A Polyclonal HaloTag® Antibody for Western Blotting and Immunocytochemistry

ABSTRACT Here we describe a polyclonal antibody generated against a purified HaloTag® protein. Ligand binding does not significantly interfere with antibody binding epitopes and thus allows co-labeling with HaloTag® Ligands. The Anti-HaloTag® pAb can be used in traditional Western blotting applications with conjugated anti-rabbit secondary antibodies for colorimetric or chemiluminescent detection or immunocytochemistry.

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Anti-HaloTag® pAb can be used in traditional Western blotting applications or for co-labeling with HaloTag® Ligands.

INTRODUCTION

The HaloTag® Interchangeable Labeling Technology[a,b] is a fusion protein reporter system that is multi-faceted and flexible (1–4). For imaging in cultured cells, HaloTag® fusion proteins can be fluorescently labeled, visualized, and resolved from other labels in a manner that is analogous to the use of antibody labeling for immunocytochemistry (ICC). In most cases, ICC requires fixation and permeabilization of cells prior to the use of immunolabeling reagents. However, HaloTag® ligands are also useful in vivo, enabling a wider range of cell imaging applications.

Because the binding is covalent, HaloTag® ligands do not dissociate from the HaloTag® reporter after protein denaturation, and thus fusion proteins can be analyzed by SDS-PAGE and fluoroimaging. This direct "cell-to-gel" application provides a rapid means of detecting HaloTag® fusions in complex lysates or reaction mixtures. This alternative to Western blotting eliminates the need for transferring proteins to nitrocellulose or immunolabeling steps.

While the HaloTag® technology offers these advantages over antibody-based applications, we sought to produce a polyclonal antibody (pAb) to the HaloTag® reporter protein to provide an additional tool for detection of HaloTag® fusion proteins by traditional Western blotting or in situ labeling by ICC. The pAb may be used as the sole means of detecting HaloTag® fusion proteins or to confirm the specific labeling achieved by HaloTag® ligands in vivo.

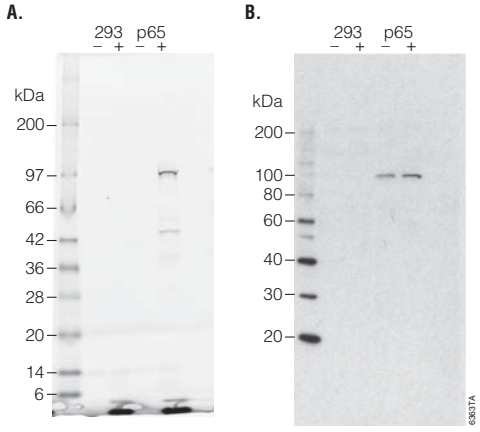

Figure 1. Immunoreactivity of Anti-HaloTag® pAb by Western blotting. 2HEK293-p65-HT2 cells (p65), or non-transfected control cells (HEK293) were labeled with 5µM HaloTag® TMR ligand for 15 minutes, or mock labeled, washed gently with PBS and lysed in SDS-gel sample buffer. Aliquots (10µl) of protein were run on SDS-PAGE. **Panel A.** The gel was scanned on a Typhoon® fluoroimager (GE HealthCare) using a 543nm laser and a 580/30 bandpass emission filter. Lane 1, DyLight™ molecular weight markers (Pierce). Adjacent lanes are unlabeled (293 –) and labeled (293 +) lysates of control cells, and unlabeled (p65 –) and labeled (p65 +) lysates of HEK293-p65-HT2 cells. Residual unbound HaloTag® TMR Ligand is visible at the bottom of lanes labeled +. **Panel B.** Proteins were transferred to nitrocellulose membranes (BioRad) at 30 volts for 4 hours. The membrane was blocked with TBST containing 2% non-fat dry milk (BioRad) for 1 hour and then incubated with 1µg/ml Anti-HaloTag® pAb (a 1:1,000 dilution in blocking solution) for 1 hour. The membrane was washed two times in blocking solution and then incubated in HRP-conjugated donkey anti-rabbit IgG secondary antibody (Part# V795A, 1:10,000 dilution in blocking solution) for 45 minutes. The membrane was washed two times in TBST and then once in TBS. All antibody reactions and wash steps were performed at room temperature. The membrane was processed for chemiluminescent detection using the ECL™ Plus Detection System (GE-Amersham). BioMax® XAR Film (Kodak) was exposed to the membrane for 20 seconds. Lane 1, MagicMark™ molecular weight markers (Invitrogen). Experimental samples correspond to those described in Panel A.

IMMUNOREACTIVITY OF ANTI-HALOTAG® pAb BY WESTERN BLOTTING

A purified preparation of HaloTag® protein was injected into rabbits, and sera was later pooled and applied to a Protein G column. The purified sample was tested for activity by Western blotting and ICC using a human cell line stably expressing a HaloTag®-p65 fusion protein (HEK293-p65-HT2).

HEK293-p65-HT2 cells were labeled with the HaloTag® TMR ligand, lysed and analyzed by SDS-PAGE (Figure 1). The results show the p65-HT2 fusion protein, which has a predicted size of approximately 98 kDa, was specifically labeled and detected using standard settings for fluoroimaging (Figure 1, Panel A). A smaller, weaker band (~47kDa) was also labeled; this represents a degradation product of the fusion protein. When the same samples were probed with the Anti-HaloTag® pAb by Western blotting, immunoreactivity against the 98kDa fusion protein was demonstrated (Figure 1, Panel B). In addition, in HEK293-p65-HT2 cells, weak immunoreactivity was also detected in a 47kDa protein with longer exposure times of the X-ray film (data not shown). The

absence of labeled proteins in the control cells indicates the specificity of both ligand labeling and pAb labeling. For each application, labeling was restricted to the stably transfected cell line, and there was no significant cross-reactivity to unrelated proteins.

IMMUNOREACTIVITY OF HALOTAG® pAb IN SITU

The p65 protein is a cytoplasmic molecule involved in the NFκB signaling pathway. We have demonstrated previously that HaloTag®-p65 fusion protein is properly compartmentalized and physiologically active in stably transfected cells (5).

After labeling with HaloTag® TMR ligand, the HEK293-p65-HT2 cells were processed for ICC using the Anti-HaloTag® pAb. The results demonstrated co-localization of the ligand and antibody binding activities in the cytoplasm of the stably transfected cells (Figure 2). Control labeling reactions with the ligand or the pAb, or both, in HEK293 cells showed negligible nonspecific labeling (data not shown). In addition, Figure 2 includes an internal negative control, as a small percentage of cells (see arrows) are not labeled with the TMR ligand or the pAb. These cells are not expressing HaloTag®-p65 protein, or the level of expression is below detection.

CONCLUSIONS

The results suggest attaching the p65 protein to HaloTag® protein does not significantly interfere with Anti-HaloTag® pAb binding epitopes. Furthermore, sequential labeling of this fusion protein with the HaloTag® TMR Ligand (in vivo) followed by the Anti-HaloTag® pAb (in situ) is possible. Multiplexing HaloTag® ligand labeling with ICC (using unrelated primary antibodies) will also enable two-color labeling for the detection of HaloTag® protein fusions and a second protein target in situ.

The availability of Anti-HaloTag® pAb adds another important reagent to the HaloTag® toolbox. It makes possible traditional Western blot applications and the use of numerous enzyme-conjugated anti-rabbit secondary antibodies for colorimetric or chemiluminescent detection. Similarly, this antibody can be used in combination with various conjugated anti-rabbit secondary antibodies for immunocytochemistry.

REFERENCES

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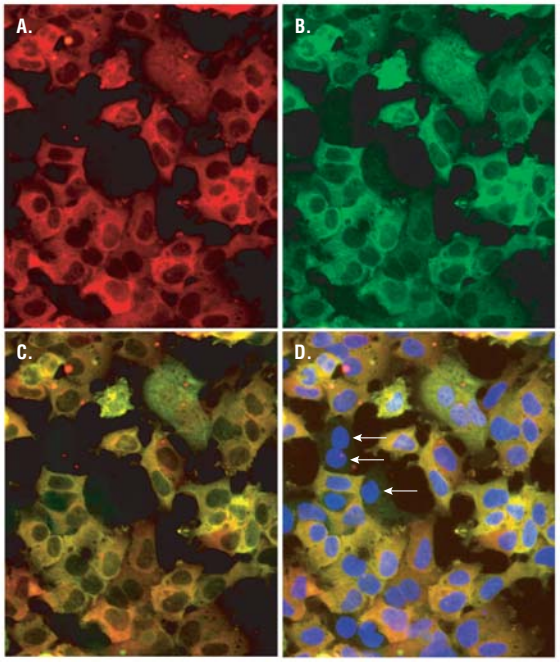

Figure 2. Co-labeling of HaloTag®-p65 fusion protein with HaloTag® TMR ligand and the Anti-HaloTag® pAb. HEK293-p65-HT2 cells were seeded onto collagen-coated 8-well chambered coverglass slides (Nunc), grown for three days to near confluency, then labeled with 5µM HaloTag® TMR Ligand for 15 minutes. The cells were gently washed with warm PBS, then fixed with 4% paraformaldehyde for 10 minutes, then permeabilized with 0.2% Triton®, blocked with 2% donkey serum, then incubated with 2µg/ml Anti-HaloTag® pAb (1:500 dilution). The cells were washed with the blocking solution and then incubated with 2µg/ml AlexaFluor® 488-conjugated anti-rabbit IgG (Invitrogen). Finally, cells were counterstained with DAPI (Vector Laboratories). Images were collected on the Olympus IX81 epi-fluorescent microscope with a 20x oil objective and filter sets appropriate for rhodamine and AlexaFluor® 488. **Panel A.** Cytoplasmic (red) labeling of HEK293-p65-HT2 cells by HaloTag® TMR Ligand. **Panel B.** Cytoplasmic (green) labeling by Anti-HaloTag® pAb. **Panel C.** Co-localization of ligand and antibody binding activities. **Panel D.** Merger of red and green fluorescence with counterstaining of the nucleus by DAPI (blue). Arrows denote rare cells that show little or no expression of HaloTag®-p65.

ORDERING INFORMATION

Product	Size	Cat.#
Anti-HaloTag® pAb	200µg	G9281

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[a] Patent Pending.

[b] For research use only. With respect to manufacture or sale of research products, or any diagnostic, therapeutic or prophylactic uses, please contact Promega for supply and licensing information.

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