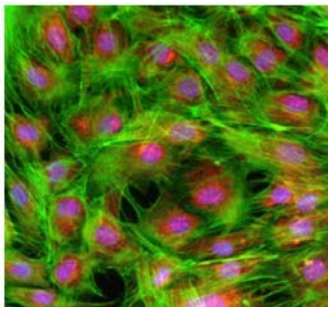
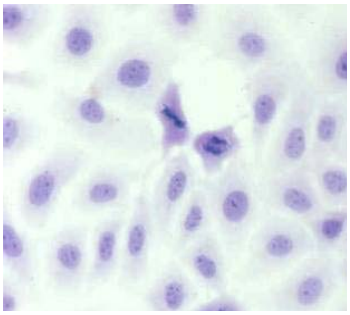
Cell Cloning by Serial Dilution in 96 Well Plates

Protocol





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Introduction

This technique is widely used for clonal isolation of hybridomas and other cell lines that are not attachment dependent. However, it is also very useful for cloning attachment dependent cells when the cell plating efficiency is very low, unknown or unpredictable. This method is fast and easy; however, like most clonal isolation methods, there is no guarantee that the colonies arose from single cells. Recloning a second time is advised to increase the likelihood that the cells originated from a single cell.

Supplies

Nonsterile

1. Pipetting aids - Corning Cat. No. 4910 (1)
2. Disposal tray or bucket for used pipettes (1)
3. Marking pen (1)
4. 200µL pipettor - Corning Cat. No. 4963 (1)
5. 8-channel 200µL micropipettor- Corning Cat. No. 4888 (1)

Sterile

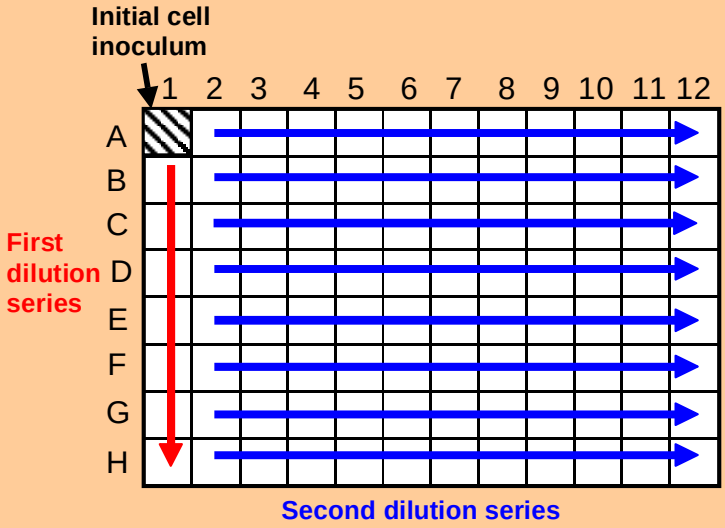
1. Cell culture medium – (Appropriate culture medium for the cells that will be cloned) (30mL)
2. Cell suspension at $2x10^4$ cells/mL (200µL needed per microplate)
3. 96 well cell culture microplate - Corning Cat. No. 3585 (1) This plate is designed for cloning cells that you want to attach to the well bottom; for cloning cells you want to stay in suspension use a 96 well round bottom **Ultra-Low Attachment** microplate - Corning Cat. No. 7007.
4. Sterile pipettor tips - Corning Cat. No. 4711 or 4810
5. Reagent dispensing reservoir/tray - Corning Cat. No. 4870 or 4871 (1)
6. 1, 5, and 10mL pipettes - Corning Cat. No. 4485, 4487 and 4488

Corning offers 8- and 12-channel adjustable 200µL micropipettors that are ideal for working in 96 well plates.

Procedure

1. Fill the reagent dispensing tray with 12mL of the appropriate culture medium, then using an 8-channel micropipettor add l00µL medium to all the wells in the 96 well plate except well **Al** (see diagram below) which is left empty.

Figure 1. Initial plate setup.



Adding 4000 cells in well A1 ($2x10^4$ cells/mL) is a good starting cell concentration. Increase this concentration for more difficult to grow cell lines.

2. Add 200µL of the cell suspension to well **A1**. (See Figure 1.) Then using a single channel pipettor quickly transfer 100µL from the first well to well B1 and mix by gently pipetting. Avoid bubbles. Using the same tip, repeat these 1:2 dilutions down the entire column, discarding 100µL from **H1** so that it ends up with the same volume as the wells above it.
3. With the 8-channel micropipettor add an additional l00µL of medium to each well in column 1 (giving a final volume of cells and medium of 200µL/well). Then using the same pipettor quickly transfer l00µL from the wells in the first column (**Al** through **H1**) to those in the second column (**A2** through **H2**) and mix by gently pipetting. **Avoid bubbles**!
4. Using the same tips, repeat these 1:2 dilutions across the entire plate, discarding l00µL from each of the wells in the last column (**A12** through **H12**) so that all the wells end up with 100µL of cell suspension.

Transferring clones directly from a well in a 96 well plate into a T-25 flask is not recommended. The cells may be unable to grow due to their inability to condition the larger volume of medium in the flask. Using some conditioned medium when subculturing the cells for the first time will also help them survive and grow.

5. Bring the final volume of all the wells to 200µL by adding 100µL medium to each well. Then label the plate with the date and cell type. Adding filtered conditioned medium (medium in which cells have been previously grown for 24 hours) to the wells can increase the success rate (cloning efficiency) for difficult to grow cells.
6. Incubate plate undisturbed at 37°C in a humidified CO_2 incubator.
7. Clones should be detectable by microscopy after 4 to 5 days and be ready to score after 7 to 10 days, depending on the growth rate of the cells. (See Figure 2 on page 3.) Check each well and mark all wells that contain just a single colony. These colonies can then be subcultured from the wells into

larger vessels. Usually each clone is transferred into a single well in a 12 well or 24 well plate.

Figure 2. Example of a fixed and stained (70% ethanol fixation followed by 1% crystal violet staining) plate containing a dilution plating of CHO-K1 cells. The highest cell densities occur in the wells immediately surrounding the A1 position. Wells A10, D10, E6, E11, F7, G6 and H4 appear to contain single colonies.

Acknowledgements

This protocol has evolved from a protocol I developed for cell culture training courses at 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.

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