EXPERIMENTS WITH NORMAL AND TRANSFORMED CELLS

A laboratory manual for working with cells in culture

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This manual is composed of a series of experiments performed during June, 1978 in the Transformed Cell Course at Cold Spring Harbor Laboratory. In preparing this manual, we have incorporated materials from manuals prepared for courses given in previous years. We are especially grateful to Bob Pollack, Richard Hynes, and William Topp for their suggestions and assistance in the formulation of both the course and the laboratory manual. The assistance of Keith Burridge, Seung-il Shin, Mei-Hui Teng, and Janet Gross is also gratefully acknowledged.

Essentially all of the techniques used in the experiments described in the manual are standard and have been described in publications cited in the manual. The cells employed are either readily available from the American Type Tissue Culture Association or can be obtained from numerous laboratories, including our own. In some cases, substitutions can be made depending on the interests of the instructors and class. For clarity, we have presented each experiment as a separate and complete entity. It is possible, however, to do several experiments simultaneously, in which case, all of the experiments can be performed within a three week period.

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INTRODUCTION

SOME CELL CULTURE TERMINOLOGY

Cell Line: A cell line is derived from a primary culture by subculture. The terms "finite" and "continuous" are given to cultures of known status. In any published description of a culture, one must give an accurate characterization and history of a culture; already published information should be referenced. Original designations of cultures obtained from other laboratories must be maintained.

Cell Strain: A cell strain can be derived from a primary culture or a cell line by the selection or cloning of cells with specific markers. Publishing procedures are analagous to those for a cell line.

Clone: A clone is a population of cells derived from a single cell by mitoses.

Plating Efficiency: Plating Efficiency (PE) refers to the percentage of inoculated cells which give rise to colonies. This term is often given as percentage of individual cells which give rise to colonies. It is a measure of cell autonomy.

Primary Culture: A primary culture is started from cells,tissues,or organs taken directly from organisms. A culture is regarded as such until it is subcultured for the first time.

Saturation Density: Saturation density refers to the maximum cell number attainable under specified culture conditions in a culture vessel. It is usually expressed as the number of cells per square centimeter (or per cubic centimeter in a suspension culture).

GUIDELINES FOR TISSUE CULTURE

General Safety and Procedures

These rules are mostly common sense and are designed to protect people and experiments.

No eating, drinking or smoking in the laboratory.

No storage of food.

If you have cuts on your hands, wear gloves. In any case, wash your hands after tissue culture work.

No mouth pipetting.

Work cleanly; wipe up any spills immediately and disinfect. Use osyl or 70% EtOH. This applies to medium as well, even if it contains no cells or virus; yeasts, molds and bacteria will still grow in it.

Clean up after yourself and leave the area ready for someone else to work. Dispose of your waste (see below) and wipe down the working surfaces with 70% ethanol or dilute bleach.

Tissue Culture Hoods

Each hood should have TWO traps: a 2 liter flask containing 100-200 ml of clorox and a smaller one to protect the vacuum line from overflow. Maintain clorox level so that medium is bleached. Add more clorox when necessary.

Traps should be emptied into the sink when ½ - 2/3 full and refilled with clorox. Make sure final clorox concentration is at least 10%; i.e., add extra clorox before emptying and allow solution to stand for 10 minutes.

When finished, wipe down the hood with 70% alcohol, or dilute bleach. Wipe up spills as soon as they occur.

When hoods are not in use the UV lights should be on (where available) and the doors closed.

Do not leave bunsen burners and vacuum lines on when not in use.

Close pipette cans after use.

Incubators

Do not spill medium. If you do, mop it up and wipe area with ethanol.

Remove contaminated plates immediately.

Incubators should not be run with excessive humidity; this increases the risk of contamination. Dampness enhances mold growth.

Disposal

Medium: Aspirate into clorox traps.

Plasticware: Place all disposable plastics (e.g. dishes) in plastic bags for autoclaving.

Contaminated Dishes: Do not open them. Seal in small plastic bags and add to plasticware waste for autoclaving.

Pipettes: Place in 4 liter beakers filled halfway with a solution of 7-X detergent or osyl. They will be autoclaved.

Pasteur pipettes: Place directly in plasticware bags for autoclaving and disposal.

Glassware: Empty when necessary, taking appropriate precautions with medium. Fill with 7X solution from tap and place in autoclavable plastic pans for collection.

Bring all items to autoclave corridor. Do not let them accumulate overnight.

STERILE TECHNIQUE

Animal cells are cultured in a variety of complex media, supplemented with serum. The media contain salts, sugars, amino acids, vitamins and other nutrients. In order to avoid growth of unwanted microorganisms, media and sera are sterilized by Millipore filtration. The media also routinely contain antibiotics and can be supplemented with anti fungal agents. Despite these precautions and the use of sterile glassware and pipettes, contamination of tissue culture with yeasts, molds, and bacteria is a frequent occurrence. It is essential that you learn, at an early stage, how to handle medium and cells using sterile techniques.

Procedures for Handling Cells and Medium

Use sterile glassware and pipettes.

When you open a bottle and after use, flame the top in a bunsen flame.

Flame pipettes.

Use bulbs to control pipettes. DO NOT MOUTH PIPETTE. This is to protect both you from the cells and the cells from contamination.

NEVER insert a pipette which may have contacted cells back into your stock bottle of medium. Use a fresh pipette.

Thus, to change the medium on a dish, the procedure is as follows:

Prewarm medium and serum to 37^{o}C in a waterbath.

Dry off the bottles and transfer to the tissue culture hood (which you should have wiped with ethanol, equipped with pipettes, clorox trap, etc.).

Open bottles, flame tops, replace caps loosely but so they won't fall off.

Transfer desired quantity of serum to the medium, or mix in separate (sterile) container. Use bulb and flamed pipette for transfer.

Move dish(es) of cells to the hood.

Using sterile, flamed, but NOT plugged, pasteur pipet, aspirate the medium into the clorox trap. Flame the pasteur pipette between dishes. Use a separate pasteur for different cells. Don't do too many dishes at a time.

Using sterile, flamed pipette, transfer desired amount of medium and serum to the dish(es). Do not reuse the pipette.

Return dishes to incubator.

Reflame tops of bottles and close tightly. Close pipette cans.

EQUIPMENT and SUPPLIES

Still air hoods serve to delineate a sterile area and prevent drafts, which carry dust, spores, bacteria, etc. Hoods should be sterilized by UV irradiation when not in use.

Sterile glassware, Sterile pipettes } Autoclaved

Sterile medium, Sterile serum } Millipore - filtered (0.45μ)

Ethanol (70%)

Clorox traps — To collect liquid waste

Tissue culture dishes — Sterile from manufacturer

Bulbs — For use with pipettes. Mouth-pipetting is forbidden.

Bunsen burner

Beakers for collection of used pipettes.

Procedure

Work only in designated tissue culture area.

Wipe surfaces with ethanol before starting.

Wipe bottles dry (e.g. if they have been standing in a water bath) before moving them into tissue culture hood.

Work cleanly, mopping spills immediately.
Discard used medium, time-expired and especially contaminated plates carefully. Otherwise, they serve as sources of contamination.

Label bottles and dishes with initials, cell type, date, etc.
When you open a new bottle of medium or serum, write the date on it and indicate how much has been removed. If you add anything to it, indicate this on the bottle. If there is only a small amount left in a bottle, discard it.

After you have finished, remove your belongings; put them away or discard properly. Wipe the working surfaces with ethanol.

GROWTH CONTROLS : EXPERIMENTS 1 and 2.

Transformed cells acquire a number of *in vitro* properties which differentiate them from their normal counterparts. We will determine three of these properties:

(a) saturation density, expressed as the number of cells per surface area that a given cell line can reach. This number is generally higher for transformed cells than for normal cells.

(b) serum dependence; i.e., the serum requirements for growth of a given cell line. These are lower for transformed cells.

(c) growth in suspension in semisolid medium (anchorage independence). Normal cells generally can not grow under these conditions, while transformed cells can.

Each team will find a different set of dishes in its incubator. Properties of each line are on your charts. The set is composed of a normal and a transformed line. On the first day we will set up experiments that will run the length of the course. This will entail trypsinizing and passaging cells on the first day, feeding and counting them through two weeks, and fixing and staining the remainder at two weeks for examination of colonies. We want, for all three assays, a measure of growth.

In the third week, we will pool our data to determine the generality of the differences between normal and transformed cells.

EXPERIMENT 1 : GROWTH CURVES
SERUM DEPENDENCE AND SATURATION DENSITY

DAY 0

Observe 1 dish each of normal and transformed cells. Note morphologies and densities. Any differences? Look for mitotic cells. Check the appearance of other teams' cells. Do you see any consistencies?

Trypsinize one 100mm dish of either the normal or transformed cell type, using 1ml of trypsin. Stop trypsinization with 9ml of DME + 10% CS. Go through the calculations necessary to set up the growth curves using the cell number of this dish (see below). With that information, you will trypsinize a sister dish and use it for setting up the cultures; this will minimize the time that the cells are exposed to trypsin.

Determine the number of cells in the dish by counting an aliquot in the hemocytometer or the Coulter counter (see accompanying instructions). Calculate how much of the cell suspension would be needed to make 10 dishes at 10^5 cells per dish. You will need 10^6 cells total. That amount will be taken and diluted to 50ml with DME + 10%CS (A).

You will then do two serial dilutions, 1:10, to give proper dilutions for 10^4 and 10^3 cells/dish. That is, dilute 5ml of solution (A) to 50ml with DME + 10%CS (B) and 5ml of solution (B) to 50ml (C).

Prepare 8 x 60mm dishes of each dilution by pipetting 5ml of solutions (A), (B), and (C) onto each of 8 labelled dishes.

Do you have enough cells from one dish? With calculations in hand, go ahead and trypsinize a sister dish(es) and set up cells as above.

Repeat for other cell type.

GROUP ASSIGNMENTS

I. A31

SV-T2

II. A31

B77/3T3

III. A31

SV813 $TG^{R}O^{R}$

IV. A31

Clone H

DAY 1

Look at yesterday's plating.

Change medium on 4 dishes of each dilution to DME + 0.5%serum.

Change medium on 4 dishes of each dilution to DME + 10%serum.

DAY	
1	- START.
2	
3	
4	- Change medium (with appropriate serum). Count
5	one dish at each density for each serum concentration.
6	
7	- Change medium. Count.
8	
9	
10	
11	- Change medium. Count.
12	
13	
14	- Count.

Graph growth curves on semi-log paper.

Calculate saturation density.

Calculate doubling time.

TRYPSINIZATION FOR COUNTING

Rinse dishes in PBS.

Use 0.5ml of trypsin. Stop trypsinization with 0.5ml of DME-10%CS. Mix well so that all the cells are dislodged from the plate and are evenly dispersed. Solution is then ready to be diluted for use in the Coulter counter or used directly in the hemocytometer (higher cell densities may require further dilution with medium). Note that different cell types adhere to plastic with different strengths. Which cells are dislodged most rapidly with trypsin? Which are removed most slowly? What determines strength of adhesion?

Determination of cell number in a suspension of cells

Introduction

There are a number of different experimental situations in which the experimenter might want to determine the number of cells in his sample. Such is the case when one wants to determine the activity of a certain enzyme per cell or the case in which one wants to follow the growth of a population of cells.

Growth of a population of cells can be followed directly by estimating the concentration of cells at various times or by measuring the protein, DNA, or RNA concentration in the culture. If the appropriate growth medium is used, the cell number, DNA, RNA and protein will all increase exponentially. As a result, the average composition of the cells will remain constant, and growth is said to be balanced.

Haemocytometer

This method consists of microscopically counting the number of cells in a very small volume of cell suspension. The haemocytometer is a microscope slide with grids on it to give squares of various size; it is constructed so that the space between the slide and a coverslip is 0.1 mm. A suspension of cells is applied to the slide by capillarity and cells within certain squares are counted microscopically. Knowing the size of the square and consequently the volume of suspension included, one can calculate the cell concentration of the suspension.