Labeling Cells Expressing HaloTag® Fusions with HTag Ligands

Before You Start

The protocols below are designed for staining and analyzing cells that express HaloTag® fusions. Prior to labeling, seed cells at the desired density, using a standard culture medium optimal for that line. For transfection, follow the manufacturer's instructions for the transfection reagent.

Note: Cells that have grown to very high densities (i.e., a confluent monolayer) may produce elevated labeling background.

Handling and Storage

- HTag ligands are supplied freeze-dried in 150 nmol quantities per vial. Upon arrival, the substances can be stored for up to two years at -20 °C.
- Immediately before staining, prepare the ligand stock solution. To do this, add 150 µL of DMF or DMSO to the contents of the 150 nmol tube and vortex.
- Once dissolved, stock solutions should be kept at -20 °C, protected from light and moisture.

Protocol

A. Rapid Live-Cell Labeling

1. Immediately before use, prepare a 1:200 dilution of HTag ligand in warm culture medium. This serves as a 5× working stock.
2. Label the cells by replacing 1/5 of the culture medium with the 5× HTag ligand working stock and gently mixing.
3. Incubate cells with HTag ligand for 15 minutes at 37 °C in a CO_2 cell culture incubator.
4. Change the ligand-containing medium with an equal volume of warm fresh medium twice. Being cell-impermeant, AF HTag ligands do not require washing to remove unbound ligand.
5. Transfer to a microscope and capture images.

B. Pulse-Chase Cell Labeling

This protocol enables the investigation of the turnover or trafficking of HaloTag® fusion proteins using pulse-chase labeling.

1. Immediately before use, prepare a 5× working stock of the pulse-labeling ligand by diluting the HTag stock 1:200 in warm culture medium.
2. Label the cells by replacing 1/5 of the culture medium with the 5× HTag ligand working stock and gently mixing.
3. Incubate cells with pulse-labeling ligand for 15 minutes at 37 °C in a CO_2 incubator.

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4. If the chase follows immediately, proceed directly to Step 5. If not, simply replace the ligand-containing medium with an equal volume of warm fresh culture medium and either allow time to elapse before the chase or carry out your intended biology as required, before moving to Step 6.
5. Immediately before use, prepare a 1:1,000 dilution of chase ligand (1× working solution) in warm culture medium. Gently change the medium with an equal volume of this 1× chase-labeling solution.
6. Incubate cells with chase-labeling ligand for 15 minutes at 37 °C in a CO_2 incubator.
7. Change the ligand-containing medium with an equal volume of warm fresh medium twice.
8. Transfer to a microscope and capture images.

C. Fixing Cells after Labeling

The covalent bond between the ligand and the HaloTag® protein enables subsequent fixation, permeabilization, and washing of cells under standard conditions without significant loss of specific fluorescent signal.

1. Label cells with an AF HTag ligand by following Steps 1–3 of Section A (Rapid Labeling) or Steps 1–6 of Section B (Pulse-Chase Labeling).
2. Change the medium with an equal volume of 4% paraformaldehyde in 1× PBS (pH 7.5) and incubate for 10 minutes at room temperature.
3. Change the fixative with an equal volume of 0.1% Triton X-100 in 1× PBS and incubate for 10 minutes at room temperature.
4. Change the detergent solution with an equal volume of 1× PBS, then either transfer to a microscope and capture images, store at 4 °C, or proceed to Section D for the immunocytochemistry protocol.

D. Immunocytochemistry

Fixed cells, after labeling with the HTag ligand, can be additionally stained immunocytochemically with any antibody of interest. The procedures described here may be used as a starting point. The protocol for a specific antibody may vary; therefore, it is necessary to follow the manufacturer's instructions.

1. Change the 1× PBS with an equal volume of blocking solution (2% normal serum from the same host as the secondary antibody and 0.1% Triton X-100 in 1× PBS) and incubate cells for 1 hour at room temperature.
2. Prepare the primary antibody solution in 1% normal serum in 1× PBS at the manufacturer's recommended concentration.
3. Change the blocking solution to the primary antibody solution. Incubate cells for 1 hour at room temperature.
4. Wash cells twice with 1% normal serum in 1× PBS for 10 minutes per wash at room temperature.
5. Dilute the secondary antibody in 1% normal serum in 1× PBS, following the manufacturer's recommendations.
6. Incubate cells in the secondary antibody solution for 30 minutes at room temperature.
7. Wash cells twice with 1% normal serum in 1× PBS for 10 minutes per wash at room temperature.

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8. Replace the wash solution with 1× PBS.
9. Transfer to a microscope and capture images.

E. SDS-PAGE Analysis

The covalent bond between the HaloTag® protein and the ligand withstands denaturation, enabling the quantitative determination of the labeled fusion protein via SDS-PAGE using a fluorescence scanner.

1. Prepare 4× SDS Sample Buffer (0.24 M Tris, 2% SDS, 50.4% glycerol, 0.4 M DTT, 3 mM Bromophenol Blue). Titrate it to pH 6.8 using HCl. To prepare 1× SDS Sample Buffer, dilute 4× Buffer 1:4 with filtered water.
2. Label cells following Steps 1–3 of Section A (Rapid Labeling) or Steps 1–6 of Section B (Pulse-Chase Labeling).
3. Replace the ligand-containing medium with 1× PBS (pH 7.5) to avoid a prominent band arising from the complete medium.
4. Lyse cells by replacing the 1× PBS with approximately 100–150 µL of 1× SDS Sample Buffer per cm^2 of cell growth area.
5. Collect the cell lysate and incubate for 5 minutes at 95 °C.
6. Perform SDS-PAGE by loading approximately 10 µL (5–10 µg total protein) of each sample per gel well, or store samples at -20 °C for later use.
7. Analyze the gel on a fluorescence scanner.

   Note: The dye front may contain fluorescent material (unbound ligand and/or tracking dyes from the sample buffer) that can complicate detection. To eliminate these sources of nonspecific fluorescence, simply run the gel until the dye front migrates off the gel, or cut the dye front from the bottom of the gel before scanning.

F. Flow Cytometry Analysis

1. Label cells expressing HaloTag® fusion protein with HTag ligand following Steps 1–3 of Section A (Rapid Labeling) or Steps 1–6 of Section B (Pulse-Chase Labeling).
2. Prepare controls:
   a. Label cells not expressing HaloTag® protein with HTag ligand to assess background from the HTag ligand.
   b. Prepare nonlabeled cells expressing HaloTag® protein to assess background from endogenous cell fluorescence or morphological changes due to expression.
3. If adherent cells with the HaloTag® express a fusion protein on the cell surface, rinse them twice with an equal volume of 1× PBS, then incubate in 1× PBS with 3 mM EDTA or another non-enzymatic solution at 37 °C for 5–15 minutes to gently lift the cells. If adherent cells express a HaloTag® fusion protein intracellularly, suspend them using an appropriate trypsin-containing solution.
4. Collect and resuspend cells in culture medium at 37 °C to a concentration of 0.5–1 × 10^6 cells/mL.
5. Analyze cells by flow cytometry.

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Troubleshooting

Although the recommendations above should ensure a good signal-to-noise ratio, specific labeling results depend on the expression level of the HaloTag® fusion protein, cell type and condition, fixation, unbound-ligand wash, etc. Listed below are potential labeling issues and methods for resolving them:

Weak or absent fluorescent signal in labeled cells

- To prevent proteolysis during cell lysis in PBS, include protease inhibitors and perform lysis at 4 °C.
- Allow cells to grow for a longer period prior to labeling to ensure adequate protein expression and cell density.
- Maintain optimal cell health. Throughout labeling and imaging, expose cells only to complete culture medium (i.e., containing serum) at 37 °C under appropriate CO_2 conditions.
- Optimize cell-labeling protocols by extending the labeling incubation time.
- Store HTag ligands desiccated at -20 °C and protected from light. Dispense the ligand into single-use aliquots to avoid repeated freeze-thaw cycles.
- Use freshly diluted HTag ligand and add it to cells immediately.
- Confirm that the correct filter set is being used for imaging.
- Adjust the settings on your fluorescence detection instrument (e.g., laser power, PMT gain, and aperture for a confocal microscope).

High background fluorescence in live cells

- Reduce the HTag ligand concentration.
- Extend the wash duration and perform washes using complete medium.
- Image cells in complete medium without Phenol Red.
- Adhere closely to the recommended labeling temperatures.
- Adjust instrument settings (e.g., lower laser power, reduce PMT gain or aperture on a confocal microscope).

High background fluorescence in fixed cells

- Lengthen wash times and/or perform washes at a higher Triton X-100 concentration or with an alternative detergent.
- Lower the staining concentration.

Cells detached from the surface

- Handle cells gently to keep them attached to the surface and consider reducing the number of medium replacements.
- Perform all labeling, washing, and imaging steps using complete culture medium, keeping cells in a 37 °C, CO_2 incubator whenever possible.

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- Increase seeding density or extend culture time to allow cells to proliferate and adhere more firmly.
- Apply an attachment matrix such as poly-L-lysine, fibronectin, or collagen.

Cell death or toxicity

- Adjust the transfection protocol or switch to a less toxic transfection reagent.
- Verify that the DNA is endotoxin-free.

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