Zenon® Pacific Orange™ Mouse IgG Labeling Kits

Table 1. Contents and Storage Information

Material	Amount	Concentration	Storage	Stability
Zenon® Pacific Orange™ mouse IgG_1 labeling reagent (Component A) or Zenon® Pacific Orange™ mouse IgG_{2a} labeling reagent (Component A)	250 µL	400 µg Fab fragment/ mL in 0.1 M sodium phosphate, 0.1 M NaCl, 5 mM sodium azide, pH 7.5	• 2–6˚C • Protect from light • For long-term storage, divide into aliquots and freeze at ≤–20˚C	3 months
Zenon® blocking reagent (mouse IgG) (Component B)	500 µL	5 mg/mL in PBS, 5 mM sodium azide, pH 7.2		
Number of Labelings: Sufficient Zenon® labeling reagent is supplied for 50 labelings. One labeling is defined as the amount of Zenon labeling reagent required to label 1 µg of an intact, affinity-purified mouse IgG antibody at a Fab:antibody molar ratio of 6:1.				
Spectral Data: Pacific Orange™ dye ~400/551 nm. See Figure 1.				

Introduction

The Zenon® Pacific Orange™ Mouse IgG Labeling Kits provide a fast, versatile and reliable method for producing antibody conjugates, even with very small (submicrogram) amounts of starting material. Antibody conjugates formed using Zenon® technology may be used to stain cells in any application where a directly labeled primary antibody is suitable, including flow cytometry, imaging, high throughput, and other applications. Moreover, this technology simplifies applications that previously were time consuming or not practical, such as the use of multiple mouse-derived antibodies in the same staining protocol.

Each of the Zenon® Pacific Orange™ Mouse IgG Labeling Kits is designed for use with a particular mouse monoclonal antibody isotype: IgG_1 or IgG_{2a}. Zenon® labeling technology utilizes a fluorophore-, biotin- or enzyme-labeled Fab fragment directed against the Fc portion of an intact IgG primary antibody in order to form a labeling complex (Figure 1). The labeled Fab fragments have been affinity purified during their preparation to ensure their high affinity and selectivity for the Fc portion of the primary antibody. Because this labeling is based on immunoselectivity, the Zenon® labeling method does not require the removal of exogenous proteins such as serum albumin or amine-containing buffers from the antibody prior to forming the complex. Crossreactivity is low with antibodies from other species.

Formation of the Fab–antibody complex occurs in less than 5 minutes, and nearly all of the primary antibodies in the mixture are labeled. Complexes formed using this technology display fluorescence intensity or enzymatic activity similar to that of directly labeled primary antibodies. In addition, the extent of antibody labeling (and thus the fluorescence intensity or 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.

Molecular Probes maintains an e-mail address (probeszenon@invitrogen.com) specifically dedicated to the Zenon® labeling technology. For technical questions and/or comments about these products, please send an e-mail to the above address, or contact our Technical Service department. For a complete list of Zenon® Mouse IgG Labeling Kits offered by Molecular Probes, please see the *Appendix*.

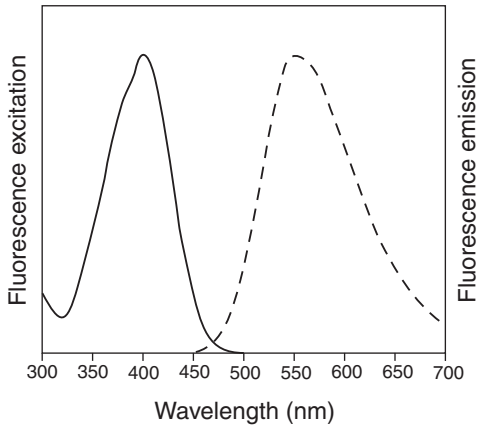

Figure 1. Normalized excitation and emission spectra of Pacific Orange™ dye conjugated to goat anti-mouse IgG in pH 7.2 buffer.

Before You Begin

Before beginning the labeling protocol, verify that the isotype of the mouse antibody matches that of the Zenon® Mouse IgG Labeling Kit. The Zenon® Pacific Orange™ mouse IgG labeling reagents in the kits are isotype specific and are not recommended for labeling antibodies that are not of the corresponding mouse IgG isotype.

Please note that the Fab:antibody ratio is the important factor when determining the amount of Zenon® Pacific Orange™ mouse IgG labeling reagent to use in the labeling protocol. In all Zenon® Pacific Orange™ Mouse IgG Labeling Kits, the Zenon® labeling reagent is provided at a concentration of 400 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 6:1.

When adjusting either the amount of antibody to be labeled or the Fab:antibody molar ratio, it is important to always use the recommended proportion of Zenon® labeling reagent and Zenon® blocking reagent (2 volumes of blocking reagent to 1 volume of labeling 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 be increased to 10 µL. Note that adding 20 µL of the Zenon® labeling reagent for each microgram of antibody (yielding a molar ratio of 12:1) will often increase the measured signal intensities by approximately 50%. Further increases in the molar ratio tend to yield smaller increases in intensity.

Materials Required but not Provided

- 4% formaldehyde solution in PBS
- 0.1% Triton X-100
- normal goat serum (NGS)

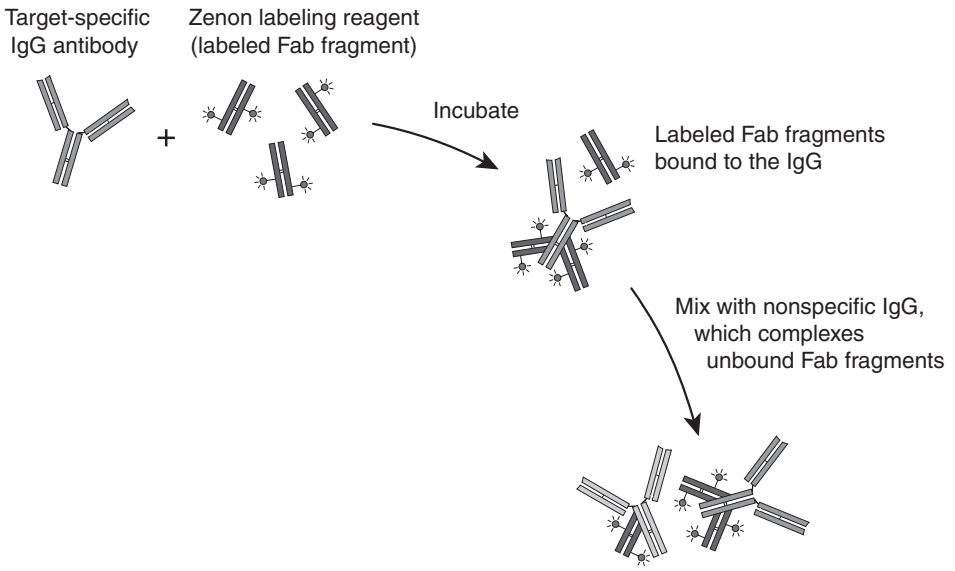

Figure 1. The Zenon® labeling scheme. An unlabeled IgG is incubated with the Zenon® labeling reagent, which contains a fluorophore-labeled Fab fragment (A). The labeled Fab fragment binds to the Fc portion of the IgG antibody (B), and excess Fab fragment is neutralized by the addition of a nonspecific IgG (C). The addition of nonspecific IgG prevents cross-labeling of the Fab fragment in experiments where multiple primary antibodies of the same type are present. Note that the Fab fragment used for labeling need not be coupled to a fluorophore, but could instead be coupled to an enzyme or to biotin.

General Application Tips

Conjugate utility

Mouse IgG antibodies labeled using Zenon® technology are expected to be suitable in all applications where a directly labeled antibody can be used. Multiple mouse IgG antibodies labeled with Zenon® reagents can be used in one experiment either sequentially or as a single staining mixture.

Conjugate stability

Once the conjugates have been formed and excess Fab taken up by the blocking reagent, the labeled complexes should be used within approximately 30 minutes.

Working concentration

For applications where a directly labeled primary antibody is typically used, the antibody labeled using Zenon® technology can generally be used at a similar or higher concentration (1.5–3-fold).

Low or no signal

If suitable controls verify that the primary antibody is binding to the expected target but no signal is observed with the antibody labeled using Zenon® technology, the signal may be increased by adding more of the Zenon® labeling reagent to increase the Fab:antibody molar ratio and/or by increasing the final concentration of the primary antibody. **While a molar ratio of 6:1 is suitable in many cases, the molar ratio can be increased up to 12:1. Further increases in the molar ratio will not result in a significant increase in the signal strength.**

Label loss or transfer

Because the labeled Fab fragment is not covalently coupled to the primary antibody, some loss of the labeled Fab fragments may occur over time. In applications with multiple mouse IgG primary antibodies, the Fab fragments may transfer slowly from one primary antibody to another. Samples should be subjected to an additional post-staining fixation step with formaldehyde in order to reduce Fab dissociation (see *Staining Applications*, below).

Zenon ® Complex Formation

The following protocol is for labeling 1 µg of antibody with a Zenon® Pacific Orange™ mouse IgG labeling reagent to obtain a 6:1 molar ratio of Fab to antibody target. This molar ratio is a suggested starting point and represents the minimum ratio for adequate labeling in most applications; individual experiments may require higher molar ratios in order to obtain satisfactory signal. For larger or smaller quantities of antibody, the amounts of the reagents specified in this protocol can be scaled accordingly. 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 contained within ascites fluid or hybridoma supernatants can also be directly labeled and do not require purification of the antibody either prior to or after labeling.

1.1 Prepare 1 µg of antibody in a suitable buffer, such as phosphate-buffered saline (PBS). The volume is not crucial, provided it is ≤20 µL.

NOTE: If using an antibody where the concentration is not specified, contact the supplier and obtain at least an approximate IgG content. If a concentration cannot be obtained from the supplier, a titration with the Zenon® labeling reagent will need to be performed to obtain the optimum labeling conditions..

If using an impure antibody preparation, e.g. ascites fluid or hybridoma supernatant, please note that such primary antibodies can still be labeled using the Zenon® Pacific Orange™ 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 1.2 should be determined by using the total IgG mass in the sample to be labeled; thus, 5 µL of the Zenon® Pacific Orange™ mouse IgG labeling reagent should be used for each µg of IgG. Nonspecific IgGs will be labeled in addition to the specific IgG; however, the labeled nonspecific IgGs should not stain the sample appreciably and will be washed away during the staining procedure.

1.2 Add 5 µL of the Zenon® Pacific Orange™ mouse IgG labeling reagent (Component A) to the antibody solution.

1.3 Incubate the mixture for 5 minutes at room temperature.

1.4 Add 10 µL of the Zenon® blocking reagent (Component B) to the reaction mixture. The blocking reagent is not isotype specific. If the application uses only a single labeled antibody, steps 1.4 and 1.5 of the labeling protocol (addition of the blocking reagent) can be omitted. The level of uncomplexed Zenon® labeling reagent is sufficiently low that it should not cause high background levels.

1.5 Incubate the solution for 5 minutes at room temperature.

The complexes are now ready and should be applied to samples within approximately 30 minutes. If longer storage is required, stop the labeling procedure at step 1.3 of the protocol. At this stage the complexes can be stored at 4°C for several weeks with the addition of 2 mM sodium azide as a preservative. When ready to use the conjugate, complete steps 1.4 and 1.5 of the protocol before applying the conjugate to the experimental sample.

Staining Applications

The following protocols were developed at Molecular Probes for the staining of cells and tissues using primary antibodies labeled with Zenon® Pacific Orange™ mouse IgG labeling reagents. The protocols may not be applicable to all experimental situations, and users are encouraged to adapt these protocols to their needs.

Immunocytochemisty

This protocol has been successfully used to stain a number of cell lines, including bovine pulmonary artery endothelial (BPAE), Muntjac, and Madin-Darby canine kidney (MDCK) cells.

2.1 Culture cells in suitable growth medium. Adherent cell lines intended for observation by microscopy can be grown directly on slides or coverslips; however, do not allow the slides or coverslips to dry out at any point during the staining protocol.

2.2 Fix the cell sample in 4% formaldehyde solution in PBS for 15 minutes at room temperature. Alternatively, the sample can be incubated for 15 minutes at 37°C under 5% CO_2. This latter fixation method has been observed to better preserve cytoskeletal morphology.

2.3 Wash the cell sample one or more times with PBS.

2.4 Permeabilize the cells by incubating the sample in PBS containing 0.1% Triton X-100 for 5 minutes at room temperature. Remove the detergent solution.

2.5 Block nonspecific binding sites in the cell sample for 30 minutes in PBS containing 10% normal goat serum (NGS).

2.6 Dilute the Zenon® labeling complex (prepared according to *Zenon® Complex Formation*, above) to the desired working concentration in PBS containing 10% NGS, and apply a sufficient volume to immerse the cell sample. If staining with more than one Zenon® labeling complex, all complexes can be combined into a single staining solution; sequential labeling is not necessary. In systems where the concentration of primary antibody has already been optimized for use with a secondary detection reagent, use the primary antibody at a concentration 1.5–3-fold higher with the Zenon® staining method. Otherwise, the appropriate working concentration will need to be determined empirically.

2.7 Incubate the cell sample for 30–60 minutes at room temperature. Zenon® labeling reagents with fluorophores should be protected from light during the incubation. When staining is complete, wash the cell sample one or more times with PBS.

2.8 Perform a second fixation of the cell sample in 4% formaldehyde solution in PBS for 15 minutes at room temperature. When the fixation is complete, wash the cell sample one or more times with PBS. Without this second fixation step, the Zenon® Fab fragment, because it is not covalently coupled to the primary antibody, can dissociate with time, and can even transfer to another primary antibody in multicolor applications. This step is optional for experiments using only a single primary antibody for labeling purposes, but signal intensity is generally superior with the additional fixation.

2.9 If desired, counterstain the cell sample 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 with PBS after any additional staining procedure.

2.10 If necessary, deposit the cells on a slide or coverslip, or place in a flow tube. For a sample to be viewed by microscopy, mount in a suitable antifade mounting medium (e.g., use the ProLong® Gold antifade reagent; P36930). Analyze or view the sample by the desired method.

Immunohistochemistry

This protocol is designed for 10–12 µm frozen sections of tissues fixed in 4% formaldehyde via transcardial perfusion. *Do not use glutaraldehyde-containing fixatives.* The use of glutaraldehyde increases sample autofluorescence and results in excessive crosslinking of proteins, which can impede antibody access and can denature antigens. Where possible, use thinner sections to promote thorough and more rapid antibody penetration into the tissue. Zenon® labeling complexes have not been tested or optimized for sections embedded in paraffin. Note that certain low-abundance antigens may not be detected as easily using Zenon® labeling technology as they would be using conventional secondary detection methods, and the Zenon® method is not recommended for very low-abundance target molecules in tissue samples.

In parallel to the labeling of an experimental sample, a negative-control sample should be processed in an identical protocol, but with the omission of the primary antibody, in order to assess nonspecific labeling. A positive control sample labeled by conventional secondary detection methods can be included during pilot studies to confirm the specificity of the staining pattern observed using the Zenon® labeling method.

3.1 Allow the slide containing the frozen tissue section to thaw at room temperature (usually 15–30 minutes). Draw a rectangle around the section with a PAP pen, if desired.

3.2 Rehydrate the section in PBS for 15 minutes at room temperature. Do not allow the section to dry at any point during the remaining steps of this staining protocol.

3.3 Permeabilize the section in PBS containing 0.2% Triton X-100 (PBT) for 20 minutes at room temperature.

3.4 Block nonspecific binding sites with PBT containing 0.2% BSA and 5% heat-inactivated normal goat serum for 30 minutes at room temperature. Alternatively, 1% BSA can be used in place of the serum. Remove the blocking buffer once the incubation is complete.

3.5 Dilute the Zenon® labeling complex (prepared according to *Zenon® Complex Formation,* above) in PBT to the desired working concentration. In a horizontal staining dish, apply 100–250 µL of the working solution to cover the section. For initial experiments, test a range of different primary antibody concentrations and different molar ratios of Zenon® labeling reagent (Fab) to antibody. A molar ratio of 6:1 is the recommended starting point for tissue staining, and, in general, Zenon® labeling methods require a higher concentration of primary antibody than would be used in a standard secondary detection scheme. It is important to include detergent in the staining buffer in order to promote antibody penetration. Buffer PBT, as described above, contains 0.2% Triton X-100; however, the detergent choice and concentration (generally 0.1–0.5%) may need to be optimized to strike a balance between tissue permeabilization and the possibility of the antigen being sensitive to detergent extraction.

3.6 Incubate the section with the staining solution in a humidified chamber for 1–2 hours at room temperature. Zenon® labeling reagents with fluorophores should be protected from light during the incubation. If staining with more than one Zenon® labeling complex, all complexes can be combined into a single staining solution; sequential labeling is not necessary. *Do not use longer incubation times.* With longer incubation times, the Zenon® Fab fragment can dissociate from the primary antibody, resulting in poor signal-to-noise ratios and possible crosslabeling of primary antibodies in multicolor staining applications.

3.7 Remove the staining solution and wash the section in PBT for 10–15 minutes at room temperature. Repeat the wash an additional two times. The wash steps should be performed in an upright Coplin staining jar.

3.8 Wash the stained tissue section for 5 minutes in PBS at room temperature. Repeat.

3.9 Perform a second fixation of the tissue section in 4% formaldehyde solution in PBS for 15 minutes at room temperature. When the fixation is complete, wash the section one or more

times with PBS. Without this second fixation step, the Zenon® Fab fragment, because it is not covalently coupled to the primary antibody, can dissociate with time, and can even transfer to another primary antibody in multicolor applications.

3.10 If desired, counterstain the tissue sample 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 with PBS after any additional staining procedure.

3.11 Mount the section in a suitable antifade mounting medium (e.g., use the ProLong® antifade reagent; P36930) and observe the fluorescence using appropriate filters.

Flow Cytometry

The following protocol was developed for the staining of peripheral blood mononuclear cells and can be readily adapted to other cell types or to whole blood samples. Note that an Fc blocking step is not required when using antibodies labeled with Zenon® technology.

4.1 Prepare the cell suspension or blood sample as preferred.

4.2 Add 100 µL of cultured cells (at 1 × 106 cells/mL) or 100 µL of a whole blood sample to a flow cytometry tube.

4.3 (Optional) Fix the cell sample in 4% formaldehyde solution in PBS for 15 minutes at room temperature. Wash the cell sample with PBS, centrifuge the sample to pellet the cells, and decant the supernatant. Resuspend the cells in the residual buffer.

NOTE: This and the following step are required only when detecting internal cell markers. If using antibodies against cell surface and internal markers in the same experiment, perform the staining of the cell surface markers first, then follow this step and steps 4.4–4.7 to stain the internal markers.

4.4 (Optional) Permeabilize the cell sample by incubating the sample in PBS containing 0.1% Triton X-100 for 5 minutes at room temperature. Wash the cell sample with PBS, centrifuge the sample to pellet the cells and decant the supernatant. Resuspend the cells in the residual buffer.

4.5 Label 1 µg of the primary antibody according to *Zenon® Complex Formation,* above.

4.6 Add the entire Zenon® labeling mixture (prepared in step 4.5) to the sample tube. **NOTE:** If using whole blood samples, the lysis of red blood cells can be performed either before or after cell staining (steps 4.6–4.7).

4.7 Incubate the sample for 30 minutes at room temperature. Zenon® labeling complexes with fluorophores should be protected from light during the incubation. When complete, wash the cells once with PBS, centrifuge the sample to pellet the cells, and decant the supernatant. Resuspend the cells in the residual buffer. A post-staining fixation step, required for imaging applications, is not required for flow cytometry applications.

4.8 Analyze the sample using appropriate instrument parameters. Pacific Orange™ dye has fluorescence excitation/emission maxima of 400/551 nm.

Appendix

Molecular Probes' Zenon® Mouse IgG Labeling Kits.

Label	Ex/Em *	Catalog Number		
		Mouse IgG_1	Mouse IgG_{2a}	Mouse IgG_{2b}
Alexa Fluor® dyes				
Alexa Fluor® 350	346/442	Z25000	Z25100	Z25200
Alexa Fluor® 405	402/421	Z25013	Z25113	Z25213
Alexa Fluor® 430	434/539	Z25001		
Alexa Fluor® 488	495/519	Z25002	Z25102	Z25202
Alexa Fluor® 532	531/554	Z25003		
Alexa Fluor® 546	556/573	Z25004	Z25104	Z25204
Alexa Fluor® 555	555/565	Z25005	Z25105	Z25205
Alexa Fluor® 568	578/603	Z25006	Z25106	Z25206
Alexa Fluor® 594	590/617	Z25007	Z25107	Z25207
Alexa Fluor® 647	650/668	Z25008	Z25108	Z25208
Alexa Fluor® 660	663/690	Z25009	Z25109	Z25209
Alexa Fluor® 680	679/702	Z25010	Z25110	Z25210
Alexa Fluor® 700	696/719	Z25011		
Alexa Fluor® 750	752/779			
Conventional dyes				
Marina Blue®	365/460	Z25040		
Pacific Blue™	410/455	Z25041	Z25156	
Pacific Orange™	400/551	Z25256	Z25257	
Fluorescein	494/518	Z25042		
Oregon Green® 488	496/524	Z25043		
Texas Red®-X	595/615	Z25045		
Biotins				
Biotin-XX	NA	Z25052	Z25152	Z25252
DSB-X™ † biotin	NA	Z25053		
R-phycoerythrin, allophycocyanin and tandem constructs				
R-phycoerythrin (R-PE)	496 ‡/578	Z25055	Z25155	Z25255
Alexa Fluor® 610–R-PE	496 ‡/630	Z25020		
Alexa Fluor® 647–R-PE	496 ‡/668	Z25021	Z25121	Z25221
Alexa Fluor® 680–R-PE	496 ‡/702	Z25022		
Allophycocyanin (APC)	650/660	Z25051	Z25151	Z25251
Alexa Fluor® 700–APC	650/723	Z25030		
Alexa Fluor® 750–APC	650/775	Z25031		
Enzymes				
Horseradish peroxidase	NA	Z25054	Z25154	Z25254
Alkaline phosphatase	NA	Z25050	Z25150	Z25250

***** Approximate fluorescence excitation and emission maxima, in nm. **†** DSB-X is our trademark for desthiobiotin-X.
‡ Additional absorption peaks are present at 546 and 565 nm. NA = not applicable.

Table 2. Molecular Probes' Zenon® Tricolor Mouse IgG Labeling Kits.

Tricolor Labeling Kit	Labels *	Catalog Number †		
		Mouse IgG_1	Mouse IgG_{2a}	Mouse IgG_{2b}
Kit #1 (for imaging)	Alexa Fluor® 488 Alexa Fluor® 555 Alexa Fluor® 647	Z25060	Z25160	Z25260
Kit #2 (for imaging)	Alexa Fluor® 350 Alexa Fluor® 488 Alexa Fluor® 594	Z25070	Z25170	Z25270
Kit #3 (for flow cytometry)	Alexa Fluor® 488 R-phycoerythrin (R-PE) Alexa Fluor® 647–R-PE	Z25080	Z25180	Z25280

* See Table 1 for fluorescence excitation and emission maxima.

Product List **Current prices may be obtained from our website or from our Customer Service Department.**

Cat #	Product Name	Unit Size
Z25256	Zenon® Pacific Orange™ Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25257	Zenon® Pacific Orange™ Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit

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Order Phone: (800) 438-2209
Order Fax: (800) 438-0228

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