Laboratory 7:
Mammalian Cell Culture

Objective

- To learn basic cell culture techniques
- To transfect mammalian cells
- To determine transfection efficiency

1. Cell culture

Most mammalian cells divide a limited number of times, usually less than 30 doublings. In contrast, immortalised cells have the ability to divide continuously in cell culture. The cell line HEK293T is a standard, multi-purpose cell line used for example in protein production, vaccine development or cancer research. It was derived from human embryonic kidney cell cultures (hence HEK) followed by immortalization via transfection with viral DNA.

Some cell lines can grow in suspension, while adherent cell lines grow as a single cell layer (monolayer) on specially treated plastic surface. In both cases it is necessary to subculture (passaging) them at regular intervals to keep cells healthy and actively growing. For passaging, adherent cells must be detached from the culture dish. This can be achieved with the proteolytic enzyme trypsin as it digests extracellular matrix (ECM) molecules. The solution contains EDTA (Ethylenediaminetetraacetic acid) a chelator of divalent cations (Ca^{++} and Mg^{++}). It serves two purposes: to 1) enhance trypsin activity and 2) to disrupt cell adhesion mediated by integrins,a family of transmembrane proteins involved in cell-extracellular matrix interactions.

After the cells have been dissociated into a suspension of single cells, they are diluted and transferred into fresh culture vessels. Cells will reattach and begin to grow and divide, and after a period of incubation again reach near confluency (there is still some growing space available on the culture dish). Cell cultures grow best if they are subcultured before confluency during log phase of growth. HEK293T cells have a doubling time of 16-18 h and confluent cultures need to be split 1:5 every second day. Since cells are very sensitive to cell density they should not be diluted too much during passaging.

The culture medium contains phenol red as a pH indicator. An appropriate medium has a pH between 7.0 and 7.2 with a color between red-orange and red. The colour change to yellow indicates a low acidic pH and can be caused by contaminants or high cell density. A shift to purple indicates a basic pH. Tissue cell culture dishes are incubated at 37°C in a 5% CO_2 atmosphere (to regulate the pH in the buffer) and a relative humidity of 90-95% (to limit evaporation).

Task

You will subculture (split) confluent HEK293T cells grown in a T25 culture flask and transfer a given number of cells into a new flask.

Safety

When working with human cells appropriate biosafety measures must be applied. Put all waste material that was in contact with cells into a yellow biohazard bag. The liquid waste will be inactivated with bleach. After you are done, wipe the working area with 70% ethanol. Close the hood and switch UV light on.

Materials

- HEK293T cells in T25 flask (about 80% confluent)
- CO_2 incubator (5% CO_2, 37°C, 95% humidity)
- Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% Bovine Serum; Invitrogen
- PBS
- Trypsin + EDTA; Invitrogen
- Disposable cell counting chamber (hemocytometer)
- Tally counter
- Inverted light microscope
- 0.4% Trypan Blue solution; Sigma
- 15 ml tube

All solutions and equipment coming into contact with living cells must be sterile and aseptic techniques should be used.

Procedure

Cell splitting (in biosafety cabinet)

1. View cultures on an inverted microscope to check cell density (confluence) and confirm the absence of bacterial and fungal contaminants. Note the colour of the medium.
2. Remove the culture medium covering the cells using a sterile pipette from the side of the flask. Then wash with 2 ml PBS, remove PBS. Be careful not to scrape the bottom of the flask, where the cells are attached.
3. Add 1 ml Trypsin (Trypsin+EDTA) to the cell monolayer and rotate the flask to cover the entire cell surface. Put the flask in the incubator (2-3 minutes). To observe the process you may watch the cells under an inverted microscope until the cell layer is detached and floating. Tap the sides of the flask to release adherent cells.
4. Resuspend detached cell clumps in 2.5 ml DMEM supplemented with serum to inhibit trypsin activity. Pipette the cell suspension up and down 5-10 times to separate aggregated cells (without air bubbles!).
5. Transfer one fifth of the volume into a new T25 culture flask (= splitting 1:5)
6. Add 4 ml culture medium and swirl the flask gently to evenly distribute cells in medium. Put your new culture flask into a 37°C CO2 incubator. Transfer the rest of the cells to a 15 ml tube. Write down the remaining volume.

Cell counting (on the bench)

You will count the trypsinized cells (in 15 ml tube) and assess their viability using the dye exclusion method. Living cells exclude the dye Trypan Blue, whereas dead cells will take up the blue dye.

1. Combine 10 µl of cell suspension (vortex the cells before pipetting, they settle quickly to the bottom) and 10 µl Trypan Blue dye in a 1.5 ml tube (1:2 dilution).
2. Immediately transfer 10 µl of the sample to a counting chamber and count the number of blue (dead) and total cells under an inverted light microscope (20X magnification). There are grid markings on the hemocytometer. Count cells in the four corner quadrants and calculate the average number of cells and of viable cells per quadrant.
3. Determine the percentage of viable versus dead cells. Cell viability should be at least 95% for healthy log-phase cultures.
4. Determine the number of cells per ml with the formula:
   average number of cells per quadrant x dilution factor x 10'000 = Number of cells/ml.
5. Calculate the total number of cells in the initial T25 flask by multiplying the number of cells/ml by the total volume (ml) of the cell suspension. Do you have enough cells to perform a transfection experiment in a 6-well plate (seed $5x10^5$ cells/well the day before the transfection)?

Questions

Q1: You have a confluent T25 flask and prepare cells for microscopy. You trypsinized cells with 1 ml and diluted next with 4 ml medium. By counting viable cells in four quadrants you obtain the following results: 56, 53, 65, 50 cells. Next you want to transfer 1.12×10^5 cells into a 60 mm cell culture dish. Which volume of cell suspension do you transfer to the new dish? Remember to take into account the dilution factor after addition of Trypan blue dye.

Q2: After trypsinization of a T75 flask you obtain 5 ml cell suspension with 1.6×10^6 cells/ ml. For storing the cells in liquid nitrogen you will need $1x10^6$ cells/ tube. How many tubes can you prepare with this amount of cells?

Q3: You have a cell line which doubles every 32 hours under ideal culture conditions. If you plated 30 ml of such a cell culture at a concentration of 2.5×10^6 cells/ml on Friday at 5 p.m., calculate the concentration of cells expected on Monday at 9 a.m.

2. Transient expression in mammalian cells using DNA transfection

The first step of recombinant gene expression in eukaryotic cells is the transfer of nucleic acids (DNA or RNA) into a population of cells, called **transfection**. Transfection can be transient or stable. **Transient** transfection is useful for initial testing of constructs in small scale and short-term expression (hours-days) as it is simple, produces results quickly and applicable to a multitude of cell lines. **Stable** transfection is preferable for large scale expression (research or production) or long-term experiments. It is based on DNA integration into the genome which is more labour intensive (weeks) as it requires selection + screening of clones.

Different transfection methods exist:

- **Chemical**: based on cationic lipid or calcium phosphate precipitation where the DNA is taken up via endocytosis
- **Electroporation**: an electric pulse creates holes in the membrane lipid bilayer through which nucleic acids can enter
- **Viral** infection: for example by lentivirus or baculovirus; used for clinical applications and difficult to transfect cells
- **Mechanical**: microinjection or bombardment of cells with DNA coated gold particles

We will use the method of **cationic lipid-mediated transfection**, which yields high efficiencies of DNA delivery in a variety of eukaryotic cells. Specially designed lipids, such as Lipofectamine, have a positively charged head group that forms complexes (liposomes) with the negatively charged nucleic acid. The positive surface charge of the liposomes facilitates fusion with the negatively charged cell membrane and the DNA/lipid complex enters the cell through endocytosis. Once inside the cell, the DNA must enter the nucleus for gene expression, while RNA can be expressed inside the cytoplasm.

We will transfect HEK 293T cells that express SV40 Large T-antigen allowing for episomal replication of transfected plasmids containing the SV40 origin of replication, such as pcDNA6/myc-His. This expression system leads to the amplification of transfected plasmids and maintenance without selection pressure through multiple generations and thus extended temporal expression.

A plasmid encoding the green fluorescent protein (GFP) will be co-transfected with the *Amy2* carrying plasmid to determine the transfection efficiency (percentage of cells transfected in the population). The gene coding for GFP was isolated from the bioluminescent jellyfish *Aequorea victoria*. Upon excitation by UV or blue light it emits green fluorescence, which can be quantified (excitation max: 488 nm; emission max: 509 nm). GFP is an exceptionally stable protein and is resistant to most proteases and chemical reagents.

Task

You will introduce *Amy2* expression plasmid in a population of HEK293T cells in order to transiently produce recombinant protein. The cells do not express Amy2 endogenously. For affinity purification of α-amylase we cloned Amy2 into pcDNA6/myc-His expression vector with the poly-Histidine tag. Co-transfection with GFP-carrying plasmid allows determination of transfection efficiency. During the next lab session you will purify His-tagged α-amylase.

Materials

- HEK293T cells in 6-well plate (90-95% confluent)
- Plasmids:
  - pAmy2-His _____ µg/µl
  - pGFP _____ µg/µl
- Lipofectamine^{TM}2000 (Invitrogen) (keep on ice!)
- Opti-MEM Medium (Invitrogen)
- CO2 incubator (5% CO_2, 37°C, 95% humidity)

Procedure

One day before transfection, $5x10^5$ HEK293T cells were plated in a 6 well dish ($5x10^5$ cells/well), in 2 ml DMEM supplemented with 10% Bovine Serum. There are various sizes of cell culture flasks and depending on the surface area you need to plate a defined number of cells in a given volume of culture medium.

At the time of transfection, the cells should be actively dividing and 70-90% confluent (confluency= percentage of surface area of flask covered by adherent cells). If cell density is too low/ high, transfection efficiency will be significantly decreased.

First mix the solutions on an alcohol-cleaned bench (wear gloves). Second, add the DNA/Lipofectamine complexes to your cell cultures under the laminar flow hood (normally all steps are carried out in a laminar flow hood).

Each group will prepare two transfection mixes:

1. Co-transfection with pAmy2-His AND pGFP (positive control)
2. Mock transfection without DNA (negative control – without DNA)

With these, you will transfect cells for two experiments: A) microscopy to evaluate transfection efficiency B) cell lysates for Western blot. Thus, each group will do **two transfections in duplicates** (Volumes below are calculated for two wells co-transfection and two wells for mock transfection = 4 wells total).

For each transfection, prepare two mixes as follows:

- **Mix 1 :** Dilute **4 µg of each** plasmid in 500 µl Opti-MEM (medium without serum) and prepare one negative control without DNA, as indicated in the table below (calculate the amounts of DNA needed). Mix gently.

Amounts in µl	Sample 1	Sample 2 (Mock)
Opti-MEM	500	500
pAmy2-His		-
pGFP		-

- **Mix 2 :** Mix Lipofectamine gently before use, then dilute 20 µl in 500 µl Opti-MEM. **Incubate 5 minutes at room temperature**.

Amounts in µl	Sample 1	Sample 2 (Mock)
Opti-MEM	500	500
Lipofectamine	20	20

- 1. After the 5 minutes incubation, combine the mix 1 (DNA) and mix 2 (Lipofectamine). You will end up with a total volume of about 1 ml. Mix gently and incubate for **20 minutes at room temperature**. (Complexes are stable for 6 hours at room temperature)
- 2. **Add half of each transfection mix** (500 µl) to the cells on the 6-well plate under the hood. Mix gently by rocking the plate back and forth.

- 3. Incubate the cells at 37°C in a CO_2 incubator for 18-48 hours prior to testing for transgene expression.

You will next determine transfection efficiency of samples that were prepared by the assistants. In case your group is waiting for a free cell culture hood, start with part 2 and 3.

3. Determination of transfection efficiency

Transfection efficiency corresponds to the % of transfected cells. In our case the transfected cells are GFP positive, while untransfected cells are GFP negative. Since we did cotransfection, GFP positive cells also express the recombinant AMY2-HIS protein. Quantitative methods to determine transfection efficiency include for example microscopy followed by image analysis or Fluorescence-activated cell sorting (FACS).

To illustrate the principle, you will determine the transfection efficiency using the fluorescence microscope by counting GFP positive cells (more or less intense depending on expression level). 24 hours after transfection HEK293T cells were fixed by adding paraformaldehyde (PFA) to permeabilize and immobilize the cells. Next, the nucleus of all cells was stained with DAPI (4', 6-Diamidin-2'-phenylindol-dihydrochlorid), a blue-fluorescent nuclear stain. Representative microscopy images are posted on Moodle.

Task

Determine transfection efficiency using two methods. Since samples stem from different experiments results cannot be compared here.

1. Estimate the amount of GFP positive (green fluorescent) versus total cells (DAPI-stained blue fluorescent) using a **fluorescence microscope**. Excitation and emission maxima are respectively 488 nm and 509 nm for GFP and 358 nm and 461 nm for DAPI. Count the number of total cells (DAPI-positive) and GFP-positive cells in at least two distinct fields of view and calculate the transfection efficiency.
Transfection efficiency manual counting: _____ %

2. Next, use the software FIJI (FIJI Is Just ImageJ) and fluorescence microscopy images of DAPI and GFP stained cells (posted on Moodle) to determine the % of GFP positive cells, (https://imagej.net/software/fiji/downloads). See FIJI guidelines on Moodle (download zip folder, unzip, use .tiff files for analysis). For quantitative analysis, parameters need to be optimised according to cell type, density, shape, signal, background etc. (here it is not required).

Transfection efficiency FIJI: _____ %