Cell biology for Biotechnology BSc Students Practice book

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1. Laboratory work, accident and fire safety rules

In order to protect our fellow students and our own safety in the laboratory, all students are obliged to observe the following rules.

Laboratory rules:

1) Students may enter the laboratory only with the permission of the teacher, and only the supervising teacher, assistant teacher or students participating in the practical training are allowed in the room during the practice.

2) Students are only allowed to bring into the laboratory the equipment necessary for their work, e.g., writing utensils, minutes. Other equipment (bag, jacket) can be stored in the place provided by the Department during the practice.

3) **Wearing the lab coat is compulsory for all students** and must be taken care of individually. Protective gloves will be provided by the Department of Education. During the practice, it is recommended to keep long hair together.

4) It is **forbidden** to eat, drink, chew or taste any substances in the laboratory.

5) During work, order and cleanliness must be maintained, spilled liquids and chemicals must be wiped up immediately.

6) No individual experimentation is allowed, only work under the instructions of the teacher!

7) The improper use of laboratory equipment such as fire extinguishers, etc. is **forbidden.**

8) In the laboratory, it is important to use the equipment properly. Only use equipment that is necessary for the exercise.

9) It is the student's responsibility to tidy up the workbenches and keep the equipment clean at the end of the exercise.

General rules on health and safety at work and accident prevention:

1) Do not work with broken or cracked glassware!

2) Do not wash the cut with water, unless corrosive or toxic substances have been involved. Carefully apply pressure, allow the wound to bleed and make sure that no glass fragments remain, then disinfect the wound area. Apply a plaster to a small wound and sterile gauze to a large wound.

3) Use protective gloves when using flammable, corrosive or toxic substances.

4) Only take any material from a labelled bottle and store chemicals in such a container. Do not replace the lids of chemical bottles or jars, always place them on the table with the lid on. This way you do not contaminate the chemical or the table. Wipe up any spills or splashes immediately.

5) Solid chemicals should be removed only with a clean chemical spoon, which should be washed after use.

6) Do not pour any remaining solvent back into the chemical bottle from which it was removed. It must be stored in a designated collection container in the laboratory. Do not pour organic solvent down the drain!

General fire safety rules:

1) The National Fire Safety Code defines five classes of fire hazard, designated by the letters "A" "B" "C" "D" "E".

   "A" - Highly flammable and explosive

   "B" - Danger of fire and explosion

   "C" - Danger of fire

   "D"- Moderately flammable

   "E"- Not liable to catch fire

   Always be aware of the flammability class of the material you are working with.

2) When extinguishing a fire, one of the following factors must be removed or eliminated:

   -combustible material
   -combustible medium (oxygen)
   -ignition temperature

3) Extinguishing a fire should always be started as soon as possible, taking into account the properties of the burning material and the size of the fire, and by a rapid and comprehensive survey of the available suitable equipment and materials.

4) An attempt should be made to extinguish the fire with the equipment and tools available.

5) In the event of a fire in or near electrical installations, the power must be disconnected before extinguishing is started.

6) There are different ways to put out a fire:
   - for a small fire, e.g., a table fire: in case of solvent ignition (a few ml), cover the fire with a damp cloth, blanket, cooking pot, cut off oxygen, possibly extinguish with water.
   - In case of a personal fire: extinguish by lying down quickly on the ground with burning clothing in a rolling motion. If necessary, use a safety shower!

- in case of a laboratory fire:

A. Use of a fire extinguisher: a device from which extinguishing agent can be directed into the fire by the pressure in the device when it is put into operation. The most suitable devices for extinguishing small, incipient fires.

Different types of extinguishers are suitable for extinguishing different types of fire. Based on the properties of combustible materials, the different classes of fire are as follows:

Fire class A: Fire of solid organic materials

Fire class B: Fire involving solid liquids or liquids in solid form

Fire class C: Flammable gases

Fire class D: Metal fires

Division F: Fires involving oils and fats

Types of fire extinguishers:

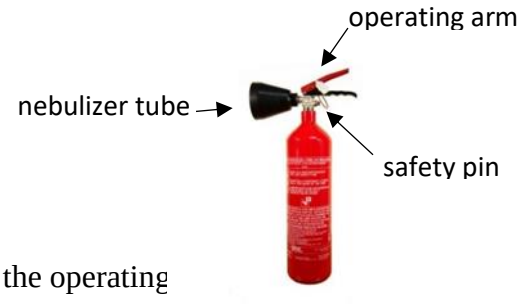
I. Powder extinguishing: The extinguisher has CO2 in the tank, the pressure is used to extinguish NaHCO3 powder. The NaHCO3 decomposes in the fire and the evolving CO2 blocks the fire from oxygen. It can be used to extinguish class A, B and C fires. Not suitable for extinguishing electrical appliances!

II. Foam extinguisher: Generally suitable for extinguishing class A and B fires, but there are extinguishers with extinguishing capacity which can also be used for class F fires. Foam extinguishing is prohibited in the presence of electricity, as foam is an extinguishing agent mixed with water.

III. Gas extinguishing: extinguishing with carbon dioxide. It is generally used to extinguish fires of fire class B or electrical fires, as it does not damage the equipment. Carbon dioxide evaporates on contact with the burning material, does not attack it, does not conduct electricity and is therefore preferably used for the transport and storage of liquids, valuable materials and objects, foodstuffs and flammable and explosive materials.

IV. Water extinguishing: Water is generally used as an extinguishing agent for the combustion of solid materials. Water extinguishing is prohibited in the presence of electricity, and water extinguishing is also prohibited for solid chemical substances that may react with water.

Extinguishing a burning person with a fire extinguisher is **FORBIDDEN!**



Operating a carbon dioxide extinguisher:

1. removing the fuse pin

2. directing the fog hose to the edge of the fire

3. apply extinguishing agent by intermittent pressure on the operating

The low temperature of the extinguishing agent may cause a risk of frostbite and therefore requires extra care.

B. Wall-mounted hydrants are water intakes for extinguishing fires and are located in a cabinet with fittings and accessories in buildings. Their location shall be indicated by the word "hydrant". Wall hydrants are not only intended to support the work of firefighters, but also, and above all, to facilitate rapid intervention and fire-fighting by building occupants and workers. They may only be used after the power has been switched off! Use must be considered in the event of water damage.

7) Immediately cool burned skin with plenty of running water, then treat with a burn ointment or spray. In severe cases, seek medical attention.

8) In the event of electric shock, first disconnect the main switch in the laboratory. In case of loss of consciousness, take the casualty to fresh air, place him in a stable side position and call a doctor.

9) Before opening the main shut-off valve of the gas line, check that the valves of each burner are closed, then put them into operation. When the work is finished, make sure that the gas burner tap on the tables and the main shut-off tap are closed.

10) Do not lean over the gas burner while working.

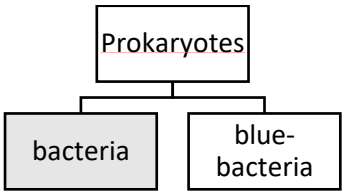
11) To prevent accidents, extinguish or light the Bunsen burners when not in use.

12) Ensure that all gas and water taps are turned off and electrical equipment is switched off before leaving the laboratory after work is completed.

In case of sickness, injury or any accident, contact the teacher immediately.

2. General characterisation of bacteria

A. Taxonomy



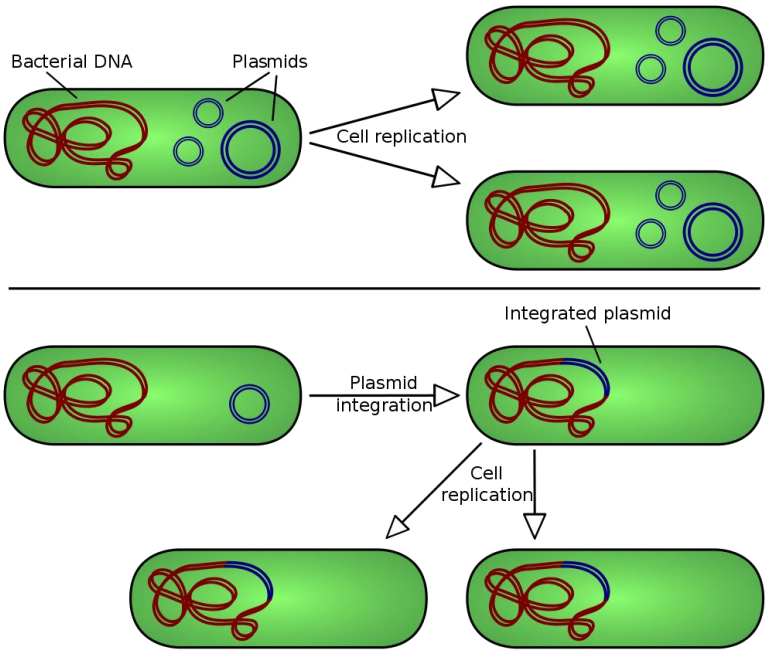
B. Structure
- Size: ~0.5–5.0 micrometres
- Shape: round, stick or twisted
- cell wall
- cell membrane
- cytoplasm
- large circular DNA
- flagella

C. Genetic material
- Nucleus: absent
- Chromosomes: one circular + plasmids
- Ploidity: haploid
- Genetic recombination: partial
- DNA wrapping on proteins: few proteins
- Gene expression: in operons
- Introns: absents
- Non coding DNA: little amount
- Ribosome localisation: free

D. Vectors/plasmids:
<u>General features:</u>
- *Functions*: Protection of DNA, transport of DNA into the cells, replication in the host cells
- Nonessential, extra chromosomal, circular, double stranded DNA molecules
- Ability to promote autonomous replication
- Plasmids are found in the nature
- Plasmids can carry antibiotic resistance genes, genes for receptors, toxins or other proteins
- Plasmids can be engineered to be useful cloning vectors

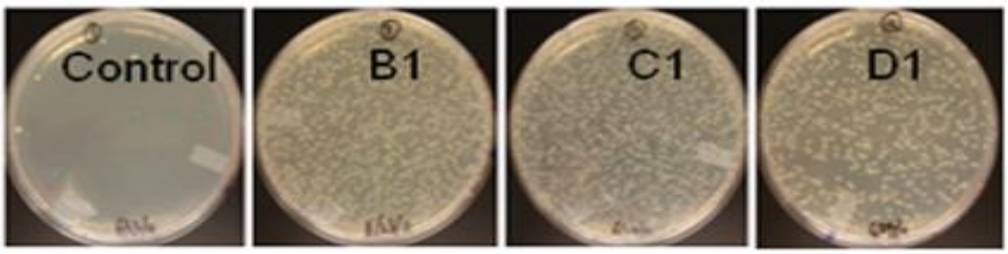
I. <u>Natural plasmids:</u>
- their size is 3-20 kB
- beneficial traits: contain resistance genes which help in the survival and/or help in the adaptation to the environment

- plasmids are able to move from one cell into another cell by conjugation
- non-integrating plasmids and episomes



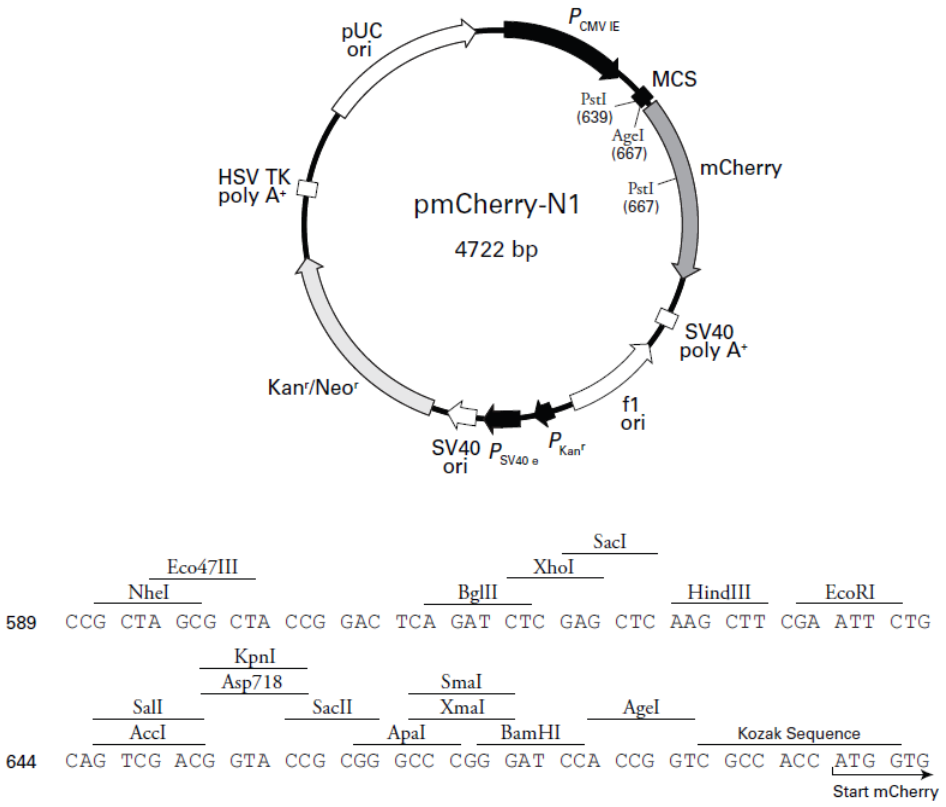
II. <u>Artificial plasmids for molecular cloning:</u>

- They are smaller: 3-6 kB
- modular structure
- multiple cloning site/polylinker region with unique restriction sites: **Cloning site** is in the vector before and after the insert, only occurring only once, short (a few bp) sequence that allows selective cloning of the insert selective excision/incorporation
- Contain a genetic marker (usually dominant) for selection: **Selection marker** allows bacteria containing only the vector to grow on the medium.



- Their 3D structures are protected against mechanical affects
- **Promoter region:** directly between the cloning site and the gene upstream of the cloner and the gene, allowing transcription, determines the amount of protein (RNA polymerase site of protein polymorphism enzyme binding).
- They are present in multiple copies (low copy or high copy plasmids)
- ATG and STOP codons

- Protein selection: tags e.g., RFP, GST, His, HA, Myc etc. Purification of the proteins with tag specific antibodies.
- fluorescent tag: *in vivo* examination of the protein



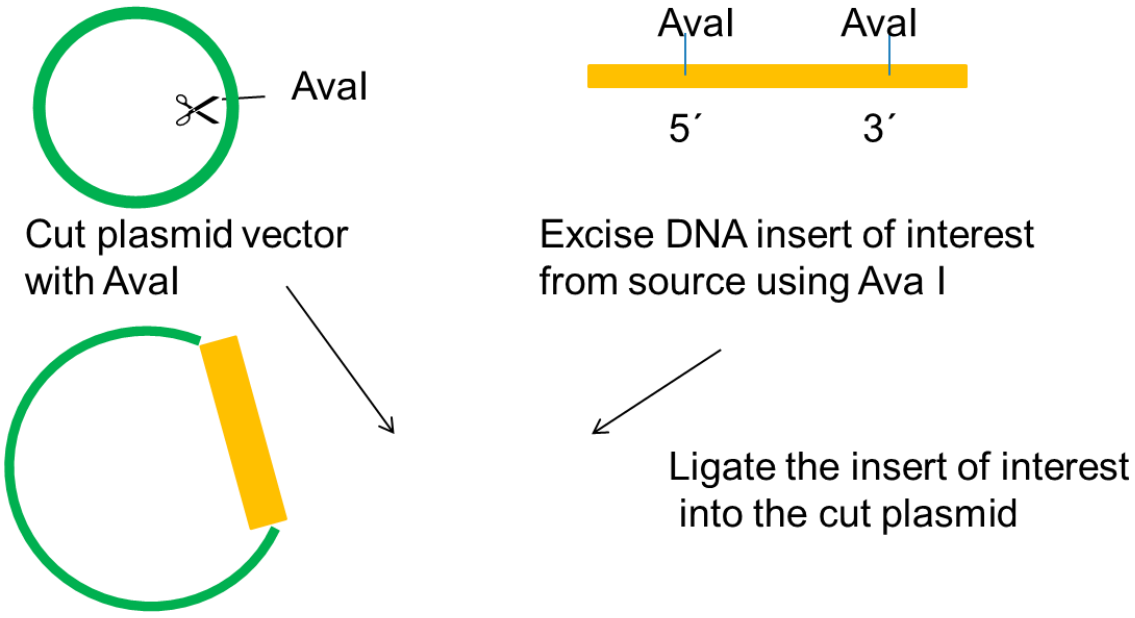
E. Competent host cells
- Not every bacterial cell is able to take up plasmid DNA.
- Bacterial cells that can take up DNA from the environment are said to be competent.
- Can treat cells (electrical current/divalent cations) to increase the likelihood that DNA will be taken up
- Two methods for transforming: heat shock and electroporation

F. Restriction enzymes
- Restriction Enzymes (also called Restriction Endonucleases) are proteins that cleave DNA molecules at specific sites, producing discrete fragments of DNA.
- Generally, restriction enzymes are thought to protect bacterial cells from phage (bacterial virus) infection. Bacterial cells that contain restriction enzymes can "cut up" invasive viral DNA without damaging their own DNA.
- Type I restriction endonucleases: e.g., EcoKI:
    1. Recognition sequence: AAC(N6)GTGC;
    2. Restriction: it does not digest within the recognition sequence, approx. ~1 kB from the recognition sequence

- Type III restriction endonucleases:
    1. Recognition sequence: AGACC 2x;
    2. Restriction: it does not digest within the recognition sequence, approx. 22-24 basepairs from the recognition sequence
- Type II restriction endonucleases:
    1. They have specific recognition sites, they digest within the recognition site at determined bases, and they are specific
    2. Recognition sequences: 4, 6, 8 bp long
    3. Palindrom structure: same base sequence at both chains
    4. They cut both strains of the DNA molecule.
    5. 3' or 5' overhang ends – sticky end (EcoRI, BamHI etc.)
    6. Cut at the middle of the recognition site- blunt end (SmaI, EcoRV)
    7. Endonuclease and methylase are two different proteins
    8. More than 3500 types (750 strains)
    9. Their names come from the host prokaryote, the numbering depends on the number in the order of discovery e.g., EcoRI, HindIII etc.

EcoRI G↓AATTC 5' overhang end/ sticky end
BamHI G↓GATCC 5' overhang end/ sticky end
EcoRV GAT↓ATC blunt end/ sticky end
SmaI CCC↓GGG blunt end
KpnI GGTAC↓C 3' overhang end/ sticky end

Cloning:



G. Bacterial culture

- Growth medium-LB: Luria-Bertani Broth (Sterile liquid)
- Components: 1 % Tripton
  0.5 % Yeast extract
  0.5 % NaCl
  for agar plates +2% agar-agar and antibiotic for selection (if needed).
- Temperature: 30-37°C
- Proliferation time: 20-60 min
- For agar plates: 16-24 h

H. Culture in liquid media

- easy access to the available nutrients
- Gentle agitation to keep the bacteria dispersed through the medium during incubation can aid this access further
- Liquid media will also dilute out waste products as they are formed
- a greater mass of bacteria may be obtained for an equivalent volume of liquid as opposed to solid media

I. Culture in solid media

- Solid media is useful when you wish to select individual colonies from a mixed culture
- for selection between bacterial cells e.g., cloning
- Inoculation onto slopes (using inoculation loop) or in stab cultures (glass vials with screw on caps fitted with rubber gaskets) can also be a convenient method for transporting strains from lab to lab without the danger of spillage of potentially infectious materials

J. Selective/differential media

- Selective media promote or suppress the growth of certain species, groups of species or strains with particular properties.
- This may be based on a strain's ability to utilize specific nutrients, produce certain by-products or resistance to certain antibiotics.
- Selection may be used in both broth and solid media.
- The ability of the strain to grow or not may be indicated by color changes in differential media
- used to identify bacterial species or subtypes

K. Antibiotic resistance

- Addition of antibiotics to liquid media will prevent the growth of non-resistant strains.
- helpful when culturing an engineered strain into which an antibiotic resistance gene has been added as a marker (cloning)
- Growth of contaminating species or colonies in which the engineering has been unsuccessful will therefore be selected against (marker negative)

- Antibiotics may be added to solid media during preparation, fulfilling a similar role to that in liquid media.
- antibiotic-infused disks may be placed onto solid media onto which a stain of interest has been inoculated.
- Where the strain is sensitive to the antibiotic, a clear zone of no growth will then be visible around the disc

Questions

1. What does the “competent bacteria” mean?

2. How do we culture the bacterial cells?

3. What is the biotechnological importance of bacterial cells?

4. What is the aim of molecular cloning?

5. What does the selection mean in case of bacterial cells?

3. Protein measurement

Basics: Photometric concentration measurements

Colored solutions absorb a determined portion of the visible light spectrum. Other materials, e.g., proteins or nucleotides are only capable of absorption of ultraviolet light. The **spectrum** of a light absorbing material can be measured by putting its water solution in the cuvette of a spectrophotometer. The **absorption maximum** of the material can be read from the spectrum, which is the wavelength of maximal light absorption.

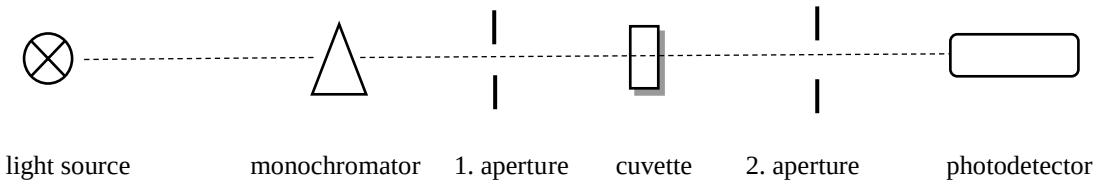


Fig. 1.: Single spectrophotometer

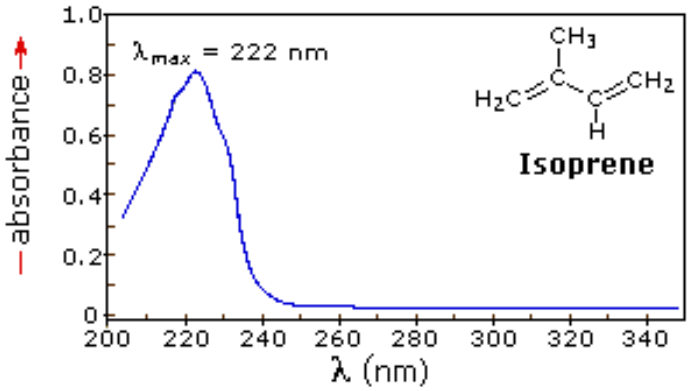


Fig. 2.: Absorption spectrum of Isoprene

The principle of light absorption is described by **Beer's Law**. Molecules absorb part of the monochromatic light travelling through the solution. The intensity of the transmitted light is measured photoelectrically. Ratio of the transmitted light (**Transmittance**) is calculated as follows:

$$T = \frac{I}{I_0} \cdot 100\ (\%)$$

where:
T – transmittance
I_0 – intensity of incident light
I – intensity of transmitted light

As the concentration of the light absorbing material is not linearly correlated to the transmittance, the concept of **Extinction** is introduced:

$$E = \log \frac{I_0}{I}$$

Extinction (or A as Absorbance or OD as Optical Density) is unitless.

If the measurement is performed on a fixed wavelength, the extinction is linearly proportional to the concentration and the thickness of layer. This leads us to the practically used formula of **Beer's Law**:

$$E = \varepsilon \cdot c \cdot l$$

where:
E – extinction (I_0/I)
c – concentration of the solution (mmol/liter = mmol/dm^3 → 1µmol/cm^3)
l – thickness of layer (cm) → Usually 1 cm cuvettes are used.
ε – (micromolar) **extinction coefficient** (cm^2/µmol)

For determining the concentration of an unknown sample, we need to use a **calibration curve** of a standard with known concentration on the given wavelength. We get a calibration curve by plotting the measured extinction values against concentration. Concentration of the unknown sample can be read from the curve if we measure its extinction photometrically.

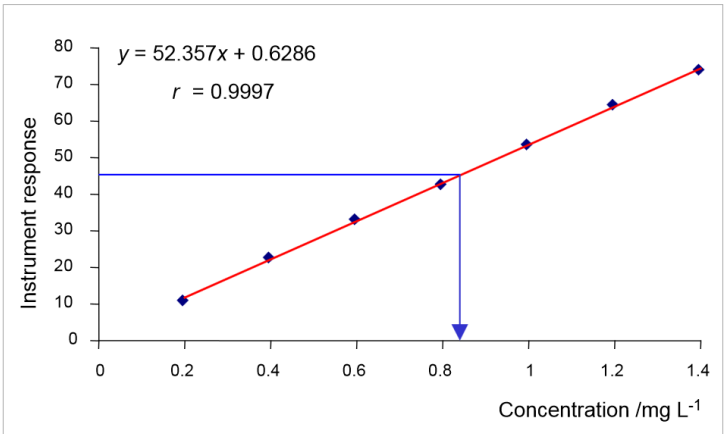


Fig. 3.: A typical calibration curve (Instrument response = Extinction). Blue line shows how the concentration of an unknown sample is determined graphically.

Types of protein concentration determinations:

- UV
- Detergent compatible (DC) protein assay
- Lowry protein assay
- Biuret assay

Determination of protein concentration using Biuret test

The principle of the determination: peptides bond with Cu^{2+} ions on high pH, and this results in purple compounds. The intensity of the color is proportional to the number of Cu-peptide complexes therefore the protein concentration. Thus, it can be used in a photometric assay.

$$Cu^{2+} + Protein \xrightarrow{OH^-} Cu - protein\ complex$$

The correlation is not linear; therefore, we can't apply Lambert's Law on it. We need to prepare a standard curve using protein samples of known concentrations and define the unknown sample's concentration by interpolation from the standard curve.

Solutions:

Work reagent:

Potassium iodide 30 mmol/l (protects Cu^{2+} ions from autoreduction)
Potassium-sodium tartrate 100 mmol/l (keeps Cu^{2+} ions in solution)
Copper sulphate 30 mmol/l
Sodium hydroxide 3.8 mmol/l

Standard:

Bovine serum albumin 60 g/l: Use this value to calculate absolute concentration values before plotting!

Sample: liver and/or muscle extract

Measurement:

1. Prepare the following solutions in 8 Eppendorf tubes:

tube #	**1.**	**2.** **6 mg/ml BSA**	**3.***	**4.**	**5.**	**6.****	**7. (liver)**	**8. (muscle)**
0.9 % NaCl	0.5 ml	-	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.4 ml	0.4 ml
1:10 standard	-	0,5 ml	0,5 ml*	-	-	-	-	-
sample	-	-	-	-	-	-	0.1 ml	0.1 ml
relative conc.	-	1	1/2	1/4	1/8	1/16	unknown	
biuret reagent	0.75 ml	0.75 ml	0.75 ml	0.75 ml	0.75 ml	0.75 ml	0.75 ml	0.75 ml

*Mix tube #3 and transfer 0.5 ml of the solution to tube #4. Mix and transfer 0.5 ml from #4 to #5, then 0.5 ml from #5 to #6 (geometrical dilution series).

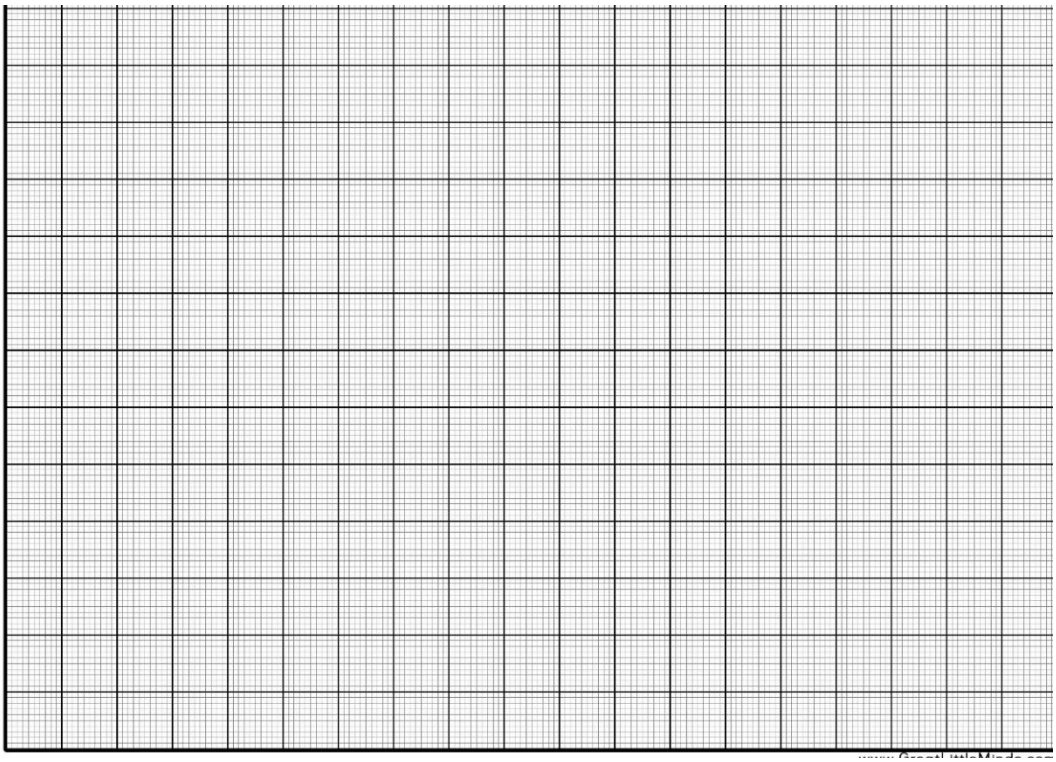
**0.5 ml from tube #6 is disposed so that every tube contains 0.5 ml solution with different concentrations.

2. Incubation on 37 °C for 30 minutes.
3. Photometrical detection at 555 nm. Tube #1 is the negative control.

Evaluation:

Plot the extinction values against absolute protein concentration to get a calibration curve.

tube #	**Absolute protein concentration (mg/ml)**	**E_{555}**
1.	control/blank	0
2.		
3.		
4.		
5.		
6.		
7.	liver	
8.	muscle	



Use the calibration curve to determine the protein concentration of the two unknown samples (graphically!). After this step, don't forget to multiply the concentration values by the dilution factor of the unknown samples (5).

Protein measurement by Detergent compatible assay kit

Reagent A: an alkaline copper tartrate solution

Reagent S

Reagent B: a dilute Folin Reagent

Protocol

1. Preparation of working reagent

Add 20 µl of reagent S to each ml of reagent A that will be needed for the run.

(This working reagent A' is stable for 1 week even though a precipitate will form after 1 day. If precipitate forms, warm the solution and vortex. Do not pipet the undissolved precipitate, as this will likely plug the tip of the pipet, thereby altering the volume of reagent that is added to the sample.)

If samples do not contain detergent, you may omit step #1 and simply use reagent A as supplied.

2. Prepare 3 - 5 dilutions of a protein standard containing from 0.2 mg/ml to about 1.5 mg/ml protein. A standard curve should be prepared each time the assay is performed. For best results, the standard should be prepared in the same buffer as the sample.

3. Pipet 5 µl of standards and samples into a clean, dry microtiter plate.

4. Add 25 µl of reagent A' or reagent A (see note from step 1) into each well.

5. Add 200 µl reagent B into each well. If microplate reader has a mixing function available, place plate in reader and let the plate mix for 5 seconds. If not, gently agitate the plate to mix the reagents. If bubbles form, pop them with a clean, dry pipet tip. Be careful to avoid cross-contamination of sample wells.

6. After 15 minutes, absorbance can be read at 675 nm. The absorbance will be stable for about 1 hour.

Sample	OD

Answer the following questions!

1. What is the wavelength of the Biuret-protein complex absorption maximum?

2. What is the monochromator?

3. How can you determine the concentration of the molecule in the cuvette, if the extinction coefficient is known?

4. Explain, how the bisecting dilution series is made.

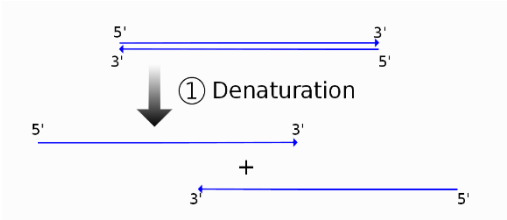
5. What is the blind sample? What can it be used for during protein determination?

4. PCR practice

Steps of polymerase chain reaction:

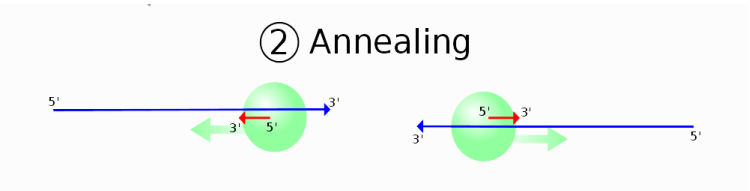
I. Denaturation

High temperature (92-98°C) denatures all the DNA.



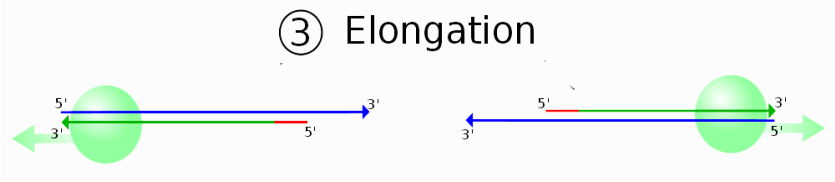
II. Annealing

Primer specific temperature (50-72°C) allows the oligonucleotides to anneal.



III. Elongation - Extension

The polymerase synthesizes the complement chain (60 or 72°C).

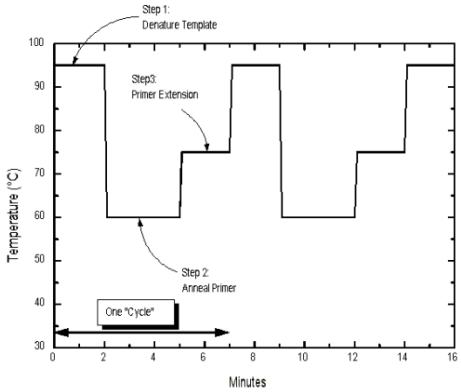


Components of the reaction:

- DNA template
- Primers
- Nucleotides (dATP, dGTP, dCTP, dTTP)
- DNA polymerase
- Buffer (including Mg^{2+})

Reaction heat profile:

92 °C 2’
92 °C 20”
56 °C 30” } 35x
72 °C 30”
72 °C 5’



Primers:

forward primer – human ß actin (Akt 11) 5’ AGA AAA TCT GGC ACC ACA CC 3’

reverse primer – human ß actin (Akt 21) 5’ GGG GTG TTG AAG GTC TCA AA 3’

Samples: negative control, positive control, sample 1 (HeLa cDNA), sample 2 (WRL68 cDNA)

Setting up the reaction:

1 reaction	**4 reactions (master mix)**
6 µl distilled water	… µl
10 µl 2x PCR buffer mix	… µl
2 µl forward + reverse primers	… µl
2 µl template	- µl
Σ 20 µl	Σ …. µl

<u>Questions:</u>

1. What can be the cause if we got products in the no template control reaction?

2. What can be the cause if we haven’t got product in the positive control reaction?

3. Why is it advised to use master mix?

4. Why do you need multiple repetitions?

5. What are the applications of PCR?

5. Agarose gel electrophoresis

Choose the suitable agarose based on the size of the DNA molecule.

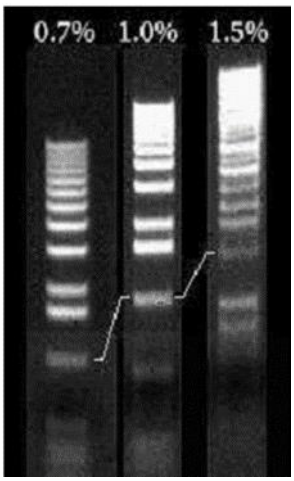
Running Buffers:

- TAE- Tris-acetate-EDTA pH 7,6-7,8
- TBE- Tris-borate-EDTA pH 8,3

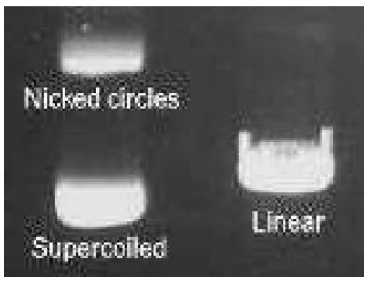
Use in appropriate dilution!

Influencing factors of DNA running:

1. Size of the DNA molecule: the speed of running is inversely proportional to the logarithm to the base ten of the molecular weight.
2. Concentration of agarose: the logarithm of the DNA electrophoretic mobility directly proportional to the gel concentration.



3. Conformation of the DNA: same sized closed circular, nicked circular and linear DNA molecules run with different speed.



4. Applied current: at low voltage the linear DNA molecule runs directly proportional to the voltage. At high voltage the relatively large fragments run faster than the smaller ones (5V/cm).

Loading buffers

Role: the loading buffer holds the DNA at the bottom of the well, doesn't let the DNA diffuse out from the gel.

- Negatively charged dye
- Bromphenol-blue, Xylen-cyanol
- Glycerol or sucrose
- Factory-made loading dyes: contain different types of dyes in one loading buffer

Visualization:

- Ethidium bromide: intercalating dye, less sensitive, carcinogen, DNA harming effect; advantage: it can be mixed into the loading, into the gel or the gel can be dyed after running, Detection wavelength: UV
- SYBR dyes (green, gold…etc.): intercalating dye, high sensitivity; advantage: only small amount is enough, its carcinogen effect is not known, it can be mixed into the loading, into the gel, detection wavelength: visible light (blue light)
- Silver dye: very sensitive, expensive

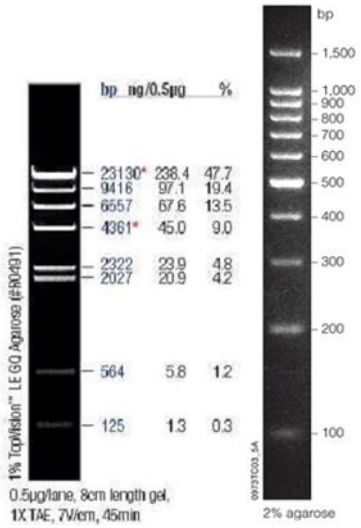
How much DNA is needed to be run on gel?

- Depends on the method of visualization
- Approx. 20 ng DNA is well seen
- If we use too much DNA, it is difficult to determine its size

Molecular weight markers/DNA ladders

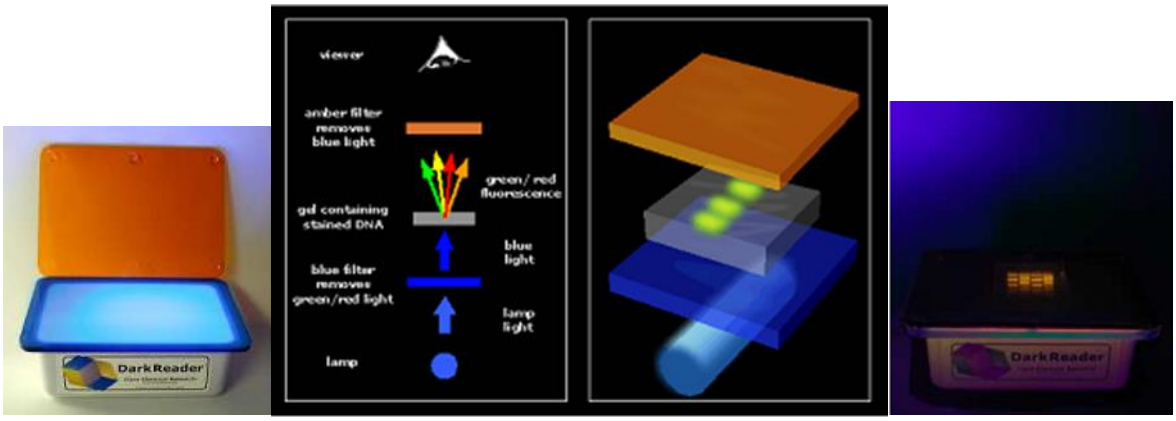
Role: determination of the size (length) of the DNA molecules

- The exact size can be only determined in case of linear or nicked circular DNA molecules
- The plasmids sometimes do not run at their exact size. Reason: coiling of circular DNA.
- Several DNA markers exist.
- The DNA marker should be appropriate to the size of the examined DNA molecule.
- The ladders already contain the loading dye; only the dye for visualization is needed to be added.
- Small amount of them are required, approx. 1,5-3 µl is enough for running.



Detection of DNA

- UV transilluminator- Excitation with UV light, UV filter!
- Transilluminator- excitation with blue light, amber screen absorbs the blue excitation light (orange filter)
- Gel documentation system – excitation with different wavelengths, detection of emission, gel photo



Questions:

1. What can affect the DNA run in a gel?

2. What is the role of the DNA ladder?

3. How can DNA be detected?

4. What can restriction endonucleases be used for?

5. What is the principle of agarose gel electrophoresis?

6. Cell culture 1.

Cell culture is the process by which cells are grown under controlled conditions, generally outside their natural environment.

Extracorporeal growth of cells in vitro.

Based on Growth Mode

- Monolayer: sticky
- Suspension

Types of cell cultures

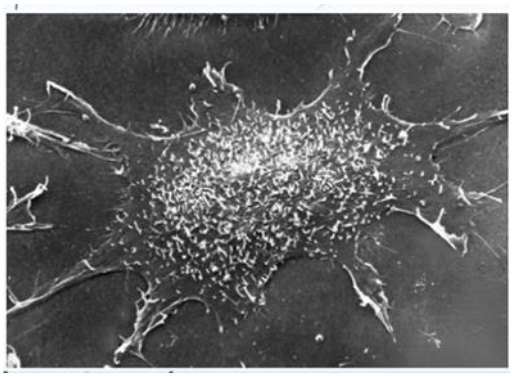
I. ***Primary cell culture****:* prepared from the original organ or tissue
   - only one type of cell presents in the culture
   - benefit: it shows the same physiological conditions that exists in the living organism

II. ***Secunder cell culture****:* prepared after one passage of the primary culture

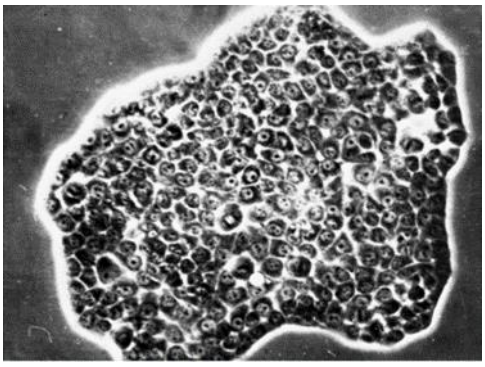
III. ***Cell strain****:* derived from primary culture after a few passage, but it has a finite division potential (40-50 passage).

IV. ***Cell line****:* these cells have the ability to proliferate indefinitely, theoretically for unlimited time and are genetically homogeneous.
   - immortalization: the ability to proliferate indefinitely
   - transformation (oncogen transfection with retrovirus)
   - tumour cells (mutation or transfection with virus)
   - spontaneous immortalization

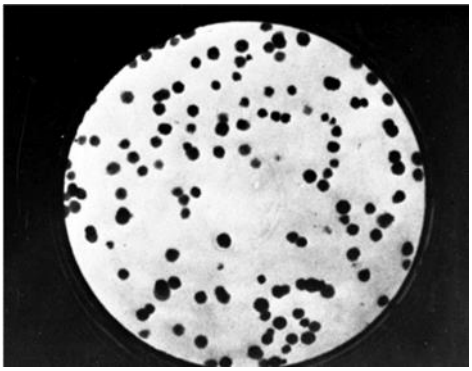
V. ***Cell banks****:* storage of cell strains and cell lines in a specific cryoprotective media and liquid nitrogen.



One cell-SEM (0.01 mm)



Cell colony (1 mm)



Tissue culture plate with cell colonies (100 mm)

Benefits of cell cultures:

- specific cells, homogenous cell population
- experiments are reproducible and planned correctly
- controlled conditions
- monitoring of cell functions (BUT! these results cannot be referred totally to the *in vivo* conditions)
- substitution of some of the animal experiments
- replacing some of the animal experiments
- investigators of human cells

Limitations of Cell Cultures

- It does not model the complexity of the organization
- Spontaneous in vitro evolution
    - Spontaneous mutations arise from a variety of sources, including errors in DNA replication, spontaneous lesions, and other more complex mechanisms.
    - Spontaneous mutations are very rare, making it difficult to determine the underlying mechanisms.
    - Even though they are rare, some selective systems allow numerous spontaneous mutations to be obtained and then characterized at the molecular level for example, their DNA sequences can be determined.
    - From the nature of the sequence changes, inferences can be made about the processes that have led to the spontaneous mutations.

Maintaining cell cultures

- STERILE work!
- **passaging***:* transferring a small and defined number of cells into a new vessel with fresh media.
- *Suspension cells*: easily passaged with a small amount of culture containing a few cells diluted in a larger volume of fresh media
- *Adherent cells*: cells first need to be detached, commonly with enzymatic digestion, then a small number of detached cells can be used to seed a new culture with fresh media
- the number of passage is very important to memorize!

Materials needed to maintain cell cultures

a) growth medium: insures the right nutrients and conditions for cell growth and proliferation.
b) sterile bench: avoid contamination with the filtration of the air
c) cell incubator: optimal temperature (37°C), 5% CO_2, optimal humidity (90-100%)
d) microscope**:** usually inverted
e) Bürker chamber: to determine the number of living cells with the help of a dye: trypan-blue
f) cell culture flasks, multi-well plates, Petri-dishes, sterile pipettes and centrifuge

Growth medium: insures the right nutrients and conditions for cell growth and proliferation.

- ions: Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, PO_4^{3-}, HCO_3^-
- glucose: energy source
- essential amino acids, that the cells cannot synthesize (NEAA if needed)
- vitamins
- buffer: maintain pH 7,2-7,4
- phenol red: pH indicator, indicates the changes in pH balance
- serum: 5-20%, fetal: FCS/FBS or adult

Storage of cells

- in icing ampule and in specific icing media (to avoid the formation of ice crystals)
- *short-term storage*: -80°C
- *long-term storage*: liquid nitrogen -196°C

Video – cell culturing

http://youtu.be/7d_kDu-P964

https://www.thermofisher.com/hu/en/home/global/forms/cell-culture-basics/cell-culture-basics-virtual-lab.html

Questions:

1. What are primary cell cultures? What is the advantage of a primary culture over a secondary culture?

2. What are the characteristics of cell lines?

3. What is typical for *in vitro* studies?

4. Why is the medium important for maintaining cell culture?

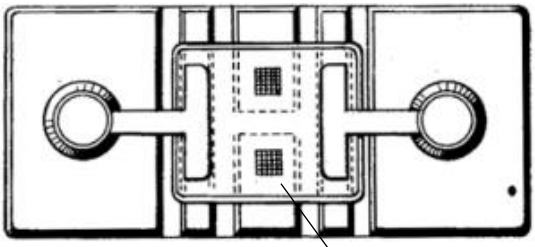
5. What does passaging of cells mean?

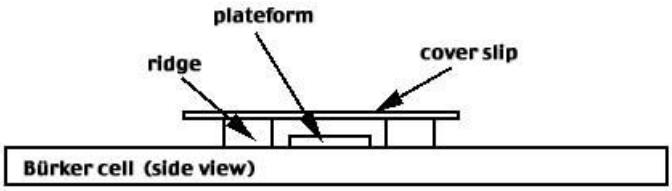
7. Cell culture 2.

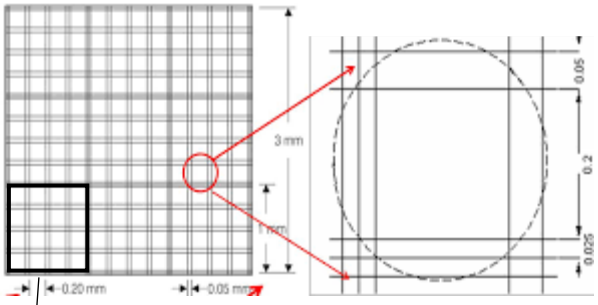
1. counting of cells

a) cells collecting from the cell culture flasks/multi-well plates/Petri-dish
   - with trypsin or cell scrapers

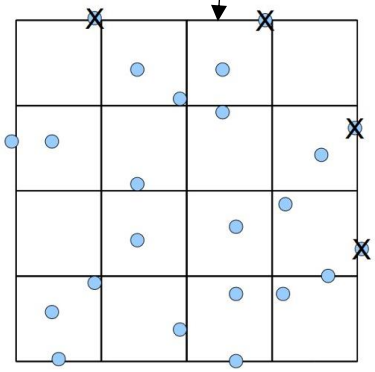
b) Bürker-chamber: to determine the number of living cells with the help of a dye: trypan-blue (only the dead cells will be blue)







c) counting of cell



$$\frac{\boldsymbol{number\ of\ cell}}{\boldsymbol{number\ of\ counted\ squares}} \times \mathbf{20} \times \mathbf{10000} = {}^{\boldsymbol{y}}/_{\boldsymbol{mL}}$$

Counting:

Distributions of cells

- Division time
- Aging
- Cell cycle
- Apoptosis

8. Viability

Cell viability is a measure of the proportion of live, healthy cells within a population. Cell viability assays are used to determine the overall health of cells, optimize culture or experimental conditions, and to measure cell survival following treatment with compounds, such as during a drug screen.

Sensitive colorimetric assays for the determination of the number of viable cells:

a) **MTT** (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide):
- quantifies the relative quantity of viable cells using this approach
- Cultures are incubated with the yellow tetrazolium dye **MTT**
- healthy cells is converted by mitochondrial enzymes into an insoluble purple formazan product

b) **XTT** (2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl):
- The yellow tetrazolium salt XTT is reduced to a highly colored formazan dye by dehydrogenase enzymes in metabolically active cells.
- That is an alternative to the MTT assay which yields a formazan product that is soluble in aqueous solutions, and thus does not require an additional solubilization step.

c) **WST-8** (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt)
- which produces a water-soluble formazan dye upon bioreduction in the presence of an electron carrier, 1-Methoxy PMS (N-methylphenazonium methyl sulfate)
- The detection sensitivity is higher than any other tetrazolium salts such as MTT, XTT or MTS.

d) **MTS** (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
- conversion of the tetrazolium salt MTS to a purple formazan in the presence of phenazine methosulfate
- The enzymes responsible are NADPH-dependent dehydrogenases, which are active in viable cells

e) **Resazurin:**
- based on the reduction of oxidized non-fluorescent blue resazurin to a red fluorescent dye (resorufin) by the mitochondrial respiratory chain in live cells
- The amount of resorufin produced is directly proportional to the number of living cells.
- rapid, simple, accurate and homogeneous/clean, high throughput assay

Performing viability measurement by resazurin assay

1. Prepare cells and test compounds in opaque-walled 96-well plates containing a final volume of 100 µl/well. An optional set of wells can be prepared with medium only for background subtraction and instrument gain adjustment.
2. Incubate for the desired period of exposure.
3. Add 20 µl resazurin solution to each well.
4. Incubate for 1 to 4 hours at 37°C.
5. Record fluorescence at a wavelength of 600 nm.

Note: Samples can be measured spectrophotometrically by monitoring the decrease in absorbance at a wavelength of 600 nm. Measure the absorbance of multiwell plates at a reference wavelength of 690 nm and subtract from the 600 nm measurement.

Alternatively, samples can be measured fluorometrically by monitoring the increase in fluorescence at a wavelength of 590 nm using an excitation wavelength of 560 nm.

Results:

9. RNA isolation with Trizol reagent

Working with RNAs requires an antiseptic, uncontaminated environment. It is important to use sterile equipment and devices. Wear a protective lab coat and gloves in order to protect the sample from contamination, and ourselves from harming chemicals of the process! RNases, which break down the RNA molecules, can be introduced accidentally into the RNA preparation at any point in the isolation procedure through improper techniques. Heat can also destroy the structure of the fragile, unstable RNA.

TRIZOL Reagent is a ready-to-use reagent for the isolation of total RNA from cells and tissues. The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate.

Caution! Trizol reagent and chloroform are corrosive and harmful! Toxic in contact with skin and if swallowed. Causes burns. After contact with skin, wash immediately with plenty of detergent and water. Wear protective lab coat and gloves! Wearing safety goggles is highly recommended! RNases can be introduced accidentally into the RNA preparation at any point in the isolation procedure through improper techniques.

TRIZOL Reagent (U.S.Patent No.5,346,994) is a ready-to-use reagent for the isolation of total RNA from cells and tissues. The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate.

Protocol summarised:

1. **Homogenisation.**
- Add 0.5 ml Trizol to 50 – 100 mg tissue then homogenise.

2. **Phase separation.**
- Incubate the sample for 3’ @ RT (room temperature) to permit the complete dissociation of nucleoprotein complexes.
- Add 0.1 ml chloroform, close the tube and shake vigorously for 15”.
- Incubate for 3’ @ RT.
- Centrifuge for 10’ @ 12 000 x g. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase.

3. **RNA precipitation.**
- Carefully pipette the upper phase into a new tube. Do no touch the interphase!
- Add 0.5 ml isopropyl-alcohol.
- Incubate for 3’ @ RT
- Centrifuge for 7’ @ 12 000 x g.
- The RNA precipitate forms a gel-like pellet on the side and bottom of the tube.

4. **RNA wash.**
- Remove supernatant by pipetting.
- Add 1 ml 75% ethanol to the pellet.
- Vortex then centrifuge for 5’ @ 7 500 x g.

5. **Redissolving the RNA.**

- Carefully remove the ethanol by pipetting
- Let the pellet dry for 5 -10' @ RT.
- Redissolve the RNA in 50 µl of distilled water.

Further steps: quantitation of RNA concentration and purity. cDNA synthesis.

Questions:

1. Why is it necessary to wear gloves during the process?

2. What are the physiological roles of RNases?

3. What can contaminate the RNA sample?

4. What are the advantage of Trizol?

10. Real-time PCR

A real-time polymerase chain reaction (real-time PCR, or qPCR) is a laboratory technique of molecular biology based on the polymerase chain reaction (PCR). It monitors the amplification of a targeted DNA molecule during the PCR (i.e., in real time), not at its end, as in conventional PCR. qPCR does not rely on any downstream analysis such as electrophoresis.

Two common methods for the detection of PCR products in real-time PCR are:

1) non-specific fluorescent dyes that intercalate with any double-stranded DNA

2) sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter, which permits detection only after hybridization of the probe with its complementary sequence

In dye-based qPCR fluorescent labeling allows the quantification of the amplified DNA molecules by employing the use of a dsDNA binding dye. During each cycle, the fluorescence is measured. The fluorescence signal increases proportionally to the amount of replicated DNA and hence the DNA is quantified in "real time". The disadvantages to dye-based qPCR are that only one target can be examined at a time and that the dye will bind to any ds-DNA present in the sample.

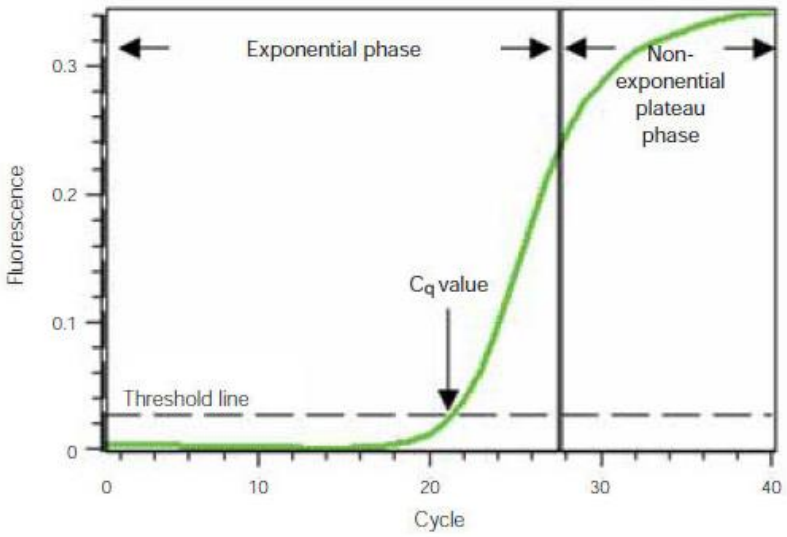
In probe-based qPCR, many targets can be detected simultaneously in each sample but this requires optimization and design of a target-specific probe(s), used in addition to primers. There are several types of probe designs available, but the most common type is a hydrolysis probe, which incorporates the use of a fluorophore and quencher. Fluorescence resonance energy transfer (FRET) prevents the emission of the fluorophore via the quencher while the probe is intact. However, during the PCR reaction, the probe is hydrolyzed during primer extension and amplification of the specific sequence it is bound to. The cleavage of the probe separates the fluorophore from the quencher and results in an amplification-dependent increase in fluorescence. Thus, the fluorescence signal from a probe-based qPCR reaction is proportional to the amount of the probe target sequence present in the sample. Because probe-based qPCR is more specific than dye-based qPCR, it is often the technology used in qPCR diagnostic assays.

Quantification cycle (Cq): When enough amplified product accumulates to yield a detectable fluorescence signal. The cycle number at which this occurs is called the Cq. Because the Cq value is measured in the exponential phase when reagents are not limited, real-time qPCR can be used to reliably and accurately calculate the initial amount of template present in the reaction based on the known exponential function describing the reaction progress.

Amplification plot

The Cq of a reaction is determined mainly by the amount of template present at the start of the amplification reaction. If a large amount of template is present at the start of the reaction,

relatively few amplification cycles will be required to accumulate enough product to give a fluorescence signal above background. Thus, the reaction will have a low, or early, Cq. In contrast, if a small amount of template is present at the start of the reaction, more amplification cycles will be required for the fluorescence signal to rise above background. Thus, the reaction will have a high, or late, Cq. This relationship forms the basis for the quantitative aspect of real-time PCR.



Amplification plot

Dyes:

- SYBR Green
- Ethidium bromide
- ROX

This method can be used in combination with reverse transcription-PCR. In the first steps in the RT-qPCR the RNA will be converted into cDNA and the cDNA will be amplificated in the presence of probes or dyes.

However, we have to use specific primers the qPCR. Synthesised primers are often used to detect specific genes of interest. For example:

Beta actin:

5’CACCATTGGCAATGAGCGGTTC3’

5’AGGTCTTTGCGGATGTCCACGT3’

Such specific primers can be used to determine the expression of a particular gene, so that the effects of different treatments n be detected and monitored.

Questions

1. Why are we using the Cq value?

2. Why are we using specific primers?

3. What is the difference between conventional PCR and qPCR?

4. What methods can we use to calculate relative gene expression?

11. Electron microscopy

Electron microscopy (EM) is a technique used to obtain ultrahigh-resolution images of individual atoms of materials and internal structures of cells. The resulting images can be used to investigate the sample properties and behaviour. It is used in biomedical research to investigate the detailed structure of tissues, cells, organelles and macromolecular complexes. Electron microscopy is used in conjunction with a variety of ancillary techniques (e.g., thin sectioning, immuno-labelling, negative staining) to answer specific questions. EM images provide key information on the structural basis of cell function and cell disease. The use of electrons as the imaging radiation source allows for greater spatial resolution (on the tens of picometers scale) as compared to the resolution achieved using photons in optical microscopy (~200 nanometers).
Electron microscopy can be divided into two main categories: scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Scanning electron microscopy (SEM):

- uses a relatively low-power electron beam for imaging and interaction with the sample
- Electron detectors identify secondary electrons at the surface and backscattered electrons in deeper regions.
- SEM requires little or no sample preparation
- There is really no limit to the sample size, other than it must be able to be introduced into and fit in the analysis chamber.
- much faster and less restrictive than other types of electron microscopy
- High spatial resolution of sample surfaces (~ 0.5 nm) providing 3-dimensional topographic information with secondary electrons.
- large samples can be directly imaged
- It provides detailed images of the surfaces of cells and whole organisms that are not possible by TEM.
- Appropriately equipped SEMs (with secondary, backscatter and X-ray detectors) can be used to study topography and atomic composition of specimens, and also, for example, the surface distribution of immuno-labels.

Transmission electron microscopy (TEM):

- used to view thin specimens (tissue sections, molecules, etc.) through which electrons can pass generating a projection image
- used, among other things, to image the interior of cells (in thin sections), the structure of protein molecules (contrasted by metal shadowing), the organization of molecules in viruses and cytoskeletal filaments (prepared by the negative staining technique), and the arrangement of protein molecules in cell membranes (by freeze-fracture)
- When samples are too thick, they must first be made thin enough for electrons to travel through them, ideally 100 nanometers or less.
- Nanomaterials can be analyzed through TEM to reveal their structure and composition information at the atomic level.

- Samples are then mounted onto a TEM grid and studied under ultrahigh vacuum conditions with a focused, intense electron beam.
- Biological samples need some form of heavy metal staining, with protocols using uranyl acetate and lead citrate being the most common.
- TEM utilizes the selected area diffraction (SAD) of electrons passing through the sample to provide crystallographic information about the sample material.

12. Fragment analysis

Fragment analysis is a genetic analysis method comprising a series of techniques in which DNA fragments are fluorescently labelled, separated by capillary electrophoresis (CE), and sized by comparison to an internal standard. Fragment Analysis (FA) is a powerful technique used in a wide range of applications: detection of mutations, genotyping, identifying short tandem repeats, and DNA profiling.

The DNA fragment analysis consists of four general steps:

- DNA extraction: Labelling fragments with fluorescent dyes. Multiple different colored fluorescent dyes can be detected in one sample. One of the dye colors is used for a labelled size standard present in each sample. The size standard is used to extrapolate the base-pair sizes of the sample product peaks.
- PCR amplification: Amplifying the labeled fragments using PCR on a thermal cycler.
- Capillary electrophoresis: Separating the fragments by size using capillary electrophoresis.
- data analysis
    - Size: The analysis software uses the size standard in each sample to create a standard curve for each sample. It then determines the relative size of each dye-labeled fragment in the sample by comparing fragments with the standard curve for that specific sample.
    - Genotype: The analysis software assigns allele calls based on user-defined makers

12. ELISA

The enzyme-linked immunosorbent assay (ELISA) is an immunological assay commonly used to measure antibodies, antigens, proteins and glycoproteins in biological samples. Some examples include: diagnosis of infection and measurement of cytokines or soluble receptors in cell supernatant or serum.
ELISA assays are generally carried out in 96 well plates. In an ELISA assay, the antigen is immobilized to a solid surface. This is done either directly or via the use of a capture antibody itself immobilized on the surface. The antigen is then complexed to a detection antibody conjugated with a molecule amenable for detection such as an enzyme or a fluorophore.

Enzyme: Horse Radish Peroxidase (HRP) glycoprotein with 4 lysine residues attaches to the secondary antibody for detection.

Substrate of HRP (chromogen): TMB (3,3',5,5'tetramethylbenzidine). It acts as a catalyst to oxidize substrate in the presence of hydrogen peroxide to produce a blue colour.

Stop solution: terminates the horseradish peroxidase reaction in the middle of the linear phase. Addition of the Stop solution results in the rapid formation of a yellow end product. The optical density of this yellow end product is measured at 450 nm in a linear range. The yellow end product is stable for sixty minutes after the addition of Stop Solution and so optical density should be measured within that time.

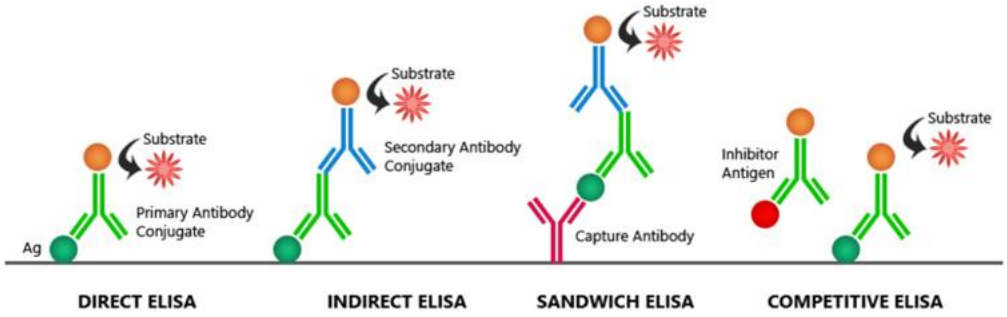
Advantages

- High sensitivity and specificity: it is common for ELISAs to detect antigens at the picogram level in a very specific manner due to the use of antibodies.
- High throughput: commercial ELISA kits are normally available in a 96-well plate format. But the assay can be easily adapted to 384-well plates.
- Easy to perform: protocols are easy to follow and involve little hands-on time.
- Quantitative: it can determine the concentration of antigen in a sample.
- Possibility to test various sample types: serum, plasma, cellular and tissue extracts, urine, and saliva among others.

Disadvantages

- Temporary readouts: detection is based on enzyme/substrate reactions and therefore readouts must be obtained in a short time span.
- Limited antigen information: information limited to the amount or presence of the antigen in the sample.

Types of ELISA:



Direct ELISA

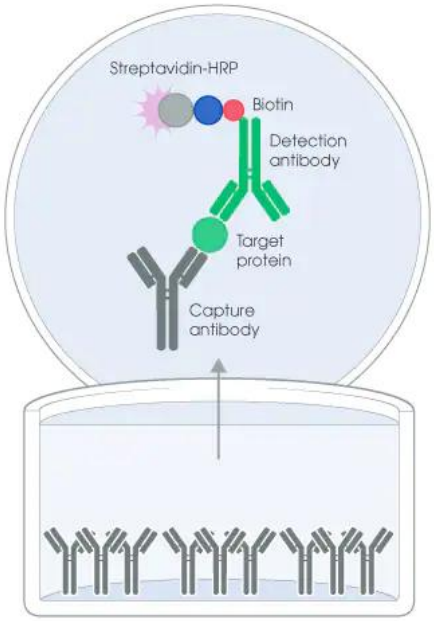
- Apply a sample containing the antigen to a solid surface.
- Enzyme linked primary antibody is added to the plate.
- Washing. After washing only antibody-antigen complexes remained to be attached to the plate.
- Apply a substrate (chromogen) that is converted by the enzyme and produces a colourful signal.

Indirect ELISA

- Antigen is added to the surface of the plate
- Buffer added.
- Primary antibody is added to the plate.
- Washing to eliminate unbound antibodies.
- Secondary antibody conjugated with HRP is applied, which recognizes the primary antibody.
- Washing to eliminate unbound antibodies.
- Addition of chromogen that is converted to a detectable form.

Sandwich ELISA

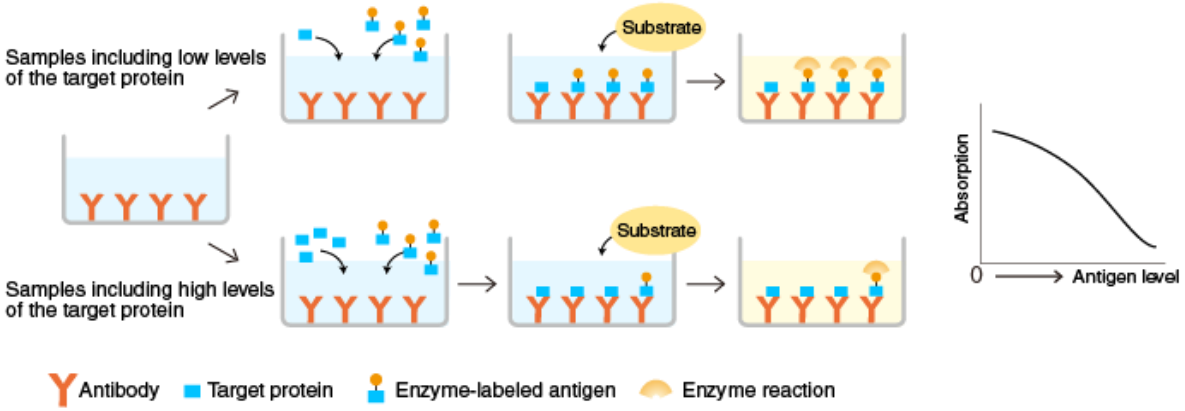
- The plate is covered with a capture antibody for the antigen.
- Buffer, then samples are added to the plate.
- Washing to eliminate disturbing materials from the samples.
- The detection antibody attaching to the antigen holds biotin (called biotinylated) as a connecting molecule for HRP.
- Washing to eliminate unbound antibodies.
- Adding HRP that is conjugated with avidin (called streptavidin HRP) that recognizes and attaches to the biotin on the detection antibody in one-one ratio.
- Washing to eliminate unbound antibodies.
- TMB substrate is added to generate a signal. The intensity of this signal is directly proportional to the concentration of the target present in the original specimen.



Competitive ELISA

Measure the concentration of an antigen by detection of signal interference.

1. Type:
    - The sample antigen competes with a reference antigen for binding to a specific amount of labelled antibody.
    - The reference antigen is pre-coated on a multi-well plate and the sample is pre-incubated with labelled antibody and added to the wells.
    - Depending on the amount of antigen in the sample, more or less free antibodies will be available to bind the reference antigen.
    - The more antigen there is in the sample, the less reference antigen will be detected and the weaker the signal.
2. Type:
    - Utilizes labeled antigen instead of a labeled antibody.
    - The labelled antigen and the sample antigen (unlabelled) compete for binding to the primary antibody.
    - The lower the amount of antigen in the sample, the stronger the signal due to more labeled antigen in the well.



Comparison of ELISAs

	Advantages	Disadvantages
Direct	• short protocol • no cross-reactivity from secondary antibody	• potential high background (all proteins in the sample bind to the surface) • no signal amplification • low flexibility: the primary antibody must be conjugated
Indirect	• signal amplification (several secondary antibodies will bind to the primary antibody) • high flexibility (the same secondary antibody may be used for several primary antibodies)	• long protocol if compared to direct ELISA • potential cross-reactivity from secondary antibody
Sandwich	• high specificity (involves two antibodies detecting different epitopes on the same antigen) • suitable for complex samples • high flexibility and sensitivity (both direct and indirect methods can be used)	• demanding design (finding two antibodies against the same target that recognize different epitopes and work well together can be challenging at times)
Competitive	• depends on base ELISA selected • suitable for small antigens	• depends on base ELISA selected

(Ref.: Basic principles and types of ELISA | Abcam)

Questions

1. What are the principles of ELISA?

2. What is the difference between direct and indirect ELISA?

3. What is the role of the avidin-biotin complex in ELISA?

4. What is the base of detection of ELISA?

5. Why the stop solution is needed for the detection?