bio-protocol www.bio-protocol.org/e3180
Vol 9, Iss 05, Mar 05, 2019
DOI:10.21769/BioProtoc.3180

An Improved Staining Method for Low Signal *LacZ* Reporter Detection in Mouse Embryos

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[Abstract] In many fields of biology, especially in the field of developmental biology, *LacZ* reporter staining is an approach used to monitor gene expression patterns. In the *LacZ* reporter system, the *LacZ* gene is inserted in the endogenous location of the target gene via gene knock-in or by constructing a transgenic cassette in which *LacZ* is placed downstream of the promoter of the target gene being examined. Currently, the most common *LacZ* staining methods used are X-gal/FeCN staining and S-gal/TNBT staining. A serious limitation of both of these methods is that they are not effective when the *LacZ* gene is expressed at a low level. In an attempt to remedy this problem, we have established a new staining protocol which combines both methods. When compared to them, the method described here is better for visualizing lowly expressed genes and it has low background with high sensitivity.

Keywords: *LacZ* reporter, Mouse embryo, Low expression, Low background, High sensitivity

[Background] The *LacZ* gene has been widely used as a reporter gene for detecting gene expression patterns (Stevens *et al.*, 1989; Bonnerot and Nicolas, 1993). The protein encoded by the *LacZ* gene is β-galactosidase, which is able to produce visible color when incubated with specific substrates. Usually, *LacZ* is placed downstream of a target gene promoter in lieu of the coding sequence of the gene. Therefore, visualizing *LacZ* expression patterns models the endogenous expression pattern of the target gene (Figure 1A). The most popular *LacZ* staining approach is the X-gal/FeCN method, in which β-galactosidase catalyzes X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) hydrolysis into 5-bromo-4-chloro-3-hydroxyindole and galactose. Then, 5-bromo-4-chloro-3-hydroxyindole is oxidized into an insoluble blue dimer with the help of potassium ferricyanide and potassium ferrocyanide, which finally displays blue color (Figure 1B) (Pearson *et al.*, 1963; Lojda, 1970). While the X-gal/FeCN staining method is highly specific and has low background, it is unable to detect genes expressed at low levels due to the low sensitivity of this stain (Gugliotta *et al.*, 1992; Sundararajan *et al.*, 2012). The S-gal/TNBT staining method was developed to study genes with low expression. S-gal (6-Chloro-3-indolyl-β-D-galactopyranoside), a chromogenic substrate like X-gal, can be hydrolyzed by β-galactosidase and TNBT (Tetranitro Blue Tetrazolium) to produce dark-brown formazan compounds under reducing

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conditions (Figure 1C). The S-gal/TNBT method is more sensitive than the X-gal/FeCN one, but a drawback is that it can have strong non-specific background staining (Sundararajan *et al.*, 2012).

By combining both the methods described above, we developed an improved *LacZ* reporter staining protocol that is highly sensitive and highly specific. This method adds an additional staining step from the S-gal/TNBT method to the X-gal/FeCN method. In our staining technique, the first staining step, which is from the X-gal/FeCN method, creates an oxidative environment which consumes non-specific enzymatic activity; the second step, which is from the S-gal/TNBT method, is a reaction that is specific for β-galactosidase and results in a sensitive and specific signal. The improved method described here has been validated by detecting several genes in different embryo stages. Furthermore, this method has been used to study relatively highly expressed genes with good results: strong and specific staining, with slightly higher background than the X-gal/FeCN method (Shen *et al.*, 2017).

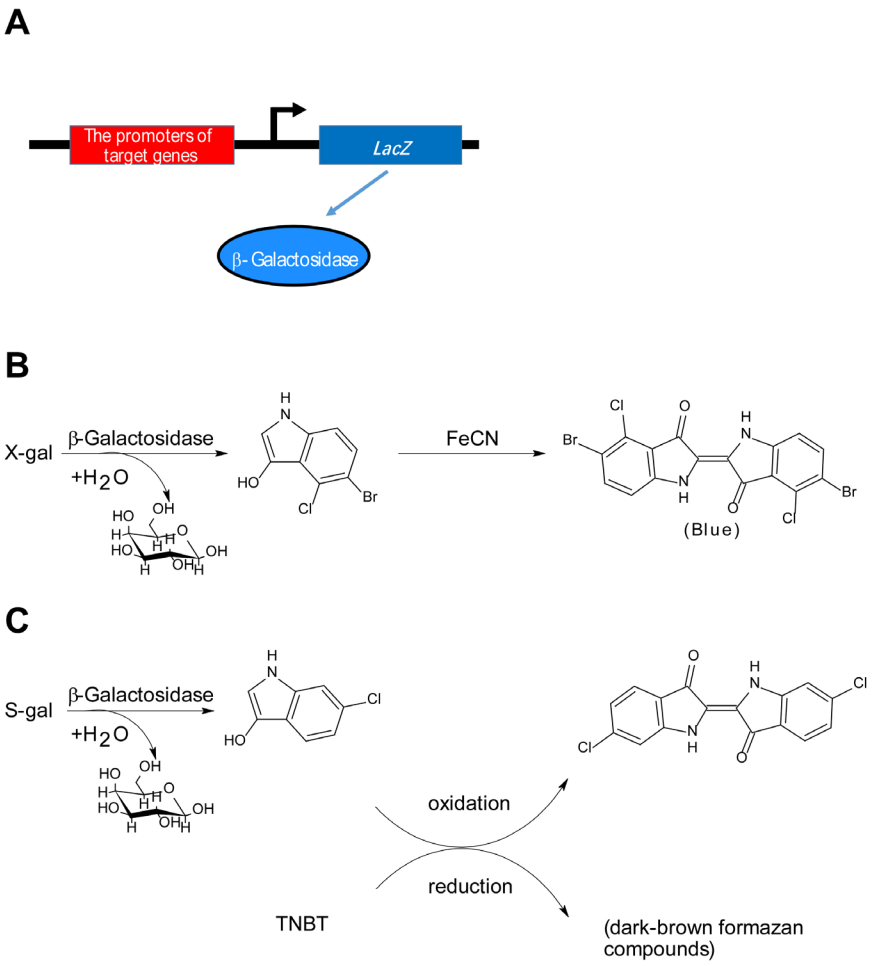

Figure 1. Schematic diagram of *LacZ* reporters and -Galactosidase reactions. (A) The *LacZ* reporter gene is placed downstream of the target gene's promoter. Thus, when the expression of the target gene is induced, the gene product of *LacZ,* β-galactosidase, is produced. The key steps of the enzymatic reactions in the X-gal/FeCN (B) and S-gal/TNBT (C) staining methods.

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Materials and Reagents

1. Sterile DNase/RNase free pipette tips 10 µl, 200 µl, 1,000 µl (Labselect, catalog numbers: T-001-10, T-001-200, T-001-1000)
2. 6-well plates (NUNC, catalog number: 140675)
3. Embryos collected from pregnant mice at E8.5, E9.5, and E10.5 stages. The mouse strain used here has been described previously in (Shen *et al.*, 2017)
4. Paraformaldehyde (PFA) (Sigma, catalog number: P6148)
5. Nonidet P-40 (NP-40) (Sangon Biotech, catalog number: A510110)
6. Sodium deoxycholate (Sigma, catalog number: D6750)
7. X-gal (Gold Bio, catalog number: X4281C)
8. $K_3Fe(CN)_6$ (Sigma, catalog number: 244023)
9. $K_4Fe(CN)_6$ (Sigma, catalog number: P3289)
10. $MgCl_2$ (Sigma, catalog number: M8266)
11. EGTA (Sigma, catalog number: E3889)
12. IGEPAL (Sigma, catalog number: I8896)
13. Salmon-gal (S-gal) (Lab Scientific, catalog number: X668)
14. Tetranitro Blue Tetrazolium (TNBT) (VWR, catalog number: TCT0250)
15. NaCl (Sigma, catalog number: S7653)
16. KCl (Sigma, catalog number: P9333)
17. Na_2HPO_4 (Sigma, catalog number: S7907)
18. KH_2PO_4 (Sigma, catalog number: P5655)
19. NaH_2PO_4 (Sigma, catalog number: S9638)
20. PBS (see Recipes)
21. 4% PFA (see Recipes)
22. 0.1 M phosphate buffer (pH 7.3) (see Recipes)
23. Wash buffer (see Recipes)
24. Staining buffer 1 (see Recipes)
25. Staining buffer 2 (see Recipes)

Equipment

1. Pipettes (Gilson, catalog numbers: F144801, F123600, F144058M, F123602)
2. Humidified chambers (for example, covered ice boxes or foam boxes with wet paper tower)
3. Dissecting forceps (Fine Science Tools, model: Dumont #5)
4. Stereomicroscope (Leica, model: MZ12.5)
5. Orbital Shaker (MIULAB, GS-20)
6. Microbiological Incubator (Thermo Scientific, PR305225G)
7. Microscope (Leica, DFC340 FX digital FireWire Camera System)

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Software

1. Leica Application Suite V3.7 (Image taking software for Leica DFC340 FX digital FireWire Camera System)

Procedure

1. Collect embryos from pregnant mice as reported previously (Shea and Geijsen, 2007) and fix the embryos in 10 ml 4% PFA (Recipe 2) in 6-well plates at room temperature (the volumes used here and in the following steps are for one embryo). The 6-well plates should be placed in humidified chambers, which are kept on orbital shakers set to 65 rpm. The fixation time varies and depends on the developmental stage of the embryos. The following table lists the recommended fixation times.

Embryo stages	E7.5	E8.5	E9.5	E10.5
Fixation time (min)	10	15	20	30

2. Use forceps to transfer the embryos to a new well of the 6-well plate filled with 10 ml Wash Buffer (Recipe 4). Wash the embryos three times with a 10 min incubation at room temperature for each wash. During the washing steps, the 6-well plates should be placed in humidified chambers on an orbital shaker set at 65 rpm.
3. Use forceps to transfer the embryos to a new well of the 6-well plate filled with 10 ml Staining Buffer 1 (Recipe 5). Incubate overnight at 37 °C in a microbiological incubator (remember to protect samples from light). The 6-well plates should be placed in humidified chambers.
4. Use forceps to transfer the embryos to a new well of the 6-well plate filled with 10 ml Wash Buffer and incubate for 10 min at room temperature. Next use forceps to transfer the embryos to a new well of the 6-well plate with Staining Buffer 2 (Recipe 6) and incubate at 37 °C in a microbiological incubator. The 6-well plates should be placed in humidified chambers. The staining should be closely monitored with a stereomicroscope until specific staining appears. The staining time could vary from several minutes to one hour.
5. Use forceps to transfer the embryos to a new well of the 6-well plate filled with 10 ml Wash Buffer and wash three times. Incubate each wash for 10 min. During the wash, the 6-well plates should be placed in humidified chambers, on an orbital shaker set to 65 rpm. After the embryos are washed, they can be imaged directly or kept at 4 °C in Wash Buffer for up to one week.
6. Take images using Leica Application Suite V3.7 on a Leica DFC340 FX microscope. We use the Z-stack option in the Leica Application Suite V3.7 because the stained embryos are relatively thick. The images generated from the Z-stack processing are used as final results. Embryos that do not express the *LacZ* gene are used as negative controls. Figure 2 shows representative staining images of X-gal/FeCN, S-gal/TNBT, and the improved method described here.

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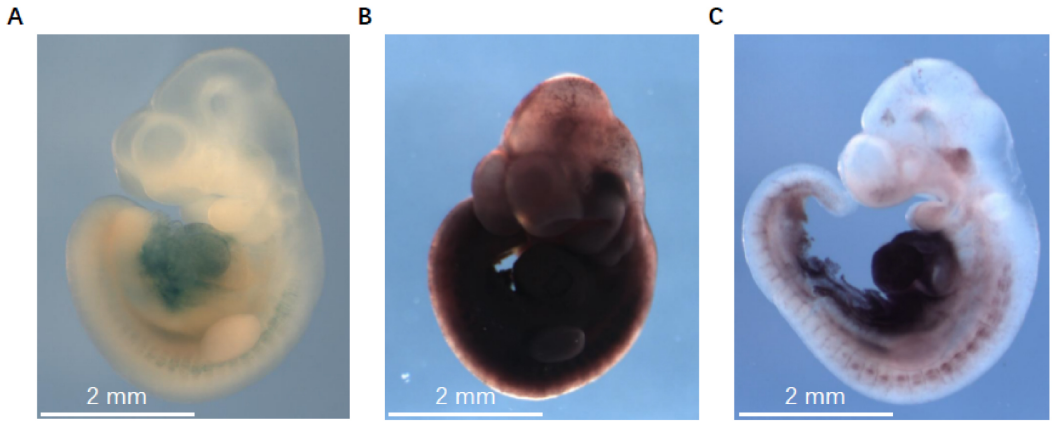

Figure 2. The representative *LacZ* staining images of X-gal/FeCN, S-gal/TNBT, and the improved method in detecting miR-322/-503's expression in E10.5 embryos. A. Embryos stained with X-gal/FeCN method. B. Embryos stained with S-gal/TNBT method. C. Embryos stained with the improved method described here. The images were taken with a Leica DFC340 FX microscope at 10x magnification.

Data analysis

Scale bars, annotations, and arrows that point at the specific stained regions are added to the embryo images.

Recipes

1. PBS
   137 mM NaCl
   2.7 mM KCl
   10 mM Na_2HPO_4
   1.8 mM KH_2PO_4
   in distilled water, pH = 7.4
2. 4% PFA
   Dissolve 4% PFA in PBS
   Heat at 50 °C and vortex until fully dissolved
   Then adjust the pH to 7.2~7.4
3. 0.1 M phosphate buffer (pH 7.3)
   23% NaH_2PO_4 solution (0.2 M NaH_2PO_4 in distilled water)
   77% Na_2HPO_4 solution (0.2 M Na_2HPO_4 in distilled water)
4. Wash buffer
   0.02% NP-40
   0.01% sodium deoxycholate in PBS

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5. Staining buffer 1
   5 mM $K_3Fe(CN)_6$
   5 mM $K_4Fe(CN)_6$
   0.02% NP-40
   0.01% deoxycholate
   2 mM $MgCl_2$
   5 mM EGTA
   1 mg/ml X-gal in PBS
6. Staining buffer 2
   1 mg/ml S-gal
   0.4 mM TNBT
   0.1% sodium deoxycholate
   0.2% IGEPAL
   2 mM $MgCl_2$ in 0.1 M phosphate buffer (pH 7.3)

Acknowledgments

We thank Wei Yu at the University of Houston for guidance in animal operations; Wenjing Bao at the Liaoning University of Traditional Chinese Medicine for technical support. This work was supported by the National Natural Science Foundation of China (No. 31701289), Anhui Provincial Natural Science Foundation (No. 1808085QH234), Educational Commission of Anhui Province of China (No. KJ2017A319), Foundation for High-level Talents in Higher Education of Anhui Province of China and Start-up Funds from the Anhui Normal University.

Competing interests

No competing interests exist.

Ethics

All animal-related work has been approved by the Institutional Animal Care and Use Committee (IACUC).

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