Staining Protocol for Flow Cytometry

Note: The SenTraGor™ Protocol for Flow Cytometry Analysis is a hybrid histochemical/immunohistochemical assay. To produce a consistent and specific signal it is required to use a primary anti-biotin antibody and a secondary antibody against your primary anti-biotin antibody and follow suggested steps. For troubleshooting regarding your staining please check our SenTraGor™ Troubleshooting Guide on our website, www.sentragortech.com , under "Protocols" page and "Documentation" page.

1. Preparation of SenTraGor™ reagent solution and biological material

Materials:

- Vial with SenTraGor™ reagent
- 100% Ethanol (EtOH)
- Parafilm
- Cells (from aspiration or cell culture)
- Ethanol solutions: 70% EtOH, 50% EtOH
- 10x Phosphate Buffered Saline (PBS) stock solution: 1.37 M NaCl, 27 mM KCl, 100 mM Na2HPO4,

18 mM KH2PO4, pH 7.4

- 0.1% Triton X/PBS: 0.1 ml Triton X diluted in 99.9 ml PBS
- Centrifuge.

Procedure:

1.1 1 Add 3.5-3.75 ml (20 mg SenTraGor™) or 7-7.5 ml (40 mg SenTraGor™) or 14-15 ml (80 mg SenTraGor™) 100% EtOH in the vial with the reagent and cover it with its cap and parafilm (Notes 3.1, 3.2 and 3.3)

1.2 2 Incubate at 56 °C in a waterbath for 120 min until the reagent is completely dissolved. Store at RT (Note 3.4)

1.3 Harvest 10^6 cells from culture

1.4 Wash 2x in 5 ml PBS, centrifuge at 1500 rpm for 5 min at RT and discard supernatant

1.5 5 Incubate with 2ml of 4% PFA for 20 min at RT

1.6 Add 3 ml of PBS and centrifuge at 1500 rpm for 5 min at 4 °C and discard supernatant

1.7 Incubate in 2ml of 0.1% Triton X/PBS for 15 min at RT

1.8 Add 2ml of PBS and centrifuge at 1500rpm for 5 min at 4 °C and discard supernatant

1.9 Wash x1 in 1ml of 50% EtOH for 5 min at RT

1.10 Centrifuge at 1500 rpm for 5 min at 4 °C and discard supernatant

1.11 Wash x1 in 1ml of 70% EtOH for 5 min at RT.

2. SenTraGor™ staining method

Materials:

- Incubator shaker
- Primary anti-biotin antibody
- Secondary antibody against biotin, fluorescent labeled
- Flow Cytometer.

Procedure:

2.1 1 After step 1.11, centrifuge at 1500 rpm for 5 min at 4 °C and discard supernatant

Antibody-enhanced detection of Senescent cells

2.2 Add 50µl of (50% diluted in 100% EtOH) SenTraGor™ reagent through a syringe attached with a 13 mm filter and membrane 0.22 µm. The pellet must be covered with the reagent. Incubate for 8 min at 37 °C in an incubator shaker with low speed.

2.3 Wash 1x in 2ml of 50% EtOH for 5 min at RT

2.4 Centrifuge at 1500 rpm for 5 min at 4 °C and discard supernatant

2.5 Repeat steps 2.3 and 2.4 2x

2.6 Add 2 ml PBS and centrifuge at 1500 rpm for 5 min at 4 °C and discard supernatant

2.7 Incubate the pellet with 100µl of the primary anti-biotin antibody, diluted 1/300 in PBS, for 60 min at 37 °C, using an incubator shaker with low speed

2.8 Add 2ml PBS and centrifuge at 1500 rpm for 5 min at 4 °C and discard supernatant
2.9 Incubate with 100µl of secondary antibody, diluted 1/100 in PBS, for 25min in the dark and on ice

2.10 Add 2ml PBS and centrifuge at 1500 rpm for 5 min at 4 °C and discard supernatant

2.11 Dilute in FACS buffer

2.12 Count senescent cells with Flow Cytometer.

3. Technical Notes

3.1 Follow accurately all safety regulations (wear gloves, mask and glasses) during manipulations and waste disposal instructions when disposing waste materials.

3.2 Prepare all solutions using deionized water (unless otherwise indicated).

3.3 The ideal concentration depends on the examined biological material and its processing and can be determined as follows: start with 3.5 ml (20 mg SenTraGor™) or 7 ml (40 mg SenTraGor™) or 14 ml (80 mg SenTraGor™) volume of 100% Ethanol. Depending on received results you can adjust final volume to 3.75 ml (20 mg SenTraGor™) or 7.5 ml (40 mg SenTraGor™) or 15 ml (80 mg SenTraGor™), respectively.

3.4 Store SenTraGor™ reagent in a non-light absorbing and airtight container at room temperature for up to 2 months. Upon longer intervals between experiments preferentially prepare a fresh solution of the dye. During the entire process the dye container must be air tightly sealed to prevent evaporation of ethanol, which in turn leads to precipitation of the saturated dye solution in cells.