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Soft Agar Colony Formation Assay as a Hallmark of Carcinogenesis

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[Abstract] Soft agar colony formation assay is established to estimate the anchorage-independent growth ability of cells. In this assay, a bottom layer of agar with complete media is poured and solidified first, followed by an upper layer containing a specified number of cells suspended in medium-agar mixture. After two weeks of incubation, the number of colonies will be counted, serving as an indicator of malignancy of tumor cells.
Keywords: Anchorage-independent growth, Colony formation, Carcinogenesis, Malignant phenotype, Agar

[Background] Anchorage-independent growth is an ability of cells to grow independently on a solid surface, and is considered as a hallmark of carcinogenesis (de Larco and Todaro, 1978). Soft agar colony formation assay is a well-established method to evaluate cellular anchorage-independent growth for the detection of the tumorigenic potential of malignant cells (Roberts *et al*., 1985), which is developed from plate colony formation assay described by Puck *et al.* in 1956 where cells were seeded on to a culture plate to assay the ability of cells to form colonies (Puck *et al*., 1956). The limitation of plate colony formation assay is that it only displays cellular abilities for anchorage-dependent growth, by which normal cells can escape from anoikis (a form of programmed cell death that occurs in anchorage-dependent cells when they detach from the surrounding extracellular matrix) and survive (Taddei *et al*., 2012). In contrast, malignant cells are capable of proliferating and growing without attachment to a substrate. Therefore, soft agar colony formation assay is developed to characterize this ability *in vitro* (Hamburger and Salmon, 1977; Yuan *et al*., 2017). The soft agar colony formation assay has been widely adapted for researches on cell differentiation, transformation and tumorigenesis as well as the efficacy evaluation of anti-tumor treatment.

Materials and Reagents

1. Cell culture disc (75-cm^2) (Corning, catalog number: 430641)
2. Cell culture plate (12-well) (Corning, Costar®, catalog number: 3513)
3. Falcon 15 ml conical centrifuge tubes (Corning, catalog number: 430791)
4. Counting slides (Bio-Rad Laboratories, catalog number: 1450011)
5. 0.1-20 ml volume pipette tips (Eppendorf, catalog number: 22492012)
6. 5-200 ml volume pipette tips (Eppendorf, catalog number: 22492039)

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7. 50-1,000 ml volume pipette tips (Eppendorf, catalog number: 22492055)
8. Human SGC7901 cell line (Cell Resource Center of the Chinese Academy of Sciences, catalog number: CC-Y1456)
9. Phosphate buffered saline (PBS) pH 7.4 (Thermo Fisher Scientific, Gibco™, catalog number: C10010500BT)
10. Trypsin-EDTA (0.25%) (Thermo Fisher Scientific, Gibco™, catalog number: 25200072)
11. RPMI 1640 medium (Thermo Fisher Scientific, Gibco™, catalog number: C11875500BT)
12. Fetal bovine serum (Thermo Fisher Scientific, Gibco™, catalog number: 10099141)
13. Penicillin-streptomycin (5,000 U/ml) (Thermo Fisher Scientific, Gibco™, catalog number: 15070063)
14. L-glutamine (Thermo Fisher Scientific, Gibco™, catalog number: 25030081)
15. Agar (Biowest, catalog number: 111860)
16. Complete 1640 medium (see Recipes)
17. 5% agar solution (see Recipes)

Equipment

1. 2-20 µl pipettes (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 4641060N)
2. 20-200 µl pipettes (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 4641080N)
3. 100-1,000 µl pipettes (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 4641100N)
4. Clean bench (Thermo Fisher Scientific, Thermo Scientific™, model: Heraguard™ ECO)
5. Autoclave (TOMY DIGITAL BIOLOGY, model: SX-500)
6. Water-Jacketed CO_2 incubators (Thermo Fisher Scientific, Thermo Scientific™, model: Forma™ Series II 3110, catalog number: 3131)
7. Thermostat water bath (Prima Technology, model: YB12)
8. Centrifuge (Eppendorf, model: 5424 R)
9. Automated cell counter (Bio-Rad Laboratories, model: TC20™)
10. Advanced microscopy group microscope (Thermo Fisher Scientific, model: EVOS)
11. Gel count colony counter (Oxford optronix, model: GelCount™)

Software

1. Statistical Program for Social Sciences 17.0 software (SPSS)

Procedure

1. Preparing 5% agar solution (see Recipes).
2. Production of the bottom layer of agar.

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   a. Add 9 ml complete medium (37 °C) to 1 ml 5% agar solution (50 °C) and mix thoroughly.
   b. Pipette 0.8 ml mixture to each well of 12-well plate and allow it to solidify for 30 min at room temperature.
3. Preparation of cell suspension.
   a. Remove the complete medium from culture dish and wash cells with 1x PBS.
   b. Add 0.5 ml 0.25% trypsin (37 °C) for 3-5 min and collect detacted cells by adding complete medium.
   c. Spin cells at 60 *x g* for 5 min and resuspend cells in complete medium, then count cells and adjust the concentration of cells to 1 x 10^3 cells/ml.
4. Production of the upper layer of agar.
   a. Add 9.4 ml re-suspended cells (37 °C) to 0.6 ml 5% agar solution (50 °C) and mix homogeneously.
   b. Pipette 0.8 ml the cell-agar mixture onto the solidified bottom layer of agar in 12-well plate and allow it to solidify for 30 min at room temperature.
   c. Add 800 µl complete medium on top to prevent drying of agar and then cells were maintained in a 37 °C humidified incubator with a mixture of 95% air and 5% CO_2.
5. Clone counting
   a. Monitor colony formation for 2-3 weeks before counting.
   b. All colonies per well were counted with a gel count colony counter, and then determine the average number of colonies of the three replicates for each group.
   c. Capture images of colonies at room temperature using an advanced microscopy group microscope (Figure 1).

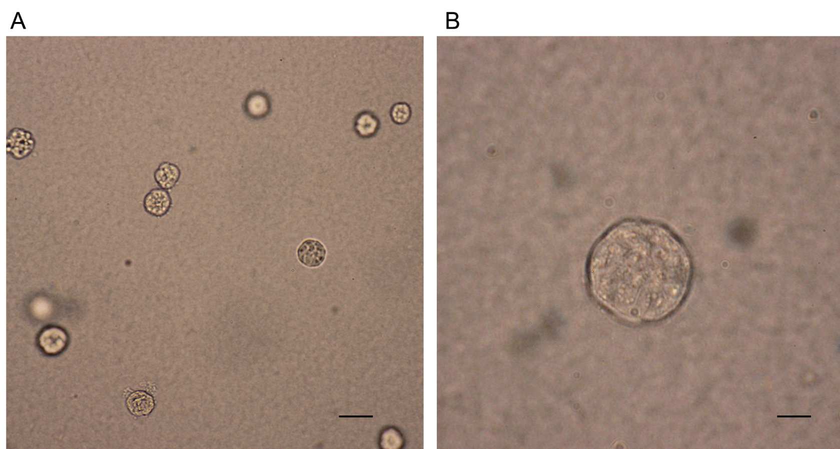

Figure 1. Photographs of representative colonies from SGC7901 cell lines. SGC7901 cells were cultured in the upper layer of agar and the formation of colonies was captured at 2-3 weeks after culture. Scale bars: A, 400 µm; B, 100 µm.

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Data analysis

All statistical data were analyzed with the Statistical Program for Social Sciences 17.0 software (SPSS). The experiments were conducted in triplicates. The results are presented as the mean ± SD. Differences between means were assessed using Student's *t*-test or one-way analysis of variance. $P < 0.05$ was considered to be statistically significant.

Notes

1. This assay was conducted using human SGC7901 cell line and is applicable to other cancer cell lines.
2. Incubate cells typically 2-3 weeks and adjust the incubation period according to the tumorgenecity of the cell line.
3. After autoclaving, agar solution should be kept sterile during the following operation.
4. Pay attention to the temperature of agar solution and complete medium. It is recommended to keep agar solution and complete medium at 50 °C and 37 °C, respectively, and mix them as soon as possible to avoid in-homogenous agglomeration (Puck *et al*., 1956).
5. Do not pour the upper layer of agar until the bottom layer of agar is coagulated completely.
6. Details of the instruction of gel count colony counter can be obtained from the website: http://www.oxfordoptronix.com/product17/page501/menu2/Colony_Counting/GelCount_/GelCount_.html

Recipes

1. Complete 1640 medium
   10% FBS
   1% penicillin-streptomycin
   2 mM glutamine
2. 5% agar solution
   a. Dissolve 5 g agar powder in 100 ml saline and autoclave at 121 °C for 15 min
   b. Place the sterile 5% agar solution in 50 °C water bath to keep it in liquid phase

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