SenTraGor™
Antibody-enhanced detection of Senescent cells

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Co-staining Protocol for Immunohistochemistry

Note: The SenTraGor™ Protocol for co-staining for Immunohistochemistry 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.

1. Preparation of the biological material

Materials:

1.1 Tissue samples (Fixed in 10% Buffered Formalin Solution and Paraffin Embedded, FFPE)
1.2 Cover glass
1.3 Incubation chambers for glass slides
1.4 Positively charged glass slides
1.5 Coplin jars
1.6 Glass beaker
1.7 Volumetric cylinder
1.8 Thin edged forceps.

Procedure:

Cut thin paraffin sections from FFPE tissues and mount them on positively charged glass slides. Incubate at 37°C overnight. Store at RT.

2. Preparation of SenTraGor™ reagent solution

Materials:

- Vial with SenTraGor™ reagent
- 100% EtOH
- Parafilm

Procedure:

2.1 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 **(Note 4.1, 4.2 and 4.3)**
2.2 Incubate at 56°C in a waterbath for 120 min until the reagent is completely dissolved. Store at RT **(Note 4.4)**.

3. SenTraGor™ staining method

Materials:

- Xylene
- Gradually decreased (96%, 80%, 70%, 50%) EtOH solutions
- Syringe
- 13 mm filter, membrane 0.22 μm
- Soft paper (dry or dipped in ethanol)
- 10x Tris Buffered Saline (TBS) stock solution: 1.5 M NaCl, 0.1 M Tris–HCl, pH 7.4. Store at 4°C
- 0.5% Triton X/TBS: 0.5 ml Triton X diluted in 99.5 ml TBS
- Primary antibody of choice
- Secondary antibody against your primary antibody of choice, HRP conjugated

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- Detection system HRP DAB kit
- Primary anti-biotin antibody
- Secondary AP conjugated antibody, specific against your anti-biotin antibody
- NBT/BCIP substrate
- 100 mM Levamisol
- KTBT buffer: 0.05 M Tris-Cl, 0.15 M NaCl, 0.01 M KCl
- Mounting media (ready to use or 40% Glycerol in TBS)
- Light microscope
- Fluorescent microscope **(optional, Note 4.5)**.

Procedure:

3.1 Deparaffinize sections by:
3.1.1: Incubation at 60°C for 20 min
3.1.2: Washing in Xylene for 15 min at RT

3.2 Gradually rehydrate in:
3.2.1: 100% EtOH for 15 min at RT
3.2.2: 96% EtOH for 10 min at RT
3.2.3: 80% EtOH for 5 min at RT
3.2.4: 70% EtOH for 3 min at RT
3.2.5: 50% EtOH for 3 min at RT

3.3 Wash x1 in TBS for 5 min at RT

3.4 Block endogenous hydrogen peroxidase according to the instructions included in the Detection system HRP DAB kit in dark conditions

3.6 Incubate with antigen retrieval buffer according to the primary antibody and incubate in cold tap water for 10-20 min

3.5 Wash 2x in TBS for 30 sec and 1x for 5 min in RT **(Note 4.6)**

3.7 Wash x2 in TBS for 30 sec and x1 for 5 min at RT

3.8 Incubate with primary antibody **(Note 4.7)**

3.9 Wash x2 in TBS for 30 sec and 1x in TBS for 5 min at RT

3.10 Incubate with the secondary antibody against your primary antibody for 1h at RT

3.11 Wash x2 in TBS for 30 sec and x1 for 5 min at RT

3.12 Proceed according to the instructions included in your Detection system HRP DAB kit

3.13 Apply DAB Chromogen according to the instructions included in the Detection system HRP DAB kit. The staining reaction is monitored under the light microscope until detection of the dark brown signal

3.14 Wash in tap water for 5 min at RT

3.15 Wash x1 in 50% EtOH for 5 min at RT

3.16 Wash x1 in 70% EtOH for 5 min at RT **(Note 4.8)**

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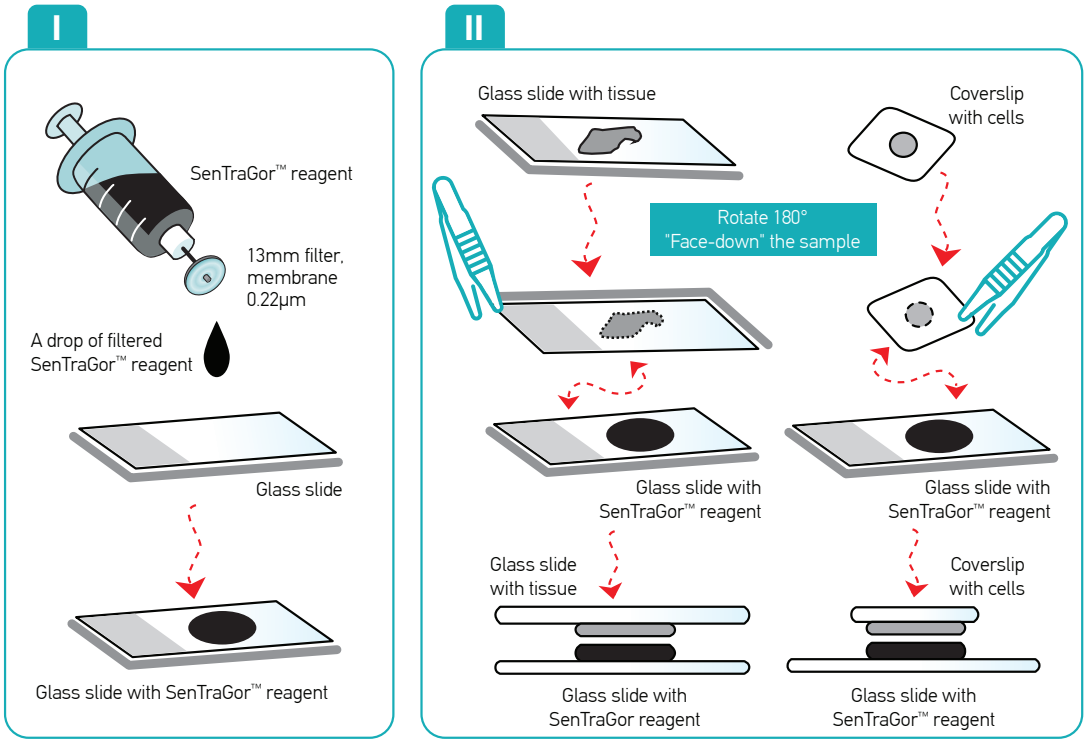
3.17 Incubate with SenTraGor™ reagent at RT. A drop of prepared reagent is placed on tissue section through a syringe attached with a 13 mm filter and membrane 0.22 μm. Then a cover glass is placed on the tissue section (using thin edged forceps) **(Note 4.9)** (see Figures I & II)



3.18 Monitor the staining reaction under the light microscope until detection of the signal (average time 5-8 min) **(Notes 4.10-4.12)**

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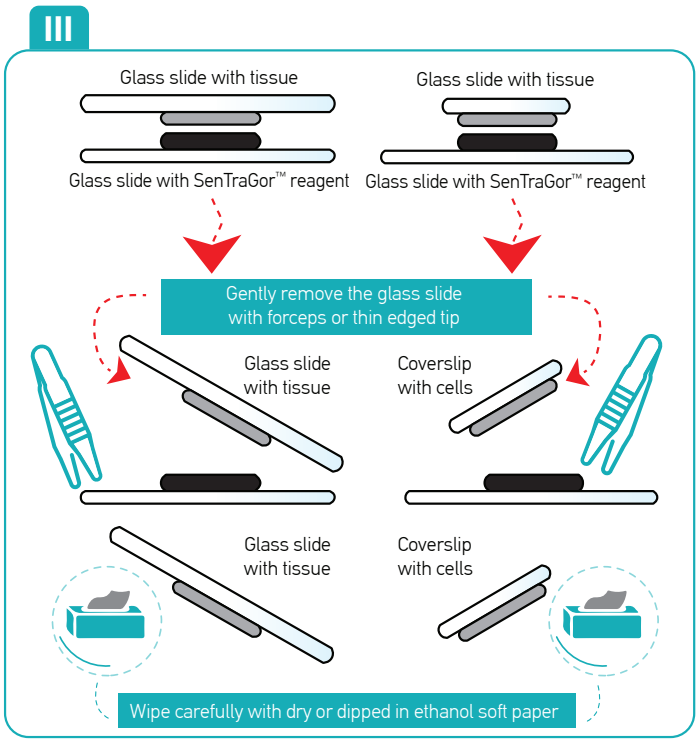
3.19 Remove gently the cover glass using thin edged forceps and clean excess SenTraGor™ reagent with soft paper (ideally dipped in ethanol) **(Note 4.13)** (see Figure III)



3.20 Wash x2 in 50% EtOH for 5 min at RT **(Note 4.13)**
3.21 Repeat washing x2 in fresh 50% EtOH for 5 min at RT **(Note 4.13)**
3.22 Wash x2 in TBS for 30 sec and x1 for 5 min at RT
3.23 Incubate with the primary anti-biotin antibody, diluted in TBS according with your standard procedure (indicative dilution: 1/300-1/500), for 60 min at 37°C **(Note 4.14 and 4.15)**
3.24 Wash x3 in TBS for 5 min at RT
3.25 Incubate with the secondary AP conjugated antibody, diluted in TBS (indicative dilution: 1/800), for 60 min at RT
3.26 Wash x3 in TBS for 5 min at RT
3.27 Wash x2 in TBS for 30 sec and x1 for 5 min at RT
3.28 Application of NBT/BCIP substrate with the addition of 20 μl of 100 mM Levamisol. Monitor the staining reaction under the light microscope until detection of the dark brown-purple signal at RT
3.29 Wash x2 in KTBT buffer for 5 min at RT
3.30 Wash x2 in tap water for 5 min at RT
3.31 Apply permanent mounting media **(Note 4.16)**
3.32 Observe under the light microscope **(Notes 4.10-4.12)**.

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4. Technical Notes

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

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

4.3 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. If non-specific ("dirt backround") reaction of the reagent is observed adjust final volume to 3.75 ml (20 mg SenTraGor™) or 7.5 ml (40 mg SenTraGor™) or 15 ml (80 mg SenTraGor™), respectively.

4.4 Store the 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 tissues.

4.5 The Fluorescent Microscope can be used in control experiments. Lipofuscin that accumulates in senescent cells is well known to exhibit autofluorescent properties that are quenched by the current SenTraGor™ reagent staining. Mount the sample in 40% glycerol/TBS medium, after its appropriate preparation, and observe by excitation at 450-490 nm, using a dichromatic mirror at 510 nm and a long-pass filter at 515 nm (Evangelou *et al.,* 2017).

4.6 In the case of liver tissue, you should use a Streptavidin/Biotin blocking kit, to block endogenous biotin. Indicative additional steps after step **3.5** are:
3.5.1: Incubate with blocking biotin (streptavidin included in the Streptavidin/Biotin blocking kit) for 15 min at RT
3.5.2: Wash in TBS x2 for 30 sec and x1 for 5 min at RT
3.5.3: Incubate with blocking biotin (biotin included in the Streptavidin/Biotin blocking kit) for 15 min at RT
3.5.4: Wash x2 in TBS for 30 sec and x1 for 5 min at RT.

4.7 Indicative dilution ratios in double staining procedures are 1/100-1/500 in TBS and incubated overnight at 4°C.

4.8 Incubation of tissue section with 70% EthOH just before addition of SenTraGor™ reagent is an essential step since SenTraGor™ is diluted in pure EthOH. Otherwise it will not be able to penetrate the tissue and staining will not be successful.

4.9 This step is crucial to avoid evaporation of the dye.

4.10 Absence of staining with SenTraGor™ reagent *per se* within 5-8 minutes does not always indicate that the sample is negative for senescence. From our experience we suggest to proceed with the AP visualization reaction. In many cases, despite it was challenging to detect the positive granules after SenTraGor™ reagent histochemical staining, we clearly detected positive (dark blue-purple) senescent cells after completion of the AP reaction. The addition of the chromogenic assay increases dramatically the sensitivity of the method.

4.11 Intracellular light blue staining can occasionally be observed when the SenTraGor™ reagent is used, and should always be taken into consideration.

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4.12 Omission of the SenTraGor™ reagent should always be performed as a negative control experiment.

4.13 This step is crucial to remove and estimate "background dirt" and clean cover glass and slides using soft paper.

4.14 The addition of the primary anti-biotin antibody is essential to obtain a consistent and specific signal. Omission of the primary anti-biotin antibody should always serve as negative control.

4.15 Incubation with solutions (BSA, blocking medium or corresponding sera) that block non-specific antibody staining is optional.

4.16 Counterstain in double staining reaction is omitted to avoid interference with the chromogen signals.