Soft–Agar Colony Formation Assay

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[Abstract] Any anchorage–independent growth of tumor cells is estimated by a soft–agar colony formation assay. This protocol provides a general workflow for establishing a soft-agar colony formation assay.

Materials and Reagents

1. Agarose-LE (Guidechem, catalog number: 9012-36-6)
2. DMEM (Life Technologies, Gibco®, catalog number: 11995)
3. Crystal violet (Matheson Coleman & BEL: catalog number: B278)
4. Fetal bovine serum (FBS) (Life Technologies, Invitrogen™, catalog number: 20140-079)
5. Phosphate buffered saline (PBS) (Life Technologies, Gibco®, catalog number: 14040)
6. Agar
7. Ethanol
8. Culture media (see Recipes)

Equipment

1. 60 mm culture dishes (Thermo Fisher Scientific, catalog number: 353002)
2. Water bath
3. Incubator

Procedure

1. Cells that have been knocked down or been treated with other procedures like drugs or some interferences are harvested and pipetted well to become single-cell suspension in complete culture media in a given concentration (*such as* 1 x 10^6/ml).
2. They are then put at room temperature and should be ready to use. At the same time, pre-warm 10% FBS DMEM at 37 °C, ready to use.

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3. Melt 4% agar (in glass bottle) by microwave and keep warm in 56 °C water bath, ready to use (remember don't melt the gel too many times, you will lose water and concentrate the gel).
4. Make bottom layer. Make 5 ml of 10% FBS DMEM containing 0.75% agar (the volume of 4% agar was calculated by: 5 ml multiply 0.75%=X multiply 4%). Therefore, you need to quickly take about 0.9 ml 4% agar from the bottle in water bath and mix it with 4.1 ml pre-warmed of 10% FBS DMEM well and put the mixture into 60-mm culture dish in the flow hood. Wait a few minutes for it to become solid.
5. Make the top layer. It contains 3 x 10^4 cells in 3 ml of 10% FBS DMEM and 0.36% agar. Dilute the single-cell suspension to 3 x 10^4 (or 1.5 x 10^4) in 3ml of 10% FBS DMEM (1 x 10^4/ml). You need to mix 2.73 ml pre-warmed 10% FBS DMEM cell suspension with 270 µl of 4% pre-warmed agar (56 °C). You have to do this fast and no bubble. To avoid this problem, you can make 6 ml (double) of this mixture and put 3 ml on the top of bottom layer. The best way to do this is to use 1 ml pipette tip to add from one side. The mixture will flow through the whole bottom layer. Do not shake the dish. It will become solid within seconds.
6. Now the dish contains 2 layers. Mark the dish and put it at 37 °C incubator for 3 weeks. Then the colonies were stained with 0.04% crystal violet-2% ethanol in PBS. Photographs of the stained colonies should be taken.
   Note: 4% Agar should be prepared in DD water then autoclave it.
7. If the colony number is too high to count, you can decrease cells to a total of 1.5 x 10^4. This would be easy to count.
8. Before you mix agarose gel with medium and cells. You should get pipette, tips ready to use, and perform it as fast as possible.

Recipes

1. Culture media
   10% FBS
   DMEM

Acknowledgments

This protocol was adapted from Kakuguchi *et al*. (2010).

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References

1. Kakuguchi, W., Kitamura, T., Kuroshima, T., Ishikawa, M., Kitagawa, Y., Totsuka, Y., Shindoh, M. and Higashino, F. (2010). HuR knockdown changes the oncogenic potential of oral cancer cells. *Mol Cancer Res* 8(4): 520-528.

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