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Use of HaloTag® Technology in Microarray-based Assays for Antibody Profiling, Small Molecule Screening and Immunogenicity Screens

ABSTRACT

Recently, several methods have been published that exploit features of HaloTag® technology perform array-based screening for immunogenic bacterial proteins, to screen denatured proteins for novel epitopes, and to interrogate small molecule inhibitors for protein interactions. This article highlights the advantages of the HaloTag® technology for these applications and briefly summarizes the details of each method.

HaloTag® Technology uses a protein fusion tag (HaloTag®) based on a small monomeric halophilic bacterial hydrolase. This modified hydrolase enzyme covalently binds chloralkane substrates, resulting in an irreversible attachment. The binding is optimized for fast kinetics and stability, and because no homolog exists in mammalian cells, background is minimal. HaloTag®-based tools can be used for virtually every step in analyzing protein function—from cloning to cellular localization to purification and identification of protein interactions. These tools include HaloTag® surfaces and resins for creating protein arrays, purifying proteins and identifying interacting proteins.

For cloning, users have a choice of HaloTag® Vectors designed for mammalian or bacterial cell expression of fusion proteins. Choices include Multi-cloning site vectors for either mammalian (e.g., pHTN HaloTag®-CMV-neo Vector; Cat.# G7721) or bacterial (e.g., pH6HTN His6HaloTag® T7 Vector; Cat.# G7971) expression, and a range of HaloTag® Flexi® Vectors that allow directional cloning and simplified transfer between vectors. Additionally, nearly 9,200 ORFs representing all major classes of proteins are available as N-terminal fusions in the pFN21A Flexi® Vector as part of a collaboration between Promega and the Kazusa DNA Research Institute. Determining whether your ORF of interest has been cloned and is available is as easy as searching the website www.promega.com/fmg with a name, keyword or sequence.

Advantages of HaloTag® Technology for Microarray-Based Applications

The HaloTag® protein has unique advantages that simplify microarray-based screening. This article reviews three recent papers describing new HaloTag®-based methods that highlight the benefits of the technology for a range of different proteomics applications. The particular advantages of HaloTag® highlighted in these papers are:

1. The covalent attachment of HaloTag® to its ligands, and consequent strong binding of proteins to arrays, means that signals are stronger and less protein is lost during washes. The covalent attachment can also survive denaturation of the attached proteins on the slide, facilitating screening for antibodies to hidden epitopes.
2. The ability to attach different fluorophores to HaloTag®-labeled proteins allows multiplexing of assays on the same array, giving more information per assay, and saving time and reagents.

A Method for Screening Bacterial Antigens

The paper *Microarray-based Method for Screening of Immunogenic Proteins from Bacteria* (1) , published in the March issue of the *Journal of Nanobiotechnology*, describes a HaloTag® array-based technique for screening bacterial proteins for immunogenicity. The detection of potential immunogenic bacterial proteins is important for vaccine development, for the identification of disease biomarkers with diagnostic applications, and for understanding bacterial pathogenesis. Conventional analysis methods involve probing nitrocellulose-bound expression libraries with polyclonal antisera, or screening whole proteome lysates after 2D gel electrophoresis. One problem with nitrocellulose-based methods for detection of bacterial antigens is potentially high levels of false positives due to cross-reactivity of the polyclonal antibodies with proteins from the expression host.

The authors developed and tested a HaloTag®-based method that eliminated this cross-reactivity problem and allowed rapid screening of whole cell lysates without requiring a protein purification step. They used known immunogenic proteins of *Camplylobacter jejuni* to illustrate the utility of their method.
C. jejuni is a pathogen that causes gastroenteritis. There is a need to identify *C. jejuni* immunogens in order to develop tests to detect bacterial contamination of food sources, and for use in rapid tests for point-of-care diagnosis. The authors cloned four known *C. jejuni* immunogenic proteins and one protein of unknown immunogenicity into the pFN18A Flexi® Vector, which attaches HaloTag® to the N-terminus of the protein of interest. The cloned proteins were expressed in *E. coli* KRX cells, allowing tight control of expression. After induction, whole-cell lysates were directly spotted onto HaloLink™ Array slides, which contain a ligand that covalently binds the HaloTag® protein. The HaloTag® fusion proteins were tightly bound to the slide, allowing washing to remove any non-specifically bound proteins. The arrays were then probed with anti-*C. jejuni* polyclonal antisera followed by a secondary antibody labeled with a fluorophore, and the results compared side-by-side with nitrocellulose-based Western blot and dot-blot methods.

The known *C. jejuni* immunogens gave the expected positive results, validating the accuracy of the new method. The authors propose that their HaloTag®-array based method is a rapid and useful alternative to traditional screening approaches for bacterial antigens based on the following advantages:

- The **specificity and strength** of HaloTag®-ligand binding minimized the possibility of false positives due to cross-reactivity between host *E. coli* and test *C. jejuni* antigens. Host proteins can be washed away without also washing away bound proteins or antibodies.
- **No purification required**. The ability to bind specific proteins to the array directly from whole cell lysates eliminated the need for a protein purification step. The process from cultivation to screening took 30 hours with the HaloTag® method, compared to 3 days for nitrocellulose-based screens.
- **Ease of expression in KRX cells**. Use of KRX cells allowed tighter control of expression of fusion proteins. Expression could be turned on or off by adding glucose to the growth medium. The non-leaky expression made it possible to express proteins in KRX that could not be expressed in BL21 cells.

HaloTag®-Based Small Molecule Screening

The identification of proteins that interact with candidate small molecules is an important component of drug discovery strategies. *A HaloTag-based Small Molecule Microarray Screening Methodology with Increased Sensitivity and Multiplex Capabilities* (2) , recently published in *ACS Chemical Biology*, describes a new method for screening small molecule libraries for potential protein interactions. Standard methods for screening for these interactions involve printing small molecules on microarrays followed by incubation of the array with target proteins, which are then detected using labeled antibodies.

The new method involves fusion of target proteins with HaloTag® and prelabeling the fusion proteins with fluorophores, eliminating the antibody detection step and saving time. The authors validated the method using the interaction between the

protein FKBP12 and two ligands—rapamycin and a “bump” ligand developed by ARIAD pharmaceuticals. The authors used a His-HaloTag®-FKBP12 fusion protein to directly compare both HaloTag® and antibody detection methods.

The ability to label HaloTag® fusion proteins with different fluorophores allowed the authors to multiplex assays. For example, they labeled wildtype and mutant HaloTag®–FKBP12 fusions green and red, respectively, and then used both to probe a single array containing both rapamycin and ARIAD “bump” spots. Rapamycin spots gave a signal in both red and green channels, whereas the bump ligand, which is specific for the mutant form, gave a signal only in red. In a different multiplex assay, the authors labeled two isoforms of the Protein Tyrosine Phosphatase 1B (PTP1B) protein with two different fluorophores and screened a 20,000-compound library for molecules that interacted with only one of the isoforms.

These authors identified the following advantages of the HaloTag®-based method:

- **Requires less protein**, possibly because the protein is covalently prelabeled with the fluorophore, avoiding the secondary antibody incubation step and subsequent washes, during which protein may dissociate from the array.
- **Is more sensitive**, detecting lower affinity interactions better than the antibody-based detection method.
- **Allows multiplexing**, thus enabling detection of different interactions on a single slide, resulting in time and cost savings.

Screening Denatured Protein Arrays

The authors of the paper *A Versatile Protein Microarray Platform Enabling Antibody Profiling Against Denatured Proteins* (3) , published in the October issue of *Proteomics Clinical Applications*, addressed the issue of screening protein arrays to detect autoantibody targets. Autoantibodies are antibodies directed against “self” antigens and are of interest as early biomarkers of certain cancers. Conventional methods for screening protein arrays for antibody reactivity typically use proteins purified from bacteria, yeast or insect cells. The proteins are bound to arrays using various attachment methodologies and screened using serum or plasma.

One problem associated with arrays probed with serum is low signal and high background, and this problem is exacerbated in autoantibody screens because the response to self proteins is typically weaker than that to a foreign antigen. The authors state that their goal was to develop an assay with reduced background and higher specificity. They also wanted to denature proteins on the array to expose linear epitopes hidden within the proteins.

Sixty-nine genes were cloned into a HaloTag® vector to create N-terminal fusions. These proteins were expressed on the array in an in vitro transcription/translation system based on HeLa cells, and linked to slides via a HaloTag® ligand. The authors were able to show that HaloTag® bound proteins could be denatured on the slide, and that arrays of denatured proteins gave different autoantibody binding results compared to native HaloTag® fusion proteins.

The benefit of the HaloTag® method reported by these authors was the **ability to perform screens on native proteins or denatured proteins**. Unlike the situation with conventional autoantibody screening methods, protein denaturation did not remove the proteins from the slides. Denaturation of the HaloTag® fusions allowed exposure of hidden epitopes that could potentially be useful biomarker candidates.

Conclusions

The HaloTag® technology provides several advantages for microarray-based applications. The covalent bond formed between the HaloTag® fusion proteins and the HaloTag® ligands means that HaloTag® fusion proteins attached to microarray slides survive washing and certain denaturing conditions better than proteins attached via conventional methods. This means that

background due to non-specific binding is reduced, and signal-to-noise ratios are enhanced. The ability to attach different fluorophores to HaloTag® fusions has also been exploited to allow multiplexing of assays on a single array. Recently, methods have been published that take advantage of these properties to perform array-based screening for immunogenic bacterial proteins, to screen denatured proteins for novel epitopes, and to interrogate small molecule inhibitors for protein interactions.

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