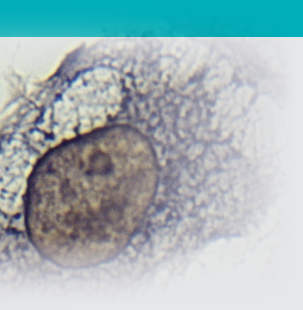

SenTraGor™
Antibody-enhanced detection of Senescent cells

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Co-staining Protocol for Immunocytochemistry

Note: The SenTraGor™ Protocol for co-staining in Immunocytochemistry 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 Cells (from aspiration or cell culture)
1.2 Coverslips and cover glass
1.3 Glass beaker
1.4 10x Phosphate Buffered Saline (PBS) stock solution: 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.4
1.5 Preparation of fixative media 1-5% (w/v) Paraformaldehyde/PBS: Dissolve 1-5 gr of paraformaldehyde (PFH) in 100 ml of PBS in a glass beaker. Heat and stir the mixture until it becomes transparent. Let the solution cool down and adjust pH to 7.4 **(Notes 4.1-4.3)**
1.6 Incubation chambers for coverslips
1.7 Positively charged glass slides
1.8 Thin edged forceps.

Procedure:

Mount cells on coverslips and fix them in 1-5% (w/v) paraformaldehyde/PBS solution for 5 min at RT. Then wash three times (approx. 1min) with PBS **(Note 4.1)**.

2. Preparation of SenTraGor™ reagent solution

Materials:

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

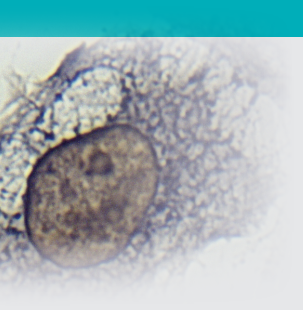
Procedure:

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 **(Notes 4.1, 4.2 and 4.4)**
2.2 Incubate at 56°C in a waterbath for 120 min until the reagent is completely dissolved. Store at RT **(Note 4.5)**.

3. SenTraGor™ staining method

Materials:

- 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 **(Note 4.6)**
- 0.5% Triton X/TBS: 0.5 ml Triton X diluted in 99.5 ml TBS
- Primary antibody of choice



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- Secondary antibody against your primary antibody, HRP conjugated
- 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 4.7)**.

Procedure:

3.1 Wash coverslips x1 in TBS for 5 min at RT
3.2 Block endogenous hydrogen peroxidase, according to the instructions included in the Detection system HRP DAB kit, in dark conditions **(Note 4.8)**
3.3 Wash x2 in TBS for 30 sec and x1 for 5 min at RT
3.4 Incubate with primary antibody according with your standard procedure **(Note 4.9)**
3.5 Wash x3 in TBS for 5 min at RT
3.6 Incubate with secondary antibody against your primary antibody, HRP conjugated, for 1 h at RT
3.7 Wash x2 in TBS for 30 sec and x1 for 5 min at RT
3.8 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
3.9 Wash in tap water for 5 min at RT
3.10 Wash x1 in 50% EtOH for 5 min at RT
3.11 Wash x1 in 70% EtOH for 5 min at RT **(Note 4.10)**

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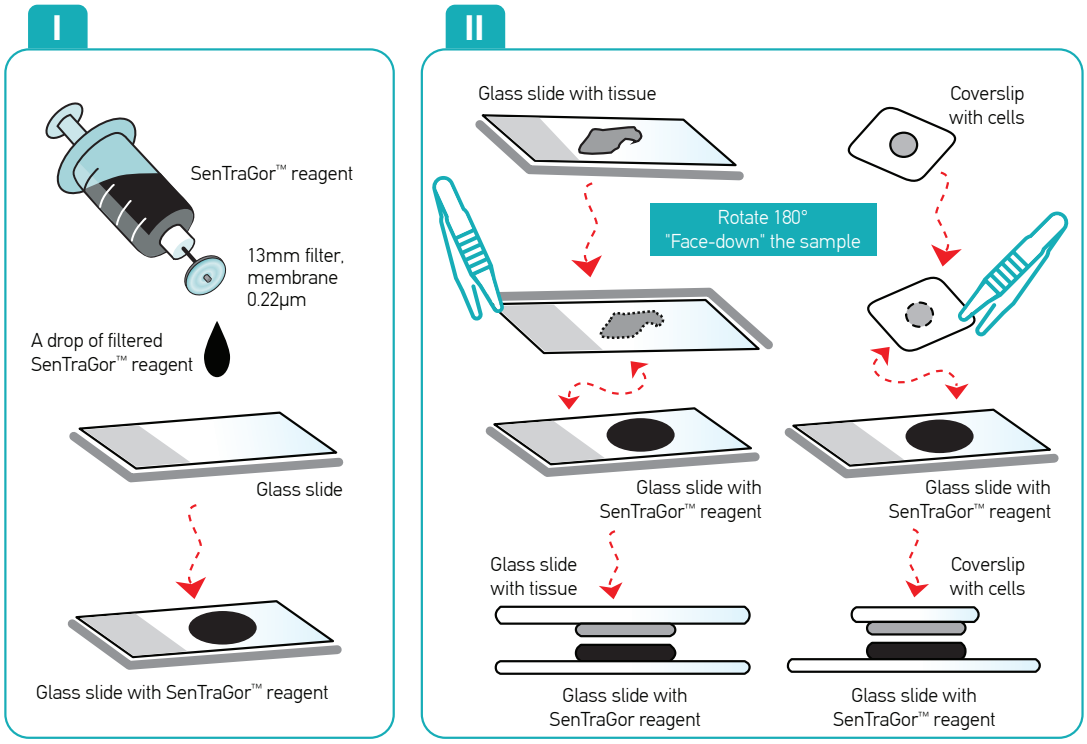
Co-staining Protocol for Immunocytochemistry

3.12 Incubate with SenTraGor™ reagent at RT. Place coverslip with cells (using thin edged forceps) on a clean glass slide (face up). A drop of prepared reagent is placed on coverslip with cells, through a syringe attached with a 13 mm filter and membrane 0.22 μm. Then a cover glass is placed on the coverslip (using thin edged forceps) **(Notes 4.8, 4.11)** (see Figures I & II)



3.13 Monitor the staining reaction under the light microscope until detection of the signal (average time 5-8 min) **(Notes 4.12-4.14)**

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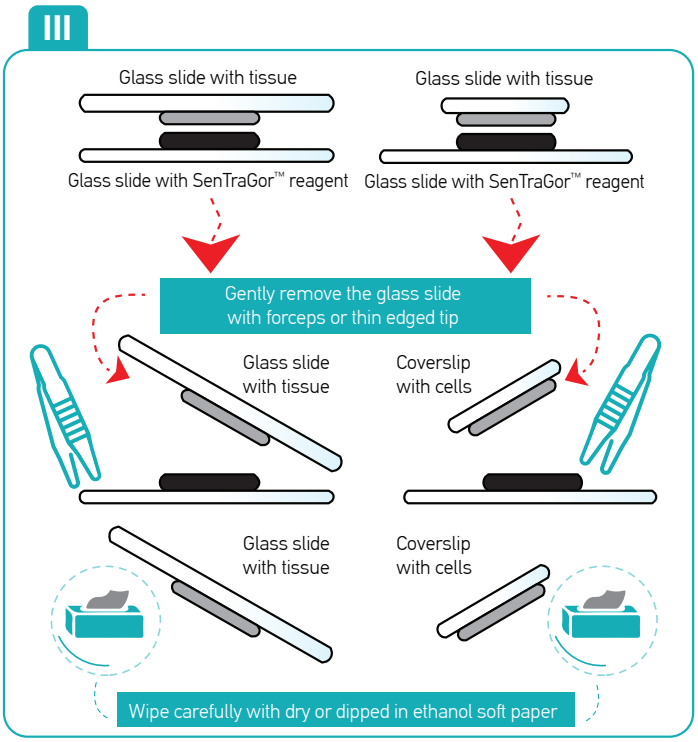
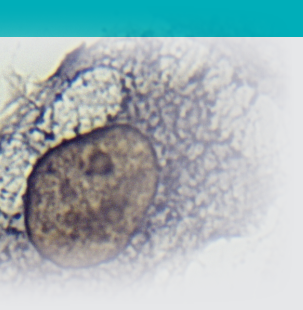
Co-staining Protocol for Immunocytochemistry

3.14 Remove gently the cover glass using thin edged forceps and clean excess SenTraGor™ reagent with soft paper (ideally dipped in ethanol) (see Figure III) **(Note 4.15)**



3.15 Wash x3 in 50% EtOH for 5 min at RT **(Note 4.15)**
3.16 Repeat washing x2 in fresh 50% EtOH for 5 min at RT **(Note 4.15)**
3.17 Wash x2 in TBS for 30 sec and x1 for 5 min at RT
3.18 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 **(Notes 4.16 and 4.17)**
3.19 Wash x3 in TBS for 5 min at RT
3.20 Incubate with the secondary AP conjugated antibody, specified against your anti-biotin antibody, diluted in TBS (indicative dilution: 1/800), for 60 min at RT
3.21 Wash x3 in TBS for 5 min at RT
3.22 Wash x2 in TBS for 30 sec and x1 for 5 min at RT
3.23 Application of NBT/BCIP substrate with the addition of 20 μl of 100 mM Levamisol. The staining reaction is monitored under the light microscope until detection of the dark blue-purple signal at RT
3.24 Wash x2 in KTBT buffer to stop the reaction, for 5 min at RT
3.25 Wash x2 in tap water for 5 min at RT
3.26 Apply permanent mounting media **(Note 4.18)**
3.27 Observe under the light microscope **(Notes 4.12 and 4.13)**.



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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 Perform preparation of the Paraformaldehyde/PBS solution in a fume hood to avoid any contact with fumes. Preferably always prepare a fresh solution before the experiments.

4.4 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.

4.5 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 cells.

4.6 Instead of TBS you can use PBS all the way.

4.7 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).

4.8 Perform all incubations in chambers to avoid exsiccation of the material.

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

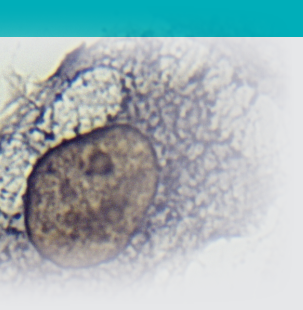
4.10 Incubation of cells with 70% EthOH just before addition of SenTraGor™ reagent is an essential step since SenTraGor™ is diluted in pure EthOH. Otherwise it will not be able to penetrate the cell and staining will not be successful.

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

4.12 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 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.

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

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



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

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

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

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