SenTraGor™

Antibody-enhanced detection of Senescent cells

arriani pharmaceuticals

a: 85 Lavriou Ave, 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

PRODUCT DATASHEET

SenTraGor™ Reagent – Antibody-enhanced detection of Senescent cells

Cat no: AR8850020, AR8850040, AR8850080

Overview

Product name

SenTraGor™ – Antibody-enhanced detection of senescent cells.

Description

A biotin linked Sudan Black B (SBB) analogue tracing lipofuscin in senescent cells.

Background

Cellular senescence is a biological process involved in normal embryonic and adult life and increases with age. It also occurs in the frame of various diseases and after therapeutic interventions. Detection and measurement of senescent cells is critical and highly desired in research and clinical practice. Senescent cells are found in a wide spectrum of age related disorders, including cancer. SenTraGor™, an SBB analogue conjugated with biotin, reacts with lipofuscin granules that have been shown to accumulate during the senescence process.

References

1. Evangelou K., Lougiakis N., Rizou S.V., et al. 2017. Robust, universal biomarker assay to detect senescent cells in biological specimens. *Aging Cell* 16, pp 192-197.
2. Georgakopoulou E.A., Tsimaratou K., Evangelou K., et al. 2013. Specific lipofuscin staining as a novel biomarker to detect replicative and stress-induced senescence. A method applicable in cryo-preserved and archival tissues. *Aging* (Albany NY). 5, pp 37-50.
3. Campisi J., d'Adda di Fagagna F 2007. Cellular senescence: when bad things happen to good cells. *Nat. Rev. Mol. Cell Biol* 8, pp 729-740.
4. Gorgoulis V.G., Halazoneitis T.D. 2010. Oncogene-induced senescence: the bright and dark side of the response. *Curr Opin Cell Biol* 22, pp 816-827.

References

5. Bujarrabal A., Schumacher B. 2017. Tracking senescent cells: A new biomarker assay opens new avenues in senescence research. *Mech Ageing Dev.* 162, pp 106-107.
6. Salmonowicz H., Passos J.F. 2017. Detecting senescence: a new method for an old pigment. *Aging Cell.* 16, pp 432-434.
7. Childs B.G., Gluscevic M., Baker D.J., et al. 2017. Senescent cells: an emerging target for diseases of ageing. *Nat Rev Drug Discov.* Jul 21.

Source

SenTraGor™ is a chemical synthetic biotinylated compound, analogue of SBB stain. Its synonym is (2-Methyl-6-((E)-(4-((E)-phenyldiazenyl)naphthalen-1-yl)diazenyl)-2,3-dihydro-1H-perimidin-2-yl)methyl 5-((3aR,4R,6aS)-2-oxohexahydro-1H-thieno[3,4-d] imidazol-4-yl)pentanoate.

Product description

Each vial contains 20 mg (Cat no: AR8850020), 40 mg (Cat no: AR8850040) or 80 mg (Cat no: AR8850080) of solid SenTraGor™ compound. To dissolve the compound, add 3.5-3.75 ml, 7-7.5 ml or 14-15 ml, respectively, of 100% Ethanol and cover with parafilm. The ideal concentration depends on the examined biological material and its processing and can be determined as follows: start with 3.5 ml, 7 ml or 14 ml volume of 100% Ethanol. If non-specific ("backgound") reaction of the reagent is observed adjust volume to 3.75 ml, 7.5 ml or 15 ml, respectively. Incubate at 56°C in a waterbath for 120 min until the compound is completely dissolved. Before use, filter with a syringe using 13 mm filter and 0.22 μm membrane.

SenTraGor™

Antibody-enhanced detection of Senescent cells

arriani pharmaceuticals

a: 85 Lavriou Ave,
190 02, Peania,
Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

PRODUCT DATASHEET

SenTraGor™ Reagent – Antibody-enhanced detection of Senescent cells

Storage

Store at room temperature. Protect from light. After reconstitution store at room temperature, protect from light and air tight seal. The reagent is stable for up to 2 months after reconstitution at room temperature.

Applications

SenTraGor™ is recommended for the recognition of senescent cells. It binds to cytoplasmic lipofuscin, a hallmark of senescent cells. It can be applied on cells from aspiration or cell cultures as well as on tissue sections from frozen or formalin-fixed paraffin embedded (FFPE) samples. It can be used for immunohistochemistry (IHC), immunocytochemistry (ICC), immunofluorescence (IF) and flow cytometry analysis (FACS). For detailed protocols refer to www.sentragor-reagent.com.

Controls

a. Positive controls:

Cellular systems that can be used as positive controls are cells undergoing replicative senescence (RS), for example primary human diploid lung fibroblasts (DLFs) at late passages of division or cells exhibiting Stress induced senescence (SISP), for example irradiated primary human DLFs, or inducible Saos2 $p21^{WAF1/CIP1}$ Tet-ON cells, when $p21^{WAF1/CIP1}$ is activated. Tissues from animal models and human clinical cases with established senescence can serve as positive controls (examples: K-RAS induced mouse lung adenomas, irradiated human laryngeal lesions).

b. Negative controls:

Cellular systems that can be used as negative controls are early proliferative passages of primary human DLFs, Saos2 $p21^{WAF1/CIP1}$ Tet-ON cell when $p21^{WAF1/CIP1}$ is OFF (not induced). Normal, non-aged, tissues can be used as negative controls.

c. References:

1) Evangelou K., Lougiakis N., Rizou S.V., et al. 2017. Robust, universal biomarker assay to detect senescent cells in biological specimens. *Aging Cell* 16, pp 192-197; 2) Galanos P., Vougas K., Walter D., et al. 2016. Chronic p53-independent p21 expression causes genomic instability by deregulating replication licensing. *Nat Cell Biol.* 18, pp 777-89; 3) Collado M., Gil J., Efeyan A., et al. 2005. Tumour biology: senescence in premalignant tumours. Nature. 436, pp 42.

SenTraGor™

Antibody-enhanced detection of Senescent cells

arriani pharmaceuticals

a: 85 Lavriou Ave, 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

PRODUCT DATASHEET

SenTraGor™ Reagent – Antibody-enhanced detection of Senescent cells

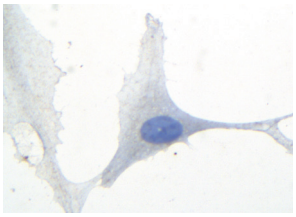
Presented data

Lipofuscin granules are present in the cytoplasm of senescent cells.

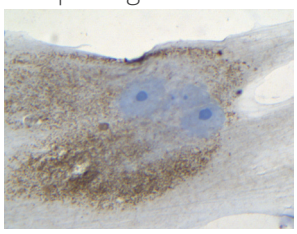
Immunocytochemistry (ICC)

Human diploid lung fibroblasts

Early passages

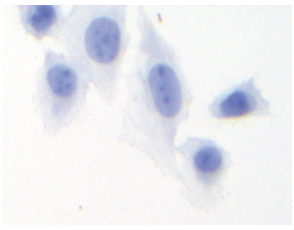


Late passages

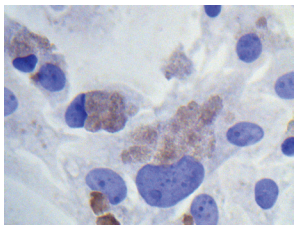


Inducible Saos2 p21$^{WAF1/CIP1}$ Tet-ON

Tet-OFF



Tet-ON

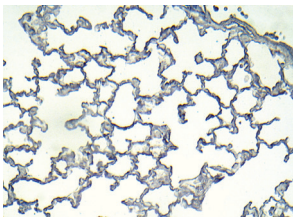


Pictures are reproduction from Evangelou K., Lougiakis N., Rizou S.V., et al., Aging Cell. 2017 Feb; 16(1): 192-197

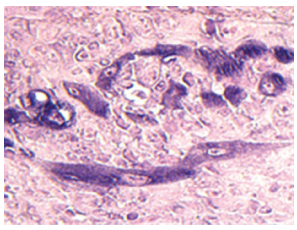
Immunohistochemistry (IHC)

Mouse lung adenoma model

Wt

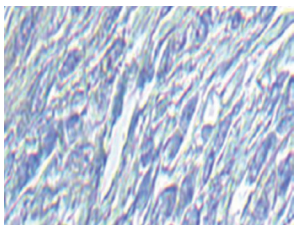


K-ras^{V12}-induction

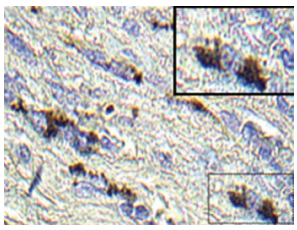


Human laryngeal tissue

Non-irradiated



Irradiated

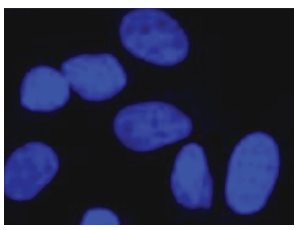


Pictures are reproduction from Evangelou K., Lougiakis N., Rizou S.V., et al., Aging Cell. 2017 Feb; 16(1): 192-197

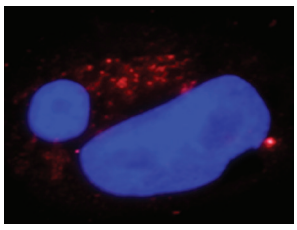
Immunofluorescence (IF)

Inducible Saos2 p21$^{WAF1/CIP1}$ Tet-ON

Tet-OFF



Tet-ON



Pictures are reproduction from Evangelou K., Lougiakis N., Rizou S.V., et al., Aging Cell. 2017 Feb; 16(1): 192-197

Use

The reagent is for research use.

Protocols

For detailed protocols you can contact us at info@sentragortechnology.com or visit our website www.sentragortechnology.com

SenTraGor™
Antibody-enhanced detection of Senescent cells

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Staining Protocol for Immunohistochemistry

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 **(Notes 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)
- Anti-biotin antibody
- Secondary antibody, HRP conjugated, against your anti-biotin antibody
- Detection system HRP DAB kit
- Hematoxylin
- 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
- Mounting media (ready to use or 40% Glycerol in TBS)
- Light microscope
- Fluorescent microscope **(optional, Note 4.5)**.

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Staining Protocol for Immunohistochemistry

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

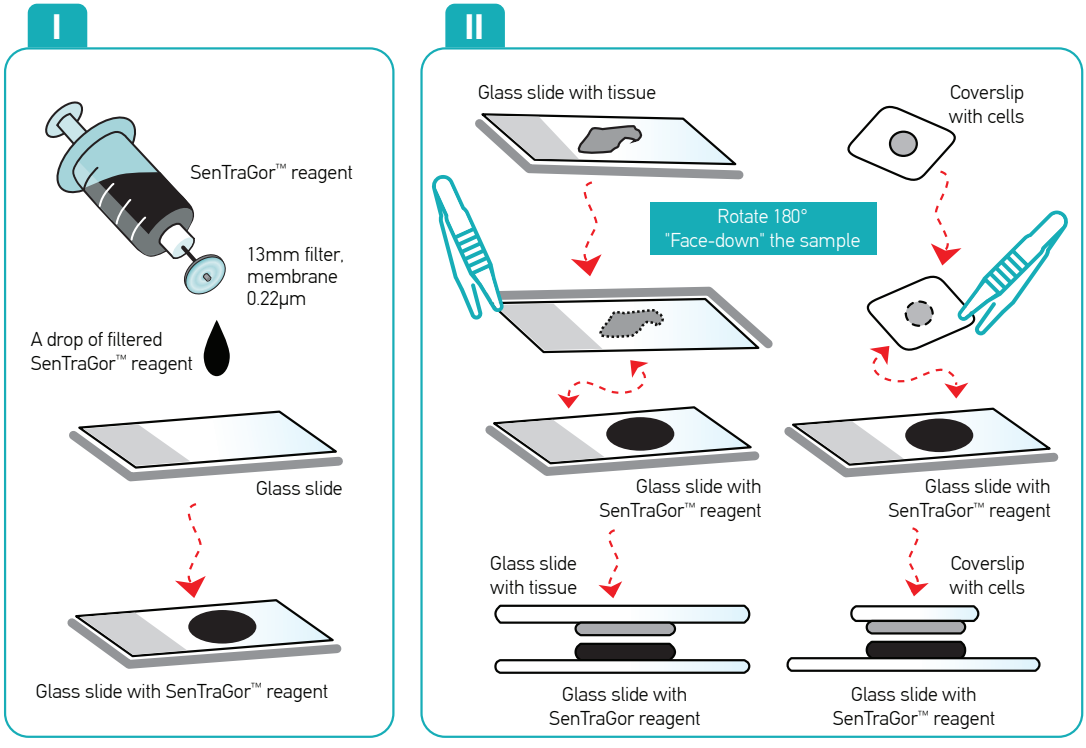
3.4 Block endogenous hydrogen peroxidase according to the instructions included in the Detection system HRP DAB kit **(Note 4.6)**

3.5 Wash x2 in TBS for 30 sec and x1 for 5 min at RT **(Note 4.8)**

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

3.7 Wash x1 in 70% EtOH for 5 min at RT

3.8 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) **(Notes 4.7-4.9)** (see Figures I & II)

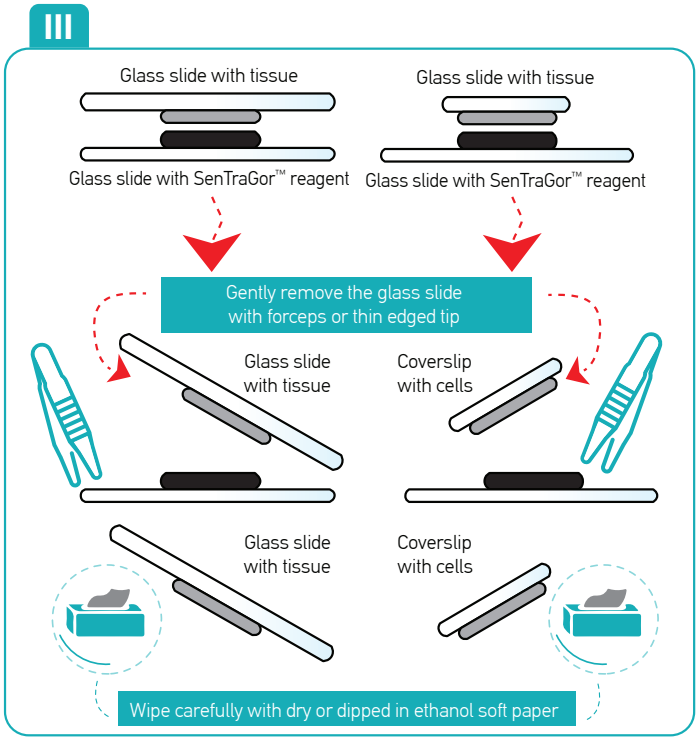


3.9 Monitor the staining reaction under the light microscope until detection of the signal (average time 5-8 min) **(Notes 4.10-4.12)**

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Staining Protocol for Immunohistochemistry

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



3.11 Wash x2 in 50% EtOH for 5 min at RT **(Note 4.13)**
3.12 Repeat washing x2 in fresh 50% EtOH for 5 min at RT **(Note 4.13)**
3.13 Wash x2 in TBS for 30 sec and x1 for 5 min at RT
3.14 Incubate with 0.5% Triton X/TBS for 3 min at RT
3.15 Wash x1 in TBS for 5 min at RT
3.16 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 or overnight at 4°C **(Note 4.14, 4.15)**
3.17 Wash x3 in TBS for 5 min at RT
3.18 Incubate with the secondary antibody against your anti-biotin antibody for 1h at RT
3.19 Wash x2 in TBS for 30 sec and x1 for 5 min at RT
3.20 Proceed according to the instructions included in your Detection system HRP DAB kit
3.21 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
3.22 Wash in tap water for 5 min at RT
3.23 Counterstain with Hematoxylin **(Note 4.16)**
3.24 Wash in tap water for 5 min at RT
3.25 Apply permanent mounting media
3.26 Observe under the light microscope.

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Staining Protocol for Immunohistochemistry

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 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 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 Instead of a DAB detection method you can use AP (alkaline phosphatase) detection assay, without the execution of step **3.4.**

4.7 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.

4.8 Perform all washing incubations in coplin jars. Perform antibody and reagent incubations in chambers to avoid exsiccation of the material.

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 DAB visualization reaction. In many cases, despite it was challenging to detect the positive granules after SenTraGor™ reagent histochemical staining, we clearly detected positive (dark brown) senescent cells after completion of the DAB 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.

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

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Staining Protocol for Immunohistochemistry

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

4.14 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 In case of using SenTraGor™ reagent *per se* in tissues use 0.1% Nuclear Fast Red as counterstain.

SenTraGor™
Antibody-enhanced detection of Senescent cells

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Co-staining Protocol for Immunohistochemistry

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 (20mg 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
- 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

SenTraGor™
Antibody-enhanced detection of Senescent cells

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Co-staining Protocol for Immunohistochemistry

- 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 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

3.6 Block endogenous hydrogen peroxidase according to the instructions included in the Detection system HRP DAB kit in dark conditions **(Note 4.6)**

3.7 Wash x2 in TBS for 30 sec and x1 for 5 min at RT **(Note 4.7)**

3.8 Incubate with primary antibody **(Note 4.8)**

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

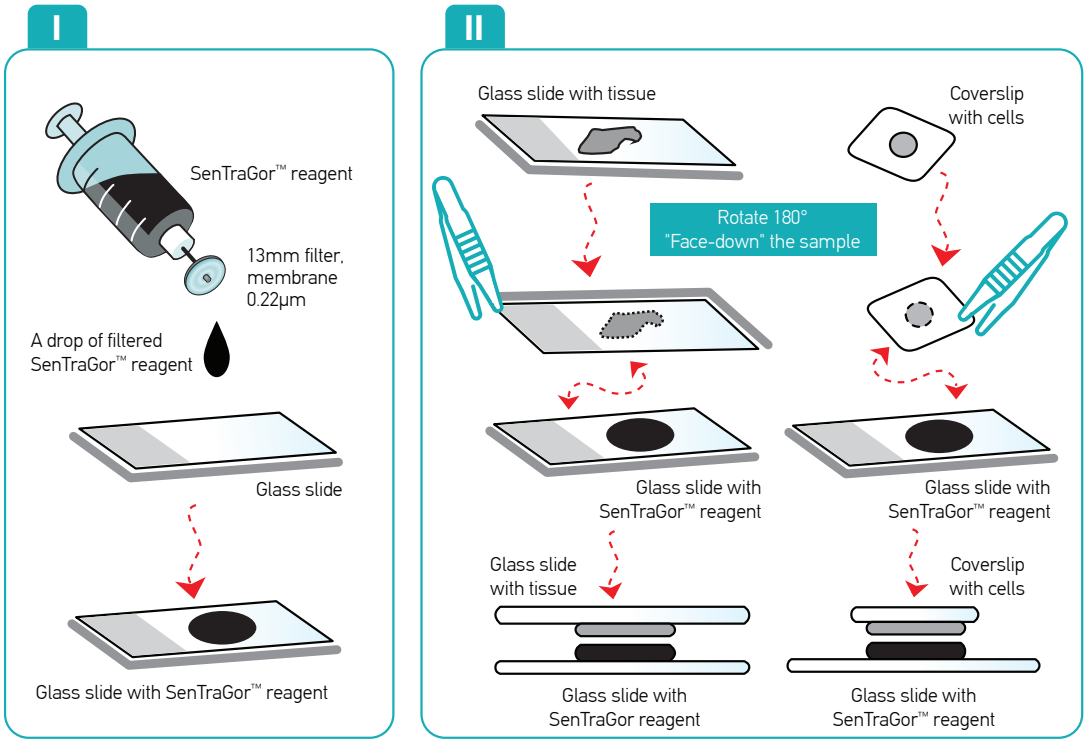
3.15 Wash x1 in 50% EtOH for 5 min at RT

3.16 Wash x1 in 70% EtOH for 5 min at RT

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Co-staining Protocol for Immunohistochemistry

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) **(Notes 4.9 and 4.10)** (see Figures I & II)

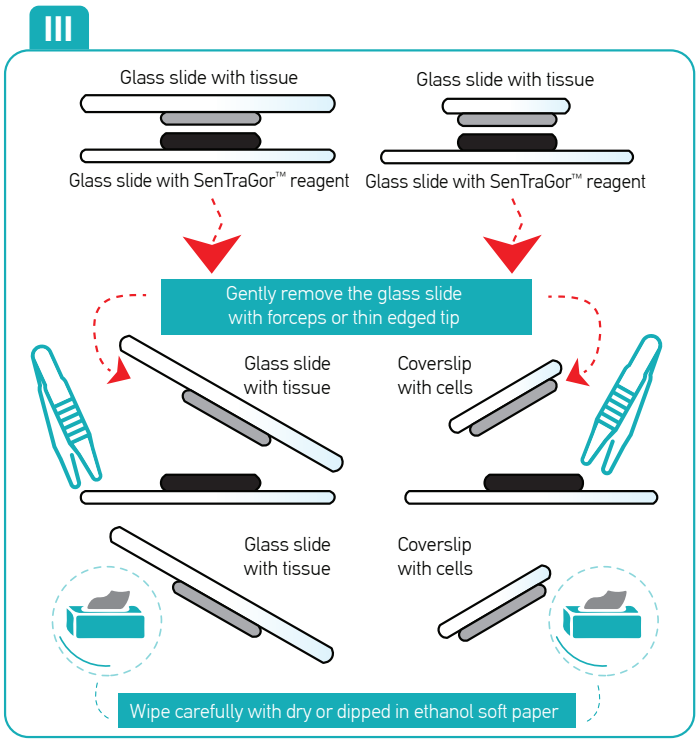


3.18 Monitor the staining reaction under the light microscope until detection of the signal (average time 5-8 min) **(Notes 4.10-4.12)**

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Co-staining Protocol for Immunohistochemistry

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)**.

SenTraGor™
Antibody-enhanced detection of Senescent cells

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Co-staining Protocol for Immunohistochemistry

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 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 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 Perform all washing incubations in coplin jars. Perform antibody and reagent incubations in chambers to avoid exsiccation of the material.

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

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 DAB visualization reaction. In many cases, despite it was challenging to detect the positive granules after SenTraGor™ reagent histochemical staining, we clearly detected positive (dark brown) senescent cells after completion of the DAB 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.

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

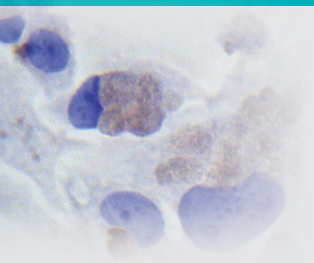
4.13 This step is crucial to remove and estimate "background dirt" and clean cover glass and slides using soft paper.

SenTraGor™
Antibody-enhanced detection of Senescent cells

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Co-staining Protocol for Immunohistochemistry

4.14 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.



SenTraGor™
Antibody-enhanced detection of Senescent cells

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Staining Protocol for Immunocytochemistry

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. 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 **(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)
- Anti-biotin antibody
- Secondary antibody, HRP conjugated, against your anti-biotin antibody
- Detection system HRP DAB kit
- Hematoxylin
- 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
- Mounting media (ready to use or 40% Glycerol in TBS)

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Staining Protocol for Immunocytochemistry

- Light Microscope
- Fluorescent microscope **(optional, Note 4.7)**.

Procedure:

3.1 Wash coverslips x1 in TBS for 5 min at RT

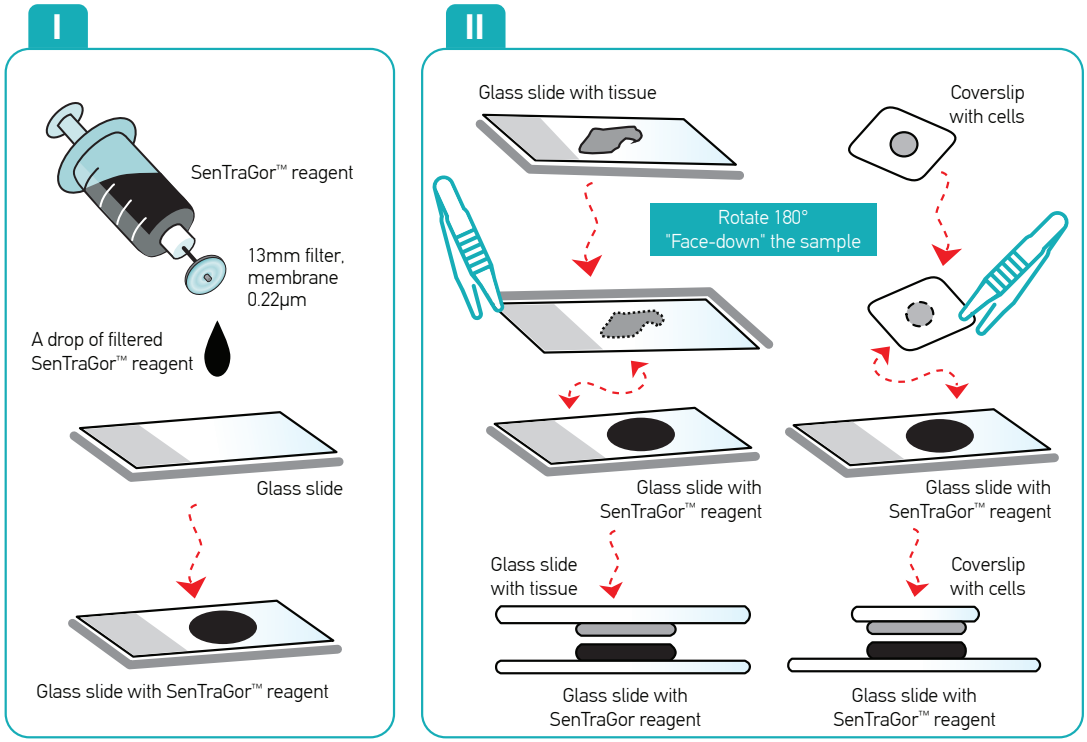
3.2 Block endogenous hydrogen peroxidase, according to the instructions included in the Detection system HRP DAB kit, in dark conditions **(Notes 4.8, 4.17)**

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

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

3.5 Wash x1 in 70% EtOH for 5 min at RT

3.6 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, 4.9)** (see Figures I & II)

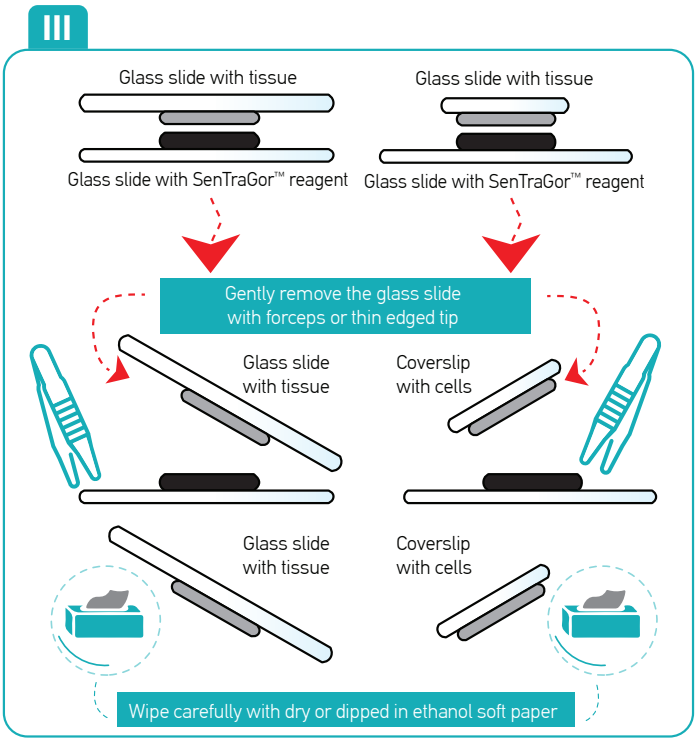


3.7 Monitor the staining reaction under the light microscope until detection of the signal (average time 5-8 min) **(Notes 4.10-4.12)**

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Staining Protocol for Immunocytochemistry

3.8 Wash x2 in 50% EtOH for 5 min at RT **(Note 4.13)** (see Figure III)



3.9 Repeat washing x2 in fresh 50% EtOH for 5 min at RT **(Note 4.13)**
3.10 Wash x2 in TBS for 30 sec and x1 for 5 min at RT
3.11 Incubate with 0.5% Triton X/TBS for 3 min at RT
3.12 Wash x1 in TBS for 5 min at RT
3.13 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 or overnight at 4°C **(Note 4.14 and 4.15)**
3.14 Wash x3 in TBS for 5 min at RT
3.15 Incubate with the secondary antibody against your anti-biotin antibody for 1h at RT
3.16 Wash x3 in TBS for 5 min at RT
3.17 Proceed according to the instructions included in your Detection system HRP DAB kit
3.18 Wash x2 in TBS for 30 sec and x1 for 5 min at RT
3.19 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 at RT
3.20 Wash in tap water for 5 min at RT
3.21 Counterstain with Hematoxylin **(Note 4.16)**
3.22 Wash in tap water for 5 min at RT
3.23 Apply permanent mounting media
3.24 Observe under the light microscope **(Notes 4.11 and 4.12)**.

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Staining Protocol for Immunocytochemistry

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 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 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 dark chambers to avoid exsiccation of the material and exposure to light.

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 DAB visualization reaction. In many cases, despite it was challenging to detect the positive granules after SenTraGor™ reagent staining, we clearly detected positive (dark brown) senescent cells after completion of the DAB 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.

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

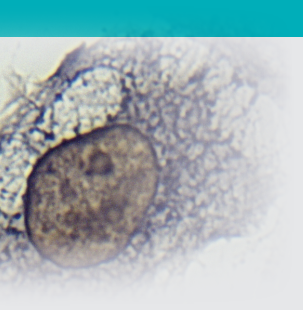
4.13 This step is crucial to remove and estimate "background dirt" and clean cover glass and slides using soft paper.

4.14 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 In case of using SenTraGor™ reagent per se in cells use 0.1% Nuclear Fast Red as counterstain.

4.17 Instead of a DAB detection method you can use AP (alkaline phosphatase) detection assay,without the execution of step **3.2.**



SenTraGor™
Antibody-enhanced detection of Senescent cells

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Co-staining Protocol for Immunocytochemistry

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

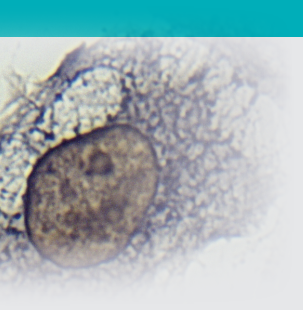
Procedure:

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
- 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



SenTraGor™
Antibody-enhanced detection of Senescent cells

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Co-staining Protocol for Immunocytochemistry

- 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)**.

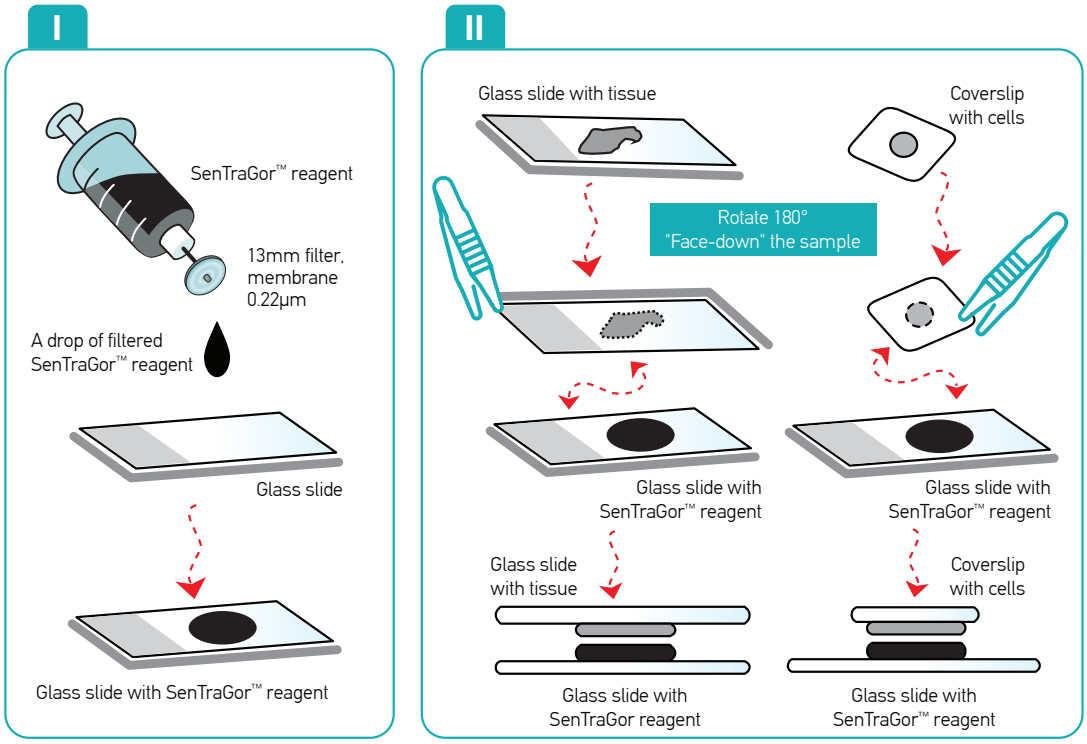
Procedure:

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

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

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, 4.10)** (see Figures I & II)

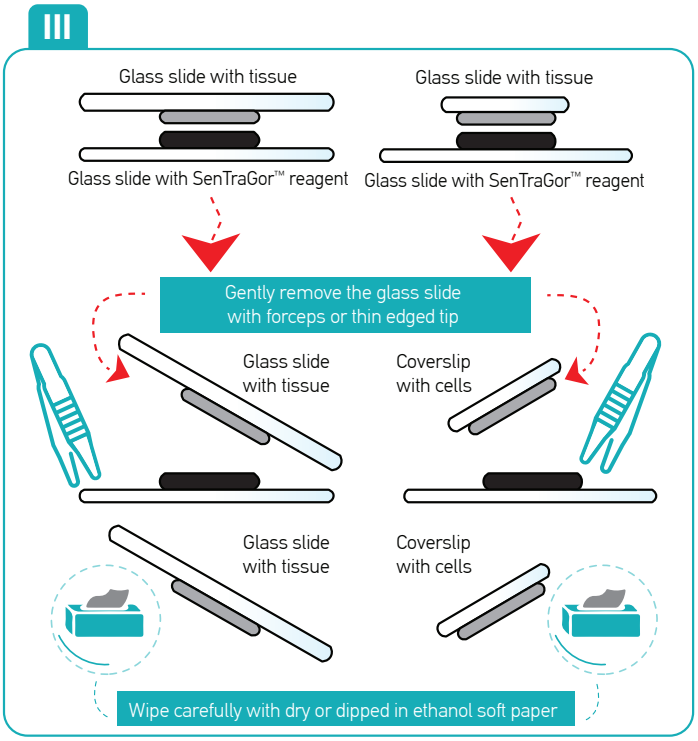


3.13 Monitor the staining reaction under the light microscope until detection of the signal (average time 5-8 min) **(Notes 4.11-4.13)**

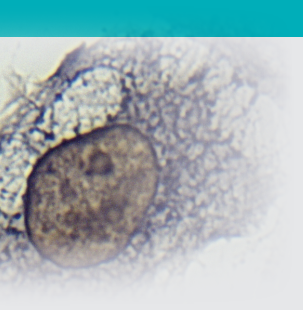
arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Co-staining Protocol for Immunocytochemistry

3.14 Wash x3 in 50% EtOH for 5 min at RT **(Note 4.14)** (see Figure III)



3.15 Repeat washing x2 in fresh 50% EtOH for 5 min at RT **(Note 4.14)**
3.16 Wash x2 in TBS for 30 sec and x1 for 5 min at RT
3.17 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 4.15 and 4.16)**
3.18 Wash x3 in TBS for 5 min at RT
3.19 Incubate with the secondary AP conjugated antibody, specified against anti-biotin antibody, diluted in TBS (indicative dilution: 1/800), for 60 min at RT
3.20 Wash x3 in TBS for 5 min at RT
3.21 Wash x2 in TBS for 30 sec and x1 for 5 min at RT
3.22 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
3.23 Wash x2 in KTBT buffer to stop the reaction, for 5 min at RT
3.24 Wash x2 in tap water for 5 min at RT
3.25 Apply permanent mounting media **(Note 4.17)**
3.26 Observe under the light microscope **(Notes 4.11 and 4.12)**.



SenTraGor™
Antibody-enhanced detection of Senescent cells

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Co-staining Protocol for Immunocytochemistry

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 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 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 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 DAB visualization reaction. In many cases, despite it was challenging to detect the positive granules after SenTraGor™ reagent staining, we clearly detected positive (dark brown) senescent cells after completion of the DAB reaction. The addition of the chromogenic 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.

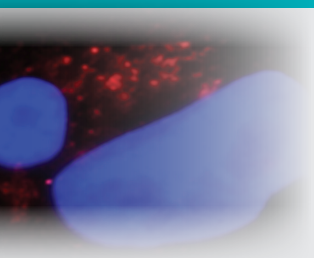
4.13 Omission of the SenTraGor™ reagent should always be performed as a negative control experiment.

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

4.15 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.

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



SenTraGor™
Antibody-enhanced detection of Senescent cells

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Immunofluorescence staining protocol for cells mounted on coverslips

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 **(Notes 4.1-4.3)**. 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
- 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)

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Immunofluorescence staining protocol for cells mounted on coverslips

- Fluorescence microscope **(Note 4.7)**
- Light Microscope.

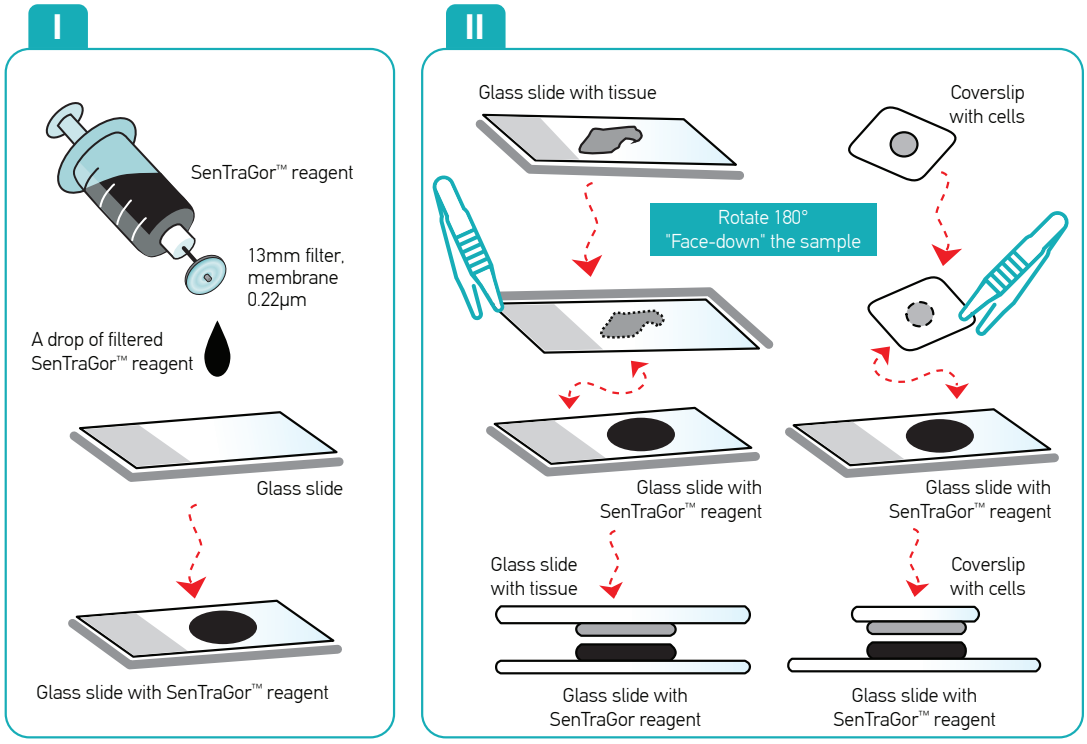
Procedure:

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

3.4 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, 4.9)** (see Figures I & II)

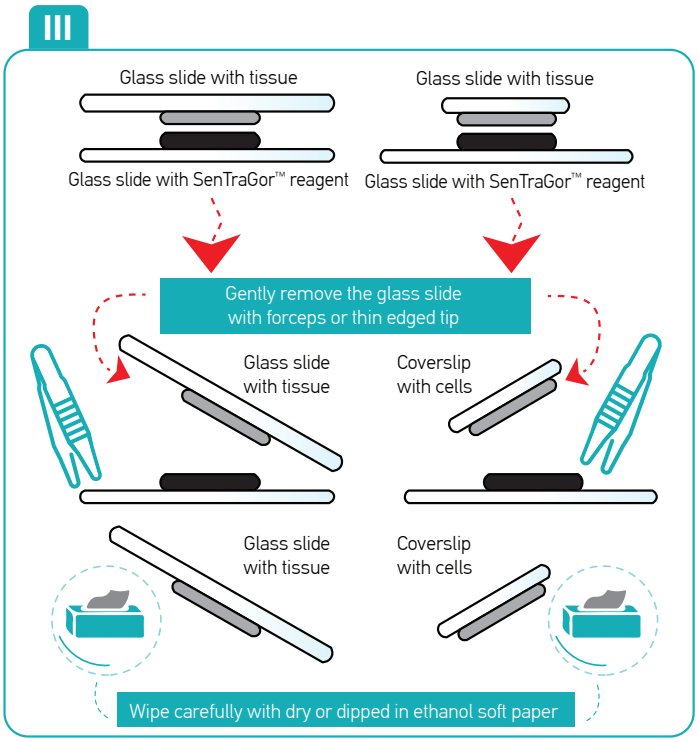


3.5 Monitor staining reaction under the light microscope until detection of the signal (average time 5-8 min) **(Notes 4.10-4.12)**

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Immunofluorescence staining protocol for cells mounted on coverslips

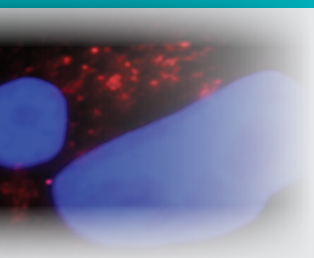
3.6 Wash x2 in 50% EtOH for 5 min at RT **(Note 4.13)** (see Figure III)



3.7 Repeat washing x2 in fresh 50% EtOH for 5 min at RT **(Note 4.13)**
3.8 Wash x2 in TBS for 30 sec and x1 for 5 min at RT
3.9 Incubate with 0.5% Triton X/TBS for 3 min at RT
3.10 Wash x1 in TBS for 5 min at RT
3.11 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 4.14 and 4.15)**
3.12 Wash x3 in TBS for 5 min at RT
3.13 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
3.14 Wash x5 in TBS for 5 min at RT
3.15 Incubate with DAPI diluted 1/1000 in TBS for 5 min at RT
3.16 Wash x2 in TBS for 5 min at RT
3.17 Apply permanent mounting medium
3.18 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.



SenTraGor™
Antibody-enhanced detection of Senescent cells

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Immunofluorescence staining protocol for cells mounted on coverslips

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

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 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.

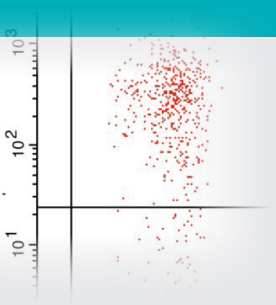
4.11 Intracellular light blue staining can occasionally be observed when the SenTraGor™ reagent is used, and should always be taken into consideration.

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 coverslips and slides using soft paper (ideally dipped in ethanol).

4.14 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.



SenTraGor™
Antibody-enhanced detection of Senescent cells

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Staining Protocol for Flow Cytometry

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 Na_2HPO_4, 18 mM KH_2PO_4, pH 7.4
- 0.1% Triton X/PBS: 0.1 ml Triton X diluted in 99.9 ml PBS
- Centrifuge.

Procedure:

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 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 1200 rpm for 5 min at RT and discard supernatant

1.5 Incubate with 5 ml 70% EtOH for 20 min at 4°C

1.6 Centrifuge at 1200 rpm for 5 min at RT and discard supernatant

1.7 Wash x1 in 5 ml PBS at RT

1.8 Centrifuge at 1200 rpm for 5 min at RT and discard supernatant

1.9 Incubate in 0.1% Triton X/PBS for 15 min at RT

1.10 Centrifuge at 1200rpm for 5 min at RT and discard supernatant

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

1.12 Centrifuge at 1200 rpm for 5 min at RT and discard supernatant

1.13 Wash x1 in 70% EtOH for 5 min at RT.

2. SenTraGor™ staining method

Materials:

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

Procedure:

2.1 After step **1.13**, centrifuge at 1200 rpm for 5 min at RT and discard supernatant

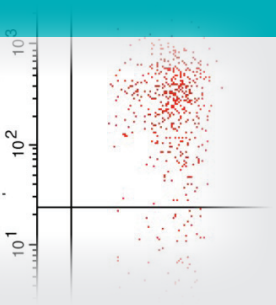
2.2 Add adequate amount of SenTraGor™ reagent through a syringe attached with a 13 mm filter and membrane 0.22 μm. The pellet must be covered with the reagent. Incubate for 8 min at RT

2.3 Centrifuge at 1200 rpm for 5 min at RT and discard supernatant

2.4 Wash 1x in 50% EtOH for 5 min at RT

2.5 Centrifuge at 1200 rpm for 5 min at RT and discard supernatant

2.6 Repeat steps **2.4** and **2.5** 3x



SenTraGor™
Antibody-enhanced detection of Senescent cells

arriani pharmaceuticals
a: 85 Lavriou Ave., 190 02, Peania, Attica, Greece
t: +30.210.66.83.000
f: +30.210 66.83.250
e: arriani@arriani.gr

Staining Protocol for Flow Cytometry

2.7 Wash x1 in 5 ml PBS for 5 min at RT
2.8 Centrifuge at 1200 rpm for 5 min at RT and discard supernatant
2.9 Incubate with the primary anti-biotin antibody, diluted 1/400 in PBS, for 60 min at 37°C
2.10 Centrifuge at 1200 rpm for 5 min at RT and discard supernatant
2.11 Wash x1 in 5 ml PBS
2.12 Centrifuge at 1200 rpm for 5 min at RT and discard supernatant
2.13 Incubate with secondary antibody, diluted 1/100 in PBS, for 20-30 min in dark and on ice
2.14 Centrifuge at 1200 rpm for 5 min at RT and discard supernatant
2.15 Wash x1 in 5 ml PBS
2.16 Centrifuge at 1200 rpm for 5 min at RT and discard supernatant
2.17 Dilute in PBS
2.18 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 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 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.