



**Promega**

Technical Manual

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# **HaloLink™ Protein Array Systems**

INSTRUCTIONS FOR USE OF PRODUCT G6140, G6180 AND G6190.



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Part# TM310

# HaloLink™ Protein Array Systems

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 Please visit the web site to verify that you are using the most current version of this Technical Manual.  
 Please contact Promega Technical Services if you have questions on the use of this product. Email: [techserv@promega.com](mailto:techserv@promega.com)

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## 1. Description

Protein arrays enable proteome-scale detection of protein:protein, protein:drug or protein:nucleic acid interactions. The HaloLink™ Protein Array Systems<sup>(a-d)</sup> provide a new way to create custom protein arrays by combining innovative HaloTag® technology, surface engineering and cell-free protein expression systems.

The HaloTag® protein is a mutated hydrolase that forms a covalent bond with HaloTag® ligands. Under physiological conditions binding is rapid and highly specific, yielding a complex that is stable even under stringent conditions (1-3). Using the **HaloLink™ Protein Array Systems**, HaloTag® fusion

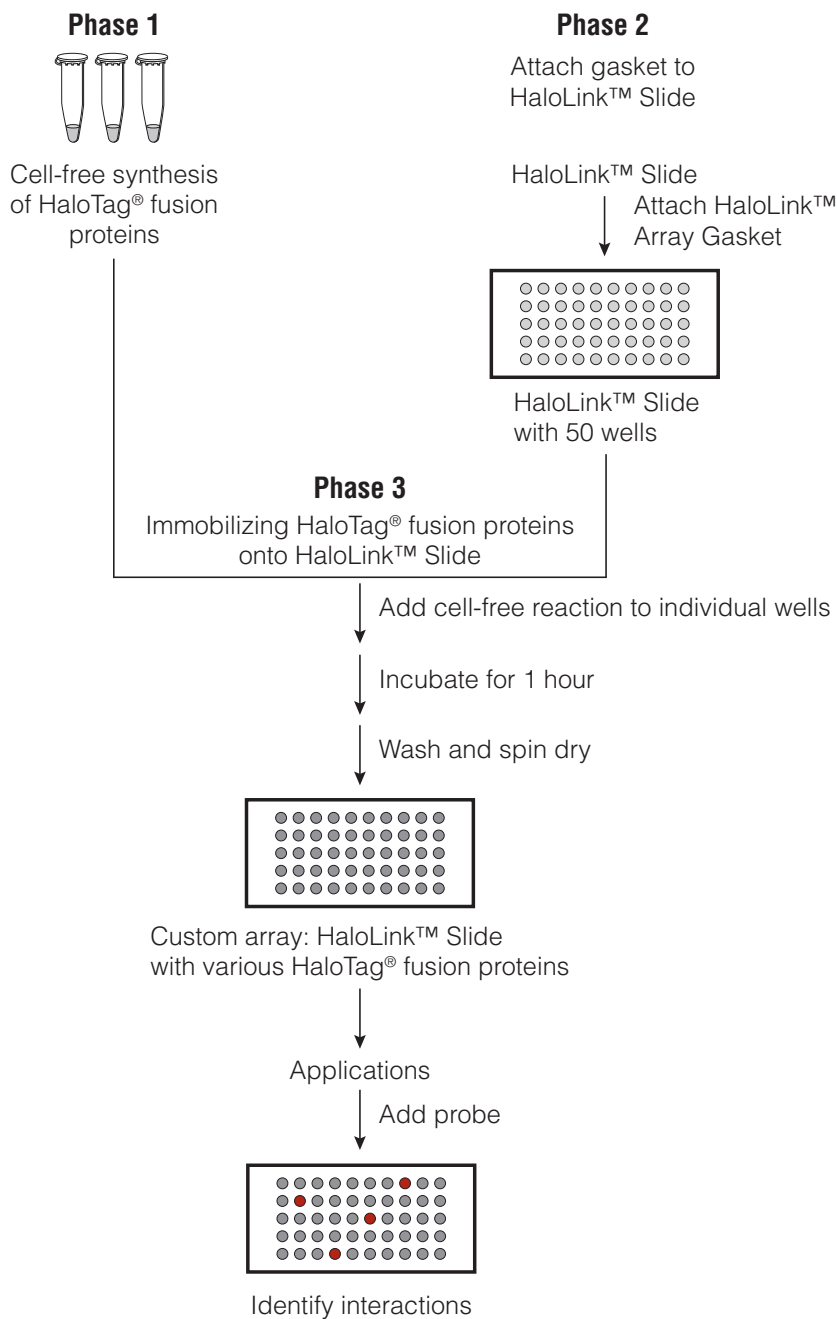
proteins are expressed in a cell-free expression system and then captured on hydrogel-coated glass slides. The fusion proteins are captured directly from the expression reaction mixture without prior purification. Using this approach, multiple fusion proteins can be rapidly synthesized and immobilized in parallel on the slide surface, and a complete experiment including protein expression, custom array formation, and protein interaction analysis can be completed in less than eight hours.

### Features of the HaloLink™ Protein Array Systems

- **Fast protein expression:** Cell-free expression systems allow quick, single-tube, coupled transcription/translation.
- **Irreversible binding of the captured protein:** Unlike other affinity tags, which tend to dissociate from the surface, HaloTag® fusion proteins are covalently bound to the HaloLink™ Slide.
- **No protein purification:** The protein of interest is immobilized directly from the cell-free expression system.
- **Reduced nonspecific binding:** HaloLink™ Slides have a unique hydrogel coating that is designed to prevent nonspecific binding while preserving the functionality of specifically captured proteins.
- **Extensive washing:** Covalent binding of HaloTag® fusion proteins to the HaloLink™ Slide allows extensive, stringent washing, that may result in reduced background and a lower incidence of false positives.
- **No need for a robotic arrayer:** The unique 50-well configuration allows multiple interactions to be studied in parallel without the need for a complex robotic arrayer.

### HaloLink™ Protein Array Systems Overview (Figure 1)

1. The desired protein-coding sequence is cloned into an appropriate HaloTag® Flexi® Vector.
2. HaloTag® fusion proteins are expressed in a cell-free expression system.
3. The supplied HaloLink™ Array Gasket is applied to the HaloLink™ Slide, creating 50 leak-free wells.
4. The cell-free expression reaction products are applied and captured on the HaloLink™ Slide, creating a custom array.
5. The array is designed for downstream applications such as protein:protein interactions and may also have potential for protein:DNA interactions, antibody screening, and enzymatic functional analysis.
6. Experimental workflow is provided in Figure 2.



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**Figure 1. HaloLink™ Protein Array Systems overview.**

## 1. Description (continued)

The HaloLink™ Array Two Slide Systems (Cat.# G6140 and G6180) contain HaloLink™ Slides, HaloLink™ Array Gaskets for creating 50-well arrays, a HaloTag® Standard Protein, Anti-HaloTag® Antibody and a cell-free expression system [either the TNT® T7 Quick Coupled Transcription/ Translation System (Cat.# G6140) or the TNT® SP6 High-Yield Wheat Germ Protein Expression System (Cat.# G6180)].

The HaloLink™ Array Six Slide System contains HaloLink™ Slides, HaloLink™ Array Gaskets and Anti-HaloTag® Antibody. Users of the Six Slide System will need either to provide their own protein expression system or order the TNT® T7 Quick Coupled Transcription/Translation System (Cat.# L1170 and L1171) or TNT® SP6 High Yield Wheat Germ Protein Expression System (Cat.# L3260 and L3261). The HaloTag® Standard Protein (Cat.# G4491) is not included with the Six Slide System but can be ordered separately. The HaloLink™ Array Systems were developed using the TNT® T7 Quick and TNT® SP6 High-Yield Wheat Germ Systems. If you choose to use a different protein expression system, you will need to optimize the experimental conditions appropriately.

This Technical Manual contains a detailed protocol for use of the HaloLink™ Protein Array Systems to create custom arrays and provides guidelines for performing protein:protein (Section 5) and protein:DNA interaction (Section 6) applications.

## 2. Product Components and Storage Conditions

Product	Cat.#
HaloLink™ Array (TNT® T7 Quick) Two Slide System	G6140

G6140 consists of two parts, which are shipped separately. Includes:

Part 1 of 2 (G6141) HaloLink™ Array (TNT® T7 Quick) Two Slide System

- 3 × 200µl TNT® T7 Quick Master Mix
- 1.25ml Nuclease-Free Water
- 50µl Methionine
- 30µg HaloTag® Standard Protein (3.0mg/ml)
- 20µg Anti-HaloTag® pAb (1.0mg/ml)

Part 2 of 2 (G4941) HaloLink™ Slides

- 2 HaloLink™ Slides
- 2 HaloLink™ Array Gaskets

<b>Product</b>	<b>Cat.#</b>
HaloLink™ Array (TnT® SP6 Wheat Germ) Two Slide System	G6180

G6180 consists of two parts, which are shipped separately. Includes:

Part 1 of 2 (G6181) HaloLink™ Array (TnT® SP6 Wheat Germ) Two Slide System

- 2 × 300µl TnT® SP6 High-Yield Wheat Germ Master Mix
- 1.25ml Nuclease Free Water
- 30µg HaloTag® Standard Protein (3.0mg/ml)
- 20µg Anti-HaloTag® pAb (1.0mg/ml)

Part 2 of 2 (G4941) HaloLink™ Slides

- 2 HaloLink™ Slides
- 2 HaloLink™ Array Gaskets

<b>Product</b>	<b>Cat.#</b>
HaloLink™ Array Six Slide System	G6190

Includes:

- 6 HaloLink™ Slides
- 6 HaloLink™ Array Gaskets
- 20µg Anti HaloTag® pAb (1.0mg/ml)

**Storage Conditions:** Store the TnT® lysates at -70°C. Store the HaloTag® Standard Protein and the Anti-HaloTag® Antibody at -20°C. The HaloLink™ Protein Array Slides should be stored at -20°C and opened just before use. After opening, unused slides should be stored at -20°C and used within one month. Store the HaloLink™ Array Gaskets at room temperature.

- Do not store the TnT® lysate at any temperature other than -70°C. Storage at other temperatures (e.g., -20°C) for even a short time will dramatically reduce activity.
- Do not freeze-thaw the TnT® lysate more than two times.
- Do not store the TnT® lysate in the presence of dry ice. Prolonged exposure to dry ice can cause significant loss of activity.

### 3. Required Materials and Experimental Considerations

#### HaloTag® Vector (not supplied in the kit)

Before using the HaloLink™ Array System to create custom arrays, the desired protein coding sequence must be cloned into a HaloTag® Flexi® Vector. See the Appendix (Section 10.A) for specific recommendations on HaloTag® Vectors available for use with this system.

**Important:** If your application requires a specificity control (HaloTag® Protein without a fusion partner), please contact Promega Technical Services for information on how to create a construct that expresses HaloTag® Protein alone.

**Note:** Difference between HaloTag® Flexi® Vectors and discontinued pHT2 Vector (Cat.# G8241). The HaloTag® protein encoded by the HaloTag® Flexi® Vectors is HaloTag® 7, which is the second-generation commercially available HaloTag® protein. It provides increase stability with regard to both temperature and denaturants, increased solubility and faster labeling kinetics, resulting in markedly improved expression compared to the HaloTag® 2 protein contained in the pHT2 HaloTag® Vector. The discontinued pHT2 Vector is not recommended for expression in bacterial cells.

#### Cell-Free Expression Systems

The TNT® T7 Quick Master Mix (supplied with Cat.# G6140) provides a single-tube, rabbit reticulocyte lysate-based eukaryotic coupled transcription/translation system designed to express 1–10µg/ml of protein. To use this system, 0.2–2.0µg of circular plasmid DNA containing a T7 promoter is added to an aliquot of the TNT® T7 Quick Master Mix and incubated in a 50µl reaction volume for 60–90 minutes at 30°C.

The TNT® SP6 High-Yield Wheat Germ Master Mix (supplied with Cat.# G6180) provides a single-tube, coupled transcription/translation reaction designed to express up to 100µg/ml of protein in two hours. To use this system, 2–12µg of circular plasmid DNA containing an SP6 promoter is added to an aliquot of the TNT® SP6 High-Yield Wheat Germ Master Mix and incubated in a 50µl reaction volume for two hours at 25°C.

We strongly recommend that you refer to the *TNT® T7 Quick Coupled Transcription/Translation Systems Technical Manual #TM045* ([www.promega.com/tbs/tm045/tm045.html](http://www.promega.com/tbs/tm045/tm045.html)) or the *TNT® SP6 High-Yield Wheat Germ Protein Expression System Technical Manual # TM282* ([www.promega.com/tbs/](http://www.promega.com/tbs/)) for specific details on use of these expression systems.

The simplicity of cell-free reactions performed using the TNT® Systems means that multiple HaloTag® fusion proteins can be expressed in parallel reactions. Other cell-free protein expression systems, as well as in vivo protein expression in mammalian cells or *E. coli*, can be used for expressing HaloTag® fusion proteins for use with HaloLink™ Protein Arrays, but the user will need to optimize any other expression system.

#### HaloLink™ Slides (supplied with all kits)

The HaloLink™ Slides are designed for protein applications. Each slide has an organic hydrogel coating activated with HaloTag® Ligand that:

- Inhibits nonspecific binding of proteins
- Specifically captures HaloTag® fusion proteins
- Provides an ideal environment for maintaining the functional integrity of captured proteins

### **HaloLink™ Array Gaskets (supplied with all kits)**

The HaloLink™ Array Gaskets are used to make 50 wells on the HaloLink™ Slides. The gasket is attached by placing the HaloLink™ Slide onto the gasket and gently pressing to make leak-proof wells that eliminate well-to-well cross contamination. Each well is 3.0mm in diameter and 1.0mm in height and has a capacity of 10µl. The gasket can be removed at the end of the assay for scanning and quantitating the signal.

### **HaloTag® Standard Protein (supplied with Cat.# G6140 and G6180) and Anti-HaloTag® pAb (supplied with all kits)**

The HaloTag® Standard Protein is a 61kDa purified HaloTag®-GST fusion protein supplied at a concentration of 3.0mg/ml. HaloTag® Standard Protein can be used to estimate the expression level of your HaloTag® fusion protein. Typical concentration range of the HaloTag® Standard Protein to use will depend on the cell-free expression system. With TNT® T7 Quick reactions, the concentration range is 1-10µg/ml (0.016-0.16 nmol/ml). With TNT® SP6 High-Yield Wheat Germ reactions, it is 10-100µg/ml (0.16-1.6 nmol/ml).

The Anti-HaloTag® pAb is a purified rabbit polyclonal antibody raised against the HaloTag® protein. The antibody is supplied at a concentration of 1.0mg/ml in PBS.

Anti-HaloTag® pAb and HaloTag® Standard Protein can be used to confirm and estimate expression levels of HaloTag® fusion proteins in cell-free systems as well as to confirm and estimate capture of the HaloTag® fusion proteins on the slide surface and to normalize for variations in:

- Day-to-day expression levels
- Expression levels in different systems
- Expression levels of panels of HaloTag® fusion proteins
- Capture efficiency of different proteins

## **4. Protocol for Expression and Capture of HaloTag® Fusion Proteins to Create a Custom Protein Array**

This section contains a protocol for expression of HaloTag® fusion proteins in cell-free systems and subsequent capture of these fusion proteins onto HaloLink™ Slides. A detailed workflow is shown in Figure 2. Guidelines for using custom protein arrays for protein:protein and protein:DNA interaction studies are provided in Sections 5 and 6, respectively.

### **Materials To Be Supplied By the User**

(Buffer compositions are provided in Section 10.B.)

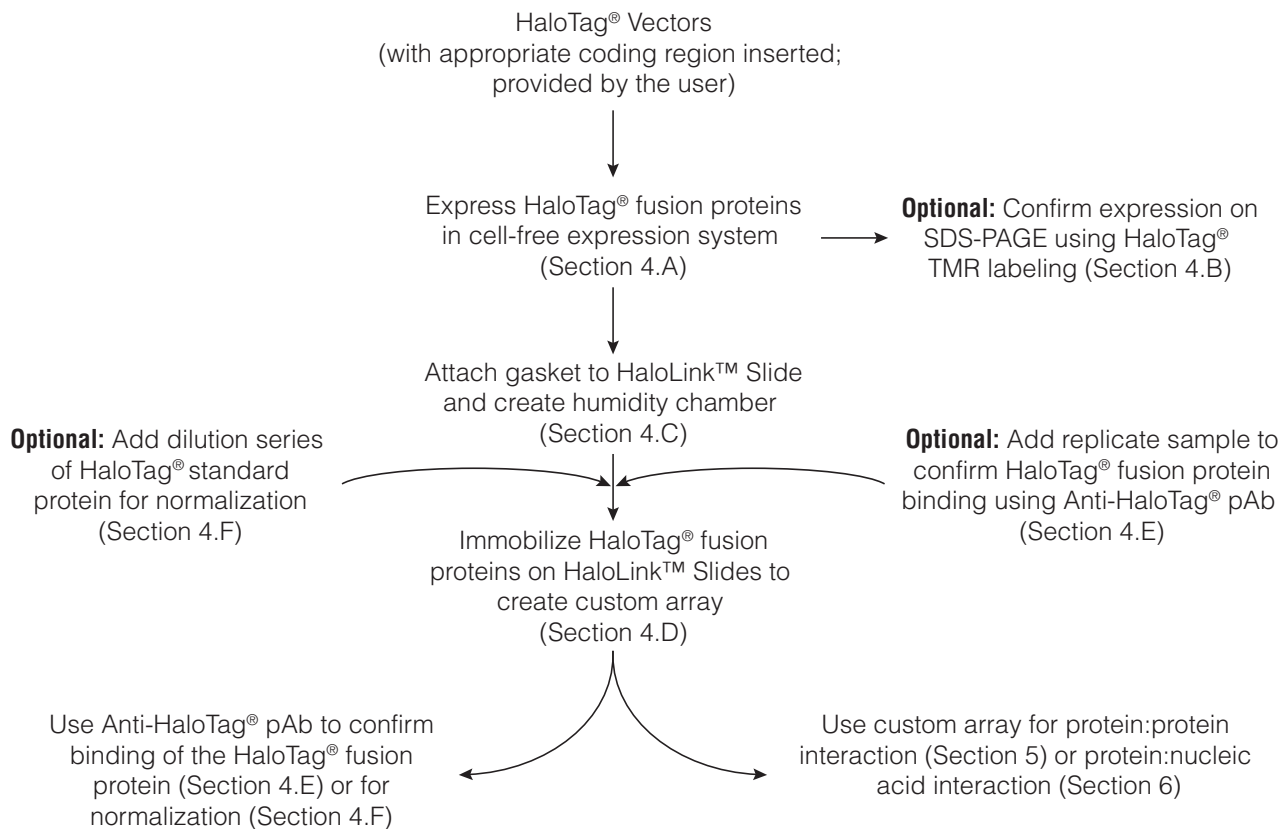
#### *Protein Expression and Verification Reagents*

- HaloTag® fusion DNA template (see Section 10.A for recommendations)  
**Note:** HaloLink™ Protein Array Systems are recommended for use with HaloTag® Protein encoded by the HaloTag® Flexi® Vectors for optimal results.
- Cell-free expression system (if using the HaloLink™ Array Six Slide System (Cat.# G6190)
- HaloTag® TMR Ligand, 5µM (Cat.# G8251, G8252)  
**Note:** The HaloTag® TMR Direct Ligand (Cat.#G2991) is too dilute for this application.)
- SDS-PAGE gels and gel-loading buffer (see Section 10.B)

#### 4. Protocol for Expression and Capture of HaloTag® Fusion Proteins to Create a Custom Protein Array (continued)

##### *HaloLink™ Slide Processing Reagents and Equipment*

- Bovine serum albumin (Blot-Qualified BSA, Cat.# W3841)
- 50ml polypropylene tube and centrifuge for 50ml tubes
- 250ml phosphate buffered saline (PBS)
- wash bottle
- fluorescent slide scanner
- dilution buffer (PBSB; See Section 10.B)
- wash buffer (PBSI; See Section 10.B; Wash buffer formulations with different salts or detergents can be used to minimize nonspecific binding and associated fluorescent background.)
- fluorescent dye labeled anti-rabbit secondary antibody. Choice of fluorescent dye will depend on the scanner. Commonly used fluorescent dyes are Cy<sup>®</sup>3 and Alexa Fluor<sup>®</sup> 532 for excitation using 532nm laser or Alexa Fluor<sup>®</sup> 633, Alexa Fluor<sup>®</sup> 647 and Cy<sup>®</sup>5 for excitation using 635nm red laser.



**Figure 2. Overview of experimental workflow for creating a custom protein array.**

## 4.A. Expressing HaloTag® Fusion Proteins

### Notes:

1. Depending on your HaloTag® fusion protein, you may need to optimize expression. Solubility, proper folding, and expression levels are some of the factors that may need to be optimized. See reference 4 for information on factors to consider when expressing proteins such as membrane proteins.
2. Five microliters/well of cell-free reaction is added to each well of the HaloLink™ Slide (each slide contains 50 wells). Scale the cell-free reaction volume to accommodate the number of assays you wish to perform.

### 4.A.1. Expression of HaloTag® fusion proteins in the TNT® T7 Quick Coupled Reaction

1. Remove the TNT® T7 Quick Master Mix from storage at -70°C. Rapidly thaw the Master Mix by hand warming and immediately place it on ice. Other components can be thawed at room temperature and stored on ice.
2. Assemble the reaction components in a 0.5ml or 1.5ml microcentrifuge tube as indicated in the table below.

Compound	Volume
TNT® T7 Quick Master Mix	40µl
Methionine	1µl
DNA Template (1µg)	Xµl
Nuclease-Free Water to a final volume of:	50µl

3. Mix gently by pipetting. If necessary, centrifuge briefly to bring the liquid to the bottom of the tube.
4. Incubate the reaction at 30°C for 60–90 minutes.
5. Proceed with capture of the protein onto the HaloLink™ Slide (go to Section 4.C).  
**Note:** We recommend that you first confirm expression of your HaloTag® fusion protein by analyzing an aliquot of the reaction by Western blot or fluorescent labeling (see Section 4.B). Store the remainder of the reaction at -70°C while you confirm expression.
6. For long-term storage, we suggest storing expression reactions at -70°C.  
**Note:** The stability of expressed proteins in frozen reactions will vary based on the particular protein involved (5).

#### 4.A.2. Expression of HaloTag® fusion Protein in the TNT® SP6 High-Yield Wheat Germ Reaction

1. Remove TNT® SP6 High-Yield Wheat Germ Master Mix from storage at -70°C. Rapidly thaw the Master Mix on ice or by hand warming and immediately place on it ice.
2. After the Master Mix has thawed, gently mix several times by pipetting.
3. Assemble the reaction components in a 0.5ml or 1.5ml microcentrifuge tube based on the volumes provided in the table below.

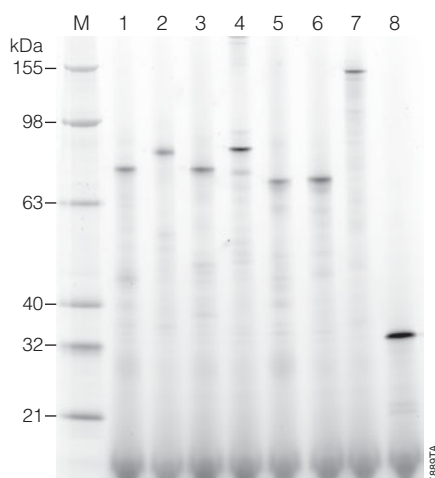
Compound	Volume
TNT® SP6 High-Yield Wheat Germ Master Mix	30µl
DNA Template (8µg)	Xµl
Nuclease-Free Water to a final volume of:	50µl

4. Gently mix after all components are added to the reaction tube.
5. Incubate the translation reaction at 25°C for 2 hours.
6. Proceed with capture of the protein onto the HaloLink™ Slide (go to Section 4.C).  
**Note:** We recommend that you first confirm expression of your HaloTag® fusion protein by analyzing an aliquot of the reaction by Western blot or fluorescent labeling (see Section 4.B). Store the remainder of the reaction at -70°C while you confirm expression.
7. For long-term storage we suggest storing expression reactions at -70°C.  
**Note:** The stability of expressed proteins in frozen reactions will vary based on the particular protein involved (5).

#### 4.B. Confirming Expression of HaloTag<sup>®</sup> Fusion Proteins with an SDS-Gel (optional)

Before creating custom arrays, we recommend that you confirm expression of your HaloTag<sup>®</sup> fusion proteins by labeling with fluorescent HaloTag<sup>®</sup> TMR ligand (not supplied; Cat.# G8251, G8252) followed by SDS-PAGE analysis. Western blotting using the supplied Anti-HaloTag<sup>®</sup> pAb and an appropriately labeled anti-rabbit secondary antibody can also be used to confirm expression. A dilution series of the HaloTag<sup>®</sup> Standard Protein can be run in parallel to estimate the expression level of your HaloTag<sup>®</sup> fusion protein. Typical dilution range of HaloTag<sup>®</sup> Standard Protein for a TNT<sup>®</sup> T7 Quick Reaction is 1.0–10 $\mu$ g/ml (0.016–0.16nmol/ml) for a TNT<sup>®</sup> SP6 High-Yield Wheat Germ Reaction it is 10–100 $\mu$ g/ml (0.16–1.6nmol/ml).

1. Prepare a 500-fold dilution of HaloTag<sup>®</sup> TMR Ligand stock solution (5mM) in PBS for a final concentration of 10 $\mu$ M. Dilute enough ligand to accommodate the number of samples you wish to analyze.
2. Mix 2.0 $\mu$ l of cell-free reaction containing the HaloTag<sup>®</sup> fusion protein with 1.0 $\mu$ l of HaloTag<sup>®</sup> TMR Ligand (10 $\mu$ M).
3. Add 7.0 $\mu$ l of PBS to a final volume of 10.0 $\mu$ l. Incubate at room temperature, protected from light, for 30 minutes.
4. Remove 5.0 $\mu$ l of the reaction, add 5.0 $\mu$ l of 2X SDS gel loading buffer and heat to 70°C for 2 minutes. Separate on an SDS-polyacrylamide gel. Analyze on a fluorescent scanner.
6. Scan the gel using a fluorescent scanner (e.g., Typhoon<sup>®</sup>, Amersham Biosciences, Ltd., 555nm<sub>Ex</sub>; 585nm<sub>Em</sub>). The HaloTag<sup>®</sup> fusion protein will appear as a fluorescent band on the gel (Figure 3). If a dilution series of the HaloTag<sup>®</sup> Standard Protein was used, HaloTag<sup>®</sup> fusion protein expression levels can be estimated by comparing the band intensity with those of the standard samples.



**Figure 3.** HaloTag<sup>®</sup> fusions were expressed in a TNT<sup>®</sup> T7 Quick Reaction, labeled with HaloTag<sup>®</sup>-TMR Ligand and analyzed on a SDS gel. Lane M: molecular weight markers, lane 1: HaloTag<sup>®</sup>-cJun, lane 2: HaloTag<sup>®</sup>-cFos, lane 3: HaloTag<sup>®</sup>-PKA, lane 4: HaloTag<sup>®</sup>-R1 $\alpha$ , lane 5: HaloTag<sup>®</sup>-p53, lane 6: HaloTag<sup>®</sup>-p65, lane 7: HaloTag<sup>®</sup>- $\beta$ Gal, lane 8: HaloTag<sup>®</sup> Protein alone, no fusion (specificity control).

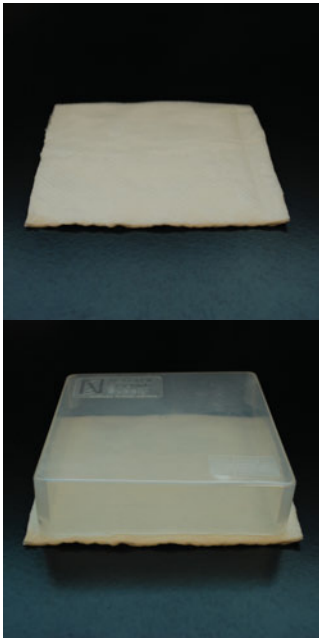
#### 4.C. Attaching the HaloLink™ Array Gasket to the HaloLink™ Slide

**!** **Important!** Read all the instructions before attempting to attach the slides to the gaskets.

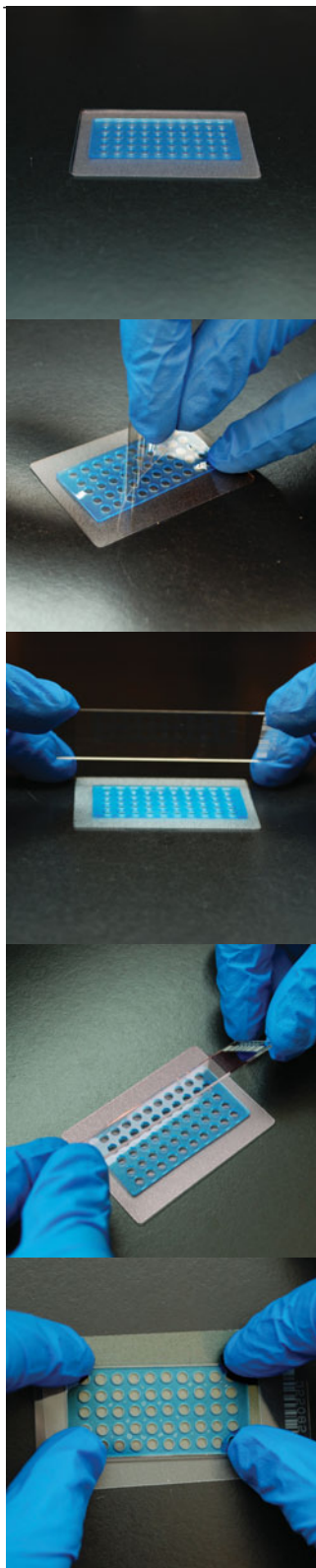
##### Precautions for Handling Slides:

- Work in a dust-free environment and always wear powder-free gloves. Dust and particles from powdered gloves can interfere with scanning.
- Only one side of the slide is coated. The coated side is the one from which you can read the bar code number correctly.
- Do not touch the coated slide surface, and hold slides only at the edges.
- Equilibrate the slide box to ambient temperature before taking a slide out. Any remaining slides can be returned to  $-20^{\circ}\text{C}$  for up to one month.
- Only one side of the HaloLink™ Array Gasket is sticky. The gasket is sandwiched between a clear and an opaque plastic sheet. The sticky side is the side covered with the clear plastic sheet.
- Do not remove an attached gasket and then reattach it to the slide. If the gasket misaligns, use another gasket and slide. Do not reuse a slide on which a gasket has been misaligned.
- All experiments on HaloLink™ Slides must be performed in a humidity chamber. A method for making a humidity chamber is provided in the protocol below.

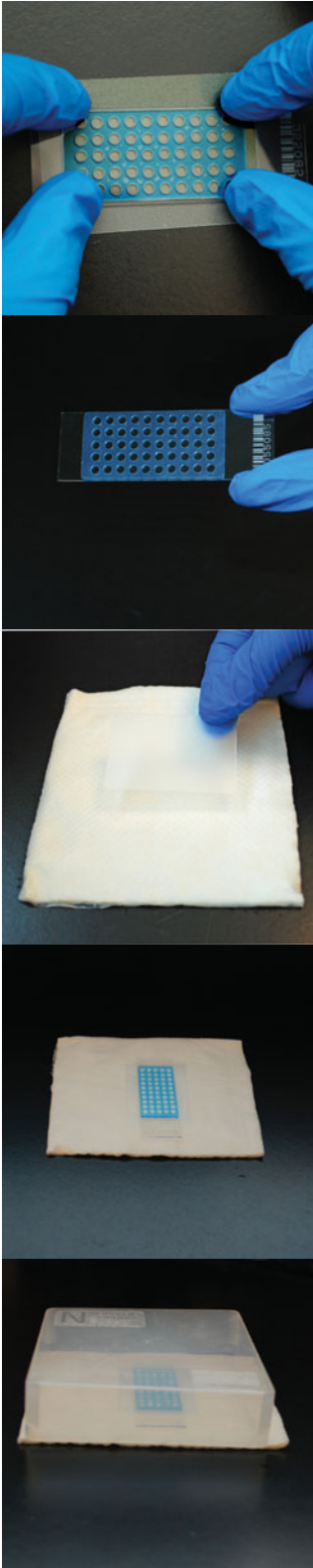
##### Protocol



1. Humidity chambers are needed for HaloLink™ Slide experiments.
  - a. Cut or fold 4 or 5 paper towels into approximately  $5 \times 5$  inch squares and soak them in water. Place them on the lab bench.
  - b. Cover the towels with a plastic lid to form a humidity chamber. (A lid from a 1.0ml pipette tip box works well.) Humidity inside the box will be approximately 90%.



2. Remove the gasket from the bag, and place it on the lab bench with the clear plastic sheet facing up.
  
3. Remove the top clear plastic sheet by holding down the gasket at one corner and gently peeling off the clear plastic sheet with the other hand.
  
4. Remove the HaloLink™ Slide from the box, being sure to hold the slide at the edges. Face the coated side toward the blue gasket. The coated side is the one from which you can read the bar code number correctly.
  
5. Hold the slide at an approximately 45° angle with the long edge of the slide aligned with the edge of the gasket. Make sure that the gasket is in the center of the slide.
  
6. Lower the other edge of the slide carefully over the gasket. The long edge of the gasket should not extend beyond the glass slide.



7. Press the back of the slide and along the edges of the slide to make a water-tight seal between the gasket and the glass.
8. Pick up the glass slide with the gasket attached.
9. Look at the slide from the back and make sure no air bubbles are trapped. Air bubbles will cause leaks. Trapped air bubbles can be removed by placing the slide gasket-side-down on the opaque plastic sheet and gently pressing down on the back of the glass slide.
10. Place the opaque plastic sheet on the wet paper towel in the humidified chamber.
11. Place the HaloLink™ Slide on the plastic sheet, gasket-side-up.
12. Cover with a plastic lid.

#### 4.D. Immobilization of HaloTag® Fusion Proteins on HaloLink™ Slides

A custom protein array is made by adding the cell-free reaction containing the expressed HaloTag® fusion protein into individual wells on the slide. The HaloTag® fusion protein binds to the HaloLink™ Slide without any need for prior purification.

##### Controls

1. **Capture Controls** should be performed to confirm capture of HaloTag® fusion proteins on the slide. Dedicate one replicate well as a control for each fusion protein being analyzed. Capture of HaloTag® fusion proteins can be confirmed by probing with the supplied Anti-HaloTag® Antibody, followed by detection with a labeled anti-rabbit secondary antibody (see Section 4.E for details).
2. **Normalization Control:** The HaloTag® Standard Protein (provided with Cat.# G6140 and G6180) can be used to normalize for variations in expression level and capture efficiency between different HaloTag® fusion proteins. A concentration series of the HaloTag® Standard Protein can be used to estimate the amount of HaloTag® fusion protein expression and capture for normalization purposes (see Section 4.F for details).
3. **Specificity Control:** HaloTag® Protein expressed without an attached fusion can serve as a control to evaluate specificity of interactions in downstream applications. A protocol for creating a construct that expresses only HaloTag® Protein can be obtained by calling technical services.
4. Additional controls may be needed for downstream applications (e.g., protein:protein or protein:DNA interaction studies). We suggest including an **Extract Control** (extract that does not contain expressed protein) to evaluate nonspecific binding of proteins in the translation lysate. We also suggest including a **PBSB-only control** to evaluate background, plus appropriate known binding partners as positive controls.




##### Warning

- Maintaining the conformational integrity of captured proteins is essential for successful downstream applications using custom arrays. The characteristics of each HaloTag® fusion protein will depend on the individual protein that is fused to the HaloTag® protein. You will need to optimize the conditions for your individual custom array. For example, proteins subject to denaturation during slide washing and drying may be stabilized by adding sucrose, trehalose or glycerol to the wash buffer (5).
- We recommend making the custom array and performing the downstream application on the same day. Long-term stability of custom arrays depends upon the characteristics of the bound protein fusion and will need to be determined for each custom array (5).
- The HaloLink™ Slide is coated with a unique hydrogel that can be damaged if it is scratched by pipette tips. Use extreme care when pipetting samples into the wells. During pipetting, keep the pipette tip just above the slide surface. Support the pipette tip on the gasket edge, and keep it above the slide surface.

## Protocol

1. Prepare samples for HaloLink™ Slide experiment and apply to wells.
  - a. If you have frozen your cell-free expression reactions, bring them to room temperature before beginning this procedure. Pipet 5.0µl of cell-free reaction (from Section 4.A.1 or 4.A.2) containing the HaloTag® fusion proteins into the wells. We recommend preparing replicate wells for each sample. One replicate well may be used to confirm HaloTag® immobilization (Section 4.E). Additional replicate wells may be necessary for downstream applications.
  - b. If you are planning to normalize the data, prepare a concentration series of HaloTag® Standard Protein (see Section 4.F) in parallel.
  - c. Include appropriate controls (e.g., extract control, PBSB-only control). You may also need to design additional controls specific for your experiment.
2. Incubate the slide in the humidity chamber for 1 hour at room temperature.
3. Following incubation, hold the slide at a 45° angle, and wash the wells with a gentle stream of wash buffer (PBSI) using a wash bottle.

 **Note:** Do not forcefully squirt the wash buffer directly onto the glass surface because you may damage the coating.
4. Fill a 50ml polypropylene tube with 40ml of PBSI. Place the slide into the 50ml tube, cap the tube, and wash the slide by inverting the tube 10 times.
5. Drain the wash buffer and dry the slide by centrifuging the tube for 3 minutes at 350 × g at room temperature.
6. Take the slide out of the tube using plastic tweezers (do not scratch the surface), and **immediately** place it in the humidity chamber. Overdrying the slide may lead to protein denaturation.
7. The slide is now ready for use in your downstream application (Sections 5 and 6 provide guidelines for protein:protein and protein:DNA interaction applications, respectively).

#### 4.E. Confirming Capture of HaloTag® Fusion Proteins on HaloLink™ Slides (optional)

We recommend that you confirm binding of your HaloTag® fusion proteins to the HaloLink™ slide. Use one replicate well from Section 4.D for this step. **This step should be performed at the same time as your chosen downstream application.**

1. Dilute the Anti-HaloTag® pAb 1:200 in PBSB to give a final concentration of 5.0µg/ml. You will use 8µl of this solution per well. Prepare sufficient volume for the number of wells used.
2. Add 8.0µl/well of Anti-HaloTag® pAb to wells containing HaloTag® fusion proteins. Perform no-protein control by adding 8.0µl of the diluted antibody solution to a well containing no HaloTag® protein. Perform an extract control by adding diluted antibody solution to wells containing extract that does not contain expressed protein.
3. Incubate for 1 hour at room temperature in the humidified chamber.
4. Following incubation, hold the slide at a 45° angle and, using a wash bottle, wash the wells with a gentle stream of wash buffer (PBSI).



**Note:** Do not forcefully squirt wash buffer directly onto the glass surface because this may damage the coating.

5. Fill a 50ml polypropylene tube with 40ml of PBSI. Place the slide into the 50ml tube, cap the tube and wash the slide by inverting the tube 10 times.
6. Drain the wash buffer, and dry the slide by centrifuging the tube for 3 minutes at 350 × g at room temperature.
7. Take the slide out of the tube using plastic tweezers, and immediately place it in the humidity chamber.
8. Dilute fluorescent-dye-labeled, anti-rabbit secondary antibody in PBSB. 10µg/ml is a good starting concentration; however, the concentration may have to be optimized for individual secondary antibodies.
9. Add 8.0µl of the diluted anti-rabbit secondary antibody to wells incubated with Anti-HaloTag® pAb.  
**Note:** Concentration and volume of secondary antibody will vary. A good starting point is 8.0µl of a 10µg/ml secondary antibody.
10. Incubate for 1 hour in the humidity chamber, protected from light (cover the chamber in foil).
11. Wash the slides as described in Steps 4.
12. Remove the gasket using tweezers. Once removed, the gasket can not be reattached.
13. Wash and spin dry the slides as described in Steps 5 and 6.
14. Scan the slides. See Section 7 for details. The stability of the slides will depend on the dye used with the secondary antibody. Follow the recommendations of the dye manufacturer. In our experience, slides that are protected from light may be stored overnight before scanning.

#### 4.F. Using HaloTag® Standard Protein for Normalization (optional)

We recommend that you normalize your experiment to account for differences in expression and capture of various HaloTag® fusion proteins. **This experiment can be done on the same slide used for the capture of HaloTag® fusion proteins and downstream application or on a separate slide depending on the number of samples being processed.**

1. Prepare a dilution series of HaloTag® Standard Protein in PBSB. A range of 0.1–10µg/ml is a good starting point. Use PBSB alone containing no HaloTag® Standard Protein as a blank.
2. Add 5.0µl of each concentration of HaloTag® Standard Protein into individual wells on the HaloLink™ Slide. For the blank, use 5.0µl of PBSB.
3. Dilute the cell-free expression reaction in PBSB to bring the concentration of the HaloTag® fusion proteins within the concentration range of the HaloTag® Standard Protein (0.1–10.0µg/ml). Typical expression level of HaloTag® fusion protein is 1.0–10.0µg/ml in TNT® T7 Quick Reaction and 10–100µg/ml in TNT® SP6 High-Yield Wheat Germ Reaction.

**Note:** Run on an SDS gel with the TMR Ligand for detection or make 2 to 3 dilutions to be sure that at least one falls within the calibration range,

4. Add 5.0µl of each diluted cell-free expression reaction sample into a separate well on the HaloLink™ Slide.
5. Incubate the slide in the humidity chamber for 1 hour at room temperature.
6. Following incubation, hold the slide at a 45° angle and, using a wash bottle, wash the wells with a gentle stream of wash buffer (PBSI).



**Note:** Do not forcefully squirt wash buffer directly on the glass surface because this may damage the coating.

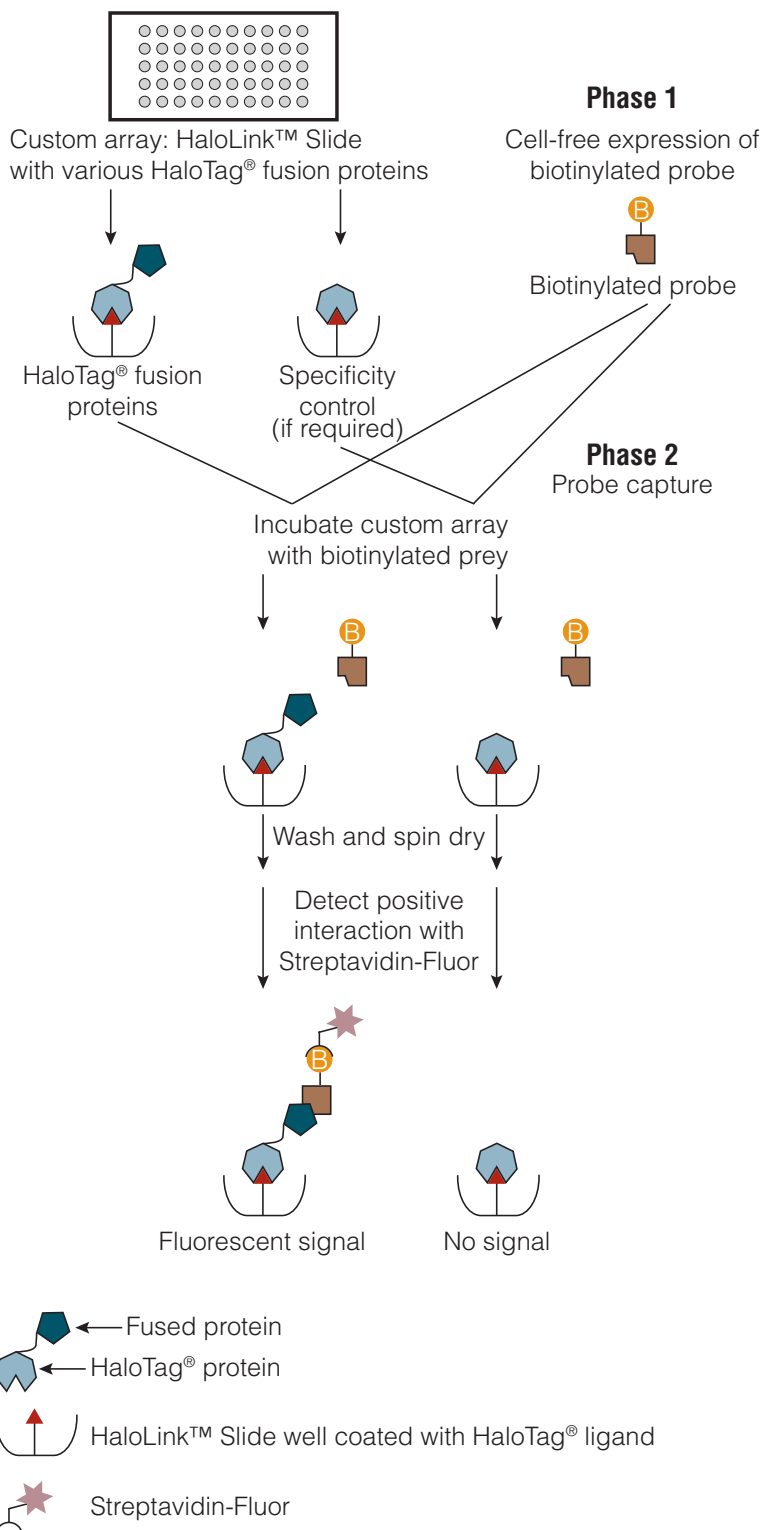
7. Fill a 50ml polypropylene tube with 40ml of PBSI. Place the slide into the 50ml polypropylene tube, cap the tube and wash the slide by inverting the tube 10 times.
8. Drain the wash buffer, and dry the slide by centrifuging the tube for 3 minutes at 350 × g at room temperature.
9. Take the slide out of the tube using plastic tweezers, and immediately place it in the humidity chamber.
10. Dilute the Anti-HaloTag® pAb 1:200 in PBSB to give a final concentration of 5.0µg/ml. You will use 8µl of this solution per well. Prepare sufficient volume for the number of wells used.
11. Add 8.0µl/well of Anti-HaloTag® pAb to wells containing HaloTag® fusion proteins. As a no-protein control, add 8.0µl of the diluted antibody solution to wells containing no HaloTag® protein. Perform extract control by adding diluted antibody to wells containing extract that does not contain expressed protein.
12. Incubate for 1 hour at room temperature in the humidity chamber.
13. Wash and spin-dry the slides as described in Steps 6–9.

14. Dilute fluorescent-dye-labeled, anti-rabbit secondary antibody in PBSB. 10.0µg/ml is a good starting concentration; however, the concentration may have to be optimized for individual secondary antibodies. You will use 8µl of this solution per well. Prepare sufficient volume for the number of wells used.
15. Add 8.0µl of the diluted anti-rabbit secondary antibody to the wells incubated with Anti-HaloTag® antibody.
16. Incubate for 1 hour protected from light at room temperature in the humidity chamber (e.g, cover the chamber with foil).
17. Wash the slides as described in Step 6.
18. Carefully remove the gasket using tweezers. Once removed, the gasket cannot be reattached.
19. Wash and spin dry the slides as described in Steps 7 and 8.
19. Scan the slides (see Section 7 for details). The stability of the slides will depend on the dye used with the secondary antibody. Follow the recommendations of the dye manufacturer. In our experience, slides protected from light may be stored overnight before scanning.

## 5. Guidelines for Protein:Protein Interaction Analysis

Custom arrays containing a panel of HaloTag® fusion proteins can be interrogated with a probe protein to identify key protein:protein interactions. Probe proteins can be obtained from several sources including cell-free systems, purified proteins and cell lysates. This manual contains guidelines for performing protein:protein interaction studies using a probe protein expressed in a cell-free system (Figure 4). The example protocol provided is divided into two phases: 1) the probe protein is expressed in a cell-free expression system and labeled in vitro with biotin during expression; and 2) the biotinylated probe protein is added to the custom array on the HaloLink™ Slide, and protein:protein interactions are detected by adding fluorescently labeled streptavidin. Optimization of protein:protein interaction and washing conditions may be required for your specific HaloTag® fusion and probe protein pair (5).

**Phase 1: Expression of Probe Protein.** The probe is the protein used to interrogate the array slide containing the expressed HaloTag® fusion protein. Probe protein is expressed in a cell-free system and labeled by incorporating biotinylated lysine residues into the nascent protein during in vitro translation using Transcend™ Non-Radioactive Translation Detection System (Cat.# L5080, L5070). See Technical Bulletin #TB182 for instructions for using this system (available online at: [www.promega.com/tbs/](http://www.promega.com/tbs/))



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**Figure 4. Schematic of HaloTag® fusion and probe protein interaction analysis on HaloLink™ Slides. There are several methods for expressing, binding and detecting probe protein interactions. This schematic uses expression and labeling of probe protein in a cell-free system for illustration purposes.**

## 5. Guidelines for Protein:Protein Interaction Analysis (continued)

### Phase 2: Capture of Probe Protein

Biotinylated probe protein is added to the custom protein array created on HaloLink™ Slides. Positive HaloTag® interaction is detected using fluorescently labeled streptavidin. Depending on the protein pair involved, different degrees of optimization of binding conditions may be required. The addition of cofactors, salts or detergents may be required for certain protein interactions. It is important to consider the following points before designing any protein:protein interaction experiment:

- a. Affinity of interaction
- b. Concentration of probe protein
- c. Functional conformation of HaloTag® fusion protein and probe protein
- d. Detection method used for interactions

Appropriate controls should be included in the experiment, including Extract Controls, Specificity Controls, and Normalization Controls (see Section 4.D).

### 5.A. Expression and Cotranslational Labeling of Probe Protein in a Cell-Free System

#### Materials to be Supplied by the User

- custom protein array on HaloLink™ Slide (from Section 4.D)
- DNA template for expressing probe protein
- Transcend™ Non-Radioactive Translation Detection System (Cat.# L5080, L5070)
- cell-free expression system: TNT® T7 Quick or TNT® SP6 High-Yield Wheat Germ System
- fluorescent dye-labeled streptavidin. (Choice of fluorescent dye will depend on the scanner.)

Commonly used fluorescent dyes are Cy®3 and Alexa Fluor® 532 for excitation using a 532nm laser or Alexa Fluor® 633, Alexa Fluor® 647 and Cy®5 for excitation using a 635nm red laser.

**Note:** 5µl/well of a cell-free reaction containing probe protein is added to each well of the custom HaloLink™ Slide. Scale up the reaction volume to accommodate the number of replicates and assays you wish to perform.

#### 5.A.1. Expression of Probe Protein in the TNT® T7 Quick Reaction

1. Remove the TNT® T7 Quick Master Mix from storage at -70°C. Rapidly thaw the Master Mix by hand-warming and immediately place on ice. Other components can be thawed at room temperature and stored on ice.
2. Assemble the reaction components in a 0.5ml or 1.5ml microcentrifuge tube based on the volumes provided below.

Compound	Volume
TNT® T7 Quick Master Mix	40µl
Methionine	1µl
DNA Template (0.2–2µg)	Xµl
Transcend™ tRNA	<u>1–2µl</u>
Nuclease-Free Water to a final volume of:	50µl

## 5.A. Expression and Cotranslational Labeling of Probe Protein in a Cell-Free System (continued)

### 5.A.1. Expression of Probe Protein in the TNT® T7 Quick Reaction (continued)

**Note:** 5µl/well of cell-free reaction containing probe protein is added to each well of the custom HaloLink™ Array Slide. Scale up the reaction volume to accommodate the number of replicates and assays you wish to perform.

3. After adding of all the components, gently mix by pipetting. If necessary, centrifuge briefly to return the reaction to the bottom of the tube.
4. Incubate the reaction at 30°C for 60–90 minutes.
5. Optional: Confirm the expression for biotin labeled probe protein using Western blot (see *Transcend™ Non-Radioactive Translation Detection Systems Technical Bulletin #TB182*).

### 5.A.2. Expression of Probe Protein in the TNT® SP6 High-Yield Wheat Germ Reaction (Phase I)

1. Remove the TNT® SP6 High-Yield Wheat Germ Master Mix from storage at -70°C. Rapidly thaw the Master Mix on ice or by hand-warming and immediately place on ice.
2. After the Master Mix has thawed, gently mix several times with a pipette tip or by pipetting.
3. Assemble the reaction components in a 0.5ml or 1.5ml microcentrifuge tube based on the volumes provided below.

Compound	Volume
TNT® SP6 High-Yield Wheat Germ Master Mix	30µl
DNA Template (2–12µg)	Xµl
Transcend™ tRNA	<u>1–2µl</u>
Nuclease-Free Water to a final volume of:	50µl

**Note:** 5µl/well of a cell-free reaction containing probe protein is added to each well of the HaloLink™ Slide. Scale the reaction volume to accommodate the number of replicates and assays you wish to perform.

4. Mix gently after all components are added to the reaction tube.
5. Incubate the translation reaction at 25°C for 2 hours.
6. Optional: Confirm the expression for biotin-labeled prey protein using Western blot (see *Transcend™ Non-Radioactive Translation Detection Systems Technical Bulletin #TB182*).

## 5.B. Protein:Protein Interaction Analysis

In phase 2, the biotinylated probe protein is incubated with the custom protein array created as described in Section 4.D.

1. Add 5.0µl of cell-free expression reaction containing probe protein to the wells containing immobilized HaloTag® fusion protein. Add probe to appropriate control wells (Section 4.D).
2. Incubate the slide in the humidity chamber for 1 hour at room temperature.
3. Following incubation, hold the slide at a 45° angle, and wash the wells with a gentle stream of wash buffer (PBSI) using a wash bottle. **Note:** Do not forcefully squirt wash buffer directly onto the glass surface because this may damage the coating.
4. Fill a 50ml polypropylene tube with 40ml of wash buffer. Place the slide into the tube, cap it, and wash the slide by inverting the tube 10 times.
5. Drain the wash buffer, and dry the slide by centrifuging the tube for 3 minutes at 350 × g at room temperature.
6. Take the slide out of the tube using plastic tweezers, and immediately place it in the humidity chamber.
7. Detection Reagent: Fluorescent-dye-labeled streptavidin is used for detecting protein:protein interactions. The optimal concentration will have to be determined by the user; however, a concentration of 10.0 µg/ml labeled streptavidin in PBSB is a good starting point.
8. Add 8.0µl of dye-labeled streptavidin to each well containing immobilized HaloTag® bait and negative controls.
9. Incubate for 1 hour protected from light at room temperature in the humidity chamber (cover the chamber with foil).
10. Wash the slides as described in Step 3, and remove the gasket using tweezers.
11. Place the slide into a 50ml tube, and fill it with 40ml of wash buffer. Cap the tube and wash the slide by inverting the tube 10 times.
12. Drain the wash buffer, and dry the slide by centrifuging for 3 minutes at 350 × g at room temperature.
13. Scan the slide (see Section 7 for details).

## 6. Guidelines for Protein:DNA Interaction Analysis

Custom arrays containing panels of HaloTag® fusion proteins can be queried with DNA probes to identify key protein:DNA interactions.

Key points to consider before performing protein:DNA interaction analysis are:

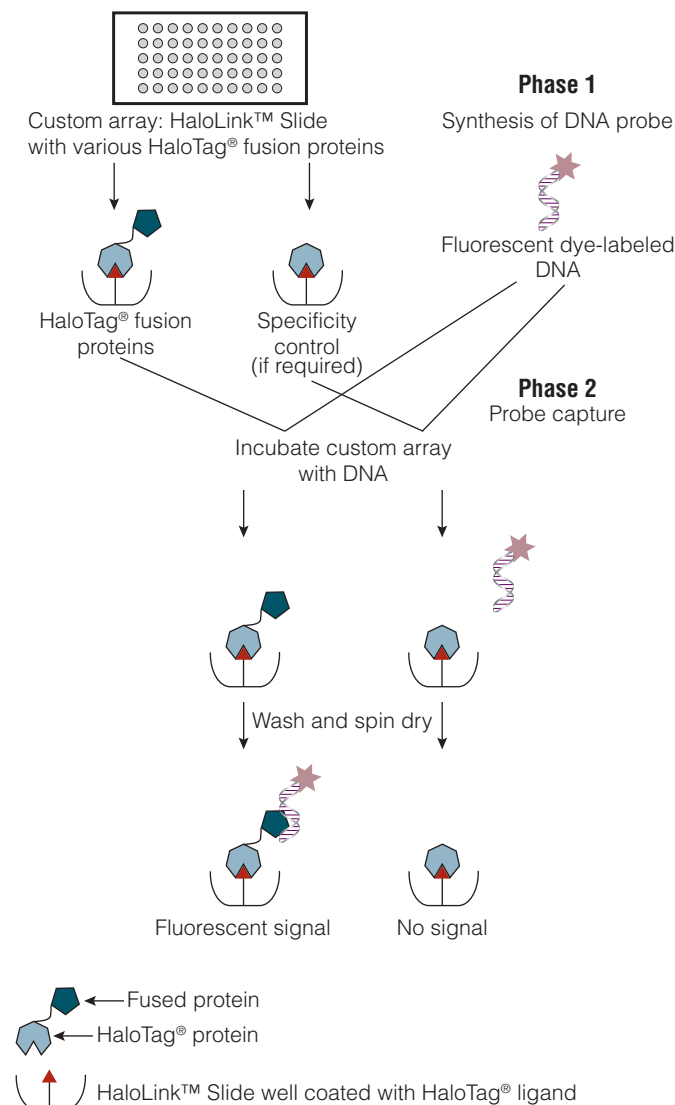
- a. Affinity of interaction
- b. Concentration of probe DNA
- c. Functional conformation of HaloTag® fusion proteins and DNA probe
- d. Method used to detect positive interactions

## 6. Guidelines for Protein:DNA Interaction Analysis (continued)

A schematic for performing protein:DNA interaction analysis on HaloLink™ Slides is shown in Figure 5. The protocol is divided into two phases:

**Phase 1:** 5' Labeling of the DNA probe with fluorescent dye. The 5' end-labeled DNA used for the probe should be of the highest purity. 5' end-labeled nonspecific DNA can be prepared as a specificity control for HaloTag® fusion protein:probe interaction. Starting concentration of DNA will depend on specific protein:DNA interaction. For an initial experiment, we suggest running a titration series to optimize the DNA concentration. Add 5-10µl/well of labeled DNA probe to each well of the HaloLink™ Slide. Scale the reaction volume to accommodate the number of assays you wish to perform.

**Phase 2:** DNA Capture. The DNA probe is added to custom arrays on the HaloLink™ Slide. Appropriate controls should be included in the experiment (e.g., extract control, specificity control and normalization controls). See Section 4.D for more information.



**Figure 5. Schematic of DNA:protein interaction analysis on HaloLink™ Slides.**

## DNA Capture on the HaloLink™ Slide

This protocol uses DNA labeled with fluorescent dye to probe proteins immobilized on HaloLink™ slides. The protocol provides general guidelines for protein:DNA interaction assays. Optimization of conditions for your specific protein:DNA interaction may be required.

### Materials to be Supplied by the User

- DNA labeled with fluorescent dye at the 5' end. Labeled DNA should be of highest purity.
  - custom protein array on a HaloLink™ Slide (Section 4.D)
1. Dilute the DNA in PBS. The final DNA concentration used will depend on the individual protein-DNA interaction and will have to be optimized.  
**Note:** As an example, in an experiment with a HaloTag®-p65 fusion, 100fmol/μl of labeled DNA proved to be optimal. For the competition experiments for p65 binding, cold DNA was titrated into 100fmol/μl of labeled DNA at the following ratios (labeled to unlabeled) 1:0, 1:0.1, 1:0.5, 1:1, 1:3, 1:6, 1:20, 1:40, 1:80.
  2. Add 5.0μl of labeled DNA to wells containing immobilized HaloTag® fusion protein.
  3. Incubate the slide at room temperature for 1 hour protected from light in the humidity chamber (cover the chamber with foil).
  4. Following incubation, hold the slide at a 45° angle and, using a wash bottle, wash the wells with a gentle stream of wash buffer (PBSI). Do not forcefully squirt wash buffer directly on the glass surface as it may damage the coating.
  5. Remove the gasket using tweezers.
  6. Fill a 50ml polypropylene tube with 40ml of PBSI. Place slide into the tube, cap it, and wash the slide by inverting the tube 10 times.
  7. Drain the wash buffer, and dry the slide by centrifuging the tube for 3 minutes at 350 × g at room temperature.
  8. Scan the slide (see Section 7 for details).

## 7. Scanning and Data Analysis of HaloLink™ Slides

### 7.A. Scanning

Suggestions below for scanning and data analysis of HaloLink™ Slides are made based on our use of a Genepix® 4000B scanner. However, any other conventional microarray scanner can be used, provided that it can accept 1 x 3-inch slides and is compatible with the fluorescent dye used on the slide.

1. Insert the slides face-down in the scanner.
2. Adjust the laser and photomultiplier tube (PMT) setting based on the fluorescent dye used in the experiment.
3. If the dye is photosensitive, use a lower laser power to avoid photobleaching.

4. We recommend doing a preview scan to identify whether the detector and laser settings are set to maximize dynamic range.
5. Scan the slide at 10 $\mu$ m resolution.
6. For data analysis, use the scanner software to calculate the median fluorescence intensity from a 2.0mm diameter area in the center of each well.

## 7.B. Data Analysis

### Data Normalization

To obtain reproducible results from protein array experiments, you need to control for experimental and random variations. Experimental factors that will introduce variability in HaloLink™ Protein Array include:

1. Variations from expression and capture of HaloTag® fusion proteins:
  - a. Differences in expression levels of various HaloTag® fusion proteins
  - b. Day-to-day and batch-to-batch variation in expression level of a single HaloTag® fusion protein
  - c. Differences in protein expression based on the choice of protein expression systems
  - d. Variation in capture efficiency of different HaloTag® fusion proteins
2. Variations coming from specific probe binding to HaloLink™ Protein Arrays
  - a. Source of probe generation; for example, protein probes can be expressed in cell-free systems (Section 5) or in cells or purified protein.
  - b. Detection method
  - c. Binding affinity

Random variations may be introduced by the operator and may depend on reagents (wash buffer, slide processing steps) as well as time and place of experiments.

HaloTag® Standard Protein can be used to normalize for the difference in expression and capture levels of various HaloTag® fusion proteins (Sections 4.B. and 4.F). For normalization as described in Section 4.F., prepare a standard curve by plotting the blank corrected median RFU values against concentration in  $\mu$ g/ml for the HaloTag® Standard Protein. Use the standard curve to calculate the concentration of the diluted HaloTag® Fusion proteins. Multiply the concentration determined from the standard curve by dilution factor to get the expression level in the cell-free expression system. To normalize the results from downstream application for variations in expression level and capture, fluorescent signal from protein-interaction studies can be divided by amount of protein calculated from standard curve (6).

There are several excellent articles on normalization of microarray data that can be adapted for HaloLink™ Protein Array Systems (7-9).

## 8. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: [www.promega.com](http://www.promega.com). E-mail: [techserv@promega.com](mailto:techserv@promega.com)

Symptoms	Causes and Comments
HaloTag <sup>®</sup> fusion protein not detected with SDS gel using HaloTag <sup>®</sup> TMR Ligand	DNA quality was poor, or wrong DNA quantity was used. Use only highly purified plasmid DNA. We strongly recommend that you refer to the <i>TNT<sup>®</sup> T7 Quick Coupled Transcription/ Translation Systems Technical Manual #TM045</i> or the <i>TNT<sup>®</sup> SP6 High-Yield Wheat Germ Protein Expression System Technical Manual # TM282</i> for specific details on use of these expression systems.
	Fluorescent scanner does not have appropriate filters and/or sensitivity. Use a fluorescent scanner with appropriate sensitivity (e.g., Typhoon <sup>®</sup> , GE Healthcare). For scanners with lower sensitivity, sample volume may need to be increased.
	Samples may have been overheated. Reduce the denaturation temperature to 60°C for 5–10 minutes.
Gasket not sticking to the glass	Only one side of the gasket is sticky. Make sure the sticky side is toward the glass slide.
Gasket came off of the glass during centrifuging in 50ml tube	Make sure the gasket does not extend beyond the edges of the slide.
No signal or weak signal with HaloTag <sup>®</sup> Standard Protein	Wrong secondary antibody used. Use an anti-rabbit secondary antibody labeled with fluorescent dye.
	Incorrect scanner setting. Make sure the laser and photomultiplier tube (PMT) setting matches the fluorescent dye used for detection.
	Photobleaching. Avoid prolonged exposure of the fluorescent-dye-labeled secondary antibody to light. Avoid multiple scans of the slide.
No signal or weak signal when HaloTag <sup>®</sup> -fusion protein is probed using Anti-HaloTag <sup>®</sup> pAb	HaloTag <sup>®</sup> -fusion protein not expressed or poorly expressed. Confirm HaloTag <sup>®</sup> -fusion protein expression by HaloTag <sup>®</sup> -TMR labeling or Western blot.
	Assay not performed correctly. Confirm that HaloTag <sup>®</sup> Standard protein gives a positive signal.
	Incorrect scanner setting. Make sure the laser and photomultiplier (PMT) setting matches the fluorescent dye used for detection.
Scan shows dark area or areas with very bright spots within the wells	Scratches on slide surface. The slide is coated with a thin hydrogel layer that can be scratched during processing (pipetting, washing, centrifuging). Be careful not to touch the slide surface during the assay and downstream processing steps.
No signal after protein:protein interaction experiment	No or low expression of probe protein. Confirm expression of the probe protein by Western blot. Optimize conditions to maximize expression of probe protein.
	No incorporation of biotin during prey protein expression. Biotin incorporation is protein dependent. Confirm biotin incorporation by Western blot.
	Weakly interacting HaloTag <sup>®</sup> fusion and probe pairs may dissociate during a multistep assay. Optimize washing and incubation steps to minimize dissociation.

## 8. Troubleshooting (continued)

Symptoms	Causes and Comments
No signal after protein:DNA interaction analysis	Low incorporation of fluorescent dye in DNA. Check incorporation of the fluorescent dye.
	Increase the concentration of the starting DNA solution.
	Binding and wash buffers may need to be optimized for each protein:DNA pair.
	Weakly interacting protein:DNA pairs may dissociate during a multistep assay. Optimize washing and incubation steps to minimize dissociation.
High background; high background fluorescence	Insufficient washing. Follow the proper washing procedure to minimize background.  Nonspecific binding. HaloTag <sup>®</sup> protein binding to the HaloLink <sup>™</sup> Slide is covalent. Hence nonspecific binding during capture of HaloTag <sup>®</sup> fusion protein from a cell-free system can be reduced by increasing the stringency of washing. Add detergent and/or higher concentration of salt to the wash buffer. Increase the number or duration of washes.

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## 10. Appendix

### 10.A. HaloTag® Vectors

The desired protein coding sequence must be cloned into a HaloTag® vector according to the cloning recommendations supplied with that vector. We recommend using the HaloTag® Flexi® Vectors. The pFN19A or pFN19K can be used to prepare a construct that expresses HaloTag® Protein alone. Contact technical services for more information. Vectors suitable for use with this system include the following:

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

#### Compatibility with Protein Expression Systems

All of the above vectors include both T7 and SP6 promoters and so can be used to express proteins in either the TnT® T7 Quick Coupled Transcription Translation System or the TnT® SP6 High-Yield Wheat Germ Protein Expression System.

#### Vector Nomenclature

The designation “pFN” indicates an N-terminal fusion vector, and “pFC” indicates a C-terminal fusion. The designation “A” or “K” in a vector name indicates that the vector carries ampicillin or kanamycin resistance.

#### Cloning Strategies

Fusing HaloTag® protein at the C- or N- terminal to the protein of interest may impact the expression of HaloTag® fusion proteins, as well as the downstream application of custom arrays created using these fusions. Please refer to the *Flexi® Vector Systems Technical Manual # TM254* ([www.promega.com/tbs/tm254/tm254.html](http://www.promega.com/tbs/tm254/tm254.html)) for detailed instructions on cloning into the HaloTag® Flexi Vectors.

### 10.B. Composition of Buffers and Solutions

#### 1X PBS

1.9mM	sodium phosphate monobasic
8.1mM	sodium phosphate dibasic
150mM	sodium chloride

Adjust pH to 7.4

#### 4X SDS Gel Loading Buffer

0.24mM	Tris-HCl (pH 6.8)
3mM	bromophenol blue
50.4%	glycerol
0.4M	dithiothreitol
2%	SDS

#### PBSB

1X	PBS
10mg/ml	BSA

#### PBSI

1X	PBS
0.05%	IGEPAL® CA-630 (Sigma Cat# I 3021 or I 8896)

Due to the solubility properties of IGEPAL® CA-630, we recommend that you first prepare a 10% stock solution in water and use this stock solution to prepare the PBSI wash buffer.

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**10.C. Related Products**

<b>Product</b>	<b>Cat.#</b>
Anti-HaloTag® pAb	G9281
HaloTag® Standard Protein	G4491
HaloTag® TMR Ligand	G8252
TNT® T7 Quick Coupled Transcription/Translation System*	L1170
TNT® T7 Quick Coupled Transcription/Translation System, Trial Size*	L1171
TNT® SP6 High-Yield Wheat Germ Protein Expression System (4 × 300µl)	L3260
TNT® SP6 High-Yield Wheat Germ Protein Expression System (1 × 300µl)	L3261
Flexi® Enzyme Blend (Sgf1 and PmeI)	R1851

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<sup>(b)</sup>U.S. Pat. No. 7,429,472 and other patents pending.

<sup>(c)</sup>U.S. Pat. Nos. 5,324,637 and 5 492,817, Australian Pat. No. 660329, Japanese Pat. No. 2904583 and other patents pending.

<sup>(d)</sup>For Research Use. Any use of the product for diagnostics requiring clearance or approval by the FDA may require a license under Mayo Clinic U.S. Pat. Nos. 6,027,913 and 6,361,949.

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