Immmunofluorescence staining protocol for cells mounted on coverslips

Note: The SenTraGor™ Protocol for Immunofluorescence 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 Troubleshooting page.

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 Dark 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. 1 min) with PBS **(Notes 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 **(Note 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)
- Primary anti-biotin antibody
- Secondary antibody against your anti-biotin, fluorescent labeled
- DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) 1 mg/ml stock solution

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- 10x Tris Buffered Saline (TBS) stock solution: 1.5 M NaCl, 0.1 M Tris–HCl, pH 7.4. Store at 4°C **(optional, Note 4.6)**
- 0.5% Triton X/TBS: 0.5 ml Triton X diluted in 99.5 ml TBS
- Mounting media (ready to use or 40% Glycerol in TBS)
- Fluorescence microscope **(Note 4.7)**
- Light Microscope.

Procedure:

3.1 Wash coverslips x1 in TBS for 5 min at RT

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

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

3.4 Incubate with SenTraGor™ reagent at RT. A drop of prepared reagent is placed on a clear glass slide through a syringe attached with a 13 mm filter and membrane 0.22 μm. Then the coverslip with the cells is turned upside down on the slide and placed on top of the drop as shown in Figures 1 & 2 (using thin edged forceps). In this manner, evaporation of ethanol is prevented while the reagent penetrates the cells. Alternatively, the drop of the SenTraGor™ reagent can directly be placed on the coverslip with the cells and then a clear glass slide is used to cover the cells with the reagent (using thin edged forceps) **(Notes 4.9 and 4.10)**.

Figure 1

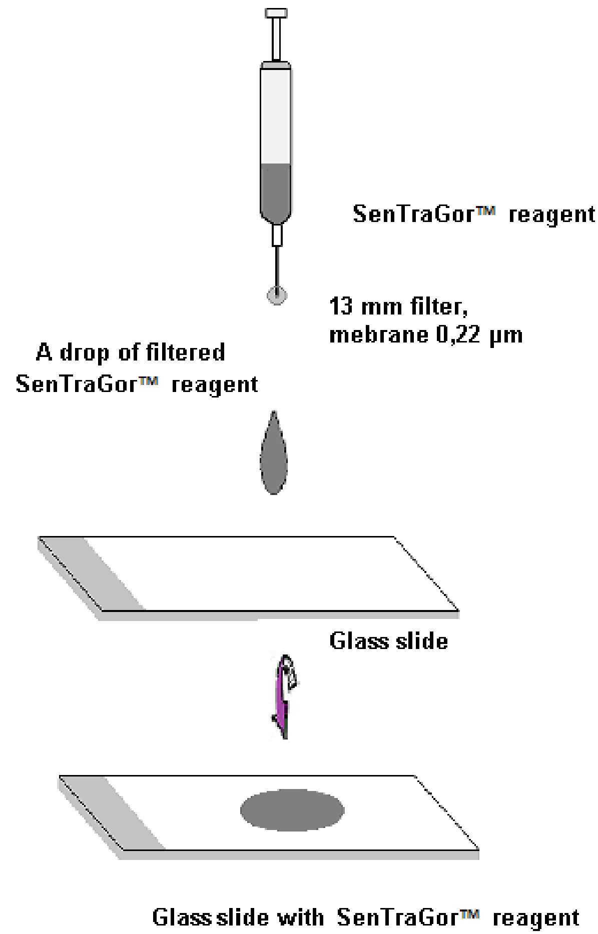


Figure 2

Glass slide with tissue

Coverslip with cells

Rotate 180°
"Face-down" the sample

Glass slide with SenTraGor™ reagent

Glass slide with SenTraGor™ reagent

Glass slide with tissue

Coverslip with cells

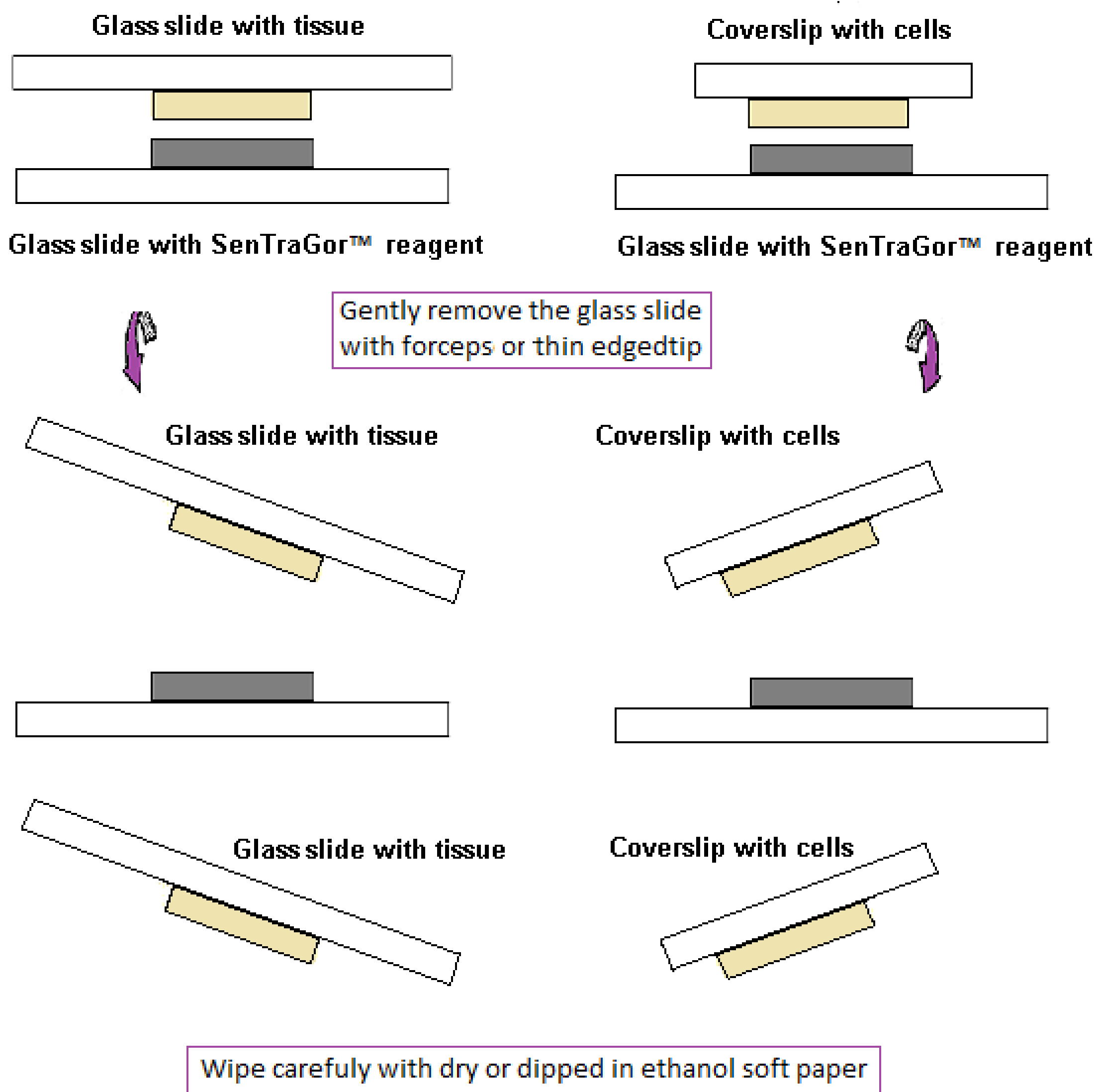
Glass slide with SenTraGor™ reagent

Glass slide with SenTraGor™ reagent

3.5 Incubate 3-8 min at room temperature or 8-10 min at 37˚C (average time). Monitor the staining reaction under the light microscope until detection of the signal (average time 5-8 min) (Notes 4.11-4.13)

3.6 Remove gently the cover glass using thin edged forceps and clean excess SenTraGor™ reagent with soft paper (ideally dipped in ethanol) (see Figure 3) **(Note 4.14)**

Figure 3



3.7 Wash x2 in 50% EtOH for 5 min at RT **(Note 4.14)**
3.8 Repeat washing x2 in fresh 50% EtOH for 5 min at RT **(Note 4.14)**
3.9 Wash x2 in TBS for 30 sec and x1 for 5 min at RT
3.10 Incubate with 0.5% Triton X/TBS for 3 min at RT
3.11 Wash x1 in TBS for 5 min at RT
3.12 Incubate with the primary anti-biotin antibody, diluted in TBS according with your standard

procedure (indicative dilutions: 1/300-1/500), for 60 min at 37°C **(Note 4.15 and 4.16)**

3.13 Wash x3 in TBS for 5 min at RT

3.14 Incubate with fluorescent secondary antibody specific against your primary anti-biotin antibody, diluted in TBS, according with your standard procedure (indicative dilution: 1/200) for 60 min in RT

3.15 Wash x5 in TBS for 5 min at RT

3.16 Incubate with DAPI diluted 1/1000 in TBS for 5 min at RT

3.17 Wash x2 in TBS for 5 min at RT

3.18 Apply permanent mounting medium

3.19 Observe under the fluorescent microscope.

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 Preparation of the Paraformaldehyde/PBS solution must be performed 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 and tissues.

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 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 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.

4.9 Perform all incubations in dark chambers to avoid exsiccation of the material and exposure to light.

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

4.11 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 immunofluorescence reaction. In many cases, despite it was challenging to detect the positive granules after SenTraGor™ reagent histochemical staining, we clearly

detected fluorescent signal in senescent cells after completion of the immunofluorescent reaction. The addition of the immunofluorescence assay increases dramatically the sensitivity of the method.

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

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

4.14 This step is crucial to remove and estimate “background dirt” and clean coverslips and slides using soft paper (ideally dipped in ethanol).

4.15 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.

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