Cell Culture Protocol

Creative Diagnostics

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01 Introduction to the Cell Culture Protocol

The following are general guidelines for cell line culture. All cell culture operations should be performed in a microbiological safety cabinet using aseptic techniques to ensure a sterile environment.

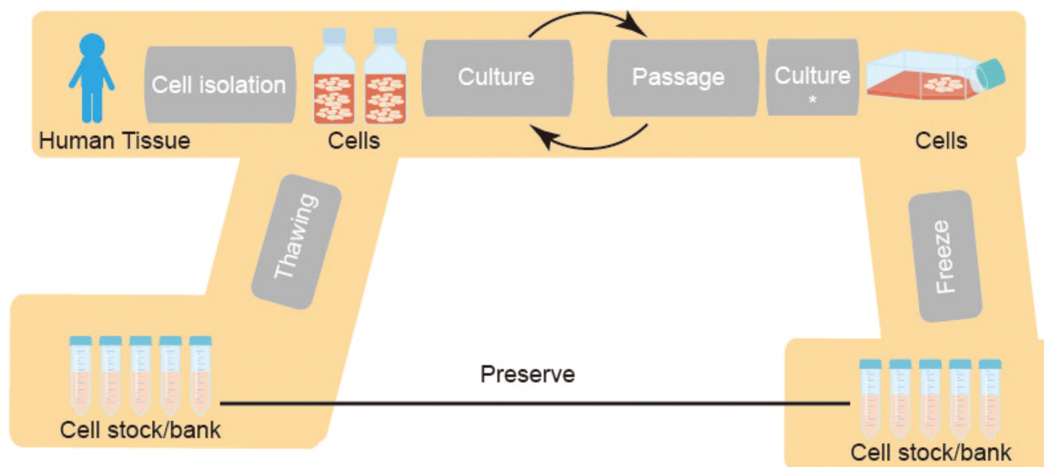

Figure 1. Cell culture procedure.

02 Aseptic Environment

- Hood regulations
  a) Please close the hood sash to the proper position to maintain proper laminar airflow.
  b) Avoid clutter.

Figure 2. Fume hood.

- Autoclaving
  a) Pipette tips (or can be purchased pre-autoclaved, DNAse/RNAse free)
  b) Glass 9″ Pasteur pipettes
  c) 70% ethanol. Be sure to coat all surfaces.

Tips: All media, supplements, and reagents must be kept sterile to prevent microbial growth in cell cultures. For some reagents and supplements that are not processed sterile, filter sterilization steps should be performed.

03 Cell Growth Medium

Before starting work, review the information provided with your cell line to clarify the type of media required, supplements, and recommended practices.

Typically, most cell lines can be cultured in DMEM or RPMI containing 10% fetal bovine serum (FBS), 2 mM glutamine, and antibiotics if necessary. Be sure to check the specific requirements of the cell line you are using for appropriate media and culture supplements.

Figure 3. Cell culture medium.

Tips: Always confirm the sterility of media and supplements before starting a culture. To ensure a sterile environment, try to purchase aseptically prepared reagents and use them only under sterile conditions in a culture hood.

04 Create the Right Growth Environment

Most cell lines are usually grown in culture flasks without the use of special substrates. However, for some cells, especially primary cells, it may be necessary to grow on specific matrices, such as collagen, to facilitate cell attachment, differentiation or growth. It is recommended that you consult the relevant literature for more information on the cell type you are culturing.

Here are examples of endothelial and epithelial cells:

For human cells, a 1% gelatin coating can be added to the flask. Alternatively, for other cell types (such as BAEC), the inside of the flask can be coated with 1% fibronectin.

Here are the steps:

- Prepare 10 mL of 1% gelatin or 1% fibronectin coating solution by dilution and filtration. This is sufficient to coat about 5 flasks.
- Add the coating solution to the flask and shake gently to distribute the solution evenly on the bottom of the flask. Place the flask in the incubator and incubate for 15-30 minutes.
- Before seeding the cells, completely remove the coating solution with a suction device.

Tips: Please note that a sterile environment must be maintained during the procedure to ensure the normal cell growth.

05 Cell Status Check

Daily inspection of cells under a microscope is recommended to ensure their health and expected growth characteristics. Adherent cells should adhere primarily to the bottom of the flask, appear round, plump, or elongated, and appear to refract light around the cell membrane. Suspended cells should have a rounded, plump appearance and show refracted light around the cell membrane. Some suspended cells may naturally aggregate. The color of the medium should be pinkish orange.

Discard cells if:

- Extensive shedding (failure of attachment) and/or shriveled, grainy, dark features.
- In a quiescent state, *i.e.,* no visible signs of growth.

Tips: Be sure to follow these instructions to ensure a healthy and proper cell culture.

06 Cell Subculture

Subculture should always be performed under sterile conditions using aseptic technique.

1. Subculture ratios can be used to prepare for experiments on specific dates, or to maintain cell cultures continuously for future use or as a backup. Suspension cell lines often have recommended subculture seeding densities, and be sure to check the guidelines for the cell line you are using. Some slow growing cells may not adapt to high subculture ratios. Similarly, some rapidly growing cells may require higher subculture ratios to avoid overgrowth. It should be noted that the subculture ratio of most cells should not exceed 1:10 to avoid low seeding density and difficult cell survival.

   As a general guideline, when collecting cells from cell flasks:

- Using a 1:2 subculture ratio, cells are ready for experiments within 1 to 2 days of reaching 70-80% confluence.
- Using a subculture ratio of 1:5, cells are ready for experiments within 2 to 4 days of reaching 70-80% confluence.
- Using a subculture ratio of 1:10, cells are ready for subculture or aliquots within 4 to 6 days when cells reach 70-80% confluence.

2. If the cells are less than 70-80% confluent, but you want to subculture the cells at a specific time (such as the weekend before Friday), you should choose a lower subculture ratio to ensure that the cells are subcultured at a high enough density to survive. For example, a subculture ratio of 1:2 or 1:5 can be used.

07 Cells Splitting

1. When the cells reach approximately 80% of the bottom of the flask (approximately 80% of the bottom of the flask is covered by a monolayer of cells), it should be ensured that they are still in the logarithmic growth phase and need to be subcultured. (Do not overcultivate the cells as this will cause them to die and be difficult to recover).
2. When subculturing, first heat the fresh medium to 37°C in a water bath or incubator for at least 30 minutes. Then choose one of the appropriate steps below to follow.
3. During the passaging, please be sure to label the culture flask with the cell line name, passage number, subculture ratio, date, operator's initials, and cell bottle number. Put the labeled culture bottle directly into the 37°C CO2 incubator. Record the subculture information in detail on the Culture Record Table. There should be separate record sheets for the recovery and usage of each bottle.

Figure 4. Carbon dioxide constant temperature incubator.

08 For the Growth of Loosely Adhesive Cell Lines, Cells Need to Be Scraped for Subculturing

1. When ready, carefully pour the media from the desired cell flask into a waste container with approximately 100 ml of 10% sodium hypochlorite, being careful to avoid drips that pose any risk of contamination.
2. Carefully pour the same volume of pre-warmed fresh medium into the flask and replace it immediately.

3. Using a cell scraper, gently scrape the cells from the bottom of the flask into the medium. Check the bottom of the flask to ensure all cells have detached before proceeding.

Figure 5. Use the cell scraper to scrape the cells.

4. Using a pipette containing serum, extract the desired split ratio of the cell suspension.

   e.g., for 1:2 split from 100 ml, take 50 ml into a new flask

   1:5 split from 100 ml, take 20 ml into a new flask

   1:10 split from 100 ml, take 10 ml into a new flask

5. Fill a new flask to the required volume (taking into account the split ratio) with pre-warmed fresh medium.

   e.g., 25 cm^2 flask approx. 5-10 ml

   75 cm^2 flask approx. 10-30 ml

   175 cm^2 flask approx. 40-150 ml

09 The Adhesive Cell Lines of The Subculturing Culture Require Insulin-Protease Treatment

Please note: Trypsinization is not necessary for all cells and may be toxic for some cells. In addition, it may lead to transient internalization of certain membrane proteins. Take these factors into consideration when designing your experiments. In these cases, other methods, such as gentle cell scraping or the use of very mild cleansers, can often be used instead.

1. When ready, carefully pour the medium from the cell flask into a waste container with approximately 100 ml of 10% sodium hypochlorite, being careful to avoid drips that pose any risk of contamination.

2. Using aseptic technique, add or pipette a sufficient volume of sterile PBS to the flask to wash the cells and remove any FBS from the residual media. Rinse the cells by gently tilting the flask several times, then carefully decant or pipette the PBS back into a waste container.

Repeat 1 or 2 times if necessary (as some cell lines take longer to trypsinize and thus require more washes to remove residual FBS to aid the action of trypsin).

3. Using a pipette, add enough liquid containing trypsin and EDTA to cover the cells at the bottom of the flask.

   e.g., 25 cm^2 flask approx. 1 ml

   75 cm^2 flask approx. 5 ml

   175 cm^2 flask approx. 10 ml

4. Gently swirl the flask to ensure that the trypsin contacts all cells. Then place the flask in a 37°C incubator. Different cell lines require different trypsinization times, and this needs to be checked every few minutes to avoid damage to the cells from over digestion.

5. After the cells have detached (it may be necessary to tap the flask a few times), add some culture medium (fetal bovine serum in it will inactivate trypsin) to the flask.

6. Using the cell suspension, transfer an appropriate amount of the cell suspension to a new flask according to the desired split ratio. These flasks were then filled with medium to the desired volume.

   e.g., 25 cm^2 flask approx. 5-10 ml

   75 cm^2 flask approx. 10-30 ml

   175 cm^2 flask approx. 40-150 ml

Allow the cells to recover and settle overnight. The medium is then changed to remove any residual trypsin.

10 Subculturing of Suspension Cells

1. Consult your cell line guide for recommended split ratios or subculture cell densities.
2. Use a pipette to remove the required amount of cell suspension from the culture flask and transfer it to a new culture flask.

- For example, for a 1:2 split, remove 50 ml from 100 ml of cell suspension.
- For a 1:5 split, remove 20 ml from 100 ml of cell suspension.

3. Add the required amount of pre-warmed cell culture medium to a new flask.

- For example, for a 1:2 split, add 50 ml of fresh medium to 50 ml of cell suspension.
- For a 1:5 split, add 80 ml of fresh medium to 20 ml of cell suspension.

11 Changing the Culture Medium

1. If cells have grown well for a few days but have not reached confluence (e.g., 1:10 split), replace the medium to replenish nutrients and maintain proper pH. If adherent cell lines begin to float or if the medium starts to change color from pink to orange, the medium must be changed as soon as possible to prevent them from changing.

2. When the medium needs to be replaced, please place the fresh medium in a water bath or incubator and heat it at 37°C for at least 30 minutes. Carefully pour the medium in the flask into a waste container with some disinfectant attached. Subsequently, quickly replace the medium with 100 ml of pre-warmed fresh medium and return flask to the 37°C CO_2 incubator.

12 Passage Number Record

The number of passages refers to the number of subcultures a cell has undergone. It is necessary to record the number of passages and ensure that it is not too high. This is to avoid using cells that have undergone genetic drift and other variations.

References

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