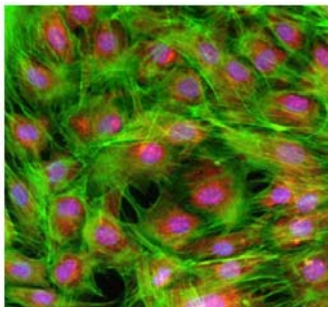
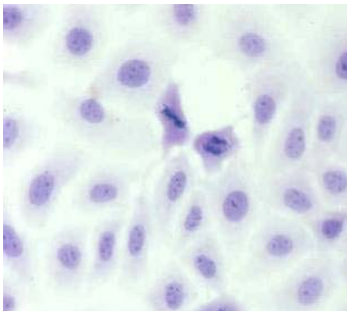
Clonal Growth of Cells in Semisolid Media

Protocol





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Introduction

Established cell lines such as HeLa and L-cells, as well as normal cells transformed by viruses, can form colonies suspended in soft agar-based media. Most normal cells will not grow under these conditions although there are exceptions (e.g. cartilage). The baby hamster kidney line, BHK-21, will not grow in agar, but will after transformation by polyoma virus (1, 2). In contrast, if agarose is used, (a purified agar, free of sulfated polysaccharides) BHK-21 will grow in suspension. If all these factors are taken into consideration and standardized, this system can be used to measure “transformation." It must be remembered, however, that morphological transformation and ability to grow in suspension are not necessarily correlated with the ability to form tumors in appropriate hosts.

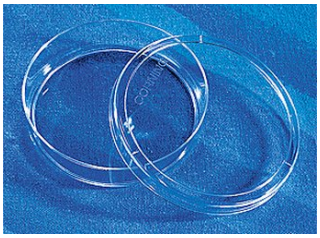
Growth of cells in semisolid medium, whether agar, agarose, or methylcellulose, offers a second advantage. The spherical bacteria-like colonies that form from monodispersed cell suspensions offer a means of isolating clones with a minimal amount of effort. Using a finely drawn pipette, single, well isolated colonies can be removed from the suspended state and subcultured. However, there are variations that must be used depending upon cell types. Clones in which there is loose intercellular bonding can be dissociated into a monodispersed population through gentle pipetting. However, many cell types require further enzymatic treatment to disperse them or must be treated as explants.

Materials

1. 20mL of 2.5% Bacto-Agar (Difco) in distilled water in 100mL glass bottle - Corning Cat. # 1395-100 or 1396-100. Solution should be sterilized by autoclaving. Agar needs to be melted at 100°C prior to use and kept at 45°C until mixed with Nutrient Mix.
2. 50mL 1X base medium - sterile. This should be made from the standard medium (no serum) used to grow the cells that will be cloned.
3. 20mL 2X base medium - sterile. Reconstituting 10X-powdered standard medium with only half the required water (no serum) is used to make the 2X medium.
4. 50mL complete growth medium – sterile. (This should be made from base medium plus 10% fetal bovine serum) for dilutions. Base medium should be the same medium that is normally used to grow the cell culture.
5. Fetal bovine serum (10mL)
6. 80mL Nutrient Mix in 100mL glass bottle - Corning Cat. # 1395-100 or 1396-100 Make up by combining:

   2X base medium (20mL)
   Fetal bovine serum (10mL)
   1X base medium (50mL)
   } **Mix well and place in 45°C water bath 30 minutes prior to using.**

7. 60mm plastic dishes - Corning Cat. # 430166 (8)
8. 15mL plastic centrifuge tubes - Corning Cat. # 430055 or 430789 (16)
9. 10mL pipettes - Corning Cat. #4488 or 4101 (1 bag)
10. 1mL pipettes - Corning Cat. #4485, 4011 or 4012 (1 bag)
11. Water baths at 45°C and 100°C
12. Cell suspension for plating (1mL at 10^6 cells/mL)



Corning offers a variety of **tissue culture treated dishes** suitable for growing cells in semisolid media.

Procedure

Label and prepare all plates and dilution tubes in advance.

1. Prepare 1X nutrient agar medium:
   a) Melt 2.5% agar in autoclave, microwave oven or boiling water bath, then place in 45°C water bath. **The agar temperature must be allowed to cool to 45°C before proceeding.**
   b) Warm nutrient mix in 45°C water bath. **The temperature of the nutrient mix must be allowed to reach 45°C before proceeding.**
   c) Pour contents of 2.5% agar into nutrient mix to create the osmotically balanced 1X nutrient agar medium with a 0.5% agar concentration. Mix gently but **AVOID BUBBLES**. Do not allow mixture to cool. **Keep nutrient agar medium in the 45°C water bath when not being used.**
2. Pipette 7mL of the 1X nutrient agar medium per 60mm dish. Allow agar to cool and harden. Once hardened, return the plates to the incubator. This agar layer will provide a base nutrient layer to support cell growth for at least one week. It will also keep the cells from reaching and attaching to the plastic on the bottom of the dish.

The key to success for this procedure is to keep the agar medium at 45°C until the cells are added and then to plate them immediately before the agar medium clumps or solidifies, or the cells are heat-shocked and damaged.

These concentrations will result in plates with $5x10^4$, $5x10^3$, $5x10^2$, and $5x10^1$ cells/dish. If different cell concentrations are required, then adjust dilutions in Step 4a accordingly.

3. Distribute 1mL aliquots of 1X nutrient agar medium in eight 15mL centrifuge tubes. **Keep tubes at 45°C in water bath and do not allow mixture to cool or it will begin to harden and develop clumps.**
4. Prepare the cell suspension in complete growth medium. When first plating a new cell type, we recommend that tubes be set up with the following cell concentrations: $1x10^5$, $1x10^4$, $1x10^3$, and $1x10^2$ cells/mL. Use 0.5mL cell suspension added to 4.5mL complete growth medium (no agar) to make these 1:10 dilutions.
    a) Add 0.5mL of each dilution to individual tubes of the nutrient agar mixture. Mix gently (but avoid bubbles) and **immediately** pour contents of the tube (0.33% agar) on top of the bottom agar layer in one of the dishes. Work rapidly. If the nutrient agar is lower than 45°C prior to mixing, then the cell suspension may form clumps when plated. If the medium is too warm, the cells will be heat-shocked and may not survive.
    b) Repeat the process for the seven remaining tubes and plates, setting up each cell concentration in duplicate.
5. Allow agar in plates to harden for 15 to 30 minutes on the bench top and then place them in a CO_2 incubator. If the resulting medium is too soft, try increasing the initial agar concentration to 3.5%. This will give a final agar concentration in the base layer of 0.7%.
6. Examine plates every two or three days until colonies are large enough to see with the unaided eye.

Acknowledgements

This protocol has evolved from protocols developed for cell culture training courses at the former W. Alton Jones Cell Science Center in Lake Placid, New York; Manhattan College, New York City; and the University of Connecticut, Storrs, Connecticut. I would like to thank all of my colleagues and students who, over the years, have contributed ideas and suggestions to its development.

References

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