No 7:Cell cloning via limiting dilution

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There are few people who haven't experienced the feeling of loneliness if for example they have to spend time alone in a strange hotel room in an unknown city. This is exactly how Susi Pensa and Adi Herence feel during cloning.

"Just imagine", says Susi to Adi, "in cloning, a single cell is placed in the well of a cell culture plate and a clone is made from it". Susi trembles in horror. "But why should people do such horrible things to us", answers Adi angrily. "Well, there are a lot of scientific reasons", says Susi and continues, "and subsequently the one antibody-producing cell must be separated from all the others".

"Well, I would continue to divide, if only to stop being lonely", answers Adi. "No, I would go on strike against misuse and would refuse to divide", says Susi and continues, "because each different cell type behaves differently and this leads to different cloning efficiencies. For this reason, 50, 300 and 1,000 cells respectively are distributed over the 96 wells of a culture plate."

"Things are not as bad as you think Susi", says Adi. "If my room were to be nicely fitted out, with a carpet made of feeder cells or with a mini-bar containing that nice medium additive cocktail that they advertise or a conditioned medium as a gift from my friends, I would divide and enjoy life". "Okay, you have convinced me", says Susi. "If they give me macrophages or thymocytes like the antibody-producing hybrodoma cells as feeder, then I'll stop striking and agree to cloning".

Procedure:

- Successful cloning can only be carried out if the cells have been completely and individually separated before being inoculated. If the cells are aggregated, the following procedure must be carried out:
- The cells should be washed in phosphate buffer (2x10E6) and incubated for 1 to 5 minutes in 1 ml of a 0.025% trypsin solution in phosphate buffer at 37 °C; subsequently, serum (10% final concentration - or trypsin inhibitor in the case of serum-free cultures) should be added to stop the trypsin reaction.
- The partially digested cells are then mechanically separated into individual cells by drawing them carefully through a syringe fitted with a thin cannula (e.g. No. 17); air bubbles should be prevented at all costs.
- Once the cells have been completely separated (this can be checked by microscope - otherwise the procedure should be repeated), the number of cells should be determined and the cell density adjusted to 1x10E6/ml.
- Dilution x 100 should then be carried out by transferring precisely 200 ml of cell suspension to 20 ml of medium; the solution should be mixed well by inverting twice. The cell density will now be 10,000 cells in 1 ml or 10E6 cells per ml.5 ml (= 50 cells), 30 ml (= 300 cells) and 100 ml (= 1000 cells) of the cell suspension should be pipetted respectively into three tubes, each containing 10 ml of culture medium. These should be mixed by inverting twice.
- A 96-well cloning plate should be prepared and labelled with 50, 300 and 1,000 (cells). 100 µl of cell culture medium per well should be available, possibly with additives (see below) and pre-warmed in the incubator.
- For inoculation, the cell suspension should then be inverted and

transferred to a conical medium vessel. Using an 8-channel multi-Electrapette (INTEGRA Biosciences), 100 µl should be transferred to the wells of the plate. N.B.: Avoid the formation of bubbles at all costs. If the inoculation procedure is started with the smallest cell density, all amounts can be taken from the same medium vessel and the same syringe tips used. This helps to save material and money.

- In order to reduce evaporation, the plates should be packed in commercially available foil or bags and placed in an incubator.
- After 1 to 2 hours, the cells will have sedimented and the plates can now be investigated under the microscope and the presence of individual cells can be confirmed. This procedure, however, is somewhat tedious and time consuming.
- A more simple procedure is to wait for one week and then to investigate the plates for the formation of cell colonies. Those wells with visible groups of cells should not be taken into account due to insufficient clonability. Those wells containing precisely one group of cells (clones) should be marked and used for further observation. This can best be done by observing the plate from below against a light source.
- Medium should only be changed if strong cell growth is observed or if the medium itself becomes yellowish in colour, i.e. when the pH is lower. N.B.: A small amount of the old medium should always be left as the cells will have conditioned themselves even when growth factors have been added:
- In cloning hybridoma cells, the supernatant can be removed in order to test for antibodies; this can be done as soon as cell colonies become visible.
- The probability of successful cloning is higher if fewer cells are inoculated. For this reason, cloning should be performed preferentially using plates with lower cell densities (labelled with 50). Statistically, one cell can be found in every second well (50 cells in 96 wells).
- Cells should not be transferred too early in order to prevent dilution of the cells. Cells should be transferred in sequence from the 96-well plate into a 48-well, 24-well, 12-well etc. until it is possible to continue cultivation in bottles.
- In order to guarantee clonability, at least 2 cloning cycles should be carried. out

Additives:

Cloning efficiency can be increased by adding growth factors or feeding cells:

1. Commercially available medium additives for hybridoma cultures

contain growth factors enabling blood cells to survive and grow.

2. Self-conditioned medium: The medium in which the cell line to be cloned has grown is added to 10 to 15% fresh culture medium. N.B. The medium must be passed through a 0.2 µm sterile filter (MediaKap, INTEGRA Biosciences) in order to be quite certain that no cells are transferred.

3. Feeder cells: Cells releasing the factors necessary for growth of the cells to be cloned are cultivated with these cells. In the case of hybridoma cells, thrombocytes (10^6 per well) obtained from mice or macrophages from bone narrow or abdominal cavity (10^4 per well) are normally used.

These cells have a limited division activity only. If cell lines are used as feeders, cell division must be prevented by radiation (5000 rad with cobalt 60 or X-ray) or by treatment with mitomycin C (0.25 mg/ml for 10^5 cells

overnight).

Source:

James W. Goding: Monoclonal Antibodies: Principles and Practice, Academic Presstice, Academic Press.

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