

Culturing A549 cells

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1.1	09/12/2016	3	1. additional equipment (water bath, paragraph 5.2) 2. concentration of L-glutamine (paragraph 5.4.1) 3. cell viability calculation formula adapted (paragraph 6.6)	Cordula Hirsch

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1 Introduction

The A549 cell line (ATCC: CCL-185) was established in 1972 by D.J. Giard, et al. (Giard et al., 1973). The cells originate from an explant culture of lung carcinomatous tissue from a 58-year-old Caucasian male. This adenocarcinomic human alveolar basal epithelial cell line has been used as a Type II pulmonary epithelial cell model e.g. for drug metabolism (Foster et al., 1998 and others). A549 cells grow as an adherent monolayer and can be used as a transfection host (ATCC.org).

2 Principle of the Method

A549 cells are grown in T75 cell culture flasks under standard growth conditions (see 6 Procedure). At about 70-90% confluency cells are detached from the flask by Trypsin/EDTA treatment, pelleted and reseeded at a concentration of 5×10^5 to 1×10^6 cells in 20 ml complete cell culture medium per T75 flask (equaling 2.5×10^4 cells/ml to 5×10^4 cells/ml, respectively).

3 Applicability and Limitations

Before starting any experiment A549 cells have to be passaged at least three times after thawing. Furthermore a maximum of 20 passages (after thawing) must not be exceeded.

4 Related Documents

Table 1: Documents needed to proceed according to this SOP and additional NM-related interference control protocols.

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5 Equipment and Reagents

5.1 Cell culture type

A549 cells are supplied by American Type Culture Collection (ATCC); ordering number: CCL-185.

5.2 Equipment

- Cell freezing container
- Centrifuge (for cell pelleting; able to run 15 ml as well as 50 ml tubes at 200 x g)
- Conical tubes (15 ml and 50 ml; polypropylene or polystyrene; e.g. from Falcon)
- Cryotubes
- Hemocytometer
- Laminar flow cabinet (biological hazard standard)
- Light microscope (for cell counting and cell observation)
- Microreaction tubes (1.5 ml; e.g. from Eppendorf)
- Pipettes (volumes of 10 µl up to 20 ml)
- T75 cell culture flasks

5.3 Reagents

- Dimethyl sulfoxide (DMSO) [CAS number: 67-68-5]
- Fetal Calf Serum (FCS)
- L-glutamine
- Neomycin¹⁾
- Penicillin¹⁾
- Phosphate buffered saline (PBS)
- Roswell Park Memorial Institute medium (RPMI-1640)
- Streptomycin¹⁾
- Trypan blue solution (0.4%) [CAS number: 72-57-1]
- Trypsin-EDTA (0.05%)

¹⁾ bought as a 100x concentrated mixture of Penicillin, Streptomycin and Neomycin (PSN) e.g. from Gibco.

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5.4 Reagent Preparation

5.4.1 Complete cell culture medium

Basic medium:

- RPMI-1640

supplemented with:

- 10% FCS
- 1x PSN, which results in final concentrations of:
 - 50 µg/ml Penicillin
 - 50 µg/ml Streptomycin
 - 100 µg/ml Neomycin
- 0.2 mM L-glutamine

5.4.2 Freezing medium

The stock solution of the freezing medium has to be freshly prepared and pre-cooled on ice for at least one hour before usage. It is prepared as a 2x concentrated stock and will finally be diluted with the cell suspension.

Stock:

- 60% complete cell culture medium
- 20% FCS
- 20% DMSO

Final concentration after mixing cell suspension (in complete cell culture medium) with freezing medium at a 1:1 ratio:

- 80% complete cell culture medium (containing cells)
- 10% FCS
- 10% DMSO

6 Procedure

6.1 General growth conditions

A549 cells are grown in T75 cell culture flasks in a total volume of 20 ml of complete cell culture medium. They are kept at 37°C, 5% CO₂ in humidified air in an incubator (hereafter referred to as “standard growth conditions”).

6.2 Cell thawing

Remove cryovial from the liquid nitrogen storage. Thaw cells in a 37°C-water bath until only a small frozen piece is visible. Decontaminate by spraying the vial with 70% Ethanol and transfer cryovial to a sterile hood. All following steps are performed under sterile conditions.

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- Transfer entire content of the cryovial into 20 ml pre-warmed (37°C) complete cell culture medium.
- Spin cells down for 5 min at 200 x g at room temperature (RT).
- Discard supernatant, resuspend cells in 20 ml pre-warmed complete cell culture medium and transfer cells into one T75 cell culture flask.
- Incubate at standard growth conditions over night (ON).
- Check confluency the next day. Cells have to be subcultured (see 6.4 below) starting from 70% confluency. Less confluent cultures receive 20 ml of fresh pre-warmed complete cell culture medium per T75 flask and are grown until reaching approximately 70% confluency.
- Culture cells as described below. Passage at least three times before usage in any experiment.

6.3 Cell freezing

To prepare a liquid nitrogen stock A549 cells are expanded until passage three after thawing. Ten T75 flasks are recommended to obtain enough cells for freezing. Grow A549 cells in these 10 T75 flasks until subconfluency (~70% confluency) and harvest and freeze them as follows:

Prearrangements:

- Prepare freezing medium (see 5.4.2) and cool down on ice at least one hour before cell harvesting.
- Make sure to have a freezing container on hand.

Cell harvesting:

- Detach cells from the 10 flasks using 2 ml Trypsin-EDTA per flask (as described in 6.4 “Subculturing”).
- Add 8 ml complete cell culture medium per flask and transfer cell suspension of 5 flasks into one single 50 ml conical tube (resulting in two 50 ml conical tubes filled with cell suspension).
- Centrifuge cells for 5 min at 200 x g at RT, resuspend both pellets in 10 ml complete cell culture medium each and merge the two times 10 ml into one tube.
- Count cells as described in 6.5 “Cell counting” and calculate the number of cryovials needed considering that each vial has to contain 1×10^6 cells.
- Label each cryovial with cell name, passage number, date and cell number.
- Adjust cell concentration to 2×10^6 cells/ml in complete cell culture medium. If the initially calculated concentration is higher the suspension is diluted with an appropriate amount of complete cell culture medium. If the initially calculated concentration is lower (e.g. 5×10^5 cells/ml) cells have to be centrifuged again and resuspended in the correctly calculated volume of complete cell culture medium.
- Mix cell suspension (2×10^6 cells/ml) at a 1:1 ratio with pre-cooled freezing medium (e.g. 10 ml cell suspension + 10 ml freezing medium). This results in a final cell concentration of 1×10^6 cells/ml.
- **Cells have to be kept at 0°C (e.g. on ice) from now on!**

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- Mix well by gently inverting the tube. A homogenous distribution of DMSO as well as cells is important to assure proper freezing and cell viability after thawing.
- Distribute 1 ml of the cell suspension in freezing medium into each cryovial.
- Place cryovials in a freezing container and place this into a freezer (-70°C to -80°C) for 24 h. This leads to a freezing rate of approximately 1°C/min.
- After 24 h place the frozen cryovials into liquid nitrogen for long term storage.

6.4 Subculturing

Routinely A549 cells are cultured in T75 flasks in 20 ml complete cell culture medium. They are subcultured every three to four days (twice a week) at approximately 70% confluency. This situation is achieved by seeding 5×10^5 cells per flask.

- Pre-warm complete cell culture medium and PBS at 37°C and thaw Trypsin-EDTA
- Discard cell culture medium.
- Wash cells twice with 20 ml of pre-warmed PBS (be careful not to flush away cells from the bottom of the flask).
- For detachment incubate cells with 2 ml Trypsin-EDTA in the incubator for 3-5 minutes.
- Tapping and shaking of the cell culture flask releases the cells from the bottom.
- Add 18 ml of complete cell culture medium, rinse the bottom of the cell culture flask 3-5 times and transfer the resulting 20 ml of cell suspension into a 50 ml conical tube.
- Centrifuge cells for 5 min at 200 x g at RT.
- Discard supernatant.
- Resuspend cell pellet in 10 ml of complete cell culture medium by 3-5 gentle pipetting steps. Avoid bubble formation and assure single cell suspension.
- Count cells as described in 6.5 “Cell counting”.
- Take 5×10^5 cells directly from the cell suspension (volume calculated as described in 6.5 “Cell counting”). Add up to 20 ml with complete cell culture medium and transfer into one T75 flask.

6.5 Cell counting

Note: Cell counting can be performed according to each laboratories regular practice. The Neubauer counting chamber is described in brief below.

- Harvest cells as described above (6.4 “Subculturing”).
- Take 10 µl of the cell suspension and pipette underneath the coverslip of the hemocytometer (see Figure 1, sample introduction point).

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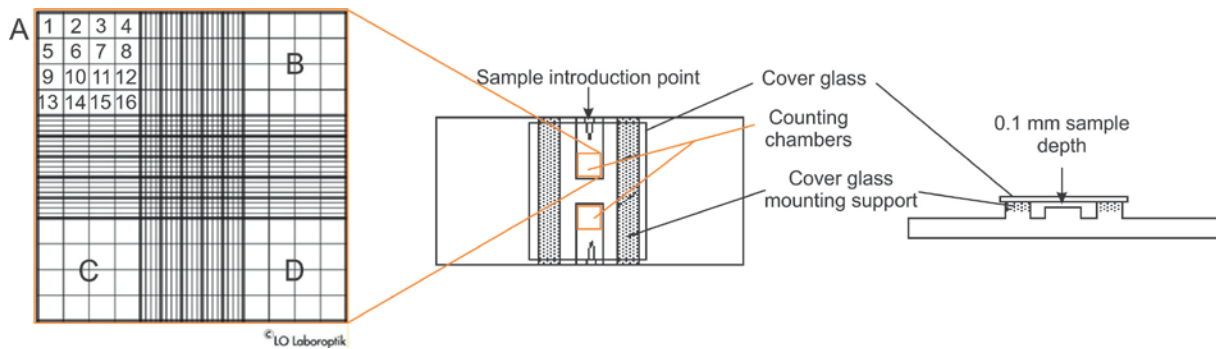


Figure 1: Schematic illustration of a hemocytometer.

Picture modified from: www.ruf.rice.edu/~bioslabs/methods/microscopy/cellcounting.html

- Count all cells in areas A, B, C and D (each area made up of 16 squares as detailed for area A in figure 1).
- Calculate the average cell count per area $[(A+B+C+D)/4]$.
- Calculate the number of cells per unit volume (cells/ml) using the following equation.

$$\text{Number of cells/ml} = \text{average cell count} \times 10^4 \times \text{dilution factor}^1$$

- Resuspend the required number of cells (taken directly from the cell suspension) in the required volume of complete cell culture medium and seed into appropriate cell culture flasks (e.g. T75 flasks for subculturing, multi-well plates for certain experiments (see respective SOPs)).

6.6 Determination of cell viability by Trypan blue

Trypan blue is a diazo dye and cell impermeable. Viable cells are thus not stained by Trypan blue while dead cells (with disintegrated plasma membranes) appear blue in the light microscope.

To determine cell viability by Trypan blue, harvest cells as described in 6.4 “Subculturing” and proceed for cell counting as follows:

- Take 30 μl of the cell suspension and add 30 μl of 0.4% Trypan blue solution in a microreaction tube (dilution factor = 2). Mix well by pipetting 3-5 times with a 100 μl pipette.
- Take 10 μl of the cell suspension and pipette underneath the coverslip of the hemocytometer (see figure 1, sample introduction point).
- Count the total number of cells in areas A, B, C and D (figure 1) as well as the number of dead cells (blue) in the same areas.
- Calculate the cell viability [%] using the following equation.

$$\text{Cell viability} = (\text{number of dead cells}/\text{total no of cells}) \times 100$$

- Calculate the number of viable cells per unit volume (cells/ml) using the equation in 6.5 “Cell counting”.

¹ Only necessary if you dilute your sample before counting.

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Note: Here you need to calculate a dilution factor of 2.

- Seed the required number of viable cells as described above.

7 Quality Control, Quality Assurance, Acceptance Criteria

A549 proved to be a robust cell line with an approximate population doubling time of 24 h. This can be calculated from total cell counts during each subculturing procedure and monitored over time as a quality criterion. If this value changes or fluctuates over time cell viability after trypsinization can be checked as an additional quality control parameter. A healthy culture should contain at least 80% viable cells.

8 Health and Safety Warnings, Cautions and Waste Treatment

All procedures have to be carried out under sterile conditions in a laminar flow cabinet (biological hazard standard). Only sterile equipment must be used in cell handling. Operators should wear laboratory coat and gloves (according to laboratory internal standards). During handling of cryovials in liquid nitrogen, a full-face mask and appropriate gloves must be worn.

Discard all materials used to handle cells (including remaining cells themselves) according to the appropriate procedure for special biological waste (i.e. by autoclaving).

9 Abbreviations

ATCC	American Type Culture Collection
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
FCS	fetal calf serum
g	constant of gravitation
PBS	phosphate buffered saline
RPMI	Roswell Park Memorial Institute medium
RT	room temperature
ON	overnight

10 References

ATCC.org:

http://www.lgcstandards-atcc.org/products/all/CCL-185.aspx?geo_country=ch#generalinformation

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Giard DJ, Aaronson SA, Todaro GJ, Arnstein P, Kersey JH, Dosik H, Parks WP; 1973: In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *J. Natl. Cancer Inst.* 51(5): 1417-1423

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