

## Enhanced Zenon® Mouse IgG<sub>1</sub> Labeling Kits

Z25090 Zenon® Alexa Fluor® 488 Mouse IgG<sub>1</sub> Labeling Kit \*enhanced with TSA™ technology\*

Z25091 Zenon® Alexa Fluor® 568 Mouse IgG<sub>1</sub> Labeling Kit \*enhanced with TSA™ technology\*

### Quick Facts

#### Storage upon receipt:

- 2–6°C
- Protect from light

#### Ex/Em:

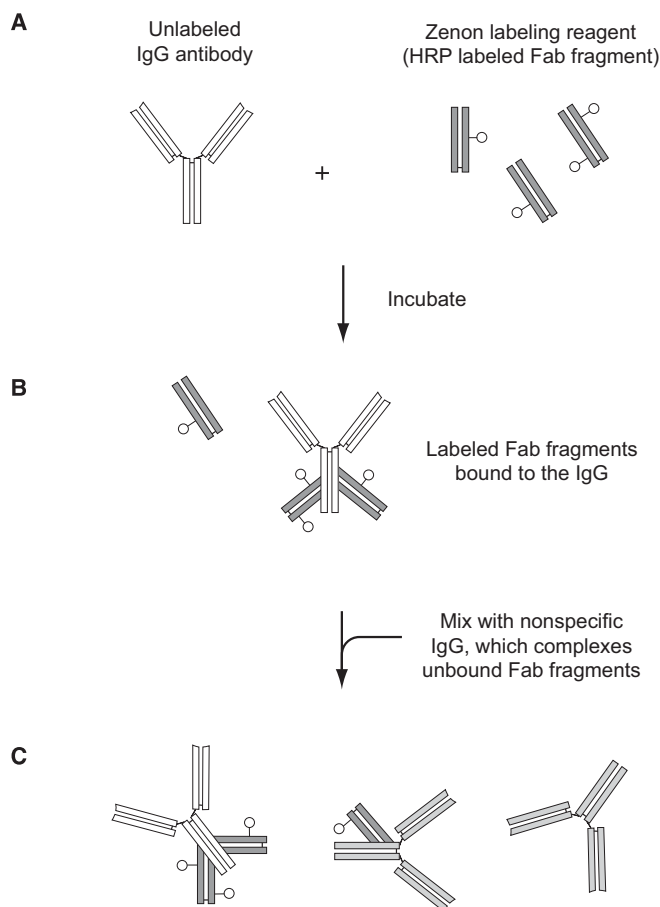
- 495/519 nm for Alexa Fluor® 488 dye
- 579/604 nm for Alexa Fluor® 568 dye

### Introduction

The Zenon® Mouse IgG<sub>1</sub> Labeling Kits enhanced with TSA™ technology combine Zenon® antibody-labeling technology, TSA (tyramide signal amplification) technology, and popular Alexa Fluor® dyes to provide exceptional target-identification capabilities. These kits pair portions of the Zenon® Horseradish Peroxidase Mouse IgG<sub>1</sub> Labeling Kit (Z25054) and the Alexa Fluor® 488 or Alexa Fluor® 568 TSA Kits (T20912 or T20914, respectively) into a single kit for researchers who want the ease of labeling mouse IgG<sub>1</sub> antibodies with Zenon® labeling reagents and the signal amplification afforded by the use of fluorescent tyramides.

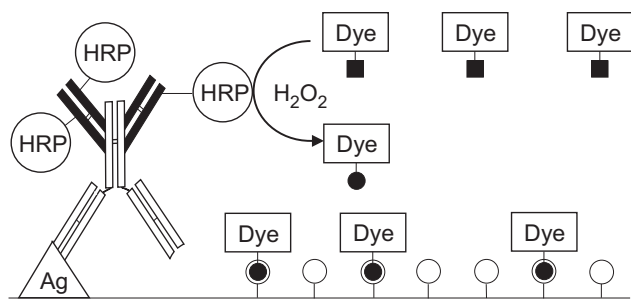
Zenon® technology is a fast, versatile, and reliable method for labeling antibodies, and the method can be used even with submicrogram amounts of starting material. Antibodies labeled with Zenon® labeling reagents can be used to stain cells in applications where directly labeled primary antibodies are suitable, such as flow cytometry, imaging, and high-throughput analysis. This technology provides a simple alternative to time-consuming or impractical approaches for using multiple mouse-derived antibodies in the same staining protocol.

The Zenon® horseradish peroxidase mouse IgG<sub>1</sub> labeling reagents are Fab fragments of goat IgG antibodies directed against the Fc portions of intact mouse IgG<sub>1</sub> primary antibodies. The Fab fragments are affinity purified to ensure their selectivity for the Fc portion of the primary antibody and then labeled with horseradish peroxidase (HRP). To form a Zenon® HRP mouse IgG<sub>1</sub> labeling complex with any mouse IgG<sub>1</sub> primary antibody, the labeling reagent is simply mixed with the antibody (Figure 1). Formation of the labeled Fab–antibody complex occurs in less than 5 minutes, and nearly all of the primary antibody in the

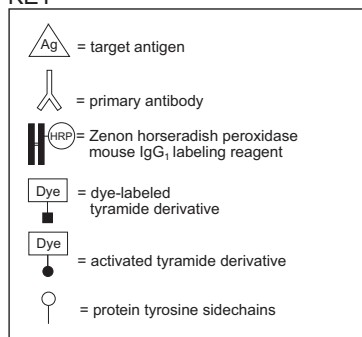


**Figure 1.** The Zenon® labeling scheme. An unlabeled mouse IgG<sub>1</sub> is incubated with the Zenon® labeling reagent, which contains a HRP-labeled Fab fragment (A). The labeled Fab fragment binds to the Fc portion of the IgG antibody (B), and excess Fab fragment is neutralized by the addition of a nonspecific mouse IgG (C).

mixture is labeled. The extent of antibody labeling (and thus, the enzymatic activity of the probe) can be adjusted by varying the amount of Zenon® labeling reagent that is added, i.e. by varying the molar ratio of labeled Fab fragment to primary antibody. Because the labeling is based on immunoselectivity, complex formation does not require the removal of exogenous proteins, such as serum albumin or amine-containing buffers, from the primary antibody prior to forming the complex. Complexes formed using this technology display HRP activity similar to that of primary antibodies directly labeled with HRP, and the cross-reactivity with antibodies from other species is low.



#### KEY



**Figure 2.** Schematic representation of TSA detection applied to immunolabeling of an antigen.

TSA technology is an enzyme-mediated detection method that uses HRP to generate high-density labeling of a target protein *in situ*<sup>1-5</sup> using tyramide. The HRP of the target-bound Zenon<sup>®</sup> HRP labeling complex activates multiple copies of the Alexa Fluor<sup>®</sup> tyramide, resulting in highly reactive, short-lived tyramide radicals (Figure 2). The signal is localized because activated tyramide is highly reactive with nucleophilic residues near the interaction site, which minimizes diffusion of the signal.

## Materials

### Contents

- **Zenon<sup>®</sup> horseradish peroxidase mouse IgG<sub>1</sub> labeling reagent** (Component A), 125  $\mu$ L
- **Zenon<sup>®</sup> blocking reagent (mouse IgG)** (Component B), 125  $\mu$ L
- **Alexa Fluor<sup>®</sup> dye-labeled tyramide** (Component C), one vial
- **Dimethylsulfoxide** (DMSO; Component D), 100  $\mu$ L
- **TSA blocking reagent** (Component E), 1.5 g
- **TSA amplification buffer** (Component F), one vial (contains thimerosal at 0.02%)
- **Hydrogen peroxide** (Component G), 100  $\mu$ L of a 30% stabilized solution

Sufficient reagents are provided for 25 labelings. One labeling is defined as the amount of Zenon<sup>®</sup> labeling reagent required to label 1  $\mu$ g of an intact, affinity-purified mouse IgG<sub>1</sub> antibody at a Fab:antibody molar ratio of 3:1 (see note A).

The Zenon<sup>®</sup> mouse IgG<sub>1</sub> labeling reagent conjugated to HRP is supplied at 200  $\mu$ g Fab fragment/mL in 0.1 M sodium phosphate,

0.1 M NaCl, pH 6.8, and 0.02% thimerosal. The Zenon<sup>®</sup> blocking reagent (mouse IgG) is supplied at 5 mg/mL in phosphate-buffered saline, pH 7.2, containing 5 mM sodium azide.

### Materials Not Provided

- Phosphate-buffered saline (PBS)
- Peroxidase quenching buffer: 1–3% H<sub>2</sub>O<sub>2</sub> in PBS

### Storage Conditions

Upon receipt, store the Zenon<sup>®</sup> Mouse IgG<sub>1</sub> Labeling Kits, enhanced with TSA technology, at 2–6°C, protected from light. The kit components should be stable for at least 6 months.

## Protocol

### Solution Preparation

**1.1 Prepare the tyramide stock solution.** Dissolve the Alexa Fluor<sup>®</sup> 488 or Alexa Fluor<sup>®</sup> 568 tyramide (Component C) in 75  $\mu$ L of DMSO (Component D). Store unused portions of this stock solution in small aliquots at  $\leq -20^{\circ}\text{C}$ , desiccated and protected from light.

**1.2 Prepare TSA blocking solution.** Assuming  $\sim 200$   $\mu$ L/cover-slip, make a 1% (10 mg/mL) tyramide blocking solution by dissolving an appropriate amount of tyramide blocking reagent (Component E) in PBS. Although the unused solution can be stored frozen at  $\leq -20^{\circ}\text{C}$  for 1 month, it is best to prepare only as much as is needed for immediate use.

**1.3 Prepare TSA amplification buffer with H<sub>2</sub>O<sub>2</sub>.** Add the appropriate amount of 30% hydrogen peroxide (Component G) to TSA amplification buffer (Component F) to obtain a final concentration of 0.0015% H<sub>2</sub>O<sub>2</sub>. For a working solution of 100  $\mu$ L per sample, use the following two step process. Step 1: Add 1  $\mu$ L of 30% H<sub>2</sub>O<sub>2</sub> to 200  $\mu$ L of TSA amplification buffer to get an intermediate concentration of 0.15% H<sub>2</sub>O<sub>2</sub>. Step 2: Add 1  $\mu$ L of the solution from step 1 to 100  $\mu$ L of amplification buffer to get a final concentration of 0.0015% H<sub>2</sub>O<sub>2</sub>.

### Cell Fixation

Follow customary cell fixation procedures, making sure that the slides or coverslips do not dry out at any point during the staining protocol. The following procedure is provided only as a guide.

**2.1 Culture cells in a suitable growth medium.** For adherent cell lines, grow the cells directly on slides or coverslips.

**2.2 Fix the cells.** Incubate the cells in 4% formaldehyde solution in PBS for 15 minutes at room temperature. Rinse the cells several times with PBS.

**2.3 Permeabilize the cells.** Incubate the sample in PBS containing 0.1–0.2% Triton<sup>®</sup> X-100 for 5–10 minutes at room temperature, or with acetone at  $\leq -20^{\circ}\text{C}$  for 10 minutes. Rinse the cells several times with PBS.

## Quenching and Blocking

**3.1 Quench endogenous peroxidase activity.** If desired, incubate the cells in peroxidase quenching buffer (~200 µL/cover slip) for 60 minutes at room temperature. This buffer (1–3% H<sub>2</sub>O<sub>2</sub> in PBS) is not provided.

**3.2 Block the cells.** Incubate the specimen with 1% TSA blocking solution (~200 µL/cover slip) for 60 minutes at room temperature or 37°C.

## Zenon® Complex Formation

The following protocol is for labeling 1 µg of a mouse IgG<sub>1</sub> primary antibody with a Zenon® mouse IgG labeling reagent using a 3:1 molar ratio of Fab to antibody target (see note A). This molar ratio is a suggested starting point and represents the minimum ratio for adequate labeling in most applications. Higher molar ratios may be necessary to obtain a satisfactory signal (see note B). The amounts of the reagents specified in this protocol can be scaled up or down for larger or smaller quantities of antibody. The Zenon® mouse 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 in ascites fluid or hybridoma supernatants can also be directly labeled without purification of the antibody either prior to, or after, labeling.

**4.1 Prepare the antibody.** Transfer 1 µg of antibody in a suitable buffer, such as PBS, to a microfuge tube. The volume should be ≤20 µL. If the concentration of the antibody is not specified, determine an approximate concentration (see note C). If an impure antibody preparation, such as ascites fluid or hybridoma supernatant, is used, then use a volume equivalent to 1 µg of IgG (see note D).

**4.2 Form the complex.** Add 5 µL of the Zenon® mouse IgG labeling reagent (Component A) to the antibody solution and mix well. Incubate the mixture for 5 minutes at room temperature.

**4.3 Block the complex.** Add 5 µL of the Zenon® blocking reagent (Component B) to the reaction mixture (see note E). Incubate the solution for 5 minutes at room temperature. The complex is now ready and should be applied to the samples within approximately 30 minutes. If longer storage is required, pause after step 4.2 and

do not block the complex until shortly before use (see note F).

## Zenon® HRP Labeling

**5.1 Dilute the complex.** Dilute the Zenon® labeling complex (prepared according to Zenon® Complex Formation, above) to the desired working concentration in PBS containing 1–10% BSA or normal goat serum (NGS). If desired, the TSA blocking solution (prepared in step 1.2) can be used as the diluent. For optimal results, the final concentration of the primary antibody in this solution must be determined empirically (see note G).

**5.2 Label the cells.** Apply a sufficient volume (50–200 µL) of the diluted complex to immerse the cell sample. Incubate the cell sample for 30–60 minutes at room temperature. Wash the cells several times with PBS. Because the Zenon® Fab fragment is not covalently coupled to the primary antibody, the Fab fragment can dissociate with time; proceed with the tyramide labeling soon after labeling the cells with HRP.

## Tyramide Labeling

**6.1 Prepare a working solution of Alexa Fluor® 488 or Alexa Fluor® 568 tyramide.** Dilute the tyramide stock solution 100-fold (see note H) in 0.0015% H<sub>2</sub>O<sub>2</sub>/TSA amplification buffer (prepared in step 1.2) just prior to tyramide labeling. Prepare 100 µL of working solution per sample. This quantity is sufficient to cover a standard 18 mm × 18 mm coverslip.

**6.2 Stain the cells.** Add the tyramide working solution to the cells and incubate for 5–10 minutes at room temperature. Rinse the cells several times with PBS.

**6.3 Counterstain the cell sample, if desired.** Counterstain with a nucleic acid stain (e.g., Hoechst 33342, DAPI or SYTOX® Green stains) or with other labeling reagents (e.g., fluorophore-labeled phalloidin), and wash several times with PBS after additional staining procedures.

**6.4 Mount the specimens on slides.** Mount the coverslips in a suitable antifade mounting medium, such as ProLong® Antifade (Molecular Probes, P7481) (see note I) and examine by fluorescence microscopy using the appropriate filter sets. The excitation and emission maxima for Alexa Fluor® 488 tyramide are 495 nm and 519 nm, respectively. The excitation and emission maxima for Alexa Fluor® 568 tyramide are 579 nm and 604 nm, respectively.

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## Product Notes

**[A]** The Fab:antibody ratio is critical for determining the amount of Zenon® mouse IgG labeling reagent to use in the labeling protocol. In all Zenon® Mouse IgG Labeling Kits, the Zenon® labeling reagent is provided at a concentration of 200 µg/mL based upon the mass of the Fab fragment. A Fab fragment has a molecular weight of ~50 kDa, compared to ~150 kDa for an intact IgG; thus, 5 µL of any Zenon® labeling reagent mixed with 1 µg of mouse IgG antibody produces a Fab:antibody molar ratio of 3:1.

**[B]** When adjusting either the amount of antibody to be labeled or the Fab:antibody molar ratio, always use equal volumes of Zenon® labeling reagent and Zenon® blocking reagent. For example, if the amount of Zenon® labeling reagent used for a reaction is increased to 10 µL, then the amount of Zenon® blocking reagent should also be increased to 10 µL. Note that adding 10 µL of the Zenon® labeling reagent for each microgram of antibody (yielding a molar ratio of 6:1) will often increase the measured signal intensities by approximately 50%. Further increases in the molar ratio tend to yield smaller increases in intensity.

**[C]** If the concentration of mouse IgG is not specified, contact the supplier and obtain an approximate IgG content. If a concentration cannot be obtained from the supplier, a titration with the Zenon® labeling reagent should be performed to obtain the optimum labeling conditions.

**[D]** Monoclonal antibodies from suppliers are generally provided as a purified IgG fraction, or in ascites fluid or hybridoma supernatant. Primary antibodies that have not been purified can be labeled using the Zenon® mouse IgG labeling reagents and do not require the removal of nonspecific IgGs or serum proteins. The appropriate amount of the Zenon® labeling reagent to add

in step 4.2 should be determined by using the total IgG mass in the sample to be labeled; thus, 5  $\mu$ L of the Zenon<sup>®</sup> mouse IgG labeling reagent should be used for each microgram of IgG. Nonspecific IgGs will be labeled in addition to the specific IgG, however, the labeled nonspecific IgGs should not stain the sample appreciably.

**[E]** If no other mouse antibodies are to be used in the application, then step 4.3 of the labeling protocol (addition of the blocking reagent) can be omitted in most cases. Nonspecific binding of the uncomplexed Zenon<sup>®</sup> labeling reagent should be minimal. However, the blocking step may still be needed to avoid labeling endogenous mouse IgG present in some mouse tissue preparations.

**[F]** If long-term storage of the labeling complex is desired, stop the labeling procedure after step 4.2 of the protocol. At this stage the complexes can be stored at  $\leq 6^{\circ}\text{C}$  for several weeks with 0.02% thimerosal as a preservative. Note that sodium azide

must NOT be used for this purpose since it is a potent inhibitor of peroxidase. To use the conjugate, complete step 4.3 of the protocol before applying the conjugate to the experimental sample.

**[G]** Typically, a final concentration of 0.5–5.0  $\mu\text{g}$  of primary antibody per mL will work well. Thus, with 1  $\mu\text{g}$  of primary antibody in the standard Zenon<sup>®</sup> labeling protocol (step 4.1), there will be 2.0–0.2 mL of working solution.

**[H]** The tyramide stock solution can be diluted 50–200-fold to produce variations of staining intensity without compromising target resolution or background signal levels.

**[I]** Coverslips can be mounted with PBS/50% glycerol medium if specimens will be examined immediately. For longer-term storage and observation, ProLong<sup>®</sup> antifade reagent/mounting medium (Molecular Probes) or Cytoseal<sup>™</sup> mounting medium (Stephens Scientific) is recommended.

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## References

1. J Immunol Methods 125, 279 (1989); 2. Cytometry 23, 48 (1996); 3. J Histochem Cytochem 45, 375 (1997); 4. J Histochem Cytochem 47, 281 (1999).

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## Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
Z25090	Zenon <sup>®</sup> Alexa Fluor <sup>®</sup> 488 Mouse IgG <sub>1</sub> Labeling Kit *enhanced with TSA <sup>™</sup> technology* *25 labelings*	1 kit
Z25091	Zenon <sup>®</sup> Alexa Fluor <sup>®</sup> 568 Mouse IgG <sub>1</sub> Labeling Kit *enhanced with TSA <sup>™</sup> technology* *25 labelings*	1 kit

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