Instruction Manual: Protocol for detecting Cellular Senescence of Human Mesenchymal Stem Cells with SenezRed™

Version 2.0

Overview

Primary human mesenchymal stem cells (MSC) can be harvested from various human tissues including bone marrow, cord blood, adipose tissue and others. Primary MSC have a great capacity for self-renewal in cell culture, and are generally collected and propagated as adherent cells in tissue culture plastic-ware.

Human MSC are multipotent stromal cells that can differentiate into a variety of cell types, including osteoblasts, myocytes, chondrocytes and adipocytes.[1] For more than 20 years, MSC have been used in clinical cell therapy, taking advantage of their innate capacity for expression of a variety of paracrine bioactive factors including angiogenic factors, growth factors, and cytokines. MSC also have a remarkable capacity for inhibiting the immune response.[2]

Cultured primary cells do not grow indefinitely, but undergo only a limited number of cell divisions, eventually reaching a state of cellular senescence. Aged MSC generally perform less well than their younger counterparts in cellular therapy, largely due to changes in the cellular physiology brought on by senescence.[2]

It is therefore of great significance to monitor the occurrence of the senescent phenotype in human MSC to ascertain their integrity and capacity for differentiation, migration and clinical therapeutic potential.[2]

This protocol describes the use of a non-toxic reagent called SenezRed™, to stain and observe cultured human MSC for the senescent state. SenezRed™ is a membrane- permeable, red-colored fluorescent probe that selectively stains living, senescent MSC.[3,4,5,6]

It is strongly recommended that you read and fully understand the entire protocol before beginning your experiments. To maintain sterility, all procedures (except as indicated) should be performed in a sterile biological safety cabinet.

Conditions of Use

This product is for research use only. It should not be used for therapeutic or diagnostic purposes. Sale of this product to a third party, or any other commercial use for this product, is prohibited without prior permission from ReproCELL (Japan) or Brilliant Research (Singapore).

Storage

The SenezRed™ reagent is supplied at a concentration of 1 mM (one millimolar) in a solution that contains DMSO (dimethyl sulfoxide). SenezRed™ can be aliquoted into smaller volumes and stored at -20°C to avoid repeated freeze/thaw cycles. The product is guaranteed stable for one year after purchase, when handled properly and stored at -20°C. When stored at 4°C, the product is stable for about 2 months.

Features of the SenezRed™ reagent

- Simple and rapid staining protocol without the need for cell fixation
- Selective staining of attached primary senescent (less potent) mesenchymal stem cells
- Stains live cells for fluorescent imaging

General Notes & Product Specifications

- Cells should be seeded in a 6-well plate for most economical use of material
- Staining and cell observation is best under conditions of 50-60% confluency
- Peak excitation / emission wavelength of SenezRed™ is at 631 / 673 nm
- A typical Cy5™ dye filter set is sufficient for observation and imaging of SenezRed™ fluorescence
  - SenezRed™ is supplied at a 1000-fold higher concentration (1 mM) than the working concentration (1 µM)
  - Senescent cells can be generated by long-term *in vitro* culture of 10 or more passages, or induced by H_2O_2 treatment
  - SenezRed™ staining can be validated by correlating to senescence associated β-galactosidase staining

Brilliant Research SenezRed™
(from ReproCELL)

Product Description	Cat. No.	Unit	Short term storage	Long term Storage
SenezRed™ (*small*)	BR-02M-020	20 uses	2 month at 4°C	1 year at -20°C
SenezRed™ (*large*)	BR-02M-100	100 uses		

Other Reagents and Equipment

Product	Supplier	Storage
6- well plate (clear polystyrene)	Cell Culture Reagent Suppliers	RT
MSC cell culture medium	Cell Culture Reagent Suppliers	4°C
Cy5™ fluorescent filter set and microscope	Instrumentation Suppliers	RT

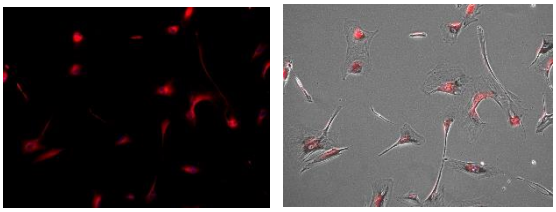
Contents

A) Staining with SenezRed™

NOTE: Human MSC should be plated in a 6-well plate (cell culture dish) and grown to the typical density at which you generally harvest and/or conduct your standard experiments. For optimal routine use and observation, a cellular density of 50-60% confluency is recommended.

1. Pre-warm enough MSC cell culture medium for your planned experiment. One milliliter (1 mL) is enough for staining one well of a 6 well cell culture plate.
2. Thaw and mix the SenezRed™ reagent.
3. Shake-down or flash-spin the tube to collect all the material to the bottom.
4. The suggested working solution for SenezRed is 1 µM. This can be obtained by making a 1:1000 dilution of the SenezRed stock solution (1 mM) with pre-warmed culture medium. Retrieve the plate of MSC cells and remove the medium from the well that you intend to stain with SenezRed™.
5. Replace the medium with 1 mL of the diluted, pre-warmed MSC culture medium containing 1 µM SenezRed™.
6. Incubate the cells for 1 hour at 37°C with 5% CO_2 and 95% humidity.
7. After staining, retrieve the plate from the incubator and remove the 1 mL of the dye-containing medium.
8. Replace the medium with 3-5 mL of fresh pre-warmed MSC culture medium. Return the plate to the incubator and allow 1 hour for "de-staining".
9. Hoechst stain can be added for visualization of cell nuclei.
10. Following the 1 hour destain, change the medium one more time with fresh, pre-warmed MSC medium and observe under a fluorescence microscope using a Cy5™ or appropriate filter set suitable for Ex/Em at 631/673 nm.

Figure 1 – Population of senescent human mesenchymal stem cells stained with SenezRed™ dye and viewed using a fluorescence microscope equipped with a Cy5™ filter set. A fluorescence image only (*left*) and the same image with the corresponding β-galactosidase stained bright-field image overlay (*right*) are shown.



B) Frequently Asked Questions

Q. **Does SenezRed™ staining require optimization?**
A. The standard protocol given here is quite simple and robust. Usually no optimization is required; however, if cells or medium or confluency states deviate much from the standard practice, some optimization may be required. Suggestions for optimization include the use of lower or higher concentrations for staining, longer de-staining times, or imaging conditions, etc.

Q. **Do I need some control wells to interpret my staining results?**
A. It is not necessary to prepare any special controls; however, multiple samples are best compared in a single experiment using identical reagents on the same day. It is critical to maintain equal staining, de-staining, and exposure times for optimal results and accurate data interpretation.

Q. **Is SenezRed toxic to the cells?**
A. As far as we know, SenezRed is not toxic to the cells. Cells can be stained repeatedly every couple of

days and over multiple passages without any effects on cell viability or functions. This is one of the significant benefits of a live fluorescent stain.

Q. **How does SenezRed compare to the traditional β-galactosidase staining procedure?**
A. SenezRed™ has been shown to detect senescent cells earlier than using traditional β-galactosidase staining for senescence. For co-staining experiments, after initial staining and de-staining with SenezRed™, cells can then be fixed and stained by following the vendor's protocol for β-gal and then viewed with a brightfield overlay as shown in Fig. 1. This validation will help the user gain confidence that they are identifying senescent cells.

Q. **How does SenezRed™ work?**
A. That is a very good question, for which we do not yet have a very good answer. The SenezRed™ staining reaction correlates very well with other markers for cellular senescence.[3,4,5,6]. At the current time, it is not known specifically to what this small molecule dye is binding within the cell. There are some evidence indicating that it is localized within mitochondria and hence may be involved in detecting some kind of senescent metabolic state for MSC; however, this is pure speculation.

Q. **Can SenezRed™ detect senescence in other cell types?**
A. This has not been investigated. We have no evidence to support or negate the activity of SenezRed™ to detect senescence on other cell types.

Q. **I observed that many apparently proliferating cells are positively stained. Does that mean that the probe is not working?**
A. There will be some background staining even among proliferative cell populations. Dead cells and cell debris can also pick up the SenezRed™ dye, giving false positive results. One hour de-staining time is highly recommended to reduce background staining. Hoechst stain can be used to distinguish living/dead cells. It is also possible to "remove" the background staining by adjusting the contrast/brightness using imaging software such as Image J. It is important to note that the same adjustments should be made to all images for a valid comparison.

Q. **What are the recommended cell density conditions for SenezRed™ staining?**
A. It is recommended to use a cell population of 60-70% confluency for the staining. To use senescence cells as control it is recommended to have cells that that require a population doubling time of more than 120 hours.

Q. **Do you have a recommended protocol to validate the SenezRed™probe?**
A. If you wish to do this with your cells, it is recommended to use a staining, de-staining and exposure settings that works well with your control senescence cells, and apply the same across proliferating cell populations of different passages for comparison. For β-gal validation, it is recommended to image the same field of cells after SenezRed™ staining and again after β-gal staining. This allows direct visual correlation of β-gal positive cells with SenezRed™ positive cells.

C) References

1. Kwon H M, Hur S-M, Park K-Y, Kim C-K, Kim Y-M, Kim H-S, Shin H-C, Won M-H, Ha K-W, Kwon Y-G, Lee, D-H, and Y-M Kim. Multiple paracrine factors secreted by mesenchymal stem cells contribute to angiogenesis. Vascular Pharmacology, Elsevier, Inc. (2014) Volume 63, Issue 1, October, pp 19-28.
2. Turinetto V, Vitale E, and C. Giachino. Senescence in human mesenchymal stem cells: functional changes and implications in stem cell-based therapy. Int J Mol Sci (2016) July 17(7), pp 1164-1182.
3. Oh, Steve et al. 'Brilliant vital fluorescent probes for detecting stem cell proliferation and senescence". Stem Cell Society Singapore (SCSS) Symposium 2015, Singapore. 17-19 Nov 2015.
4. Raghothaman, Deepak et al. "A cell permeable fluorescent probe for detecting stem cell senescence". Biology of Aging conference, Singapore. 22-24 October, 2015.
5. Raghothaman, Deepak et al. "Vital fluorescent probes for detecting live stem cell proliferation and senescence". Aging: Cellular Mechanisms and Therapeutic Opportunities, a Herrenhausen Symposium. A Nature Medicine Conference. Hanover, Germany. 28-29 September, 2015.
6. Oh, Steve. "Stem cell bioprocesses and senescence". The Rejuvenation Biotechnology Conference, San Francisco, USA. 18-21 Aug. 2015.

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