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Colorimetric Detection of Senescence-Associated β Galactosidase

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Abstract

Most normal human cells have a finite replicative capacity and eventually undergo cellular senescence, whereby cells cease to proliferate. Cellular senescence is also induced by various stress signals, such as those generated by oncogenes, DNA damage, hyperproliferation, and an oxidative environment. Cellular senescence is well established as an intrinsic tumor suppressive mechanism. Recent progress concerning senescence research has revealed that cellular senescence occurs in vivo and that, unexpectedly, it has a very complex role in tissue repair, promoting tumor progression and aging via the secretion of various cytokines, growth factors, and enzymes. Therefore, the importance of biomarkers for cellular senescence has greatly increased. In 1995, we described the "senescence-associated β galactosidase" (SA-βgal) biomarker, which conveniently identifies individual senescent cells in vitro and in vivo. Here, we describe an updated protocol for the detection of cell senescence based on this widely used biomarker, which contributed to recent advances in senescence, aging and cancer research. We provide an example of detecting SA-βgal together with other senescence markers and a proliferation marker, EdU, in single cells.

Keywords

Aging; Biomarker; Cellular senescence; EdU labeling; SA-βgal; Immunostaining

1. Introduction

In contrast to germ cells, stem cells and cancer cells, most normal human somatic cells do not express a detectable level of telomerase and have a finite replicative capacity, due to the progressive erosion of telomeres that protect the ends of the chromosome (1, 2). This finite replicative lifespan of human cells was originally described by Hayflick and colleagues in cultured human fibroblasts (3). Telomeres become shortened at each round of cell division and, when they reach the critical length for replication, cells are permanently arrested with a G1 DNA content in a state called replicative senescence or cellular senescence (4, 5). Subsequent studies showed that cellular senescence also occurs prematurely in a telomere-independent manner in response to various kinds of stress such as oncogenic insults mediated by RAS (6) or RAF (7), hyperproliferative signals mediated by E2F1 (8) or ETS2 (9), oxidative stress (10, 11), DNA damage (12, 13), or induction of tumor suppressor proteins such as ARF (8), $p16^{INK4A}$ (14), and PML (15, 16). In addition, we have previously shown that the replicative senescence of human fibroblasts as described by Hayflick is in fact mediated by a mosaic of cells that undergo either telomere-dependent or -independent senescence in culture (17). Cellular senescence has been well established as an intrinsic tumor suppressive mechanism that prevents cells from dividing with faulty short telomeres that may cause genomic instability (1, 18). Cellular senescence is also a protective mechanism against oncogene-induced DNA replication stress, and other cellular stresses

such as DNA damage and oxidative stress, to prevent cells with irreparable damage from undergoing further replication. Several studies have suggested that cellular senescence also occurs in vivo. For example, senescent cells were detected in a mouse model of liver fibrosis (19). Additionally, dysfunctional telomeres in mice lacking the RNA component of telomerase have been shown to activate a cellular senescence pathway to suppress tumorigenesis in the absence of apoptosis (20, 21). The existence of senescent cells in vivo was also demonstrated in mouse models of induction of oncogenes such as *Eμ-NRAS* (22), $KRAS^{V12}$ (23) or *BRAF* (24) as well as of loss of tumor suppressor genes such as *PTEN* (25). Importantly, the in vivo connection between aging and cellular senescence in tissues was recently demonstrated using a mouse model in which the removal of senescent cells can prevent or delay tissue dysfunction and extend health span (26). Several laboratories have recently shown that senescent cells also secrete many kinds of growth factors, proteases, and cytokines that can promote cancer progression and aging to cause detrimental effects (27), suggesting very complex roles for senescent cells in vivo (2).

To understand the role of cellular senescence in cancer and aging, developing reliable biomarkers of cellular senescence is very important. Senescent cells show a flat and enlarged morphology with increased cytoplasmic and nuclear volume. Senescent cells do not respond to mitogens. Therefore, senescent cells can be identified by their lack of DNA synthesis, or by genes that are differentially expressed. However, many somatic cells in our body consist of quiescent or terminally differentiated cells, and the DNA synthesis measurement does not distinguish these cells from senescent cells. In addition, downregulation of proliferation-associated genes and upregulation of growth inhibitory genes are common features among senescent, quiescent, and terminally differentiated cells. In 1995, we discovered that senescent cells expressed a β-galactosidase activity, which is histochemically detectable at pH 6.0 (28). We termed this activity “senescence-associated β galactosidase” (SA-βgal). This biomarker was expressed in senescent, but not in pre-senescent or quiescent fibroblasts, nor in terminally differentiated keratinocytes (28). SA-βgal also showed an age-dependent increase in dermal fibroblasts and epidermal keratinocytes in skin samples from human donors of different ages, suggesting that it could be a good biomarker to identify senescent cells in vivo (28). Although we described that this marker can be detected in a senescent-independent manner, for instance in cells cultured in confluence conditions for long periods of time or in tissue structures such as hair follicles and the lumens of eccrine glands, we showed that SA-βgal activity is tightly associated with the senescent phenotype and increases in frequency in aged tissues, consistent with accumulation of senescent cells with age in vivo (28). Several subsequent studies have reinforced the idea that SA-βgal is a useful biomarker for the detection of senescent cells in culture as well as in vivo, in rodents and primates (5, 29–37). To date, by virtue of the simplicity and its reliability, the SA-βgal assay method is cited in more than 2,400 publications and has been the most extensively utilized biomarker for senescent cells in vitro and in vivo (5, 21–26, 29–37). Interestingly, the SA-βgal assay has also been used with other model organisms such as zebrafish (38) and *Caenorhabditis elegans* (39). The SA-βgal activity has been shown to partly reflect the increase in lysosomal mass (40). Increased expression of the *GLB1* gene, encoding a lysosomal enzyme, contributes to SA-βgal activity (41). Increased levels of lysosomal enzymes and an increased lysosomal activity are known to be one of the hallmarks of cellular senescence (42, 43). Several other senescent biomarkers have also been described such as p16 overexpression, senescence-associated heterochromatic foci (SAHF), which are nuclear DNA domains densely stained by DAPI (44), DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS) (45), and the senescence-associated inflammatory transcriptome (46). However, compared to SA-βgal, these additional markers are not nearly as universal or convenient to use.

As in some exceptional cases the SA-βgal assay can stain non-senescent cells, showing that SA-βgal-positive cells are indeed not cycling is helpful. We have reported the SA-βgal staining protocol with thymidine labeling several years ago (47). BrdU labeling has been widely used to determine the percentage of proliferating cells in culture and tissues. However, BrdU labeling require cells and tissue samples to be subjected to strong denaturing conditions such as concentrated hydrochloric acid or mixtures of methanol and acetic acid. These harsh staining conditions degrade the structure of the specimen and cause poor retention of the cell morphology. Thus, BrdU labeling is not quite suitable for co-staining with SA-βgal. Recently, 5-ethynyl-2′-deoxyuridine (EdU), a thymidine analog, has been developed for labeling DNA (48). EdU labeling does not require a denaturing step because the fluorescent azide to detect EdU is 1/500 the size of the BrdU antibody. It diffuses and penetrates cells rapidly without denaturation. Therefore, EdU labeling coupled to SA-βgal staining may constitute a convenient method for both in vitro and in vivo assays.

In this chapter, we update the previous SA-βgal assay protocol in detail and describe the protocol for SA-βgal assay together with EdU labeling (marker for cell proliferation), DAPI staining (marker for SAHF), and immunostaining (various protein markers for senescence) to detect cellular senescence in single cells in multiple ways.

2. Materials

2.1. Cell Culture

1. Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum.
2. 100× penicillin–streptomycin.
3. 35-mm plates or 6-well plates (see Note 1).
4. WI-38 fetal lung normal human fibroblasts (Coriell Cell Repositories, Camden, NJ, USA) or any other types of cells.

2.2. Fixation and SA-βgal Staining of Cultured Cells

1. Phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; adjust to pH 7.4 with HCl if necessary).
2. Fixing solution: 4% formaldehyde, neutral buffered (Sigma-Aldrich, Saint-Louis, MO, USA, see Note 2).
3. Staining solution: 1 mg/mL 5-bromo-4-chloro-3-indolyl-beta-d-galactopyranoside (X-gal, Invitrogen, Eugene, CA, USA) (see Note 3), 1× citric acid/sodium phosphate buffer (pH 6.0, *see* below) (see Note 4), 5 mM potassium ferricyanide, 5

[1]For EdU labeling and immunostaining, Lab-Tek II Chamber Slide II (Nunc, Rochester, NY) can be used, although cell morphology and cell attachment are better either on a 35-mm plate or 6-well plate after EdU labeling and immunostaining. Cells should be at or less than 50–80% confluence at the time of assay. SA-βgal staining tends to increase in cells that have been plated for several days. SA-βgal staining of non-adherent cells such as lymphocytes requires a Cytospin centrifuge (Thermo Scientific, Asheville, NY, USA) to deposit cells onto glass slides.

[2]For convenience, small aliquots of neutral buffered 4% formaldehyde solution are commercially available. The solution is stored at room temperature and each small container can be used up to a month after opening. For some cells or tissues, freshly prepared 2% formaldehyde plus 0.2% glutaraldehyde in PBS preserves the cell morphology somewhat better. The 25% glutaraldehyde solution can be obtained in small aliquots from Sigma and stored at −20°C. Formaldehyde and glutaraldehyde are toxic and emit toxic vapors. Use a chemical fume hood to avoid inhalation. Wear gloves, safety glasses, and protective clothing to avoid skin contact.

[3]20 mg/mL X-gal solution: X-gal is dissolved at 20 mg/mL in dimethylformamide (DMF) and stored in dark-colored or aluminum foil-wrapped tubes to protect from light. The solution can be stored at -20°C for a few days. DMF is toxic and emits toxic vapors. Use a chemical fume hood to avoid inhalation. Wear gloves, safety glasses, and protective clothing to avoid skin contact.

mM potassium ferrocyanide, (see Note 5), 150 mM NaCl, and 2 mM $MgCl_2$ (Table 1).

4. Mounting medium (Dako, Carpinteria, CA, USA) (see Note 6).
5. 22 × 22 mm cover glasses (see Note 6).

2.3. Fixation and SA-βgal Staining for Tissue Samples

1. Fixing solution: 1% formaldehyde either freshly prepared or diluted from 4% formaldehyde, neutral buffered (Sigma-Aldrich, see Note 2) with PBS.
2. Staining solution: As indicated in Subheading 2.2.
3. Counter staining solution: Eosin (Sigma-Aldrich).

2.4. EdU Labeling

1. Fixing solution: 4% formaldehyde, neutral buffered (see Note 2).
2. Permeabilizing solution: 0.5% Triton X-100 in PBS.
3. Click-iT® EdU Alexa Fluor® 594 Imaging Kit (red fluorescence, Invitrogen) or Click-iT® EdU Alexa Fluor® 488 Imaging Kit (green fluorescence, Invitrogen).
4. Hoechst 33342 solution (included in the Click-iT® kit).
5. DAPI (Sigma-Aldrich) 1 mg/mL solution in distilled water is placed at 4°C for short-term or at −20°C for long-term storage. The solution has to be protected from light.
6. Mounting medium (Dako, see Note 6).
7. 22 × 22 mm cover glasses (see Note 6).

2.5. Immunostaining

1. Fixing solution: 4% formaldehyde, neutral buffered (Sigma-Aldrich, see Note 2).
2. Permeabilizing solution: 0.5% Triton X-100 in PBS.
3. Blocking solution: 0.5% BSA in PBS (see Note 7).
4. Antibody dilution buffer: 0.5% BSA in PBS (see Note 7).

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[4] 0.1 M citric acid solution: citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$) is dissolved at 0.1 M in water. The solution can be kept at room temperature for several months. 0.2 M sodium phosphate solution: sodium dibasic phosphate (Na_2HPO_4) or sodium dibasic phosphate dehydrate ($Na_2HPO_4 \cdot H_2O$) is dissolved in water at 0.2 M. The solution can be stored at room temperature for several months. 5× citric acid/sodium phosphate buffer (pH 6.0): mix 36.85 mL of 0.1 M citric acid solution with 63.15 mL of 0.2 M sodium phosphate (dibasic) solution. Verify that the pH is 6.0. If the pH of the buffer is not 6.0, add either citric acid buffer or sodium phosphate buffer to adjust to pH 6.0. Some cell types, such as mouse fibroblasts or human epithelial cells, stain less intensely for SA-βgal. The staining intensity can sometimes be improved by slightly decreasing the pH. Try several pH ranges from 5.0 to 6.0 to optimize the staining conditions, making sure to include positive and negative controls. Most, if not all, cells stain positive at pH 4.0 because of endogenous lysosomal β galactosidase activity regardless of senescence status; therefore, caution should be exercised when lowering the pH of the staining solution. The buffer can be kept at room temperature for several months.

[5] 50 mM potassium ferricyanide solution: potassium ferricyanide is dissolved in water at 50 mM concentration and stored at 4°C in a tube covered with aluminum foil to protect from light. 50 mM potassium ferrocyanide solution: potassium ferrocyanide is dissolved in water at 50 mM concentration and stored at 4°C in a tube covered with aluminum foil to protect from light. Both can be stored for several months. Avoid heating or adding acidic solution to prevent release of highly toxic hydrogen cyanide gas. Mixing the staining solution with other chemical waste by aspirating or dumping is not advisable because waste may contain acidic solutions. Autoclaving waste solution should be avoided.

[6] These mounting mediums and cover glasses are suitable for SA-βgal staining, EdU labeling, and immunostaining on either a 35-mm or 6-well plate.

[7] The success in co-staining for SA-βgal with immunostaining depends on the antigen and antibody. Senescent cells tend to have high background problems for immunostaining depending on the primary and secondary antibody. If this is the case, blocking with 5% nonfat milk in PBS or diluting the primary and/or secondary antibodies may help.

5. Secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA).
6. 1 mg/mL DAPI (Sigma-Aldrich), stored as described above (see Subheading 2.4).
7. Mounting medium (Dako) (see Note 6).
8. 22 × 22 mm cover glasses (see Note 6).

3. Methods

3.1. SA-βgal Staining for Cultured Cells

1. Seed 2–5×10^4 cells in either a 35-mm plate or 6-well plate, and culture for 2–3 days or more (see Note 1).
2. Wash cells twice with PBS.
3. Fix cells with neutral buffered 4% formaldehyde for 3 min at room temperature (see Note 8).
4. Wash cells twice with PBS.
5. Add SA-βgal staining solution (2 mL per 35-mm plate).
6. Incubate cells with staining solution at 37°C (*NOT* in a CO_2 incubator).
7. Blue color is detectable in some cells within 2 h, but staining is generally maximal in 12–16 h (see Note 9).
8. After blue color is fully developed wash cells twice with PBS. Add one drop of mounting medium, and place cover glasses either on a 35-mm plate or 6-well plate.
9. Count the blue SA-βgal-positive cells under a microscope (see Note 10). In general, human normal fibroblast cultures are considered to be senescent if >80% of cells are SA-βgal positive.

3.2. SA-βgal Staining for Tissue Samples

1. Obtain biopsy specimens and rinse briefly in PBS to remove any blood.
2. Place in OCT compound (Miles Scientific, Princeton, MN, USA) in a Tissue-Tek Cryomold and flash freeze in liquid nitrogen containing 2-methylbutane (see Note 11).
3. Unused samples can be stored at −80°C, but the enzyme is not stable after freezing. In general, samples should be processed immediately or within a few hours after freezing.
4. Cut 4-μm sections of the samples.
5. Place sections onto slides that have been treated with silane to make them adhesive.
6. Fix sections in 1% formaldehyde in PBS for 1 min at room temperature.
7. Wash with PBS three times.

[8]Longer fixation time such as 15 min destroys enzyme activity and significantly decreases the SA-βgal staining. 3–5 min of fixation is enough for SA-βgal staining, EdU labeling, and immunostaining.
[9]If cultured fibroblasts are confluent for long period of times, density-induced SA-βgal staining, independent of senescence, may occur (28). Such staining is generally less intense than that observed in senescent cells and disappears within 2 days after the confluent cells are replated.
[10]Blue staining is easier to be recognized under a bright field rather than with phase contrast.
[11]Direct freezing in liquid nitrogen may fracture the specimen. Thus, placing samples in OCT on top of dry ice is best. Avoid repeated freeze thawing of the samples, which will affect morphology and destroy the SA-βgal enzymatic activity.

8. Immerse sections in SA-βgal staining solution overnight.
9. Counterstain with eosin.
10. View by bright-field microscopy (see Note 12).

3.3. EdU Labeling for Cultured Cells

1. Seed 2–5×10^4 cells in either a 35-mm plate or 6-well plate, and culture for 2–3 days or more (see Note 1).
2. Aspirate culture media and add 1 mL of DMEM containing 10% serum and 3 μM EdU provided as Click-iT® EdU Alexa Fluor® (594 or 488) Imaging Kit for 24 h (see Note 13).
3. Wash cells with PBS once.
4. Fix cells with neutral buffered 4% formaldehyde for 5 min at room temperature.
5. Wash cells twice with PBS.
6. Add 1 mL of 0.5% Triton® X-100 in PBS and incubate for 5 min at room temperature for permeabilization.
7. Wash cells twice with PBS.
8. Add 0.5 mL of Click-iT® reaction cocktail prepared according to the manufacturer's instructions (Invitrogen).
9. Incubate the plate for 30 min at room temperature, protected from light.
10. Remove the reaction cocktail and then wash each well once with PBS.
11. Add 1 mL of diluted DAPI solution in PBS (1 μg/mL) to label nuclei for 5 min at room temperature, protected from light.
12. Wash cells twice with PBS, add one drop of mounting medium, and place a cover glass on a plate.
13. Observe cells under an inverted fluorescent microscope (see Note 14).
14. Determine the percent of labeled nuclei (%LN) by counting the number of total (DAPI stained) and labeled (green or red fluorescent) nuclei in several randomly chosen fields (generally 200–500 total nuclei). %LN = (labeled nuclei/total nuclei) × 100. In general, human fibroblast cultures are considered to be senescent if <10% of cells incorporate EdU over a 1-day interval.

3.4. SA-βgal Staining with EdU Labeling for Cultured Cells

1. Incubate cells with 10 μM EdU for 24 h (see Subheading 3.3, steps 1 and 2).
2. Wash, fix, and stain for SA-βgal activity as described above (see Subheading 3.1, steps 2–7 and Note 15).

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[12] Some tissues structures, such as hair follicles and the lumens of eccrine glands, show strong age-independent staining (28).

[13] EdU labeling is more convenient and less labor intensive compared with traditional thymidine labeling for cultured cells. We described protocols for thymidine labeling and co-staining SA-βgal with thymidine labeling previously (47). The protocol for EdU labeling and detection is slightly modified from manufacturer's instructions (Invitrogen). The best concentration of EdU should be determined according to each cell line and the type of experiment. A high concentration of EdU may lead to excess signals of EdU labeling that may be detected in microscope filters used for monitoring subsequent DAPI staining and immunostaining.

[14] If cells are on chamber slides, use a regular (not an inverted) fluorescent microscope.

[15] Because enzyme activity for SA-βgal activity is not stable, the SA-βgal assay must be performed before EdU detection. 3–5 min is adequate for fixation and permeabilization for SA-βgal staining with EdU Labeling, respectively. Avoid longer fixation times.

3. After blue color develops, wash, permeabilize, and add Click-iT® reaction cocktail and DAPI as described above (see Subheading 3.3, steps 5–11).
4. Wash cells twice with PBS, add one drop of mounting medium, and place a cover glass on a plate. Observe cells under an inverted fluorescent microscope (see Note 14).
5. Count cells with blue-colored staining for SA-βgal activity (% of SA-βgal-positive cells) under a bright field and with EdU labeled (%LN) under green or red fluorescence. Count DAPI-stained cells as the total number of cells.

3.5. SA-βgal Staining with Immunostaining for Cultured Cells

1. Culture cells in 35-mm or 6-well plates.
2. Wash, fix, and stain for SA-βgal activity as described above (see Subheading 3.1, steps 2–7 and Note 15).
3. Wash with PBS twice after blue color is developed.
4. Permeabilize cells with 0.5% of Triton X-100 in PBS for 5 min at room temperature.
5. Block plates with 0.5% BSA in PBS for 20 min (see Note 7).
6. Incubate plates with a primary antibody in 0.5% BSA either for 2 h at room temperature or overnight at 4°C (see Note 7).
7. Wash three times with PBS, 10 min each.
8. Incubate slides with secondary antibody in 0.5% BSA for 1 h at room temperature.
9. Wash three times with PBS, 10 min each.
10. Add 1 mL of diluted DAPI solution in PBS (1 μg/mL) to label nuclei for 5 min at room temperature.
11. Wash cells twice with PBS, add one drop of mounting medium, and place a cover glass on a plate. Observe cells under an inverted fluorescent microscope (see Note 14).
12. Count cells with blue-colored staining for SA-βgal activity under a bright field and with immunostaining under green or red fluorescence. Count DAPI-stained cells as the total number of cells.

3.6. SA-βgal Staining with EdU Labeling and Immunostaining for Cultured Cells

1. Culture cells in 35-mm or 6-well plates.
2. Add EdU, stain for SA-βgal activity, and detect EdU labeling as described above except for DAPI staining (see Sub heading 3.4, steps 1–3).
3. Wash cells twice with PBS.
4. Follow the immunostaining procedure after the permeabilization step as described above (see Subheading 3.5, steps 5–11 and Note 16).

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[16]The intensity of SA-βgal staining and EdU labeling do not decrease even if plates are kept with primary antibody at 4°C overnight. EdU labeling does not require a denaturation step like BrdU labeling and has been used for tissue samples. Therefore, staining of SA-βgal, EdU-labeled DNA, antigen, and SAHF can be done for tissue samples in a similar order shown for cultured cells. If the intensity of EdU labeling (green fluorescence) is too high, emission from a green fluorophore may be detected through a red emission filter and DAPI filter. Therefore, we recommend to use Click-iT® EdU Alexa Fluor® 594 Imaging Kit (red fluorescence) for EdU labeling together with immunostaining since red fluorophore is difficult to detect through green emission filter and DAPI filter.

5. Count cells with blue-colored staining for SA-βgal activity under a bright field microscope and with either EdU or immunostaining under green or red fluorescence. Count DAPI-stained cells as the total number of cells. An example of SA-βgal staining with EdU Labeling and immunostaining is shown in Fig. 1.

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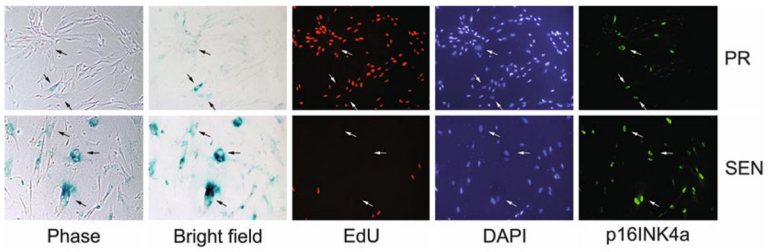

Fig. 1.

SA-βgal staining with 5-ethynyl-2′-deoxyuridine (EdU) labeling, DAPI staining, and immunostaining of cultured cells. Early passage WI-38 fibroblasts (passage 25; *upper panel*) were cultured until near-to-senescence (passage 40; *lower panel*). Co-staining was performed as described in Subheading 3.6. SA-βgal staining was visualized and photographed under phase contrast and bright field microscopy as indicated. EdU labeling, DAPI, and immunostaining of $p16^{INK4a}$ were visualized and photographed by fluorescence microscopy. Mouse monoclonal antibody against p16 (Santa Cruz) was used for immunostaining. Note that the strong $p16^{INK4a}$ staining is correlated well with SA-βgal staining and the lack of EdU labeling in both early passage proliferating and senescent cultures (shown with *arrows*). PR and SEN indicate proliferating and senescent culture respectively.

Table 1

SA-βgal staining solution

Component	Stock solution	Amount for 10 mL	Final concentration
Citric acid/sodium phosphate buffer (pH 6.0)	5×	2 mL	1×
Potassium ferricyanide	50 mM	1 mL	5 mM
Potassium ferrocyanide	50 mM	1 mL	5 mM
NaCl	5 M	0.33 mL	150 mM
$MgCl_2$	1 M	20 μL	2 mM
X-gal	20 mg/mL	0.5 mL	1 mg/mL
H_2O	–	5.2 mL	–