HALOTAG™ INTERCHANGEABLE LABELING TECHNOLOGY FOR CELL IMAGING AND PROTEIN CAPTURE

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The HaloTag™ Interchangeable Labeling Technology provides new options for rapid, site-specific labeling of proteins in living cells and in vitro. The technology is based on the formation of a covalent bond between the HaloTag™ Protein and synthetic ligands that carry a variety of functionalities, including fluorescent labels, affinity tags and attachments to a solid phase. The covalent bond forms rapidly under physiological conditions, is highly specific and essentially irreversible, yielding a complex that is stable even under denaturing conditions. The ability to create labeled HaloTag™ fusion proteins with a wide range of optical properties and functions allows researchers to image and localize labeled HaloTag™ protein fusions in live- or fixed-cell populations and isolate and analyze HaloTag™ Protein fusions and protein complexes. This article presents data obtained using three commercially available ligands and one ligand that is currently under development.

Introduction

The ability to specifically label proteins is key to revealing the dynamics and functions of proteins in living cells (1). However, many of the conventional methods used to generate fluorescently tagged proteins and image them in their native environment are time consuming and difficult, requiring expertise in protein chemistry, the successful microinjection of labeled products into cells, specialized techniques and instrumentation (2). On the other hand, de novo synthesis of proteins obtained through cloning and transfection of cells is more likely to result in native patterns of protein localization. The development of new methods for labeling proteins by genetic fusion is expanding our understanding of cellular function. Here we describe the new HaloTag™ Interchangeable Labeling Technology, a flexible system that enables efficient labeling of fusion proteins in living cells as well as in vitro.

Overview of the HaloTag™ Interchangeable Labeling Technology

The interchangeability of the ligands facilitates imaging at different wavelengths or incorporating novel functionalities without changing the underlying genetic construct. Figure 1 outlines the HaloTag™ Labeling strategy. First, the HaloTag™ pHT2 Vector[a,b,c] encoding the HaloTag™ Protein or protein fusion is introduced into cells by either transient transfection or generation of stable cell lines expressing the HaloTag™ Protein. The cells are typically incubated with an appropriate HaloTag™ Ligand[c,d] for 5–60 minutes. The HaloTag™ Ligands readily cross the cell membrane where they covalently bind to the HaloTag™ Protein. Unbound ligand is washed out, and the investigator proceeds with the desired application: fluorescence imaging (live- or fixed-cell; Figure 2), cell lysis and protein capture, or gel analysis.

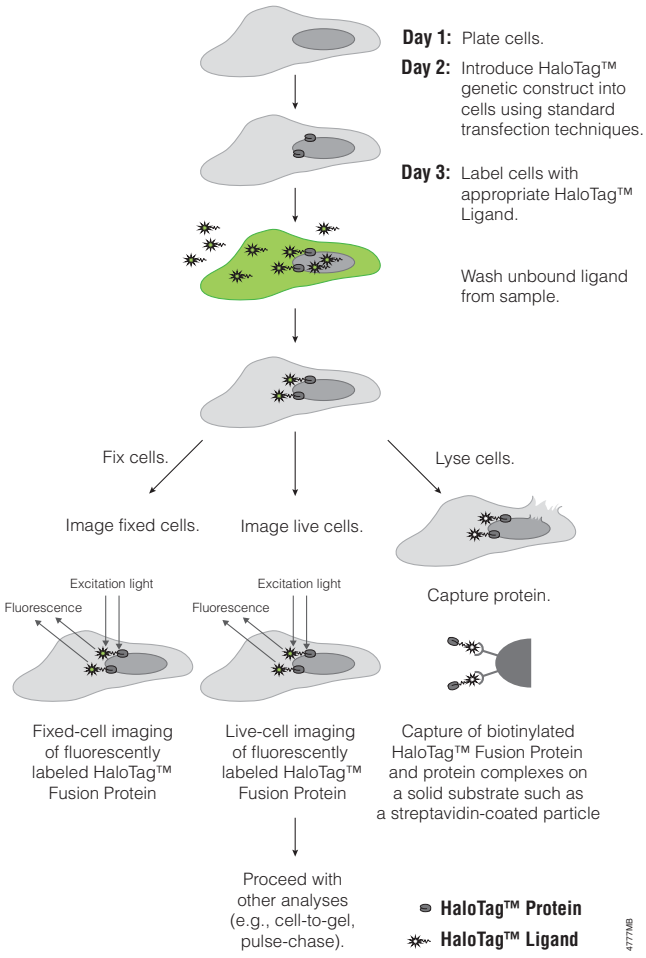

Figure 1. Overview of HaloTag™ Interchangeable Labeling Technology applications.

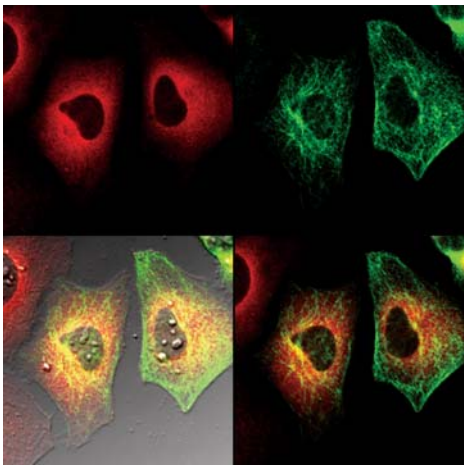

Figure 2. Fixed cells expressing p65-HaloTag™ Protein labeled with HaloTag™ TMR Ligand. HeLa cells transiently transfected with plasmid encoding the p65-HaloTag™ fusion protein were labeled with 5µM HaloTag™ TMR Ligand for 15 minutes at 37˚C according to the protocol described in the Technical Manual (#TM260). Cells were fixed with 3.7% paraformaldehyde, stained with mouse Anti-βIII Tubulin Antibody (Cat.# G7121) at 1µg/ml followed by incubation with Alexa Fluor™-488-conjugated goat-antimouse IgG (Molecular Probes). Images were generated on an Olympus FV500 confocal microscope in sequential mode using appropriate filter sets for TMR, Alexa Fluor™ or transmitted light. **Panel A.** TMR fluorescence. **Panel B.** Alexa Fluor™-488 fluorescence. **Panel C.** Overlaid AlexaFluor™-488 and TMR fluorescence and transmitted light. **Panel D.** Overlaid AlexaFluor™-488 and TMR fluorescence.

Components of the HaloTag™ Interchangeable Labeling Technology

The HaloTag™ Protein is a mutant of a hydrolase protein that efficiently forms a covalent bond with the HaloTag™ Ligands (Figure 3). This 33kDa monomeric protein can be used to generate N- or C-terminal fusions that can be efficiently expressed in a variety of cell types. Since the HaloTag™ Protein is of prokaryotic origin, endogenous activities are absent from mammalian cells. The HaloTag™ pHT2 Vector contains: a CMV enhancer/promoter for strong, constitutive expression in many cell types, a chimeric intron to minimize the use of cryptic 5´-donor splice sites, a T7 promoter for use with in vitro transcription and/or translation systems, the sequence encoding the HaloTag™ Protein, and an SV40 late polyadenylation signal.

The HaloTag™ Ligands are small chemical tags that are capable of covalently labeling the HaloTag™ Protein. These ligands contain two crucial components: 1) a common HaloTag™ Reactive Linker that initiates formation of a covalent bond with the HaloTag™ Protein, and 2) a functional reporter such as the fluorescent dyes TMR and diAcFAM or affinity handles such as biotin (Figure 4). The rate of HaloTag™ Ligand binding to the HaloTag™ Protein is remarkably fast. Fluorescence polarization analysis using a purified GST-HaloTag™ Protein fusion shows that the HaloTag™ TMR Ligand binds rapidly in vitro. The binding

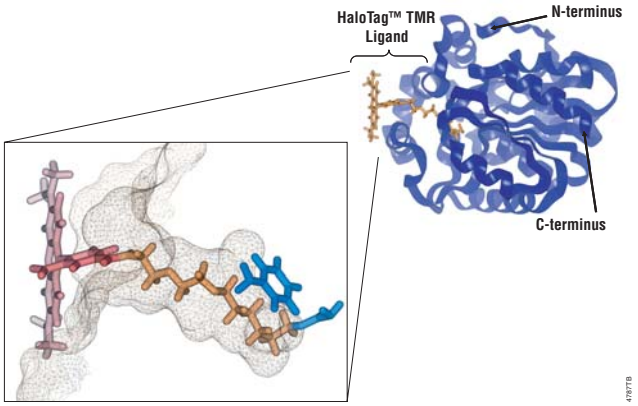

Figure 3. Molecular model of the HaloTag™ Protein with a covalently bound HaloTag™ TMR Ligand. Overview of the protein structure (top right) with close-up of the ligand tunnel outlined by a mesh Connolly surface (left). The HaloTag™ TMR Ligand (fluorescent moiety in red, reactive linker in orange) is shown covalently bound to the aspartate nucleophile (shown in blue). Replacement of the catalytic base (histidine) with a phenylalanine (also shown in blue) renders the HaloTag™ Protein inactive by impairing its ability to hydrolyze the ester intermediate, leading to the formation of a stable covalent bond.

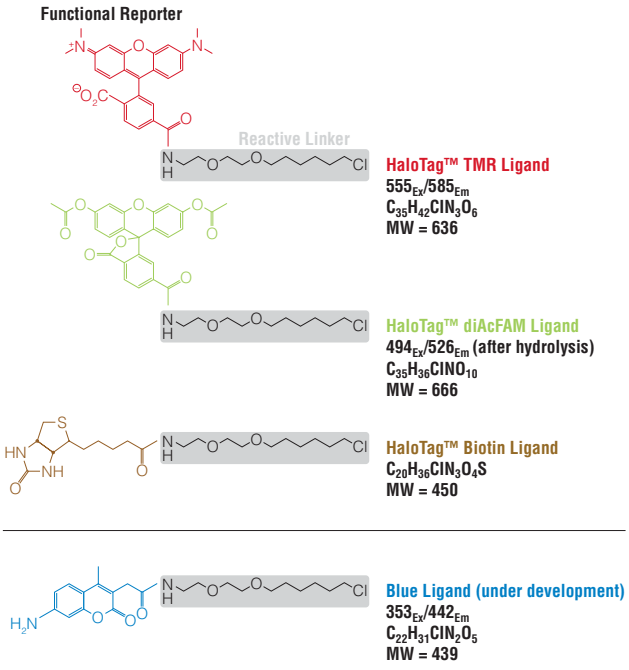

Figure 4. Structure of the HaloTag™ Ligands. Abbreviations: TMR, tetramethyl rhodamine; diAcFAM, diacetyl fluorescein.The proposed structure for the blue ligand, which is currently under development, is also shown.

kinetics are similar to the on rate measured for TMR-biotin binding to streptavidin (Figure 5). This rate is similar to the biotin-streptavidin interaction (4). The HaloTag™ TMR, diAcFAM and Biotin Ligands have shown no detectable cellular toxicity or morphological side effects under the labeling conditions described in Technical Manual #TM260.

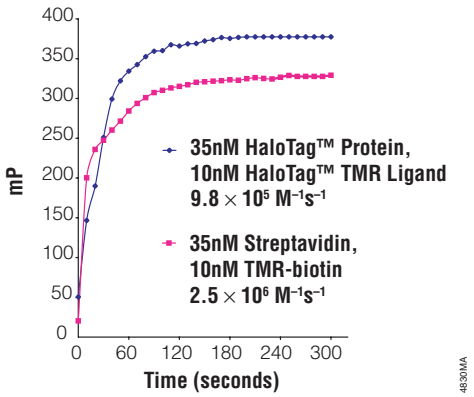

Figure 5. Fluorescence polarization (FP) analysis comparing HaloTag™ Ligand and streptavidin-biotin on rates. Measurements were performed on a Beacon 2000 instrument, and the data were used to calculate second-order rate constants (3). Qureshi *et al.* (4) have reported streptavidin-biotin on-rates of 5.0×10^6 $M^{-1}sec^{-1}$.

Imaging Live and Fixed Mammalian Cells

As shown in Figure 6 (Panels A-C) cells expressing the HaloTag™ Protein and labeled with the HaloTag™ TMR (red) or diAcFAM (green) Ligand or the blue ligand (under development) are brightly fluorescent. In contrast, cells that do not express the HaloTag™ Protein show no detectable fluorescence under the same labeling and imaging conditions (5). These results demonstrate the high specificity of cell labeling and the efficient use of the ligands for imaging live mammalian cells. The excellent signal-to-noise ratio allows detection of proteins expressed at relatively low levels.

The HaloTag™ Protein can be fused to a protein or target sequence of interest that directs the fusion protein to a specific subcellular compartment. To demonstrate this capability we generated constructs encoding a p65-HaloTag™ Protein chimera. The p65 protein (also known as RelA and NF-κB3) is a member of the eukaryotic nuclear factor κB (NF-κB) protein family. The NF-κB factor is expressed in many cell types and plays an important role in inflammation, autoimmune response and apoptosis by regulating the expression of genes involved in these processes. The NF-κB proteins contain a nuclear-localization sequence (NLS), which is rendered inactive in non-stimulated cells through the binding of specific NF-κB inhibitors, known as the IκB proteins. Binding of IκB masks the NLS, which leads to retention of the NF-κB proteins (including p65) in the cytoplasm of the cell (6–8).

HeLa cells were transfected with the plasmid encoding the p65-HaloTag™ fusion protein and labeled with the HaloTag™ TMR Ligand (Figure 7) or the HaloTag™ diAcFAM Ligand (data not shown). As expected, the p65-HaloTag™ fusion protein is excluded from the nucleus and shows a diffuse cytosolic localization.

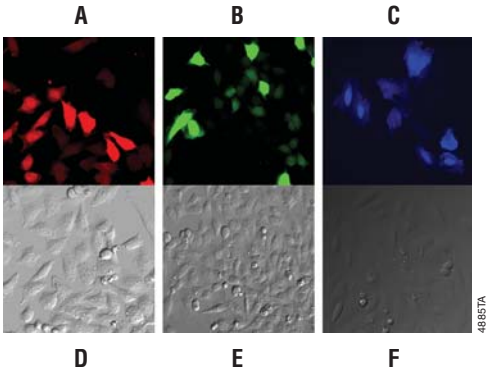

Figure 6. Live cells expressing HaloTag™ Protein labeled with three different ligands. HeLa cells transiently transfected with HaloTag™ pHT2 Vector were labeled with 5µM HaloTag™ TMR Ligand (**Panel A**); 10µM HaloTag™ diAcFAM Ligand (**Panel B**); or 25µM blue ligand (currently under development; **Panel C**) for 15 minutes at 37°C according to the protocol described in Technical Manual #TM260. Images were generated on an Olympus FV500 confocal microscope using appropriate filter sets for TMR or FAM or transmitted light (**Panels D, E and F**). The blue ligand was excited using a mercury bulb and fluorescence was detected using a Hamamatsu CCD camera.

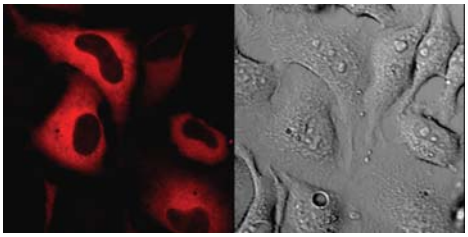

Figure 7. Cytosolic localization of the p65-HaloTag™ fusion protein labeled with HaloTag™ TMR Ligand. HeLa cells transiently transfected with vector encoding the p65-HaloTag™ fusion protein were labeled with 5µM HaloTag™ TMR Ligand (**Panel A**) for 15 minutes at 37°C according to the protocol described in Technical Manual #TM260. Images were generated on an Olympus FV500 confocal microscope using filter sets for TMR fluorescence or transmitted light (**Panel B**).

The HaloTag™ Ligands and expression of the HaloTag™ protein have shown no detectable toxicity or morphological side effects at recommended labeling conditions in the cell lines tested (e.g., HeLa and CHO-K1). This characteristic allows imaging of live cells over long periods of time, including times required for studying phenomena such as the cell cycle, cell differentiation, long-term effects of drugs and other applications. Figure 8 presents images of HeLa cells transiently expressing the p65-HaloTag™ fusion protein and labeled with the HaloTag™ TMR Ligand. Images were taken every 20 minutes for 14 hours. Cells have unaltered morphology at all time points examined. Importantly, expression of the p65-HaloTag™ fusion protein and treatment with the HaloTag™ TMR Ligand have no effect on complex cellular functions. For example, the time-lapse images in Figure 8 clearly show a cell in the process of dividing (see arrow).

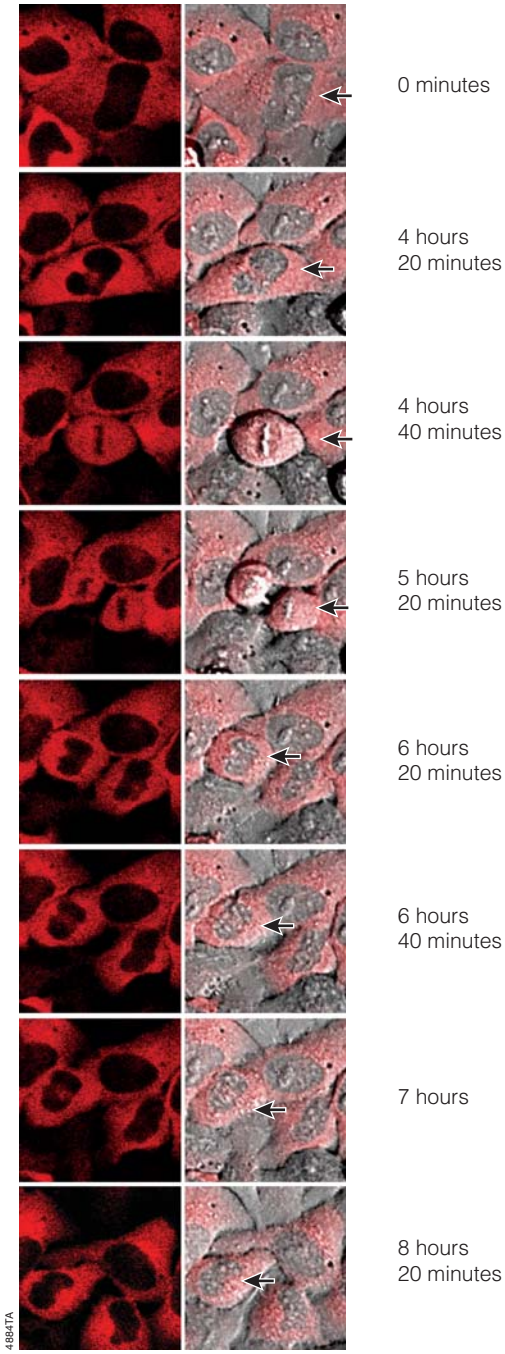

Figure 8. HeLa cells expressing the p65-HaloTag™ fusion and labeled with HaloTag™ TMR Ligand. HeLa cells transiently transfected with plasmid encoding the p65-HaloTag™ fusion protein were labeled with 5µM HaloTag™ TMR Ligand for 15 minutes at 37°C according to the protocol described in TM260. Images were generated on an Olympus FV500 confocal microscope using filter sets for TMR or transmitted light. Images were collected every 20 minutes over a period of 8 hours 20 minutes.

The stability of the covalent bond between the HaloTag™ Protein and the HaloTag™ Ligands allows users to image fixed cells. In addition, the resistance of the fluorescent signal

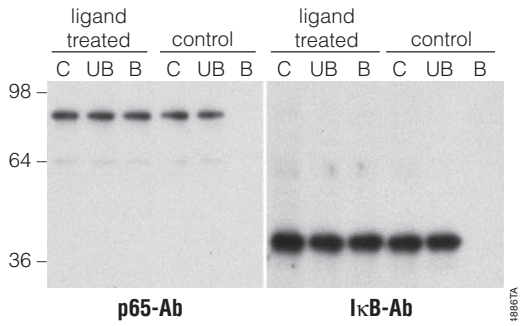

Figure 9. Efficient capture of a p65-HaloTag™ fusion protein and co-capture of IκB on paramagnetic streptavidin-coated particles. CHO-K1 cells transiently transfected with plasmid encoding the p65-HaloTag™ fusion protein were incubated in the presence or absence of 25µM HaloTag™ Biotin Ligand. Cells were lysed, and biotin-labeled proteins were captured on SA-coated paramagnetic particles (Cat.# Z5481). Proteins were resolved by SDS-PAGE and analyzed by Western blot with anti-p65 antibody (BD Bioscience, **Panel A**) or anti-IκB antibody (BD Bioscience, **Panel B**). Content of lanes for both **Panels A and B**: C = cell lysate; UB = unbound protein; B = bound protein.

to cell fixatives also allows users to multiplex the HaloTag™ Technology with different immunocytochemical and, potentially, immunohistochemical techniques. To demonstrate this capability, HeLa cells transiently expressing the p65-HaloTag™ fusion protein were labeled with the HaloTag™ TMR Ligand, fixed and counterstained with anti-βIII tubulin antibody (Figure 2). The transfected cells labeled with the HaloTag™ TMR Ligand remain brightly fluorescent after fixation (Figure 2, Panel A). HeLa cells expressing the HaloTag™ Protein and labeled with the HaloTag™ diAcFAM or the blue ligand under development also remain brightly fluorescent upon fixation (data not shown). Figure 2, Panel B shows the fluorescence pattern of cells labeled with the HaloTag™ TMR Ligand after fixation and counterstaining with the anti-βIII tubulin antibody. The resulting Alexa Fluor™ signal reveals the typical cytoskeletal architecture. The overlay of the TMR and Alexa Fluor™ signals is shown in Figure 2, Panels C and D. The ability to image fixed cells is important for applications where large numbers of cells are analyzed by automated screening methods such as those involving High Content Analysis.

Gel Analysis of HaloTag™-Based Fusion Proteins

The HaloTag™ Protein-Ligand interaction is stable and highly specific. The fluorescently labeled HaloTag™ Protein can be boiled with SDS-PAGE sample buffer and resolved by SDS-PAGE without detectable loss of the fluorescent signal (5). Analyzing labeled HaloTag™ Protein can be combined with other protein analysis techniques such as Western blotting. Our preliminary data indicate that this approach also can be used successfully to study post-translational modification of the HaloTag™ Protein-based fusions (e.g., proteolytic cleavage, data not shown).

Capture of HaloTag™-Based Fusion Proteins and Co-Capture of Protein Interaction Partners

The HaloTag™ Technology can be used to capture HaloTag™ Protein fusions expressed in mammalian cells using a ligand containing an affinity tag. CHO-K1 cells were transiently transfected with a construct encoding the p65-HaloTag™ protein fusion and incubated with or without the HaloTag™ Biotin Ligand according to the protocol described in Technical Manual #TM260. Cell lysates were incubated with Streptavidin MagneSphere® Paramagnetic Particles (Cat.# Z5481) to capture the biotin-labeled fusion protein. Proteins were resolved by SDS-PAGE and analyzed by Western blot analysis using anti-p65 antibody (BD Bioscience). As expected, both control and ligand-treated cells express the fusion protein (Figure 9, lanes 1 and 4). However, only beads incubated with the cytosol of ligand-treated cells are able to capture the p65-HaloTag™ protein (Figure 9, Panel A, lanes 3 and 6). Moreover, the immobilized p65-HaloTag™ Protein was able to capture and "pull-down" its specific interacting partner IκB (Figure 9, Panel B, lanes 3 and 6).

Conclusions

The ability to label and analyze proteins in their native environment is critical to developing a detailed understanding of biological pathways and cellular function. The newly developed HaloTag™ Interchangeable Labeling Technology is a flexible system that enables the efficient labeling of fusion proteins directly in living cells and in vitro. We have demonstrated the in vivo effectiveness of this new system by showing that the HaloTag™ Ligands readily cross the membrane where they covalently bind the HaloTag™ Protein. Fluorescently labeled HaloTag™ Protein fusions can readily be imaged in either live- or fixed-cell preparations. This capability allows researchers to examine protein translocation in real time and multiplex the HaloTag™ Technology with different immunocytochemical techniques for High Content Analysis. Furthermore, because the HaloTag™ Protein-Ligand interaction is so stable, labeled fusion proteins can be analyzed directly using SDS-PAGE. Lastly, the HaloTag™ Technology can be an important tool for confirming suspected protein:protein interactions or for identifying new interacting protein partners. ■

Acknowledgments

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Protocol

HaloTag™ Interchangeable Labeling Technology Technical Manual #TM260
(www.promega.com/tbs/tm260/tm260.html)

Ordering Information

Product	Size	Cat.#
HaloTag™ pHT2 Vector	20µg	G8241
HaloTag™ TMR Ligand	30µl	G8251
HaloTag™ diAcFAM Ligand	30µl	G8271
HaloTag™ Biotin Ligand	30µl	G8281

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