Senescence Associated β-galactosidase Staining (Senescence assay)

Principle. The identification of senescent cells is based on an increased level of lysosomal β-galactosidase activity. Cells under normal growth condition produce acid lysosomal β- galactosidase, which is localized in the lysosomes. The enzymatic activity can be detected at the optimal pH 4.0, using the chromogenic substrate 5-bromo-4-chloro-3-indolyl β D-galactopyranoside (X-gal). In comparison, upon senescence, the lysosomal mass is increased, leading to production of a higher level of β-galactosidase, termed senescence-associated β-galactosidase (SA-β-gal). The abundant senescence-associated enzyme is detectable over background despite the less favorable pH conditions (pH 6.0). The SA-β gal positive cells stain blue-green, which can be scored under bright-field microscopy (http://www.bio-protocol.org/e247).

Protocol

Assay design. The following protocol is designed for one 35 mm well of a 6-well plate and the use of Senescence β-Galactosidase Staining Kit #9860 (Cell Signaling, MA, USA)

Solutions provided in the kit:

1. **10X Fixative Solution** (15ml): 20% formaldehyde, 2% glutaraldehyde in 10X PBS
2. **X-gal** (150 mg): 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside powder
3. **10X Staining Solution** (15 ml): 400 mM citric acid/sodium phosphate (pH 6.0), 1.5 M NaCl, 20 mM $MgCl_2$
4. **100X Staining Solution Supplement A** (1.5 ml): 500 mM potassium ferrocyanide
5. **100X Staining Solution Supplement B** (1.5 ml): 500 mM potassium ferricyanide

Other necessary materials not included in the kit:

1. 1X PBS (Phosphate Buffered Saline): 17 mM KH_2PO_4, 5 mM Na_2HPO_4, 150 mM NaCl (pH 7.4)
2. N-N- Dimethylformamide (DMF)
3. Polypropylene tubes
4. 37°C dry incubator (no CO_2)
5. Phase contrast or light microscope

Setup – all solutions must be prepared just prior to use.

1) **PBS** – prepare at least 6 mL / 35 mm well

2) **Staining solution**: Re-dissolve the 10X Staining Solution by heating to 37°C with agitation. Dilute to 1X with distilled water (for ex., 1 mL of 10X + 9 mL of dH_2O). Need 930 µl /35 mm well.

3) **Fixative solution**: Dilute to 1X with distilled water (for ex., 1 mL of 10X + 9 mL of dH_2O) (work in chemical fume hood). Need 1 mL /35 mm well.

4) **X-gal**: To prepare 20X solution dissolve 20 mg of X-gal in 1 mL DMF (work in chemical fume hood). Need 50 µl /35 mm well. Use only polypropylene plastic or glass.

5) **β-Galactosidase Staining Solution**:

For each 35 mm well combine in polypropylene container:

- 930 µl of 1X staining solution
- 10 µl of staining supplement A
- 10 µl of staining supplement B
- 50 µl of 20X X-gal

Adjust pH to 5.9-6.1 with HCl.

Procedure

- **Preparation of the cells** (may vary depending on the cell lines and the purpose of the experiment).

Day 1

- Seed the cells in the 6-well plates (to have 70-80% confluence on final day)
- Let adhere overnight (ON).

Day 2

- Treat (ionizing radiation, DNA-damaging agents, etc.).
- Incubate in conditions optimal for cell growth for additional 5-7 days.

- **Fixation and staining cells with β- Galactosidase staining solution**

Final day after treatment

- Remove the growth media from the cells.
- Rinse each 35 mm well with 2 mL of 1X PBS (one time).
- Add 1 mL of 1X Fixative solution per well (work in chemical fume hood)
- Incubate 10-15 min at room temperature (RT).
- Rinse each 35 mm well with 2 mL of 1X PBS (2 times)
- Add 1 mL of β- Galactosidase staining solution per well. Seal plate with parafilm to prevent evaporation, wrap with foil and put in a plastic bag.
- Incubate the plate at 37°C ON in a dry incubator (no CO_2).

Next day

- While β-Galactosidase Staining Solution is still on the plate, check the cells under a microscope (100-200X total magnification) for the development of blue color.
- For long-term storage of the plates, remove the β-Galactosidase Staining Solution and overlay the cells with PBS. Store at 4°C (sealed to prevent evaporation).

- **Image acquisition and analysis**

- Capture images of cells in each well using a 10x or 20x objective (at least 300 cells in each experimental condition).
- Count the number of stained (SA-β-gal positive) and unstained cells in each experimental condition.
- Represent the SA-β-gal positive cells as a percentage of the total cell number in each experimental condition.

Potential applications

The SA- β gal assay is very useful for testing whether different conditions or compounds can induce or inhibit the appearance of senescent cells. Potential applications include determining the replicative potentials of normal cell populations in culture and determining the abilities of drugs or genetic manipulations to induce a senescence response in culture or in vivo (tissue samples). In addition, SA- β gal can be used to study the appearance of senescent cells after different types of stresses and to identify the potential antiaging protective effects of compounds. In cancer research, senescence assay is used to assess treatment-induced senescence of tumor cells (induced by chemotherapeutic drugs, radiation, etc).

References

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2. Debacq-Chainiaux, F. et.al. Nat Protocols. 2009; 4(12):1798-1806.

3. Itahana, K. et al. Methods Mol Biol. 2007; 371:21-31