Effects of Different Fixatives on β-Galactosidase Activity

Wenbin Ma, Keith Rogers, Berton Zbar, and Laura Schmidt
Laboratory of Immunobiology (WM,BZ), Pathology and Histology Laboratory (KR), and Intramural Research Support Program (LS), SAIC–Frederick, Inc., NCI-Frederick, Frederick, Maryland

SUMMARY β-Galactosidase (β-Gal) staining is widely used to demonstrate specific gene expression during evaluation of gene targets in vivo. This technique is extremely sensitive to fixation. Optimal fixation conditions are necessary to obtain the maximal β-Gal activity. In this experiment, Carnoy’s and three different aldehyde fixatives were used at different temperatures and over different time points. Kidneys from LacZ-stop-human alkaline phosphatase (ZA/P) double reporter mice were used to generate positive material for the experiment. The results show that glutaraldehyde combinative solution (LacZ) produced the most consistent and reliable results. Paraformaldehyde and formaldehyde were effective as fixatives only at 4°C for a period of less than 4 hr, and Carnoy’s solution destroyed β-Gal activity.

KEY WORDS
β-galactosidase
X-gal staining
fixative
temperature

Tissue Specimens
The Z/AP mice were sacrificed by CO₂ asphyxiation and the kidneys were collected and cut into 0.2 × 0.4 × 0.5-mm samples. The kidney was selected for this study because expression of β-Gal is consistent in the kidney of the Z/AP mice (Lobe et al. 1999; and unpublished data). The kidney samples were immediately transferred to the different fixatives.

Fixation
Four different fixatives were used in this study: (a) LacZ fixative solution containing 0.2% glutaraldehyde, 5 mM EGTA (pH 7.3), 100 mM MgCl₂ in 0.1 M NaPO₄ (pH 7.3) (Lobe et al. 1999); (b) 4% paraformaldehyde in PBS (Quality Biological; Gaithersburg, MD); (c) 10% neutral buffered formalin (NBF); and (d) Carnoy’s fluid (Sheehan and Hrapchak 1980). The tissues were fixed in the different fixatives for 1, 2, 4, or 8 hr at 4°C, or at room temperature (RT) with a solution change every 2 hr. The samples were transferred into 15% sucrose in PBS for 4 hr and subsequently into 30% sucrose in PBS at 4°C overnight. The tissues were embedded in OCT and 10-μm cryostat sections were cut. The sections were dried at RT for 2 hr and stored at −20°C before staining.

Correspondence to: Dr. Laura Schmidt, IRSP, SAIC–Frederick, NCI-Frederick, Building 560, Room 12-69, Frederick, MD 21702. E-mail: schmidtl@mail.ncifcrf.gov
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X-Gal Staining and Evaluation

The sections were brought to RT and washed three times in 0.1 M PBS (pH 7.4) for 5 min. The sections were transferred to X-Gal staining solution (1 mg/ml of 5-bromo-4-chloro-3-indolyl β-d-galactoside (X-Gal), 2 mM MgCl₂, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.01% sodium deoxycholate, and 0.02% Nonidet-P40) at 37°C overnight. The samples were washed in distilled water three times, counterstained by Neutral Red, and dehydrated in different concentrations of ethanol, cleared in xylene, and coverslipped.

Results

The Z/AP reporter mice were developed to test the efficiency of Cre recombinase expression temporally and spatially by a decrease in X-Gal staining (LacZ) and an increase in human AP staining in the tissues of offspring from matings between Z/AP reporter mice and the Cre transgenic mice under study. Before Cre-mediated recombination, the chicken β-actin promoter drives LacZ gene expression in most mouse tissues. After Cre-mediated recombination, the floxed lacZ gene and polyA signal are removed and human alkaline phosphatase is expressed.

In this study, the kidney was selected as the organ for testing the effect of different fixatives on β-Gal activity. Two temperatures and four time points were evaluated. The results are summarized in Table 1 and Figure 1. There was no difference in X-Gal staining between 0.2% glutaraldehyde (LacZ fix), 4% paraformaldehyde, and 10% NB formalin fixatives after 1 hr at either temperature. After a 2-hr fixation, LacZ fix with 0.2% glutaraldehyde, 10% NB formalin and 4% paraformaldehyde produced strong X-Gal staining at 4C and a slightly diminished intensity at RT with both 4% paraformaldehyde and 10% NB formalin. After a 4-hr fixation at 4C, the X-Gal staining with LacZ fix containing 0.2% glutaraldehyde was undiminished, the X-Gal staining with 4% paraformaldehyde and 10% formalin fixatives was slightly diminished, but staining was severely reduced at RT using 4% paraformaldehyde or 10% formalin fixative. There was only a slight decrease in staining with the 0.2% glutaraldehyde fixative after 8 hr at RT. However, 8 hr of fixation at RT with 4% paraformaldehyde or 10% NB formalin destroyed the β-Gal activity in the tissues. No X-Gal staining was detected after 1, 2, 4, or 8 hr in Carnoy’s fixative at either temperature.

Discussion

The purpose of fixation is to protect cells and cell components from the effects of autolytic change and from damage caused by the reagents used in various histological processes. In routine histology, fixatives are selected according to the desired properties, e.g., preservation of tissue integrity, speed of penetration, and hardening properties. For enzymatic histochemistry and immunohistochemistry protocols, fixatives are selected on the basis of their potential effect on a particular enzyme or antigen. In these experiments we selected four different fixatives widely used in histochemistry and immunohistochemistry studies. To avoid further destruction of β-Gal activity, we used frozen tissue sections instead of paraffin-embedded sections.

X-Gal staining results in kidney tissue sections from Z/AP mice indicated that the LacZ fixative containing 0.2% glutaraldehyde was an excellent fixative for evaluating β-Gal activity. Variation in time and temperature of fixation did not affect the intensity of X-Gal staining when 0.2% glutaraldehyde fixative was used. Usually, the time of fixation using 0.2% glutaraldehyde is limited to a maximum of 2 hr because it is an unstable substance and is chemically degraded by oxidation at low pH. Glutaraldehyde penetrates rather slowly, so the block size must be smaller to ensure complete fixation. To avoid these problems, the LacZ solution also contained buffered PBS (0.1 M, pH 7.4) and EGTA, which stabilized the pH and increased its ability to penetrate tissues. In these experiments, the LacZ fixative solution was changed every 2 hr. β-Gal activity was stable in LacZ fixative containing 0.2% glutaraldehyde for up to 8 hr without significant change at either 4C or RT.

Paraformaldehyde is widely used in histochemistry and immunohistochemistry because it penetrates rapidly with minimal shrinkage of the tissue. During fixation, paraformaldehyde is dissociated into formaldehyde. After prolonged fixation, paraformaldehyde and formalin results would be expected to be similar. In our experiments, X-Gal staining results with 4% paraformaldehyde in PBS and 10% NB formalin were similar. β-Gal activity survived a 2-hr fixation at 4C and 1-hr fixation at RT, but enzymatic activity decreased after 4 hr at 4C and after 2 hr at RT.

Carnoy’s fixative was created for histological procedures to balance shrinkage and distortion in tissues fixed in absolute alcohol, and acetic acid. It consists of

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<th>Table 1 Effects of fixative time and temperature on β-galactosidase activity of the Z/AP kidney</th>
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<td>Fixatives</td>
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<td>LacZ (2% glutaraldehyde)</td>
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<td>4% paraformaldehyde</td>
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<td>Carnoy’s fixative</td>
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Effects of Fixatives on β-Gal Activity

chloroform, alcohol, and acetic acid. Although Carnoy’s fixative is sometimes used for fixing tissue enzymes, our results show complete inactivation of β-Gal activity after 1 hr fixation at 4C (data not shown). The ability of Carnoy’s fixative to destroy β-Gal activity may be related to the acetic acid component. The acidic pH may be an important factor contributing to the absence of X-Gal staining.

Monitoring the activity of β-Gal by X-Gal staining has been widely used to evaluate Cre-mediated recombination in Cre reporter mice (Lobe et al. 1999; Soroiano 1999). Our results emphasize the importance of selecting the right fixative, duration, and temperature for tissue preparation before X-Gal staining methods for evaluation of Cre-mediated recombination. Overfixation and/or incorrect choice of fixative may lead to an underestimation of Cre recombinase expression, resulting in invalid conclusions.

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Literature Cited


Figure 1  Kidney slices were fixed in LacZ solution at 4C for 1 hr (A), 8 hr (B), and at RT for 8 hr (C). Kidney slices were fixed in 4% paraformaldehyde at 4C for 1 hr (D), 4 hr (E), and at RT for 4 hr (F). Kidney slices were fixed in buffered 10% formalin at 4C for 1 hr (G), 4 hr (H), and at RT for 2 hr (I). Bar = 220 μm.