Zenon™ pHrodo™ Deep Red IgG Labeling Reagents

Catalog Numbers Z25618 and Z25622

Pub. No. MAN1000088 Rev. A00

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from thermofisher.com/support.

Product description

The Invitrogen™ Zenon™ pHrodo™ Deep Red Human IgG Labeling Reagent for screening antibody internalization provides a fast, reliable method for evaluating antibody internalization. Zenon™ labeling technology uses pHrodo™ Deep Red-labeled Fab fragments (that is, labeling reagent) directed against the Fc portion of an intact IgG primary antibody to form a labeling complex. Formation of the Fab-antibody complex occurs in less than 5 minutes with no purification required. The pHrodo™ Deep Red dyes have minimal fluorescence at neutral pH and only upon entry into the late endosome and lysosome do they brightly fluoresce. Because of this, Zenon™ pHrodo™ Deep Red labeling reagents are optimal for screening the endocytosis and degradation of therapeutic antibodies, including antibody-drug conjugates.

Contents and storage

Item[1,2]	Cat. No.	Amount	Storage[3]
Zenon™ pHrodo™ Deep Red Human IgG Labeling Reagent	Z25618	250 µL	• Store at 2–8°C protected from light • Do not freeze
Zenon™ pHrodo™ Deep Red Mouse IgG Labeling Reagent	Z25622		

[1] Approximate fluorescence excitation/emission maxima for pHrodo™ Deep Red reagent are 640/655 nm.
[2] The 400 µg Fab fragment/mL is supplied in 0.1 M sodium phosphate and 0.1 M NaCl (pH 7.5).
[3] When stored properly, kit components are stable for at least 12 months.

Required materials not supplied

- Whole IgG primary antibodies
- Suspension cells at 2 x 10^6 cells/mL in cell culture medium or adherent cells in a 96-well plate at 5,000–10,000 cells/well in cell culture medium
- Cell culture medium
- 96-well plates
- Instruments (that is, flow cytometer, fluorescence microscope, or high content analysis instrument) to analyze cells probed with the Zenon™ pHrodo™ Deep Red Human IgG Labeling Reagent or Zenon™ pHrodo™ Deep Red Mouse IgG Labeling Reagent

Labeling procedure

Procedural guidelines

- Zenon™ pHrodo™ Deep Red labeling reagents are goat Fab fragments selective for the Fc portion of human or mouse IgG antibodies. They are pan reactive allowing for binding to all human or mouse subclasses and are used to non-covalently couple the Zenon™pHrodo™ Deep Red-labeled Fab fragments to the unconjugated human or mouse IgG antibodies. This leaves the antigen binding site of the antibody unmodified.
- Formation of the Fab-antibody labeling complex occurs in less than 5 minutes with most of the primary antibodies in the mixture labeled. Complexes formed using this technology display fluorescence intensity similar to that of directly labeled primary antibodies.
- The extent of antibody labeling and the resulting fluorescence intensity of the probe can be adjusted by varying the amount of Zenon™ pHrodo™ Deep Red labeling reagent that is added. This adjustment is done by varying the molar ratio of labeled Fab fragment to primary antibody.

- The protocol described here is for performing antibody binding assays with one 96-well plate of antibodies, each at 60 nM (0.9 µg/well) with Zenon™ pHrodo™ Deep Red IgG labeling reagent at 180 nM (0.9 µg/well). This molar ratio is a suggested starting point and represents the minimum ratio for adequate signal to background in most applications. Experiments with antibodies against highly-expressed antigens may obtain satisfactory signal with lower antibody concentrations as follows:
  - One 96-well plate at a ratio of 60 nM antibody to 180 nM Zenon™ labeling reagent
  - Two 96-well plates at a ratio of 30 nM antibody to 90 nM Zenon™ labeling reagent
  - Four 96-well plates at a ratio of 15 nM antibody to 45 nM Zenon™ labeling reagent
- For larger or smaller quantities of antibody, the amounts of the reagents specified in the protocol can be scaled accordingly. The Zenon™ IgG labeling reaction does not require the removal of bovine serum albumin (BSA) or other stabilizing proteins that may be present in antibody preparations. Antibodies contained within serum may also be directly labeled and do not require purification of the antibody before or after labeling.
- The pHrodo™ Deep Red dye has excitation and emission maxima of approximately 640 nm and 655 nm, respectively, and can be detected with a standard Cy5 filter.
- pHrodo™ Deep Red dye photodegrades under UV excitation. Therefore, if using stains such as DAPI or Hoechst, image with the DAPI channel following pHrodo™ Deep Red imaging.
- pHrodo™ Deep Red dye is sensitive to sodium azide at concentrations greater than 1 mM. If samples have sodium azide at concentrations greater than 1 mM, we recommend removing the sodium azide using a desalting column.

Prepare 4X antibody working solution

1. Prepare sufficient volume of 4X working solution of antibody in cell culture medium so that you can use 25 µL for each sample. For example, to fill one 96-well plate, prepare 2.5 mL of working antibody solution.

   Note: An optimal starting concentration for many antibodies is 60 nM, therefore a 4X stock will equal 240 nM.

2. Aliquot 25 µL of 4X antibody working solution to each well of a 96-well plate.

Prepare 4X Zenon™ working solution

1. Prepare 4X working solution of Zenon™ pHrodo™ Deep Red IgG labeling reagent. For example, for one 96-well plate, add 240 µL of Zenon™ pHrodo™ Deep Red IgG labeling reagent to 2.16 mL of cell culture medium to prepare 2.4 mL of Zenon™ working solution. This provides enough reagent to label 96 wells of antibody with a 60 nM concentration at a molar ratio of 3:1.

2. Aliquot 25 µL of 4X Zenon™ working solution to each well of the 96-well plate. Incubate for 5 minutes at room temperature to allow the labeling complexes to form.

Label suspension cells

1. Prepare at least 5 mL of suspension cells at 2×10^6 cells/mL in cell culture medium.

2. Add 50 µL of cells to each well of the 96-well plate containing the antibody and the Zenon™ labeling reagent.

3. Incubate the cells with the labeling complex for 1–24 hours under standard cell culture conditions. Add other antibodies or cell labels as desired.

4. Analyze cells using flow cytometry.

Label adherent cells

1. Prepare a 96-well plate containing 5,000–10,000 cells/well. After the cells adhere, adjust the volume so each well contains 50 µL of culture medium.

2. Add 50 µL of the labeling complex (step 2) to each well of the 96-well plate.

3. Incubate the cells with the labeling complex for 1–24 hours under standard cell culture conditions.

Optimization

- Depending on the use, higher antibody concentrations can lead to aggregation. If aggregation occurs, then reduce the antibody concentration.
- Background is determined by using a control with no primary antibody that is treated with only Fab-pHrodo™ Deep Red dye. While pHrodo™ Deep Red dye is non-fluorescent at neutral pH, cells can still endocytose Fab fragments through nonspecific mechanisms. Because of this, some background may be observed. While increasing the molar ratio of Fab to primary antibody can increase signal intensity, it may also increase background and therefore may need to be optimized. Incubation times also have an impact on background as these nonspecific processes are slower.
- Using more than the recommended amount of Zenon™ reagent can increase background. However, signal can be increased by reducing antibody concentration while keeping Zenon™ concentration constant. If increased signal to background is a concern, start by titrating down your antibody concentration while keeping Zenon™ concentration constant.

Limited product warranty

Life Technologies Corporation and its affiliates warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have questions, contact Life Technologies at **www.thermofisher.com/support**.

Life Technologies Corporation | 29851 Willow Creek Road | Eugene, Oregon 97402 USA
For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

Revision history: Pub. No. MAN1000088 A00

Revision	Date	Description
A00	24 April 2024	New document for Zenon™ pHrodo™ Deep Red IgG Labeling Reagents.