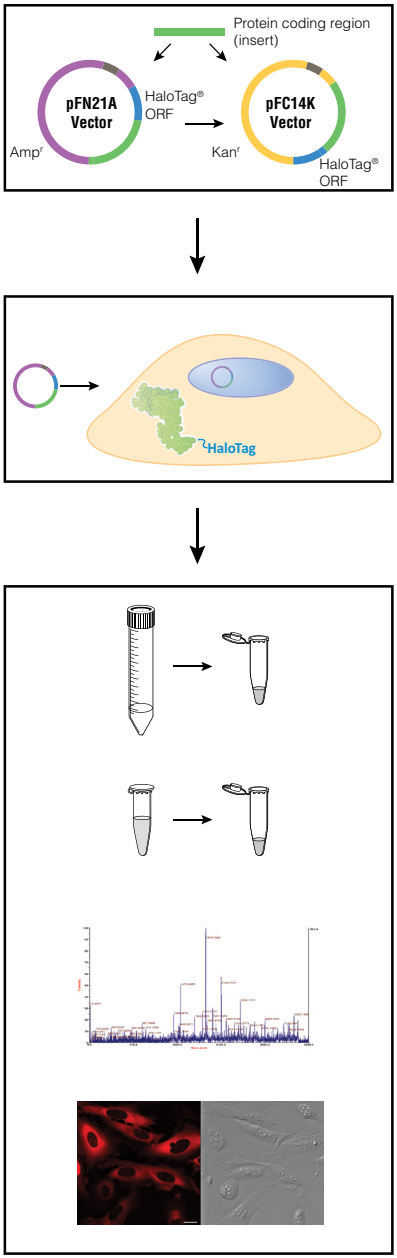
HaloTag® Complete Pull-Down System

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

1. Introduction

Higher order protein complexes and interactions carry out cellular processes. However, understanding and deconvoluting these protein interactions can be challenging. The HaloTag® Complete Pull-Down System[a–h] (Cat.# G6509) provides the necessary reagents for the isolation and identification of intracellular protein complexes from mammalian cells. Figure 1 depicts the general experimental scheme. A protein-coding region of interest is cloned initially into the amino (N)-terminal pFN21A HaloTag® CMV Flexi® Vector or carboxy (C)-terminal pFC14K HaloTag® CMV Flexi® Vector or both for expression of HaloTag® fusion proteins under a CMV promoter when transfected into mammalian cells. The expressed HaloTag® fusion protein and the endogenous interacting protein partners are isolated as a protein complex. The HaloTag® fusion protein forms a highly specific and irreversible bond with the HaloLink™ Resin, allowing rapid and efficient capture of dilute protein complexes. As a negative control for the pull-down assay, HaloTag® Control Vector is provided. To more fully characterize the function of the protein of interest, the HaloTag® TMRDirect™ Ligand allows the study of cellular localization, trafficking and protein turnover using the same HaloTag® construct.

We recommend first cloning your protein into the N-terminal pFN21A HaloTag® CMV Flexi® Vector and then transfering the insert to the C-terminal pFC14K HaloTag® CMV Flexi® Vector to obtain both N- and C-terminal HaloTag® fusion proteins. **Note:** If the initial cloning is performed into the pFC14K HaloTag® CMV Flexi® Vector, subsequent transfer to other Flexi® Vectors is not possible. A series of Flexi® Vectors allowing transfer of your protein-coding region to vectors with variable expressions levels or for expression in different systems are available separately. (See the *Flexi® Vector Systems Technical Manual* #TM254.) Additionally, a library of prepared HaloTag® N-terminal clones is available from Kazusa at: **www.promega.com/kazusa/**



Amplify insert, appending PmeI/SgfI sites, or digest insert from a Flexi® Vector clone (e.g., Kazusa).

Purify and ligate insert into the N- or C-terminal Flexi® Vector or both.

Prepare transfection-grade plasmid DNA.

Transfect mammalian expression vector into cultured cells.

Lyse transfected cells.

Capture protein complexes with HaloLink™ Resin.

Wash resin and elute protein complexes.

Analyze protein complexes using applications such as mass spectroscopy or Western blotting.

Perform complementary cell imaging studies with the HaloTag® TMRDirect™ Ligand.

Figure 1. Schematic of the HaloTag® Complete Pull-Down System protocol.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
HaloTag® Complete Pull-Down System	**1 kit**	**G6509**

For Research Use Only. Not for Use in Diagnostic Procedures. Includes:

Wizard® SV Gel and PCR Clean-Up System (Cat.# A9280)

- 5 Vacuum Adapters
- 3ml Membrane Wash Solution
- 10 Collection Tubes
- 10 Wizard® SV Minicolumns
- 1.25ml Nuclease-Free Water
- 4ml Membrane Binding Solution

HaloTag® Pull-Down Cloning System (Cat.# G6051)

- 2µg pFN21A HaloTag® CMV Flexi® Vector
- 2µg pFC14K HaloTag® CMV Flexi® Vector
- 20µg HaloTag® Control Vector
- 500u T4 DNA Ligase (HC)
- 1.25ml Nuclease-Free Water
- 1ml 5X Flexi® Digest Buffer
- 500µl 2X Flexi® Ligase Buffer
- 50µl Flexi® Enzyme Blend (SgfI & PmeI)
- 50µl Carboxy Flexi® Enzyme Blend (SgfI & EcoICRI)

Transfection Reagent

- 1ml FuGENE® HD Transfection Reagent (Cat.# E2311)

HaloTag® Mammalian Pull-Down System (Cat.# G6501)

- 10ml Mammalian Lysis Buffer
- 25ml 10X TBS Buffer
- 1.3ml SDS Elution Buffer
- 1.25ml HaloLink™ Resin

Protease Inhibitor

- 1ml Protease Inhibitor Cocktail, 50X (Cat.# G6521)

HaloTag® Ligand

- 30µl HaloTag® TMRDirect™ Ligand (Cat.# G2991)

Storage Conditions: Store the Wizard® SV Gel and PCR Clean-Up System at 15–30°C. Store the FuGENE® HD Transfection Reagent, HaloTag® Pull-Down Cloning System and HaloTag® Mammalian Pull-Down System at 2–10°C. Store the Protease Inhibitor Cocktail, 50X, at –30 to –10°C. Store the HaloTag® TMRDirect™ Ligand below –65°C.

Available Separately

PRODUCT	SIZE	CAT.#
10X Flexi® Enzyme Blend (SgfI & PmeI)	**50µl**	**R1851**
	100µl	**R1852**

3. PCR Product Purification and Cloning

Your protein-coding region must be PCR amplified prior to cloning into the N-terminal pFN21A or C-terminal pFC14K HaloTag® CMV Flexi® Vectors. If the protein-coding region initially is cloned into the pFN21A vector, it can subsequently be transferred to the pFC14K vector to obtain both N-terminal and C-terminal HaloTag® fusion proteins. If the coding sequence has been cloned into the C-terminal vector, transfer to the N-terminal vector or other Flexi® Vectors will not be possible. A library of prepared HaloTag® N-terminal clones is available from Kazusa at: **www.promega.com/kazusa/**

Cloning using the Flexi® Vector Systems is based on two rare-cutting restriction endonucleases: SgfI and PmeI. While most (>98%) known human open reading frames are not affected by the use of these restriction enzymes for directional cloning, we recommend scanning your protein-coding region for SgfI and PmeI sites. The desired protein-coding region must be amplified using PCR primers that append a SgfI site and a PmeI site to the PCR product. To append these sites, incorporate a SgfI site in your amino-terminal PCR primer and a PmeI site in your carboxy-terminal PCR primer (Figure 2).

Note: Promega provides a tool that scans your sequence for SgfI and PmeI sites to ensure these sites are absent and helps with primer design. This tool is available at: **www.promega.com/resources/tools/flexi-vector-primer-design-tool/**

Transfer of protein-coding regions into N-terminal fusion vectors results in translational read-through of the SgfI site, which encodes the peptide sequence Ala-Ile-Ala. The PmeI site is placed at the carboxy terminus, appending a single valine residue to the last amino acid of the protein-coding region. The valine codon, GTT, is immediately followed by an ochre stop codon, TAA.

C-terminal protein fusions are created by inserting the SgfI/PmeI fragment containing the protein-coding region into a C-terminal Flexi® Vector cut with SgfI and EcoICRI. The blunt end of PmeI (GTTT) is ligated to the blunt end of EcoICRI (CTC) to generate the sequence: GTT TCT CNN. This sequence translates to Val-Ser-Xaa, allowing read-through into the C-terminal sequences present on the C-terminal Flexi® Vectors. Since both the PmeI and EcoICRI sites are destroyed, the protein-coding region cannot be transferred from these vectors. For this reason, we do not advise cloning a protein-coding amplimer directly into a C-terminal Flexi® Vector if you plan to transfer the protein-coding region to a different Flexi® Vector. By cloning the PCR fragment first into an N-terminal Flexi® Vector, the ability to transfer to any other Flexi® Vector is preserved.

For more information about Flexi® Vectors, see the *Flexi® Vector Systems Technical Manual* #TM254, available at: **www.promega.com/protocols/**

3. PCR Product Purification and Cloning (continued)

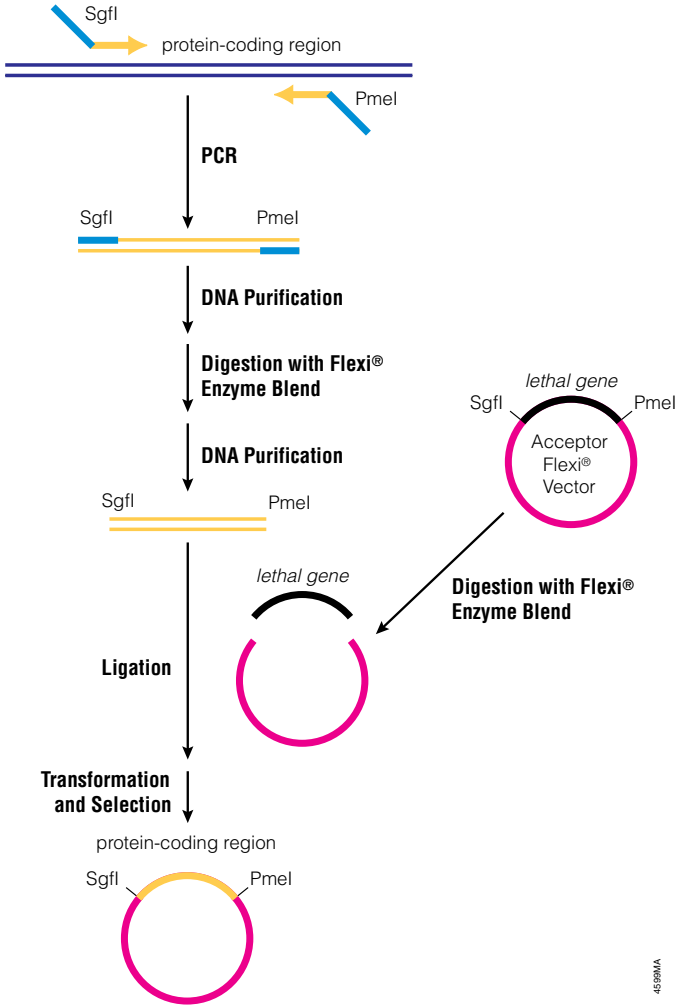


Figure 2. Cloning a protein-coding region into the Flexi® Vectors. PCR primers are designed to append SgfI and PmeI sites to the protein-coding region. After amplification, the PCR product is purified to remove the DNA polymerase and primers and digested with SgfI and PmeI. The DNA is purified again to remove the small oligonucleotides released by the restriction enzymes. The digested PCR product is ligated into an acceptor Flexi® Vector that has been digested with SgfI and PmeI. Following transformation, the cells are selected with the appropriate antibiotic for the particular Flexi® Vector used.

Materials to Be Supplied by the User
(Solution compositions are provided in Section 9.)

- 95% ethanol
- high-efficiency competent cells (≥1 × 10^8cfu/µg)
- 1.5ml polypropylene microcentrifuge tubes or 17 × 100mm polypropylene tubes (BD Falcon™ Cat.# 352059)
- LB plates with the appropriate antibiotic at the appropriate concentration
- SOC medium

3.A. PCR Product Purification

This protocol describes direct purification of DNA from the amplification reaction using the supplied Wizard® SV Gel and PCR Clean-Up System. If the amplification yields multiple PCR products, purify the desired band from an agarose gel to remove undesired PCR products. To reduce primer dimer carryover, 80% ethanol can be substituted for the prepared Membrane Wash Solution used to wash the SV Minicolumn in Section 3.A, Step 6.

! The Wizard® SV Gel and PCR Clean-Up System is not suitable for purifying transfection-grade DNA.

1. Add 15ml of 95% ethanol to the Membrane Wash Solution. Mark the bottle label to record that this addition was made. Tightly close the bottle cap after each use to prevent evaporation.
2. Add an equal volume of Membrane Binding Solution to the amplification reaction (see Notes 1 and 2).

   At this point, the DNA can be purified using microcentrifugation to force the solution through the Wizard® SV Minicolumn and to wash the DNA (proceed to Step 3). Alternatively, a vacuum can be used to pull the solution through the Minicolumn and to wash the DNA (see Technical Manual #TM254). Vacuum Adapters allow the use of a vacuum manifold (e.g., a Vac-Man® Laboratory Vacuum Manifold, Cat.# A7231) and vacuum source for DNA purification.
3. For each amplification reaction, place one SV Minicolumn in a Collection Tube.
4. Transfer the amplification reaction to the SV Minicolumn assembly and incubate for 1 minute at room temperature.
5. Centrifuge the SV Minicolumn assembly in a microcentrifuge at 16,000 × *g* for 1 minute. Remove the SV Minicolumn from the SV Minicolumn assembly and discard the liquid in the Collection Tube. Return the SV Minicolumn to the Collection Tube.

3.A. PCR Product Purification (continued)

6. Wash the column by adding 700µl of Membrane Wash Solution to the SV Minicolumn. Centrifuge the SV Minicolumn assembly for 1 minute at 16,000 × *g*. Empty the Collection Tube as before and place the SV Minicolumn back in the Collection Tube. Repeat the wash with 500µl of Membrane Wash Solution, and centrifuge the SV Minicolumn assembly for 5 minutes at 16,000 × *g*.
7. Remove the SV Minicolumn assembly from the centrifuge, being careful not to wet the bottom of the column with the flowthrough. Empty the Collection Tube, return the SV Minicolumn to the Collection Tube and centrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of residual ethanol.
8. Carefully transfer the SV Minicolumn to a clean 1.5ml microcentrifuge tube. Apply 50µl of Nuclease-Free Water directly to the center of the column without touching the membrane with the pipette tip. Incubate at room temperature for 1 minute. Centrifuge for 1 minute at 16,000 × *g* (see Note 3).
9. Discard the SV Minicolumn and store the microcentrifuge tube containing the eluted DNA on ice while assembling the restriction enzyme digests in Section 3.B.

Notes:

1. The maximum binding capacity of the column is approximately 40µg of DNA per column, and as little as 10ng has been successfully purified. The maximal capacity of a single SV Minicolumn is approximately 1ml of PCR product added to 1ml Membrane Binding Solution (2ml total). For PCR volumes >350µl, continue to pass the sample through the column until all of the sample has been processed.
2. Mineral oil does not interfere with purification.
3. The volume of eluted DNA will be approximately 42–47µl. If the DNA needs to be further concentrated, perform an ethanol precipitation. Alternatively, the DNA may be eluted in as little as 15µl of Nuclease-Free Water without a significant reduction in yield. If using an elution volume of 15µl, verify that the membrane is completely covered with Nuclease-Free Water before centrifugation. Do not elute in volumes less than 15µl (see Technical Manual #TM254).

3.B. Restriction Digest of PCR Product and Acceptor pFN21A HaloTag® CMV Flexi® Vector

Digestion reactions for the PCR product and the acceptor N-terminal Flexi® Vector, pFN21A HaloTag® CMV Flexi® Vector, can be performed concurrently.

Do **not** use the C-terminal pFC14K HaloTag® CMV Flexi® Vector if you plan to transfer the protein-coding region to a different Flexi® Vector in the future. C-terminal Flexi® Vectors lack PmeI sites and cannot serve as donors for other Flexi® Vectors. Go to Section 3.C if you are planning to clone your PCR product directly into the pFC14K HaloTag® CMV Flexi® Vector and will not move the ORF into any other Flexi® Vector.

1. Thaw the 5X Flexi® Digest Buffer, the acceptor pFN21A HaloTag® CMV Flexi® Vector and Nuclease-Free Water, and store on ice. Vortex the 5X Flexi® Digest Buffer and the acceptor Flexi® Vector before use.
2. Assemble the following reaction components to cut the PCR product with SgfI and PmeI.

Component	Volume
5X Flexi® Digest Buffer	4µl
Purified PCR product (up to 500ng)	_µl
Flexi® Enzyme Blend (SgfI & PmeI)	4µl
Nuclease-Free Water to a final volume of	20µl

Assemble the following reaction components to cut the acceptor Flexi® Vector with SgfI and PmeI.

Component	Volume
Nuclease-Free Water	12µl
5X Flexi® Digest Buffer	4µl
pFN21A HaloTag® CMV Flexi® Vector (200ng)	2µl
Flexi® Enzyme Blend (SgfI & PmeI)	2µl
Final Volume	20µl

Take care when pipetting solutions that contain glycerol, such as the Flexi® Enzyme Blend, because small volumes are difficult to pipet accurately.

3. Incubate both reactions at 37°C for 30 minutes.
4. Heat the reaction with the Flexi® Vector at 65°C for 20 minutes to inactivate the restriction enzymes. Store on ice until the PCR product and vector are ligated in Section 3.E.
5. Add 20µl of Membrane Binding Solution to the reaction with the PCR product.

Do **not** add Membrane Binding Solution to the reaction with the pFN21A HaloTag® CMV Flexi® Vector.

3.C. Restriction Digest of PCR Product and Acceptor pFC14K HaloTag® CMV Flexi® Vector

Digestion reactions for the PCR product and the acceptor C-terminal Flexi® Vector, pFC14K HaloTag® CMV Flexi® Vector, can be performed concurrently.

Note: If the protein-coding region is cloned into pFC14K HaloTag® CMV Flexi® Vector, it is not possible to transfer the ORF to pFN21A HaloTag® CMV Flexi® Vector or other Flexi® Vectors. C-terminal Flexi® Vectors lack PmeI sites and cannot serve as donors for other Flexi® Vectors. See Section 3.B to clone your PCR product into the N-terminal pFN21A HaloTag® CMV Flexi® Vector and retain the ability to transfer the insert.

1. Thaw the 5X Flexi® Digest Buffer, the acceptor pFC14K HaloTag® CMV Flexi® Vector and Nuclease-Free Water, and store on ice. Vortex the 5X Flexi® Digest Buffer and the acceptor Flexi® Vector before use.
2. Assemble the following reaction components to cut the PCR product with SgfI and PmeI.

Component	Volume
5X Flexi® Digest Buffer	4µl
Purified PCR product (up to 500ng)	_µl
Flexi® Enzyme Blend (SgfI & PmeI)	4µl
Nuclease-Free Water to a final volume of	20µl

Assemble the following reaction components to cut the acceptor Flexi® Vector with SgfI and PmeI.

Component	Volume
Nuclease-Free Water	12µl
5X Flexi® Digest Buffer	4µl
pFC14K HaloTag® CMV Flexi® Vector (200ng)	2µl
Carboxy Flexi® Enzyme Blend (SgfI & EcoICRI)	2µl
Final Volume	20µl

Take care when pipetting solutions that contain glycerol, such as the Carboxy Flexi® Enzyme Blend, because small volumes are difficult to pipet accurately.

3. Incubate both reactions at 37°C for 30 minutes.
4. Heat the reaction with the Flexi® Vector at 65°C for 20 minutes to inactivate the restriction enzymes. Store on ice until the PCR product and vector are ligated in Section 3.E.
5. Add 20µl of Membrane Binding Solution to the reaction with the PCR product.

Do **not** add Membrane Binding Solution to the reaction with the pFC14K HaloTag® CMV Flexi® Vector.

3.D. Cleanup of PCR Products After Restriction Enzyme Digestion

DNA can be purified using microcentrifugation to force the solution through the SV Minicolumn and wash the DNA. Alternatively, a vacuum can be used to pull the solution through the Minicolumn and wash the DNA. The Vacuum Adapters allow the use of a vacuum manifold (e.g., a Vac-Man® Laboratory Vacuum Manifold) and vacuum source for DNA purification. To purify the DNA using a vacuum manifold, refer to the vacuum protocol in Technical Manual #TM254.

1. For each amplification reaction, place one SV Minicolumn in a Collection Tube.
2. Transfer the PCR product prepared in Section 3.A, Step 5, to the SV Minicolumn assembly, and incubate for 1 minute at room temperature.
3. Centrifuge the SV Minicolumn assembly in a microcentrifuge at 16,000 × *g* (14,000 rpm) for 1 minute. Remove the SV Minicolumn from the SV Minicolumn assembly, and discard the liquid in the Collection Tube. Return the SV Minicolumn to the Collection Tube.
4. Wash the column by adding 700µl of Membrane Wash Solution, previously diluted with 95% ethanol (see Section 3.A, Step 1), to the SV Minicolumn. Centrifuge the SV Minicolumn assembly for 1 minute at 16,000 × *g*. Empty the Collection Tube, and place the SV Minicolumn back in the Collection Tube. Repeat the wash with 500µl of prepared Membrane Wash Solution, and centrifuge the SV Minicolumn assembly for 5 minutes at 16,000 × *g*.
5. Remove the SV Minicolumn assembly from the centrifuge, being careful not to wet the bottom of the column with the flowthrough. Empty the Collection Tube, return the SV Minicolumn to the Collection Tube, and centrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of residual ethanol.
6. Carefully transfer the SV Minicolumn to a clean 1.5ml microcentrifuge tube. Apply 30µl of Nuclease-Free Water directly to the center of the column without touching the membrane with the pipette tip. Incubate at room temperature for 1 minute. Centrifuge for 1 minute at 16,000 × *g*.
7. Discard the SV Minicolumn, and store the microcentrifuge tube containing the eluted DNA on ice while assembling the ligation reaction in Section 3.E.

   Note: Alternatively, the DNA can be stored at –20°C if you are not proceeding directly to the ligation reaction in Section 3.E.

3.E. Ligation of PCR Product and Acceptor Flexi® Vector

1. Assemble the following reaction components:

Component	Volume
2X Flexi® Ligase Buffer	10µl
Acceptor Flexi® Vector from Section 3.B or 3.C, Step 4 (50ng)	5µl
PCR product (approximately 100ng)	_µl
T4 DNA Ligase (HC) (20u/µl)	1µl
Nuclease-Free Water to a final volume of	20µl

2. Incubate at room temperature for 1 hour.

The 2X Flexi® Ligase Buffer contains ATP, which degrades during temperature fluctuations. Avoid multiple freeze-thaw cycles and exposure to frequent temperature changes by making single-use aliquots of the buffer.

3.F. Transformation

Transform high-efficiency, *E. coli* competent cells with the ligation reaction (≥1 × 10^8cfu/µg DNA). If you are using competent cells other than high-efficiency JM109 Competent Cells (Cat.# L2001) purchased from Promega, it is important to follow the appropriate transformation protocol. The recommended transformation protocol for Promega high-efficiency JM109 Competent Cells is provided in Technical Manual #TM254. Selection for transformants should be done on LB plates supplemented with 100µg/ml ampicillin or 25µg/ml kanamycin, as appropriate for the acceptor Flexi® Vector.

Note: Use 100µg/ml ampicillin for the pFN21A HaloTag® CMV Flexi® Vector or 25µg/ml kanamycin for the pFC14K HaloTag® CMV Flexi® Vector to select clones. Flexi® Vectors with the letter "A" in the name contain the ampicillin resistance gene, and Flexi® Vectors with the letter "K" in the name contain the kanamycin resistance gene.

3.G. Screening for the Desired Clone

Screen 8–12 colonies for each PCR product. For inserts in the pFN21A HaloTag® CMV Flexi® Vector, digest the construct with SgfI and PmeI to check for the correct size of the protein-coding region. The Flexi® Vector Systems contain sufficient volume of the 10X Flexi® Enzyme Blend (SgfI & PmeI) to perform the cloning steps only. The 10X Flexi® Enzyme Blend (SgfI & PmeI) (Cat.# R1851 or R1852) can be purchased separately for screening purposes. The protocol for digestion of plasmid DNA with the 10X Flexi® Enzyme Blend (SgfI & PmeI) can be found in Technical Manual #TM254.

The PmeI cut site is destroyed when cloning into the pFC14K HaloTag® CMV Flexi® Vector; therefore, you cannot screen clones using the 10X Flexi® Enzyme Blend (SgfI & PmeI). If no XbaI cut site exists in the protein-coding region, screen the constructs using SgfI and XbaI. The XbaI cut site is downstream of the PmeI site. Alternatively, use other restriction sites present in the vector.

Find the sequences for the pFN21A and pFC14K HaloTag® CMV Flexi® Vectors at: **www.promega.com/vectors/**

4. Transfer of Protein-Coding Regions Between Flexi® Vectors

To transfer your protein-coding region from one Flexi® Vector (donor) to a another Flexi® Vector (acceptor), choose the appropriate acceptor vector with the desired expression and tag options (see Table 1 in Technical Manual #TM254). The HaloTag® Pull-Down Cloning System provides the pFN21A HaloTag® CMV Flexi® Vector for initial cloning of the protein-coding region and the pFC14K HaloTag® CMV Flexi® Vector for transfer of the insert if desired. Alternatively, the protein-coding region can be cloned directly into the pFC14K HaloTag® CMV Flexi® Vector but transfer to other vectors is not possible. If you plan on transferring your protein-coding regions to other Flexi® Vectors, we recommend cloning the insert into the pFN21A HaloTag® CMV Flexi® Vector.

To transfer to other Flexi® Vectors, choose an acceptor Flexi® Vector with a different antibiotic resistance marker than the donor because antibiotic selection is the basis for selecting the desired clone (such as the transfer from ampicillin-resistant pFN21A HaloTag® CMV Flexi® Vector to kanamycin-resistant pFC14K HaloTag® CMV Flexi® Vector).

There are two basic categories of Flexi® Vectors: those containing SgfI and PmeI sites and expressing either a native (untagged) protein or an N-terminal-tagged protein, and those containing SgfI and EcoICRI sites and expressing a C-terminal tagged protein. Flexi® Vectors for expressing C-terminal-tagged proteins act only as acceptors, never as donor vectors. To transfer protein-coding regions between Flexi® Vectors expressing native protein or an N-terminal-tagged protein, the donor and acceptor vectors are digested with SgfI and PmeI simultaneously, prior to ligation of the insert, transformation and selection of the cells (Section 5.A). To create a C-terminal-tagged protein, the donor plasmid expressing native protein or an N-terminal-tagged protein is digested with SgfI and PmeI. Because EcoICRI cuts frequently in protein-coding regions, the acceptor plasmid containing the C-terminal tag is digested with SgfI and EcoICRI in a separate reaction. The two separate digests are combined for ligation of the insert, transformation and selection of the cells (Section 4.B).

The protocols in Sections 4.A and 4.B are for the transfer of protein-coding regions to other Flexi® Vectors that can allow variable expression levels and different expression systems (such as *E. coli*, cell-free, etc.). These vectors are available separately. For a complete list, visit:
www.promega.com/products/protein-expression/protein-labeling-and-detection/halotag-technology-products/halotag-vectors/

Note: For troubleshooting information, consult the *Flexi® Vector Systems Technical Manual* #TM254, available at:
www.promega.com/protocols/

4.A. Transfer of Protein-Coding Regions Between Flexi® Vectors Expressing Native or N-Terminal Fusion Proteins

Do **not** use C-terminal Flexi® Vectors, which have names starting with "pFC", as acceptors or donors since they lack PmeI sites.

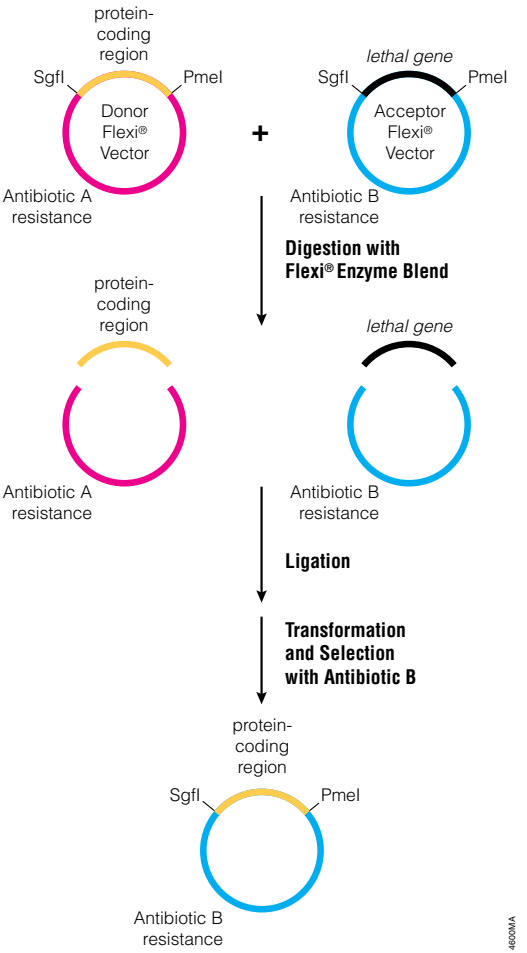


Figure 3. Transfer of a protein-coding region between N-terminal or native Flexi® Vectors. The donor Flexi® Vector containing the protein-coding region is mixed with an acceptor Flexi® Vector that has a different antibiotic resistance. The two plasmids are digested with SgfI and PmeI, and the mixture is ligated and transformed into *E. coli*. The cells are plated on the appropriate selective media for the acceptor Flexi® Vector. Protein-coding regions transferred into N-terminal fusion vectors allow translational readthrough of the SgfI site, which encodes the peptide sequence Ala-Ile-Ala.

1. Use the Wizard® *Plus* SV Minipreps DNA Purification System (Cat.# A1330), Wizard® SV 96 Plasmid DNA Purification System (Cat.# A2250) or a similar method to prepare the donor Flexi® Vector DNA. Adjust the volume, so the final DNA concentration is 50–100ng/µl.
2. Assemble the following reaction components to cut the Flexi® Vectors:

Component	Volume
5X Flexi® Digest Buffer	4µl
Acceptor Flexi® Vector (100ng)	1µl
Donor Flexi® Vector (100ng)	_µl
Flexi® Enzyme Blend (SgfI & PmeI)	2µl
Nuclease-Free Water to a final volume of	20µl

Take care when pipetting solutions that contain glycerol, such as the Flexi® Enzyme Blend, because small volumes are difficult to pipet accurately.

3. Incubate at 37°C for 15–30 minutes.
4. Heat the reaction at 65°C for 20 minutes to inactivate the restriction enzymes. Store the reaction on ice while assembling the ligation reaction in Step 5.
5. Assemble the following ligation reaction components:

Component	Volume
2X Flexi® Ligase Buffer	10µl
Digested DNA from Step 4 (100ng total)	10µl
T4 DNA Ligase (HC) (20u/µl)	1µl
Final Volume	21µl

6. Incubate at room temperature for 1 hour.
7. Transform high-efficiency, *E. coli* competent cells with the ligation reaction (≥1 × 10^8cfu/µg DNA). If you are using competent cells other than high-efficiency JM109 Competent Cells (Cat.# L2001) purchased from Promega, it is important to follow the appropriate transformation protocol. The recommended transformation protocol for Promega high-efficiency JM109 Competent Cells can be found in Technical Manual #TM254. Selection for transformants should be on LB plates supplemented with 100µg/ml ampicillin or 25µg/ml kanamycin, as appropriate for the acceptor Flexi® Vector.

4.A. Transfer of Protein-Coding Regions Between Flexi® Vectors Expressing Native or N-Terminal Fusion Proteins (continued)

8. Screen at least 4 colonies for each protein-coding region. Digest the plasmid to ensure that SgfI and PmeI cleave their recognition sites flanking the protein-coding region, so the insert can be cloned into other Flexi® Vectors.

   Screen at least 8 colonies for each protein-coding region transferred to or from the pF3A WG (BYDV) or pF3K WG (BYDV) Flexi® Vectors. Lower transfer frequencies with these vectors are due to a higher background of plasmid backbone heterodimers between the WG (BYDV) Vectors and other Flexi® Vectors. Other Flexi® Vectors share common regions flanking the SgfI and PmeI sites, such that plasmid backbone dimers are unstable. The pF3A and pF3K WG (BYDV) Flexi® Vectors lack these common flanking regions because of the inclusion of the BYDV translation-enhancing sequences.

   If you are using the pF3A WG (BYDV) or pF3K WG (BYDV) Flexi® Vectors, the number of minipreps performed can be reduced by prescreening colonies to identify those harboring plasmid backbone heterodimers. Colonies containing such heterodimers can be identified by their ability to grow in the presence of both antibiotics. Pick individual colonies and restreak on a plate containing ampicillin and a plate containing kanamycin, or inoculate two broth cultures for overnight growth, one with ampicillin and the other with kanamycin. Colonies containing the clone of interest will grow only in the medium containing the antibiotic associated with the acceptor plasmid.

4.B. Transfer Protocol from Native or N-Terminal Flexi® Vectors to C-Terminal Flexi® Vectors

Use the C-terminal Flexi® Vectors, which have names starting with "pFC", as acceptors but not as donors since they lack PmeI sites. This is the type of reaction performed when transferring the protein-region from the N-terminal pFN21A Flexi® Vector to the C-terminal pFC14K Flexi® Vector.

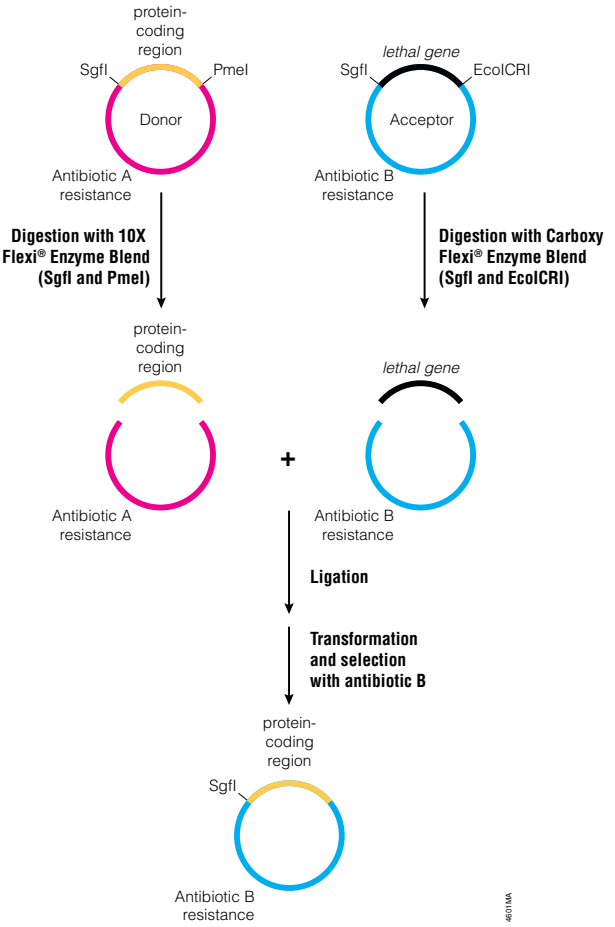


Figure 4. Transfer of a protein-coding region into the C-terminal Flexi® Vectors. The donor Flexi® Vector containing the protein-coding region is digested with SgfI and PmeI. The acceptor Flexi® Vector, which has a different antibiotic resistance, is digested with SgfI and EcoICRI in a separate reaction. The two digested plasmids are combined, the mixture is ligated and transformed into *E. coli*, and cells are plated on the appropriate selective media for the acceptor Flexi® Vector. When the blunt ends of PmeI and EcoICRI are joined, an in-frame Ser codon, which appends the downstream C-terminal protein-coding region contained on the Flexi® Vector backbone, is created.

4.B. Transfer Protocol from Native or N-Terminal Flexi® Vectors to C-Terminal Flexi® Vectors (continued)

1. Use the Wizard® *Plus* SV Minipreps DNA Purification System (Cat.# A1330), Wizard® SV 96 Plasmid DNA Purification System (Cat.# A2250) or a similar method to prepare the donor Flexi® Vector DNA. Adjust the volume, so the final DNA concentration is 50–100ng/µl.
2. Assemble the following reaction components to cut the donor Flexi® Vector:

Component	Volume
5X Flexi® Digest Buffer	2µl
Donor Flexi® Vector (100ng)	_µl
Flexi® Enzyme Blend (SgfI & PmeI)	1µl
Nuclease-Free Water to a final volume of	10µl

Take care when pipetting solutions that contain glycerol, such as the Flexi® Enzyme Blend, because small volumes are difficult to pipet accurately.

3. In a separate tube, assemble the following reaction components to cut the acceptor C-terminal Flexi® Vector:

Component	Volume
Nuclease-Free Water	6µl
5X Flexi® Digest Buffer	2µl
Acceptor C-terminal Flexi® Vector DNA (100ng)	1µl
Carboxy Flexi® Enzyme Blend (SgfI & EcoICRI)	1µl
Final Volume	10µl

Note: Acceptor C-terminal Flexi® Vectors will have names starting with "pFC".

4. Incubate both reactions at 37°C for 15–30 minutes.
5. Heat both reactions at 65°C for 20 minutes to inactivate the restriction enzymes. Store the reactions on ice while assembling the ligation reaction in Step 6.
6. Assemble the following ligation reaction components:

Component	Volume
2X Flexi® Ligase Buffer	10µl
Digested donor Flexi® Vector prepared in Step 2 (approximately 50ng)	5µl
Digested acceptor C-terminal Flexi® Vector prepared in Step 3 (50ng)	5µl
T4 DNA Ligase (HC) (20u/µl)	1µl
Final Volume	21µl

7. Incubate at room temperature for 1 hour.

8. Transform high-efficiency *E. coli* competent cells with the ligation reaction (1×10^8cfu/µg DNA). If you are using competent cells other than high-efficiency JM109 Competent Cells (Cat.# L2001) purchased from Promega, it is important to follow the appropriate transformation protocol. The recommended transformation protocol for Promega high-efficiency JM109 Competent Cells can be found in Technical Manual #TM254. Selection for transformants should be on LB plates supplemented with 100µg/ml ampicillin or 25µg/ml kanamycin, as appropriate for the acceptor Flexi® Vector.

   Note: Use 100µg/ml ampicillin or 25µg/ml kanamycin to select clones. Flexi® Vectors with the letter A in the name contain the ampicillin resistance gene, and Flexi® Vectors with the letter K in the name contain the kanamycin resistance gene.

9. Screen at least 8 colonies for each protein-coding region. If there is no XbaI restriction site present in the protein-coding region, digest with SgfI and XbaI to check for the correct insert size. The XbaI site is downstream of the PmeI site in all C-terminal Flexi® Vectors. Check the sequence of the vector used for other restriction sites that could be used for screening. Lower transfer frequencies are due to a relatively higher background of plasmid backbone heterodimers between the C-terminal Flexi® Vectors and other Flexi® Vectors. Other Flexi® Vectors share common regions flanking the SgfI and PmeI sites, such that plasmid backbone dimers are unstable. The C-terminal Flexi® Vectors may lack these common flanking regions because of the inclusion of the protein fusion tag sequence.

   The number of minipreps performed can be reduced by prescreening colonies to identify those harboring plasmid backbone heterodimers. Colonies containing such heterodimers can be identified by their ability to grow on both antibiotics. Pick individual colonies and restreak on an ampicillin plate and a kanamycin plate, or inoculate two cultures for overnight growth in media: one with ampicillin and the other with kanamycin. Colonies containing the clone of interest will grow only in the antibiotic associated with the acceptor plasmid.

5. Transfection

Successful transfection involves optimizing the FuGENE® HD Transfection Reagent:DNA ratio, amount of DNA used, complexing time, cells and medium used. For the HaloTag® Complete Pull-Down System, a 3:1 ratio of transfection reagent:DNA is recommended when using HEK293 and HeLa cells. A detailed optimization protocol and troubleshooting information are available in the *FuGENE® HD Transfection Reagent Technical Manual* #TM328.

Materials to Be Supplied by the User

- cells for transfection
- vector encoding HaloTag® fusion protein in the form of transfection-grade DNA
- HaloTag® Control Vector (Cat.# G6591) in the form of transfection-grade DNA
- cell culture medium with serum appropriate for the cell type being transfected
- serum-free cell culture medium for complex formation (Opti-MEM® I reduced-serum medium, standard medium or sterile water can be used.)
- 15cm dish or other culture plates
- 24-well culture plate to serve as a rack for FuGENE® HD Transfection Reagent

5.A. Plating Cells

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

Notes:

1. These cell densities have been used with HeLa and HEK293 cells. Other cell lines may require optimization to determine the best cell density to use. It is also possible to grow cells in suspension.
2. For low-expressing HaloTag® fusion proteins, two or three 15cm dishes can be pooled and used in the pull-down assay (see Section 7 for protocols to verify protein expression).
3. If you are using cells that are stably expressing a HaloTag® protein, plate the cells at similar densities, but skip Section 5.B and grow until the cells have reached 80–90% confluency.
4. For a list of conditions that were used to transfect various cell types, visit the FuGENE® HD Protocol Database at: **www.promega.com/resources/tools/fugene-hd-protocol-database/**

5.B. Preparing the FuGENE® HD Transfection Reagent

1. Before use, allow the vial of FuGENE® HD Transfection Reagent to reach room temperature.
2. Mix by inverting or vortexing briefly. No precipitate should be visible. If reagent has been frozen accidentally, briefly warm at 37°C to dissolve precipitate and cool to room temperature.

5.C. Transient Transfection Protocol

Note: The FuGENE® HD Transfection Reagent can be used in transfection protocols in the presence of up to 100% serum, allowing transfection of cell types that require continuous exposure to serum, such as primary cell cultures.

If not using the transfection conditions for HEK293 or HeLa cells, we strongly recommend that you optimize transfection conditions for each cell line. If you have optimized transfection parameters as described in Technical Manual #TM328, use the empirically determined conditions for your experimental transfections. For a list of conditions that were used to transfect various cell types, visit the FuGENE® HD Protocol Database at: **www.promega.com/resources/tools/fugene-hd-protocol-database/**

When using HeLa or HEK293 cells, we recommend the following conditions for transfection per 15cm dish seeded at 1–1.2 × 10^7 cells total and grown to 70–80% confluency as described in Section 6.B. If using other transfection reagents, follow the manufacturer's recommendations.

1. Add 30µg of endotoxin-free transfection-grade DNA to 1ml of minimal medium (such as Opti-MEM®, Invitrogen Cat.# 51985-091) to a sterile tube, and mix.
2. Add 90µl of room temperature FuGENE® HD Reagent directly to the medium; mix immediately. Do **not** allow undiluted FuGENE® HD Transfection Reagent to contact the sides of the tube.
3. Incubate the medium/DNA/FuGENE® HD mixture for 15 minutes at room temperature.
4. Add the mixture to a plate in a drop-wise manner and gently rock to distribute the transfection mixture.
5. Harvest cells typically 24–48 hours after transfection (or at 80–90% confluency for stable cell lines).

5.D. Stable Transfection Protocol

The goal of stable transfection is to isolate and propagate individual clones containing transfected DNA. Therefore, it is necessary to distinguish nontransfected cells from those that have taken up the exogenous DNA. This screening can be accomplished by drug selection when an appropriate drug-resistance marker is included in the transfected DNA.

Typically, cells are maintained in nonselective medium for 1–2 days post-transfection, then plated in selection medium (medium containing the appropriate drug). The use of selection medium is continued for 2–3 weeks, with frequent changes of medium to eliminate dead cells and debris until distinct colonies can be visualized. Individual colonies then are trypsinized and transferred to flasks for further propagation or to multiwell plates for limited dilution cloning in the presence of selective medium.

Several drug-selection markers are commonly used for long-term transfection studies. For example, cells transfected with recombinant vectors containing the bacterial gene for aminoglycoside (e.g., neomycin) phosphotransferase can be selected for stable transformation in the presence of the drug G-418. Similarly, expression of the gene for hygromycin B phosphotransferase from the transfected vector will confer resistance to the drug hygromycin B. Promega offers vectors conferring resistance to G-418, hygromycin B or puromycin.

Before using a particular drug for selection purposes, determine the amount of drug necessary to kill the cells you will be using. This may vary from one cell type to another. Construct a kill curve using varying concentrations of the drug to determine the amount needed to select resistant clones. The optimal drug concentration is generally the amount that induces cell death in >90% of nontransfected cells within 5–7 days.

For stable transfections, cells should be transfected with a plasmid containing a gene for drug resistance using the transfection protocols outlined in Technical Manual #TM328.

Optional: As a selection-drug-negative control, transfect cells using DNA that does not contain the drug-resistance gene.

1. Forty-eight hours post-transfection, harvest adherent cells and plate at several different dilutions (e.g., 1:2, 1:5, 1:10) in selective medium.
2. For the next 14 days, replace the selective medium every 3 to 4 days.
3. During the second week, monitor cells for distinct colonies of surviving cells. Complete cell death should occur in cultures transfected with the negative control plasmid.
4. Transfer individual clones by standard techniques (e.g., using cloning cylinders or limiting dilution cloning) to 96-well plates, and continue to maintain cultures in selective medium. **Note:** If single clones are not required, pools of stable transfectants can be maintained and frozen.

6. Protein Pull-Down

The HaloTag® pull-down scheme is depicted in Figure 5. The HaloTag® fusion protein transiently or stably expressed in mammalian cells is used as bait to capture interacting protein partners. After cell lysis, the HaloTag® fusion protein, complexed with its protein binding partners, is captured on the HaloLink™ Resin. The captured complexes are gently washed and eluted using SDS elution buffer or cleaved from the resin using TEV (Tobacco Etch Virus) protease. The recovered complexes are then suitable for analysis by a variety of methods including SDS-PAGE, Western blotting or mass spectrometry. As a negative control, we recommend using cells transfected with the HaloTag® Control Vector; alternatively, untransfected cells can be used.

For additional information including troubleshooting, consult the *HaloTag® Mammalian Pull-Down and Labeling System Technical Manual* #TM342, available at: **www.promega.com/protocols/**

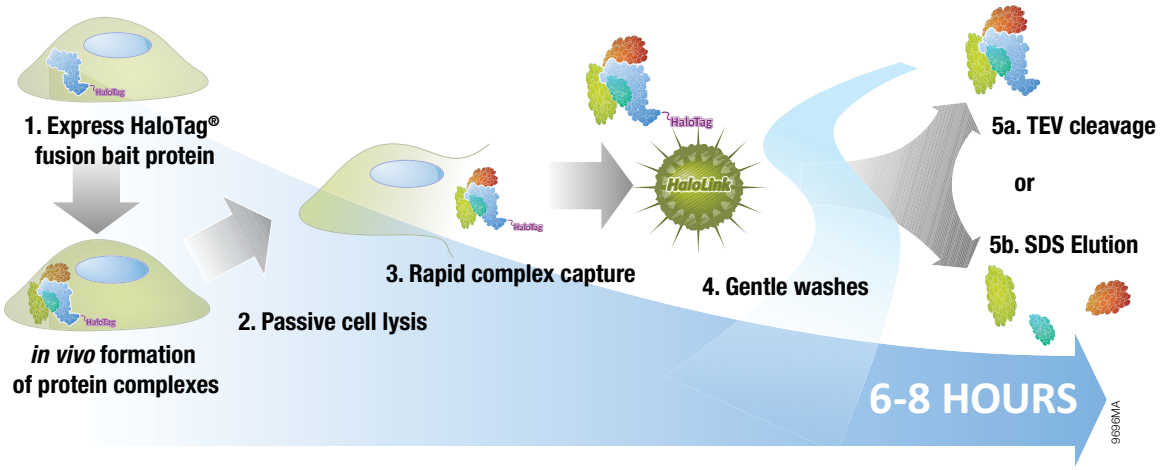


Figure 5. Schematic of the HaloTag® Mammalian Pull-Down System protocol.

6. Protein Pull-Down (continued)

Materials and Equipment to Be Supplied by the User

(Solution compositions are provided in Section 9.)

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

Additional Materials and Equipment to Be Supplied by the User for Cell Labeling (Section 8)

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

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

6.A. Preparing Reagents

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

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

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

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

6.B. Collecting Mammalian Cells Expressing HaloTag® Fusion Proteins

Use cells that were transfected using instructions in Sections 5.C and 5.D.

1. Harvest cells typically 24–48 hours after transfection (or at 80–90% confluency for stable cell lines).
2. Remove the medium and gently wash the cell layer with 20–25ml of ice-cold PBS. Aspirate to remove the PBS wash.
3. Add 25–30ml of ice-cold PBS to the cells, and gently scrape to collect cells into conical tubes. Centrifuge the cells at 4°C for 5–10 minutes at 2,000 × *g*, and discard the PBS. Store the cell pellets at –80°C for at least 30 minutes prior to lysing them as described in Section 6.C. Cell pellets expressing HaloTag® fusions can be stored at –80°C for up to 6 months.

6.C. Protein Pull-Down Protocol

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

The Mammalian Lysis Buffer is optimized for this system (see composition in Section 9). Other lysis buffers might be compatible with this system as well. In addition, nondetergent buffers may work when used in combination with a freeze-thaw. To prevent the disruption of the protein complexes, sonication is not recommended. Additives and cofactors known to be necessary to preserve the protein interaction complexes may be added at this point. We do not recommend using SDS, IGEPAL® CA-630 or Tween® 20 detergents in the lysis buffer because these inhibit capture of the protein onto the HaloLink™ Resin.

Note: The Protease Inhibitor Cocktail is optimized to work with this system (see composition in Section 9). Other protease inhibitor cocktails can reduce binding efficiency. Avoid using any cocktails that are known to contain 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) or that are of unknown composition.

The elution method used in this protocol releases the interacting protein partners and leaves behind the HaloTag® fusion protein, which is covalently bound to the resin. Alternatively, TEV Protease cleavage can be used to isolate the entire complex including the bait protein originally fused to the HaloTag® protein (see the protocol in Section 7.C).

Phase 1. Equilibrate Resin

1. Mix the HaloLink™ Resin by inverting the bottle until you have obtained a uniform suspension.
2. For each pull-down experiment, dispense 200µl of HaloLink™ Resin into two 1.5ml microcentrifuge tubes (one tube for the experimental sample and one tube for the negative control sample). Centrifuge for 1 minute at 800 × *g*. Carefully remove and discard the supernatant, leaving the resin at the bottom of the tube.
3. Add 800µl of Resin Equilibration/Wash Buffer (Section 6.A). Mix thoroughly by inverting the tube. Centrifuge for 2 minutes at 800 × *g*. Carefully remove and discard the supernatant, leaving the resin at the bottom of the tube. Repeat this wash step 2 additional times for a total of 3 washes. Do not remove the final wash supernatant until you are ready to bind lysates (Phase 3). This will prevent the resin from drying out.

Phase 2. Prepare HaloTag® Fusion Protein and Control Lysates

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

1. For each sample, grow approximately 1–1.2 × 10^7 cells as described in Section 5.C.

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

2. Thaw the frozen cell pellet prepared in Section 6.B, and resuspend the cells in 300µl of Mammalian Lysis Buffer; pipet or briefly vortex to mix.

   Notes:

   1. Mammalian Lysis Buffer is optimized for total cell lysis, including the nucleus. If cytoplasmic and nuclear fractions need to remain as separate pools, perform an initial cytoplasmic lysis as described in Section 7.B. If you are processing the cytosolic fraction only, discard the nuclear pellets.
   2. If you will be processing both the cytosolic and nuclear fractions, the nuclear pellets can be lysed subsequently with the Mammalian Lysis Buffer as described here. If you know your complex requires certain cofactors or small molecules to maintain complex integrity, please add these to the Mammalian Lysis Buffer and the wash buffer.

3. Add 6µl of the 50X Protease Inhibitor Cocktail and incubate on ice for 5 minutes.

4. To reduce lysate viscosity following the incubation, homogenize with a Dounce glass homogenizer (2ml size) on ice using 25–30 strokes using the large pestle (B). Alternatively, pass the cells through a 25- or 27-gauge needle 5–10 times.

   Note: We do not recommend sonication because protein complexes may fall apart, and overheating may reduce the HaloTag® protein activity.

5. Centrifuge the sample at 14,000 × *g* for 5 minutes at 4°C to clear the lysate.

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

6. Transfer the clear lysate to new tube, and place the tube on ice until Phase 3.

6.C. Protein Pull-Down Protocol (continued)

Phase 3. Bind Protein Complexes

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

   Note: We recommend you determine the binding efficiency (Section 7.A), and to do so you will need to set aside 10µl of the diluted lysate as the prebinding fraction. Store this fraction on ice.

2. Remove the Resin Equilibration/Wash Buffer supernatant from the equilibrated resin, and add the remainder of the diluted lysate.

3. Incubate with mixing on a tube rotator (or equivalent device) for 15 minutes at room temperature. Make certain that the resin does not settle to the bottom of the tube; settling will reduce binding efficiency.

   Notes:

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

4. Centrifuge the tubes for 2 minutes at 800 × *g*, and discard the supernatant.

   Note: To determine the binding efficiency (Section 7.A), set aside 10µl of the supernatant as the unbound fraction. Store this fraction on ice.

Phase 4. Washing

1. Add 1ml of Resin Equilibration/Wash Buffer (Section 6.A) to each tube, and mix thoroughly by gently inverting the tube several times. Centrifuge for 2 minutes at 800 × *g*. Discard the wash. Repeat three additional times, for a total of four washes.

2. Add 1ml of Resin Equilibration/Wash Buffer (Section 6.A), and mix thoroughly by inverting the tube several times. Incubate at room temperature for 5 minutes with mixing. Centrifuge for 2 minutes at 800 × *g*. Discard the wash.

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

Phase 5. Protein Elution

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

   Note: In some instances, it is possible to substitute the SDS Elution Buffer for the optional urea elution buffer as described in Section 7.D (composition in Section 9). Samples eluted in urea may be directly digested with Lys-C prior to mass spectroscopy analysis. See Section 7.D for more details.

2. Centrifuge for 2 minutes at 800 × *g*, and carefully transfer the eluate to a fresh tube, leaving the resin at the bottom.

   Notes:

   1. Resin particles in the eluted fraction could be problematic if the sample is to be analyzed directly in solution by mass spectroscopy (see Section 7.D). If resin particles are present in the eluate, we recommend transferring the eluted fraction to a spin column with a 1.5ml collection tube and spinning for 15 seconds at 10,000 × *g*.
   2. This elution method releases the interacting protein partners and leaves behind the HaloTag® fusion protein, which is covalently bound to the resin. Alternatively, TEV Protease cleavage can be used to isolate the entire complex including the bait protein originally fused to the HaloTag® protein (Section 7.C).

7. Protein Detection and Analysis Protocols

7.A. Detecting Expression and Binding Efficiency of HaloTag® Fusion Proteins

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

1. For the HaloTag® TMRDirect™ Ligand, dilute the stock solution (100µM) 1:2 in DMSO to make a 50µM working solution. For the HaloTag® TMR Ligand (Cat.# G8251, G8252), dilute the stock solution (5mM) 1:100 in DMSO for a final concentration of 50µM.
2. Mix 10µl of diluted lysate containing the HaloTag® fusion protein or the equivalent amount of unbound fraction (saved in Phase 3, Steps 1 and 4) with 19µl of water and 1µl of 50µM HaloTag® TMR Ligand.
3. Incubate at room temperature for 15 minutes protected from light.
4. Add 10µl of 4X SDS gel loading buffer (Section 9) and heat at 70°C for 5 minutes or 95°C for 2 minutes.
5. Load 5–10µl onto an SDS-polyacrylamide gel.
6. Quantitate band intensities for the before- and after-binding fractions on a fluorescent detection scanner such as the Typhoon®, GE Healthcare Bio-sciences (excitation 532nm; emission 580nm).
7. To determine the binding efficiency, divide the band intensity of the sample after binding by the band intensity of the sample prior to binding. This value represents the percentage of unbound protein. Subtract the unbound percentage from 100% to calculate the percentage bound.

7.B. Cell Lysis with Cytosolic and Nuclear Fractionation

1. Thaw the cell pellets. Add 300µl of Cytoplasmic Lysis Buffer (composition in Section 9) and pipet or vortex briefly.
2. Add 6µl of 50X Protease Inhibitor Cocktail and incubate on ice for 30 minutes.
3. Homogenize with a Dounce glass homogenizer (2ml size) using 25–30 strokes on ice using the large pestle (B). Alternatively, pass cells through a 25- or 27-gauge needle 5–10 times to complete lysis.
4. Centrifuge at 800 × *g* for 20 minutes at 4°C to separate the cytosol from the nuclear pellet.
5. To process the cytosolic fractions, transfer the clear lysate to a new tube, and place the tubes on ice until protein dilution and binding (Phase 3; Section 6.C). If you do not need the cytosolic fraction, discard the supernatant without disturbing the nuclear pellets at the bottom of the tube.
6. To process the nuclear fractions, use the standard lysis protocol (Phase 2; Section 6.C).

7.C. TEV Protease Cleavage of Fusion Proteins

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

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

1. Following the last wash (Phase 4, Section 6.C), resuspend the resin in 50µl of 1X ProTEV Buffer and 30 units of ProTEV Plus Protease.
2. To improve cleavage, incubate at 25°C with shaking for 1 hour, making certain that the resin does not settle.
3. Centrifuge for 2 minutes at 800 × *g*, and carefully transfer the eluate to a fresh tube, leaving the resin at the bottom of the centrifuged tube.

   Notes:

   1. Resin particles in the eluted fraction could be problematic if the sample is to be analyzed directly in solution by mass spectroscopy (see Section 7.D). If resin particles are present in the eluate, we recommend transferring the eluted fraction to a spin column with a 1.5ml collection tube and spinning for 15 seconds at 10,000 × *g*.
   2. This elution method releases the entire complex including the bait protein originally fused to the HaloTag® protein and interacting protein partners.

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

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

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7.D. Downstream Analysis and Applications

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

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

Mass Spectroscopy Analysis

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

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

When the protein complexes are recovered by TEV cleavage (Section 7.C) or urea elution (Section 6.C, Phase 5), it is possible to perform the tryptic digestion directly in-solution without the need to resolve the samples using gel electrophoresis. Samples eluted in urea may be directly digested with Lys-C prior to mass spectroscopy analysis. For samples that will be directly injected into mass spectroscopy apparatus, it is important that no resin particles have been carried over in the eluted fraction. If resin particles are present in the eluate, we recommend transferring the eluted fraction to a spin column with a 1.5ml collection tube and spinning for 15 seconds at 10,000 × *g*.

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

7.E. SDS-PAGE Analysis to Examine HaloTag® Protein Fusion Expression

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

Although all of the HaloTag® ligands perform in this function, the HaloTag® TMR Ligand is recommended for direct SDS-PAGE fluorescence scanning applications. Representative data are shown in Technical Manual #TM342.

Materials to be Supplied by the User
(Solution compositions are provided in Section 9.)

- cells expressing HaloTag® fusion protein
- 1X PBS (pH 7.5)
- 1X SDS sample buffer
- heat block or water bath at 95°C
- equipment and running buffer necessary for SDS-PAGE
- fluorescent scanner

1. To label cells, follow Steps 1–4 of Section 8.A or 8.B, ending with cells in 1X PBS.
2. Lyse cells by replacing the 1X PBS from the wells with ~75–100µl of 1X SDS sample buffer per cm^2 of cell growth area (e.g., 150–200µl of 1X SDS sample buffer per well of a 24-well plate that has a 1.9cm^2 growth area).
3. Collect cell lysate, and incubate for 5 minutes at 95°C.
4. Perform SDS-PAGE by loading 10µl (5–10µg total protein) of each sample per well of the gel, or store samples at –20°C for later use.
5. Analyze the gel on a fluorescence scanner.

   Note: The dye front might contain fluorescent material that can complicate detection (unbound ligand and/or tracking dyes used in sample buffers). To eliminate these sources of nonspecific fluorescence, simply run the gel until the dye front migrates off of the gel or cut the dye front off of the bottom of the gel before scanning.
6. After scanning, the proteins can be transferred to nitrocellulose for Western blot analysis if used promptly (i.e., gel is not fixed, remains moist and is not left in buffer or deionized water).

8. Cell Imaging Protocols

The same HaloTag® construct used for protein complex isolation can be used for cellular localization, protein trafficking and protein turnover studies. The following protocols use the HaloTag® TMRDirect™ Ligand provided in the HaloTag® Mammalian Pull-Down and Labeling System (Cat.# G6500). The HaloTag® TMRDirect™ Ligand and other HaloTag® Ligands for cellular imaging are also available separately and are listed in Section 10. Additional information is available at: **www.promega.com/protocols/**

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

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

Use these protocols only for complementary cellular imaging studies. Once the HaloTag® protein is labeled with the HaloTag® TMRDirect™ Ligand it cannot be used for protein complex isolation. Use the protocols described in Section 6 to perform the pull-down experiments.

8.A. Live-Cell Imaging

This protocol can be used to label adherent or nonadherent cells. Adherent cells that have been plated or are still in suspension can be used. Labeling of the HaloTag® protein can be accomplished concurrently with expression by adding the HaloTag® TMRDirect™ Ligand at the time of transfection or at the time of plating for stable cell lines. Alternatively, the HaloTag® TMRDirect™ Ligand can be added at a later time up to 15–18 hours prior to imaging. Representative data are shown in the *HaloTag® Mammalian Pull-Down and Labeling Systems Technical Manual* #TM342, available at: **www.promega.com/protocols/**

1. Plate cells in chambered cover glass or other cell culture device, and allow them to reach appropriate confluency for desired transfection method.
2. Transfect cells with the DNA construct encoding the HaloTag® fusion protein following the manufacturer's recommendations (skip this step if the cells are stably expressing the HaloTag® protein).
3. Labeling can be performed at this time or at a later time, but must be performed at least 15–18 hours before imaging.

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

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

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

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

6. Gently replace the ligand-containing medium with an equal (or greater) volume of warm fresh medium, or fix solution (for endpoint assays; see Section 8.B).

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

8.B. Fixed-Cell Imaging

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

Fixed cells can be treated with detergents, such as Triton® X-100, to further eliminate nonspecific labeling and permeabilize cells for downstream immunocytochemical applications. The conditions here are sufficient to permeabilize the plasma membrane. Alternative or additional detergents might be necessary to permeabilize other structures.

1. Label the cells following Steps 1–5 of Section 8.A.

2. Replace the medium with an equal volume of warm 4% paraformaldehyde/0.2M sucrose/1X PBS (pH 7.5), and incubate for 10 minutes at room temperature.

3. Replace fixative with an equal volume of 1X PBS + 0.1% Triton® X-100, and incubate for 10 minutes at room temperature.

4. Replace the detergent-containing solution with an equal volume of 1X PBS.

5. Transfer to a microscope, and capture images using a confocal microscope or wide-field fluorescent microscope equipped with appropriate filter sets and lasers ($555nm_{Ex}/580nm_{Em}$) or proceed to immunocytochemistry (ICC).

9. Composition of Buffers and Solutions

Supplied with System

Membrane Wash Solution
(after ethanol addition)

10mM	potassium acetate (pH 5.0)
80%	ethanol
16.7µM	EDTA (pH 8.0)

To prepare this solution, add 15ml of 95% ethanol to the supplied Membrane Wash Solution (concentrated) as described in Section 3.A, Step 1.

Membrane Binding Solution

4.5M	guanidine isothiocyanate
0.5M	potassium acetate (pH 5.0)

5X Flexi® Digest Buffer

50mM	Tris-HCl (pH 7.9 at 37°C)
250mM	NaCl
50mM	$MgCl_2$
5mM	DTT
0.5mg/ml	acetylated BSA

2X Flexi® Ligase Buffer

60mM	Tris-HCl (pH 7.8 at 25°C)
20mM	$MgCl_2$
20mM	DTT
2mM	ATP

Store in single-use aliquots at –20°C. Avoid multiple freeze-thaw cycles.

HaloLink™ Resin

25% slurry in 25% ethanol

Mammalian Lysis Buffer

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

10X TBS Buffer

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

Protease Inhibitor Cocktail
Reconstituted 50X stock will contain:

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

SDS Elution Buffer

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

To Be Supplied by User

Antibiotic stock solutions

100mg/ml ampicillin in deionized water (sterile filtered)
25mg/ml kanamycin; kanamycin sulfate in deionized water (sterile filtered)

LB plates with antibiotic

Add 15g agar to 1 liter of LB medium. Autoclave. Allow the medium to cool to 50°C before adding ampicillin to a final concentration of 100µg/ml or kanamycin to a final concentration of 25µg/ml, as appropriate for the acceptor Flexi® Vector. Pour 30–35ml of medium into 85mm petri dishes. Let the agar harden. Store at 4°C for up to 1 month or at room temperature for up to 1 week.

LB medium (per liter)

10g Bacto®-tryptone
5g Bacto®-yeast extract
5g NaCl

Adjust the pH to 7.5 with NaOH. Autoclave to sterilize. For LB plates, include 15g agar prior to autoclaving.

2M Mg^{2+} stock

20.33g $MgCl_2 \cdot 6H_2O$
24.65g $MgSO_4 \cdot 7H_2O$

Add distilled water to 100ml. Filter- sterilize.

TE buffer

10mM Tris-HCl (pH 8.0)
1mM EDTA

cytoplasmic lysis buffer

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

4X SDS-PAGE gel loading buffer

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

SOC medium (100ml)

2.0g Bacto®-tryptone
0.5g Bacto®-yeast extract
1ml 1M NaCl
0.25ml 1M KCl
1ml 2M Mg^{2+} stock, filter-sterilized
1ml 2M glucose, filter-sterilized

Add Bacto®-tryptone, Bacto®-yeast extract, NaCl and KCl to 97ml of distilled water. Stir to dissolve. Autoclave and cool to room temperature. Add 2M Mg^{2+} stock and 2M glucose, each to a final concentration of 20mM. Bring the volume to 100ml with sterile, distilled water. The final pH should be 7.0.

urea elution buffer

8M urea
100mM Tris (pH 8.5)

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

Prepare fresh for each use.

1X PBS Buffer (pH 7.5)

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

1X SDS sample buffer

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

10. Related Products

HaloTag® Flexi® Vectors for Protein Expression in Mammalian Systems

Product	Size	Cat.#
pFC14A HaloTag® CMV Flexi® Vector	20µg	G9651
pFC14K HaloTag® CMV Flexi® Vector	20µg	G9661
pFN21A HaloTag® CMV Flexi® Vector	20µg	G2821
pFN21K HaloTag® CMV Flexi® Vector	20µg	G2831
HaloTag® Flexi® Vectors—CMV Deletion Series Sample Pack	9 × 2µg	G3780
HaloTag® Control Vector	20µg	G6591

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
10X Flexi® Enzyme Blend (SgfI & PmeI)	25µl	R1851
	100µl	R1852
Carboxy Flexi® Enzyme Blend (SgfI & EcoICRI)	50µl	R1901

Protein Immobilization

Product	Size	Cat.#
HaloLink™ Resin*	1.25ml	G1912
	2.5ml	G1913
	10ml	G1914
	25ml	G1915
MagneHis™ Ni-Particles	2ml	V8560
	10ml	V8565

*Size reflects volume of settled resin.

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® Alexa Fluor® 488 Ligand	15µl	G1002
HaloTag® R110Direct™ Ligand	30µl	G3221

Protein Analysis

Product	Size	Cat.#
ProTEV Plus	1,000u	V6101
	8,000u	V6102
Trypsin Gold, Mass Spectrometry Grade	100µg	V5280

11. Summary of Changes

The following changes were made to the 8/17 revision of this document:

1. An incorrect reference to “Phase 1” was changed to “Phase 2” in Section 6.C, Phase 3, Step 1.

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[c]U.S. Pat. Nos. 7,429,472, 7,888,086 and 8,202,700, Japanese Pat. No. 4748685 and other patents pending.

[d]U.S. Pat. Nos. 7,425,436, 7,935,803, 8,466,269, 8,742,086, 8,420,367 and 8,748,148 and other patents and patents pending.

[e]U.S. Pat. Nos. 8,293,503 and 8,367,403, European Pat. No. 1685247 and other patents and patents pending.

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[g]Patent Pending.

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