Marker Gene Technologies, Inc

Product Information Sheet

MarkerGene™ Cellular Senescence Microtiterplate Assay Kit

Product M1405

Marker Gene Technologies, Inc.
University of Oregon Riverfront Research Park
1850 Millrace Drive
Eugene, Oregon 97403
1-888-218-4062
www.markergene.com


MGT

MarkerGene™ Cellular Senescence Microtiterplate Assay Kit
(Product M1405)

NOTE: The following information is given as a viable methodology for use of the MarkerGene™ Cellular Senescence Microtiterplate Assay Kit. The user may determine their own best conditions for use dependent on the specific conditions present in their experiment.

I. OVERVIEW

Many primary cell types, particularly fibroblast cells, have a limited capacity to continue to divide in cell culture. Even normal cells derived from fetal, embryonic or newborn tissue, typically undergo between 40 and 60 cell divisions, but then often stop dividing. This number is often referred to as the Hayflick Limit.

Normal diploid differentiated cells lose the ability to divide, normally after about 50 cell divisions in vitro, and some cells become senescent even before this time, because of DNA double strand breaks, the introduction of toxins or the like. This phenomenon is also known as "replicative senescence". The change to a non-dividing state is called senescence and is accompanied by changes in morphology and gene expression patterns. In addition, senescence also is accompanied by increases in intracellular and secretory enzymatic activity.

Recent studies have shown that ß-galactosidase histochemical staining at pH 6 is a useful enzymatic marker of cellular senescence (the so-called senescence associated beta-galactosidase; SA-ß-Gal). While cells entering senescence show this enzymatic activity, immortalized cell types, including tumor or other quiescent cells, are not stained under these conditions. The implications of these methods in such diverse areas as age-related pathology research, tumor analysis and tissue culture maintenance is significant. The SA-βGal assay is one of the most widely accepted markers of cell aging. Often analysis is performed using the chromogenic substrate X-Gal. However, the substrate X-Gal has limited sensitivity and is difficult to quantify. The degree of blue staining in positive cells varies both with cell type and staining conditions, and this introduces subjectivity to results. Some positively stained cells are strongly, moderately, or weakly stained blue and all may be recognized as positive cells.

The MarkerGene™ Cellular Senescence Microtiterplate Assay Kit (M1405) uses the high-sensitivity substrate fluorescein di-β-D-galactopyranoside (FDG) to quantify senescent cells. FDG turnover produces the highly fluorescent product fluorescein, which is easily detected using an appropriate filter set for fluorescein fluorescence (i.e. EX 485nm and EM 535 nm) in a microtiterplate assay format.

This assay kit contains all of the reagents for performing up to 100 assays using a 12-well tissue culture plate format using samples of cells or for cells that have been grown onto sterile cover slips for analysis from other plate sizes. The assays can also be adapted for use with 96-well, 24-well, 6-well and in 60 or 100 mm plates. In addition, the kit also contains reaction buffers, dilution solvents, a detailed protocol and references for use. Additional product information is available from our technical assistance staff, either by telephone (toll-free) 1-888-218-4062 or by e-mail at techservice@markergene.com .

II. MATERIALS

A.) 1 **Substrate Reagent:** 5mg Fluorescein di-ß-D-Galactopyranoside (FDG).

B.) 2 **Staining Buffer**

C.) 3 **Fixing Solution:** This solution contains glutaraldehyde and formaldehyde.

D.) 4 10X **PBS**, pH 7.4

E.) 5 **Dimethyl Sulfoxide** (solvent).

Storage and Handling: The substrate reagent included in this kit should be kept cold when not in use and stored, desiccated at -20˚C. Protect solutions of the substrate reagent and reference standards from light.

Additional Items Required:

- Tissue culture plate(s)
- Appropriate cell lines
- CO_2 incubator for cell culture.
- 37°C incubator, without CO_2
- Light microscope.
- Epifluorescence microscope with FITC-type filter set (optional).
- Fluorescence microtiterplate reader equipped with fluorescein filter set.

III. PROTOCOL

For 12-well plate (22mm diameter) clear bottom sterile culture plates.

NOTE: It is recommended that the SA-β-Gal assay be run first with known senescent cells (high passage number) to verify activity, if possible. Senescence can also be induced in some cell lines by addition of ROS (reactive oxygen species) activating compounds like BrdU, H_2O_2. Please see the references below for more information or consult the literature for your specific cell line.

1.) Plate healthy growing cells in a 12-well tissue culture plate (for example, Falcon 35-3043 Multiwell 12-well Tissue Culture Plate or equivalent) or onto sterile glass cover slips (18mm circular). Alternately, cells can be grown in 6-well (e.g. Corning, Costar 3516 sterile Tissue Culture plates),

or 96-well (Corning, Costar 3696 flat-bottom Sterile Tissue Culture, or Falcon, Becton-Dickinson 35-3072 Microtest 96 Sterile Tissue Culture plates) or equivalent. Cells are typically plated into the microplates approximately 24 hours prior to analysis and at a confluency of approximately 60-70 %. The number of cells per well will vary with plate size.

2.) Prepare 2mM FDG solution: Dissolve 5mg Substrate Reagent 1 provided in 38µL H_2O:DMSO (1:1) solution to make 200mM stock FDG solution. Make sure all clumps dissolve by gentle inverting or vortex mixing, but do not heat or ultrasonicate. Stock solutions should be stable for several days at -20°C. Dilute stock 100X in sterile H_20 to make 2mM FDG solution. (For example, add 5µL 200mM FDG solution to 495µL sterile water).

NOTE: *MAKE ONLY AS MUCH 2MM FDG AS NEEDED FOR THE DAYS' USE. THIS SUBSTRATE LOSES ACTIVITY WHEN STORED, EVEN AT LOW TEMPERATURES. STORE THE REMAINING SOLID OR 200 MM STOCK AT -20°C IMMEDIATELY AFTER USE. THE DEVELOPMENT OF A YELLOW COLOR / GREEN FLUORESCENCE INDICATES DECOMPOSITION OF THE SUBSTRATE.*

3.) Transfer cover slips to 12 well plate when cells are 70% confluent. Wash cells 3 times with 500µL 1X PBS, pH 7.4 (Prepare from 10X PBS 4 provided in kit.).

4.) Cells can be treated at this point with specific compounds, drugs or cofactors for specified periods. If incubation times with these compounds will be more than 24 hours, it is recommended that the concentration of cells/confluency of the cells used be lowered appropriately.

5.) Fix cells by adding 500µL Fixing Solution 3 to each well. Incubate at room temperature for 30 minutes. Aspirate Fixing Solution and wash 3 times with 500µL 1X PBS.

6.) Add 500µL Staining Buffer 2 to each well. Next, add 50µL 2mM FDG solution to each well. Incubate 2-20 hours at 37°C without CO_2. Measuring the standard kinetics every two hours is recommended.

7.) After incubation, the plates can be read directly in the microtiterplate reader, or by transferring 100µL of the supernatant from each well to a 96-well plate for fluorescence measurement in triplicates.

8.) Include a blank containing only the reaction mixture (staining buffer and FDG) without cells.

9.) Read the fluorescence at Ex/Em: 485nm/535nm or using an equivalent filter set. Consult with your instrument manufacturer for appropriate filter sets for fluorescein fluorescence if necessary. Subtract blank value from each sample well value.

NOTE 1: It is recommended that a comparison with X-Gal staining be carried out, for some data points, to validate if required. Please see our Product **M1389** for more information about this method. Representative comparison of data obtained using the **M1405** kit and X-Gal staining for Primary Human Skin Fibroblast cells (GM03440) is shown in Figure 1 (see below).

NOTE 2: Senescence Associated β-Gal activity has been found to increase slightly upon confluence with adherent cell lines (3-5% for confluent cells vs. < 1% for exponentially growing cultures). See Pieper, et al., 1999 for more information about running long-term assays in the same plate format.

NOTE 3: The use of hydrogen peroxide or t-butyl hydroperoxide as a control is not recommended with this assay. Fluorescein di-β-D-galactopyranoside (FDG) is not stable under peroxide conditions.

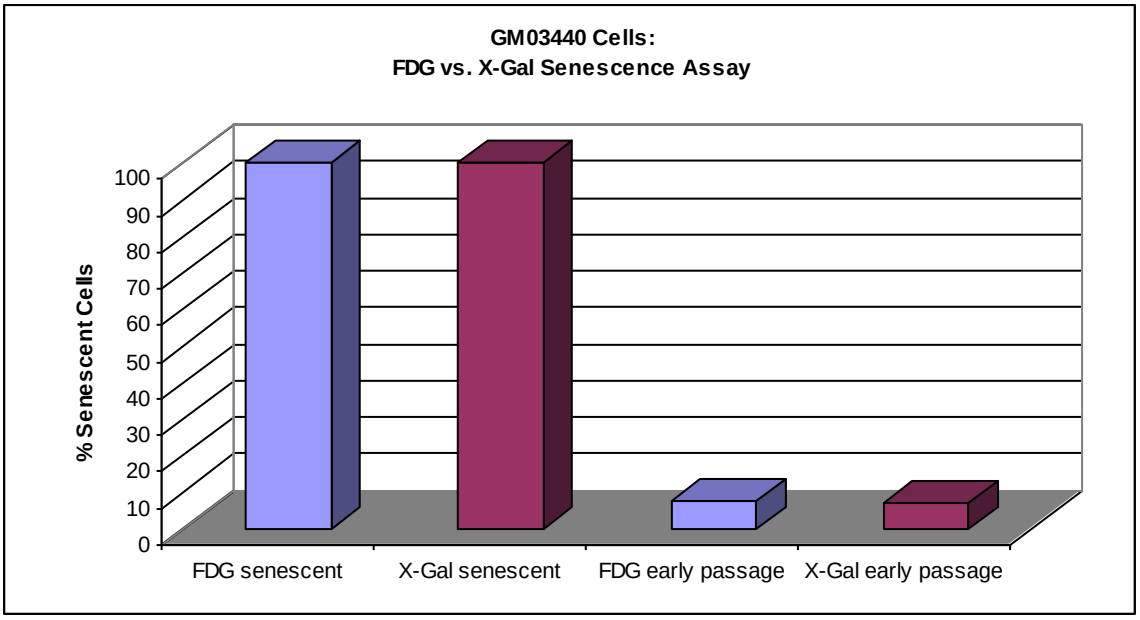

Figure 1: X-Gal vs. FDG senescence assay on human skin fibroblasts, GM03440. Known senescent and early passage human skin fibroblasts were treated with X-Gal or FDG to determine senescence. X-Gal and FDG cells were fixed with Fixing Solution 3 for 30 minutes and washed 3 times with 1X PBS. X-Gal cells were treated with a staining buffer containing ferrous and ferric ions and X-Gal (10:1) (Product **M1389**) and incubated for 16 hours at 37°C without CO_2. Senescent (blue) and healthy cells (clear) were counted and percentage senescent cells were calculated. FDG cells were incubated for 16 hours at 37°C without CO_2 in 500µL Staining Buffer 2 and 50µL 2mM FDG. Fluorescence was measured with Ex/Em: 485nm/535nm.

M1405 KIT CONTENTS			
DESCRIPTION	**QUANTITY**	**PART NO.**	**STORAGE**
REAGENTS			
1 SUBSTRATE REAGENT	1 x 5 mg	1405-001	F
2 STAINING BUFFER	1 x 100 mL	1405-002	RT
3 FIXING SOLUTION	1 x 100 mL	1405-003	C
4 10X PBS	1 x 10 mL	1405-004	RT
5 DMSO	1 x 5 mL	1405-005	RT, FL
DOCUMENTATION			
MSDS Sheets	3		
Product Information Sheet	1		

Notes: F=store at or below -20°C; RT=store at room temperature; C=store cold (4°C); L=light sensitive; FL=flammable; R=read protocol instructions carefully prior to use.

REFERENCES

1. Dimri GP, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens m, Rubeli I, Pereira-Smith O, Peacocke M, Campisi. (1995) “A biomarker that identifires senescent human cells in culture and in aging skin in vivo.” PNAS 92: 9363-9367.

2. Yang NC, Hu ML. (2004) “A fluorimetric method using fluorescein di-β-D-galactopyranodise for quantifying the senescence-associated □-galactosidase activity in human foreskin fibroblast Hs68 cells.” Anal Biochem 325: 337-343.

3. Eriko M, Nakabayashi K, Suzuki T, Kaul SC, Ogino H, Fujii M, Mitsui Y, Ayusawa D. (1999) “5-Bromodeoxyuridine induces senescence-like phenomena in mammalian cells regardless of cell type or species.” J. Biochemistry 126: 1052-1059.

4. Chen JH, Ozanne SE, Hales CN, (2007) ”Methods of Cellular Senescence Induction Using Oxidative Stress.” In “Biological Aging: Methods and Protocols” Methods in Molecular Biology 371: 179-189.

5. Katakura Y, Miura T, Uehara N, Nakata E, Shirahata S (1999) “Senescence Induction in Cancer Cells.” In Animal Cell Technology: Challenges for the 21st Century: Proceedings of the joint international meeting of the Japanese Association for Animal Cell Technology (JAACT) and the European Society for Animal Cell Technology (ESACT) 1998, Kyoto, Japan, Part XI, pages 279-282.

6. Pieper RO, Lester KA, Fanton CP (1999) "Confluence-induced alterations in CpG island methylation in cultured normal human fibroblasts." Nucleic Acids Research 27(15): 3229–3235.

AUXILIARY PRODUCTS

Product # **Name**

M0250: Fluorescein di-β-D-Galactopyranoside (5 mg)

M1194: 2', 7'-Dichlorofluorescein di-ß-D-galactopyranoside (DCFDG) (5 mg)

M1352: MarkerGene™ ß-Galactosidase Staining Kit

M0259: MarkerGene™ *in vivo lacZ* ß-Galactosidase Intracellular Detection Kit.

M0204: Fluorescein

M1405-002:Staining Buffer (100 mL); M1405-003:Fixing Solution (100 mL)

M1389: MarkerGene™ Cellular Senescence Assay Kit

Contact and Support

For questions or comments on this or any product from Marker Gene Technologies, Inc., you may contact us by phone or via our website. We welcome customer feedback and we make every effort to improve our products based on input from our clients.

To ask a question or make a comment or suggestion, you can email us at techservice@markergene.com, call us at **1-888-218-4062** or fax to **541-342-1960**.

For more information on our products and services, please visit our website at www.markergene.com, where you can find:

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Marker Gene Technologies, Inc.
University of Oregon Riverfront Research Park
1850 Millrace Drive
Eugene, Oregon 97403
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