Experiments Laboratory 7

1) Subculturing (splitting) Human embryonic kidney 293 cells (HEK293T)
2) Transient transfection of HEK293T cells with recombinant p-Amy-His plasmid and a reporter plasmid encoding Green Fluorescent Protein (GFP).
3) Determination of transfection efficiency

Working with cells in culture

- **WORK STERILE!**
- DEMO BY ASSISTANTS
- Wear gloves, lab coat
- Remember that your lab coat/gloves might carry germs!
- CHANGE pipette/tip after each manipulation or if you touch anything
- KEEP TUBES AND PLATES CLOSED AS MUCH AS POSSIBLE!!!
- Manipulate the cells in the middle of the hood (laminar flow).
- Wash with EtOH 70% hood or pipets if contaminated by cells
- When you are done flush aspiration pump with EtOH 70%, close the hood and switch UV light on.

Mammalian cells

- Model system to study tissue/organ development, diseases (e.g. cancer) and gene function
- Biotechnology: expression and production of recombinant proteins, monoclonal antibodies.

Cell types

Tissue types in the human body:

- **Epithelial tissue** (covers the organs; function in secretion, selective absorption, protection, transport)
- **Connective tissue** (structural support of the body; bone, cartilage, tendons, ligaments; stores nutriments)
- **Nervous tissue** (relays information between the brain and the muscle in the form of chemical/electrical impulses).
- **Muscle tissue** (integrated with skeleton and heart; contracts upon impulse).

Primary Cells vs Cell Lines

Primary cells

- Cells freshly derived from an organism
- Usually undergo a limited number of cell divisions
- Are very delicate to handle
- Senescence: cells stop dividing (example: shortening of telomeres)

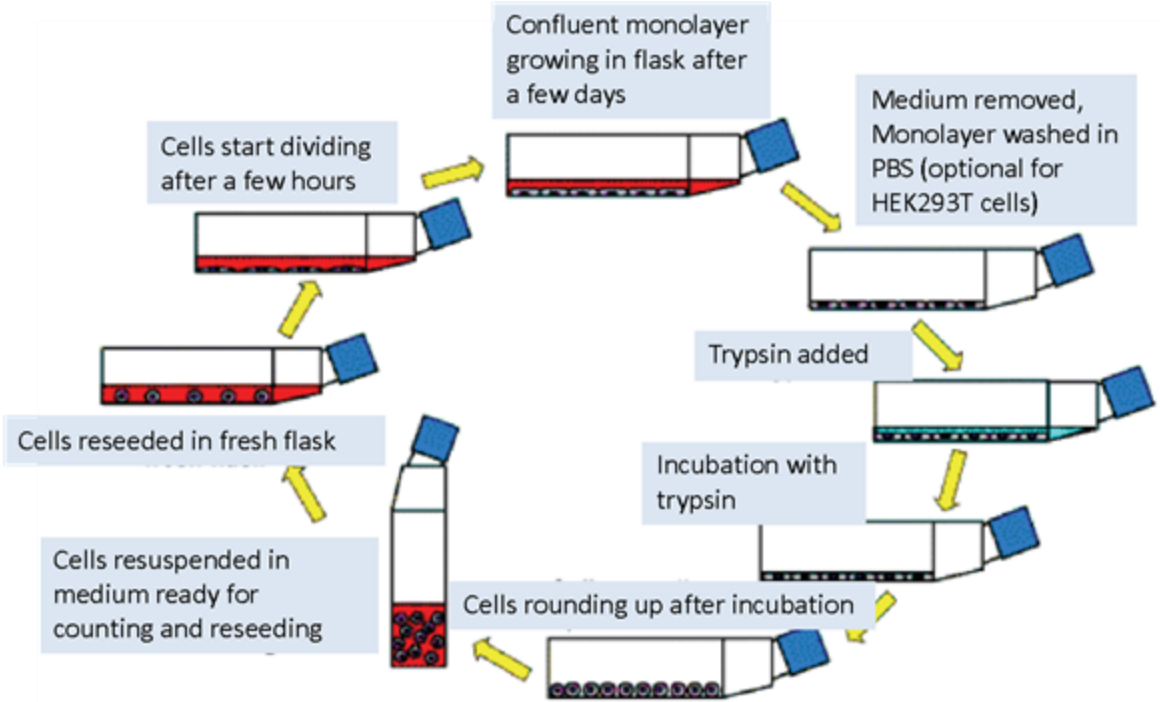
Cell lines

- Derived from embryos, tumors or transformed cells
- Undergo many cell divisions
- Generally easy to handle
- Cells continue to grow due to random mutations, expression of telomerase, viral infection

Cell line HEK 293T

- Immortalized cell line (integration of viral DNA into host chromosome).
- HEK293**T** cells express the the simian virus 40 (SV40) large **T** antigen, allowing episomal replication of transfected plasmids containing the SV40 origin of replication (e.g. pcDNA6/myc-His).
  Amplification of transfected plasmids results in high levels of expression of the desired gene products.
- Doubling time: 16-20 hours.

1) Subculturing adherent cells (also known as passaging or splitting)

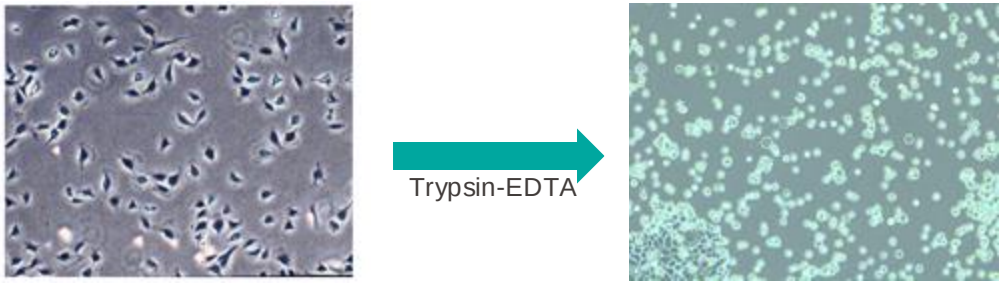


Trypsin–EDTA solution is used to detach cells from tissue culture dishes and to dissociate cells from one another (single cell suspension).

See video on Moodle

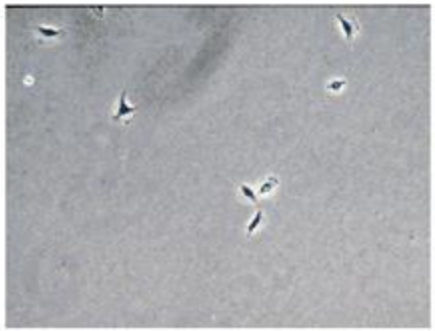
Incubation with Trypsin-EDTA

- **Trypsin** is a proteolytic enzyme that cleaves proteins that link the cells with their extracellular matrix (ECM), thereby releasing cells from the culture dish
- **EDTA** chelates bivalent cations thus inhibiting cell-cell/ECM communication that is dependent on calcium
- Detached cells are round shaped and free floating in the solution. As soon as cells have detached, add culture medium to the dish to inactivate the trypsin

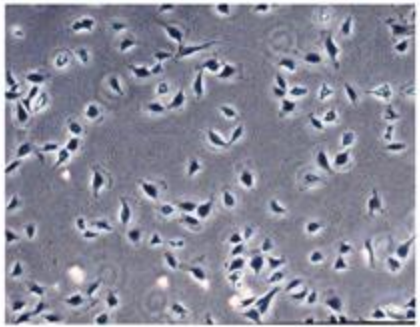


Replating cells

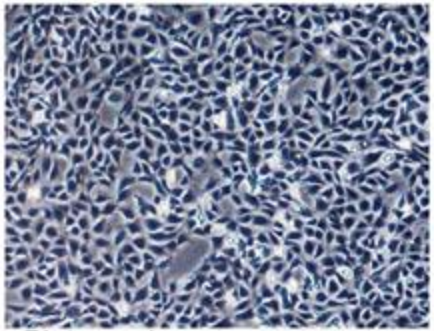
- Upon replating, cells attach to the dish and start to grow and divide
- Cells are grown until they are confluent and ready for an experiment or next subculture



Lag phase



Log phase



Plateau phase

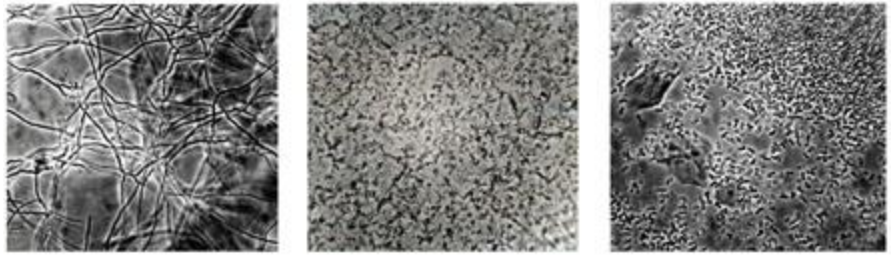
Basic constituents of cell culture medium

- **Organic salts**
  - Osmotic balance
  - Regulation of membrane potential
  - Cell attachment
  - Enzyme cofactors
  - Stabilization of pH
- **Carbohydrates**
  - Glucose
- **Amino Acids**
  - L-Glutamine (essential amino acid)
- **Fetal Calf Serum (FCS; supplement)**
  - Vitamins
  - Proteins (bovine serum albumin (BSA) is a major component of FCS)
  - Fatty acids lipids
  - Growth factors

Optional constituents

- **Antibiotics**
  - To prevent bacterial contamination
  - Despite the use of antibiotics contaminations are possible (e.g. antibiotic resistant bacteria, mycoplasma, fungi, yeast, viruses, other cells).
- **Phenol Red**
  - pH indicator
  - Color of medium changes from red to yellow if cells are overconfluent (consumption of nutrients) or contaminated with microorganism.

Acidic Neutral Basic

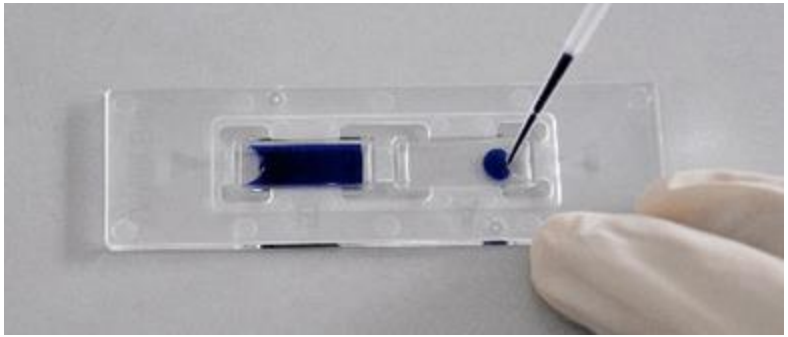


Fungi Bacteria Yeast

Tissue Culture
Dishes Flasks and Plates

Useful numbers for cell culture
https://www.thermofisher.com/ch/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/cell-culture-useful-numbers.html

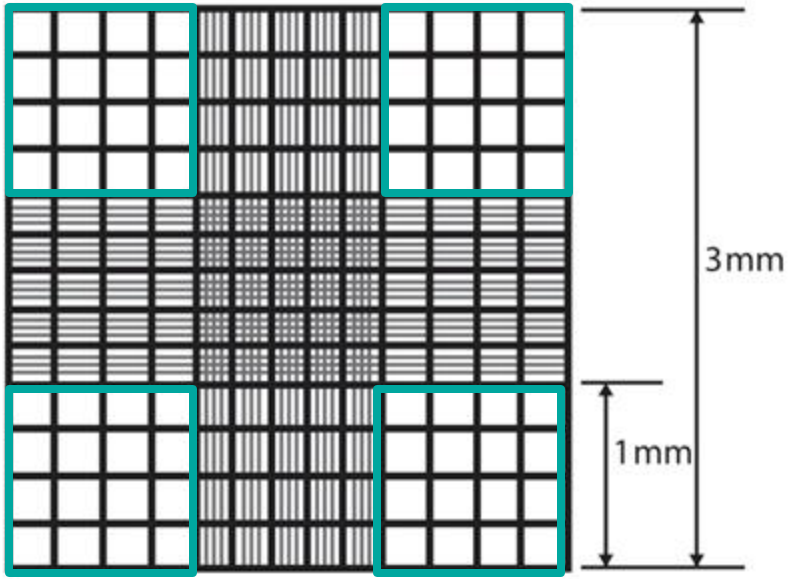
Counting Chamber



Cell concentration can be assessed by putting the cell suspension in a counting chamber (hemocytomer) with known volume.

Cell viability is assessed by mixing the cells with **Trypan blue** (vital stain; exclusion stain).

Counting Chamber Grid



The counting chamber grid contains 9 large squares (3 x 3) including 4 corner squares (divided into 16 small squares).

Count cells in four corner squares (quadrant) and calculate average.

How to calculate cell concentration

Volume in the larges squares:

The area results from the length of the edges: 1 mm x 1 mm = 1 mm^2
with the chamber depth of 0.1 mm this represents a volume of 0.1 mm^3
Conversion: 0.1 mm^3 = 0.1 µl = 10^{-4} ml

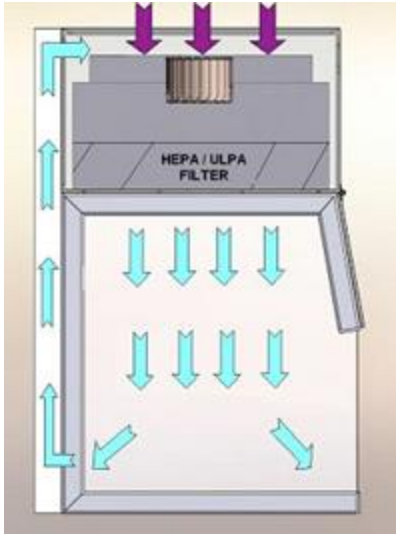
Number of cells / ml:
average # of cells per large square x dilution factor x 10^4

Inverted Microscope

Allows visualization of cells in culture from the bottom of the culture flask.

Its light source and condenser on the top, above the stage pointing down, while the objectives and turret are below the stage pointing up.

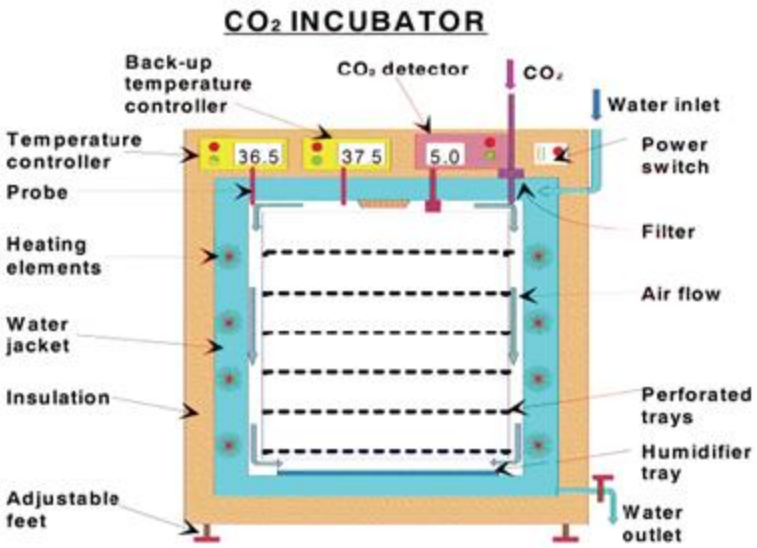
Laminar Flow Hood



Laminar Air Flow

- provides a work area with aseptic/sterile conditions for the tissue culture.
- continuous displacement of **air**.
- HEPA (High Efficiency Particulate **Air**) filter removes particles from the **air.**

Cell incubator



Standard conditions:

- 37 °C
- 5% CO_2
- 95% humidity

Transfection of mammalian cells with plasmid DNA

Transient transfection

- introduction of expression plasmids into cell population leads to transient production of recombinant protein (no selection).

Stable transfection

- selection of cells that integrated plasmid into the host genome (rare event) and recovery of clonal cell line.
  Requires plasmid-encoded selectable marker (e.g. Blasticidin).

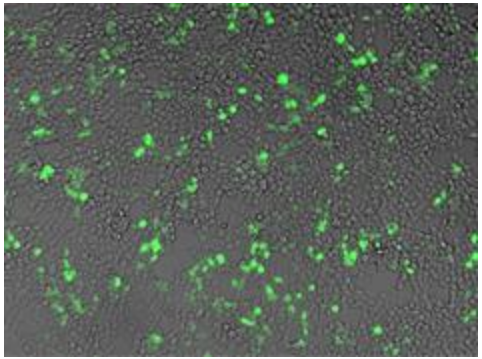
2) Transient transfection

- Objective: Expression of AMY2-His fusion protein.
- Co-transfection of pAmy2-His and a GFP encoding plasmid into HEK293T cells.
- We provide plasmid DNA
  - pAmy2-His : 0.5 µg/µl
  - pGFP : 0.5 µg/µl

Green fluorescent protein (GFP)

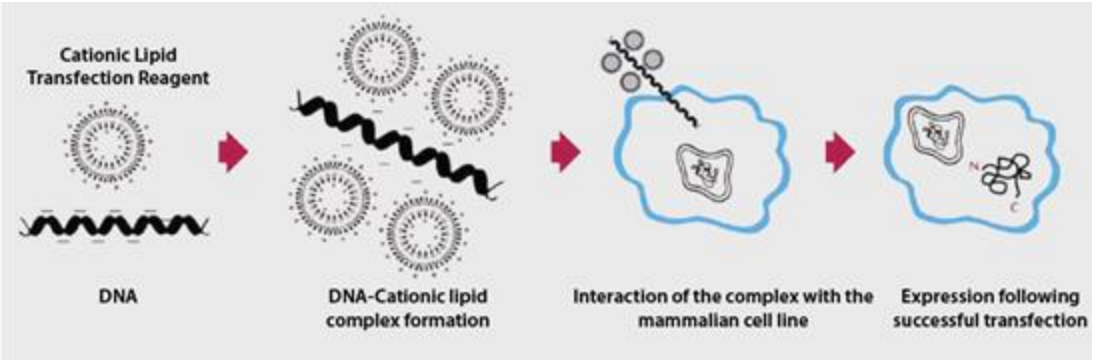
- GFP gene is frequently used as reporter of expression.
- GFP was first isolated from the jellyfish *Aequorea victoria.*
- Exhibits bright green fluorescence when exposed to ultraviolet blue light.
- We will use GFP to determine the **transfection efficiency**.

Image of GFP positive cells observed under fluorescence microscope.



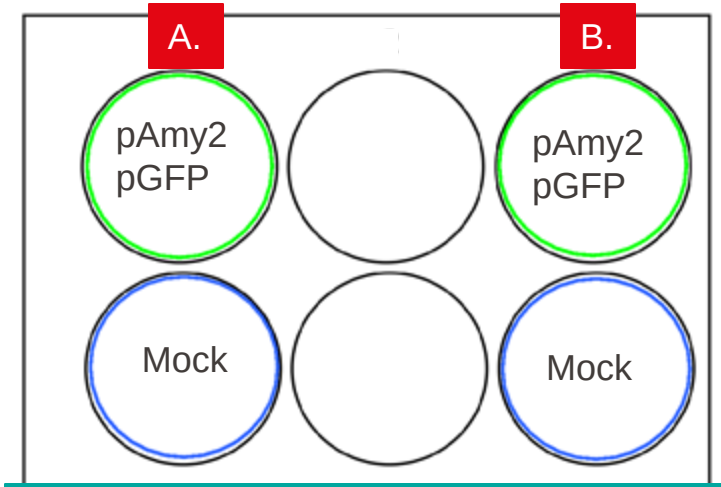
Cationic lipid-mediated transfection

- Transfection reagent: Lipofectamine™ 2000
- Clean bench with 70% EtOH before
- Prepare DNA-Lipofectamine complexes at the bench
- Complexes will be added to the cells under the laminar flow hood



Source: http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cell-Culture/Transfection/transfection-methods/Lipid-Transfection.html

Scheme for transfection in 6-well plate



Label your plate with group number and Mock versus co-transfection.

Duplicates for two separate experiments

A. Microscopy to evaluate transfection efficiency
B. Cell lysates for Western blot