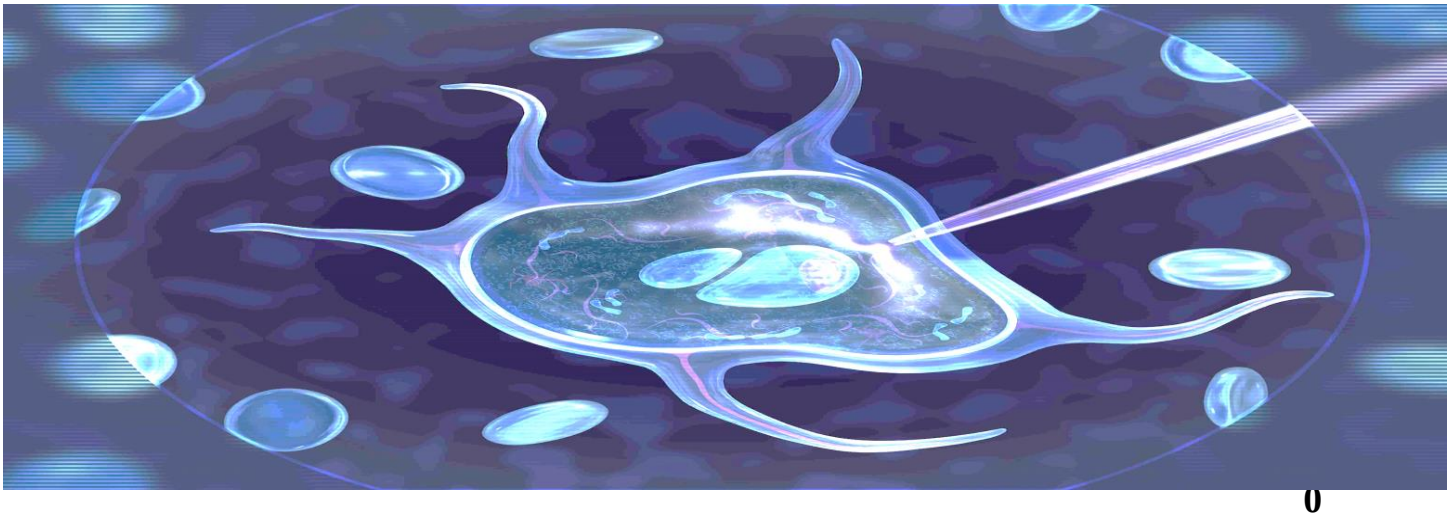
Biotechnology & Genetic Engineering
-Practical-

(BCH 462)- Handnote

Prepared by:
Ghadah A. Alamro & Jameelah M. Almusallam
Demonstrators in Biochemistry Department, College of Sciences, King Saud University



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Introduction

Brief introduction:

Biotechnology is technology that utilizes biological systems, living organisms or parts of this to develop or create different products [(1)].

Genetic engineering is the process by which scientists modify the genome of an organism. Creation of genetically modified organisms requires recombinant DNA. Recombinant DNA is a combination of DNA from different organisms or different locations in a given genome that would not normally be found in nature. The manipulation of genetic material will produce organisms with desired heritable traits or characteristics. On the other hand, this manipulation could have some potential risks, for example, the inserted genes may have unexpected harmful effects [(2)].

General lab safety and types of hazards in biotechnology lab

As shown in Safety lab sheet.

Sterile technique for Bacterial cultures.

All strains of Escherichia coli are potentially pathogenic. Before opening any culture vessel think carefully. What needs to be done before and after manipulation? What you are going to do with the culture vessel? What you are going to do with any equipment that has come into contact with its contents?

Safe sterile technique is largely a matter of common sense. It has two major aims:

1) Preventing contaminant organisms from getting into your cultures. Contamination into cultures can occur from the air when cultures are opened for manipulation, so open them for a minimum time and use smooth movements to reduce sudden air currents. It may also occur from unsterile equipment, as when a pipette-tip touches your filthy, bacteria-laden hands, so be careful with any used pipette, dispose them immediately.

2) Preventing any organisms or accidental contaminants from getting out. Escape from cultures can occur from dripping pipettes, from putting used spreaders and inoculating hoops on the bench without sterilising them first, by aerosols formed by blowing bubbles through cultures with pipettes, or by rapid air movement (particularly with dry fungal spores).

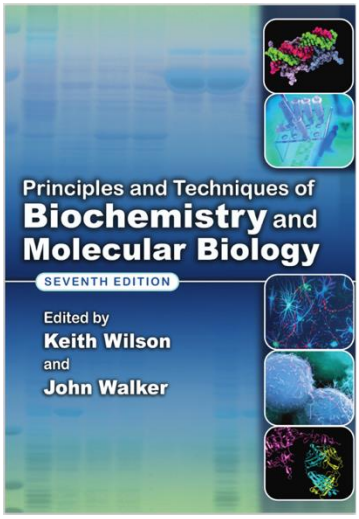
The media and glassware you have been provided will be autoclaved in steam at 121°C for 20 min. However, the mouths of culture tubes, inoculating hoops and spreaders will need to be flamed using with a Bunsen burner. Used pipettes and discarded tubes must be disposed of into disinfectant. Use proper sterile technique whatever the organisms that being used. Because you never know when a culture may have been contaminated, and you owe a duty of care to yourself and others not to filthy your lab space with potentially pathogenic bacteria.

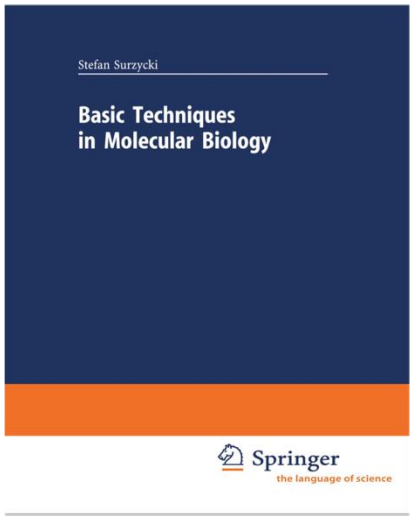
Practical hints to remember during your work in biotechnology lab:

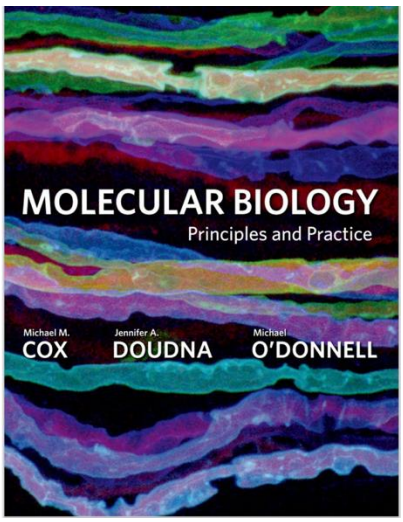
1) Always wash your hands and spray with 70% ethanol or wear gloves.
2) Always keep the caps on the polystyrene tubes loose so that air can circulate. Only cap tightly when the cells are no longer growing and are being stored in the refrigerator until the transformation efficiency has been calculated.

3) When scraping the frozen cell sample, hold the microcentrifuge tube at the top rather than at the bottom, so that the sample does not fully thaw. (one may want to keep the frozen samples on dry ice.)
4) Always have a negative control in the experiments. A negative control is used to show that the media is not contaminated and what is growing in the media are the bacteria cells and not contaminants.
5) Ampicillin can cause allergic reactions on contact with skin to those who are sensitive to penicillin. Do not touch the agar.

Sources of information







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References:

1) https://www.ntnu.edu/ibt/about-us/what-is-biotechnology
2) https://library.scotch.wa.edu.au/sciences/year10/biotechnology/geneticengineering

Experiment (1): Plasmid Isolation and Purification

Aim:

- To isolate pure plasmid DNA from E. coli using alkaline lysis method.

Introduction:

The DNA of most bacteria is contained in a single circular molecule, called the bacterial chromosome. In addition to the chromosome, bacteria often contain an extra hereditary genetic element called plasmids which are small, double stranded, closed circular DNA molecules that replicate independently from a bacterial chromosome. Every plasmid has its own origin of replication (replicon) and use the enzymes and proteins that encoded by their host for their replication and transcription. Plasmid found in a wild variety of bacterial species and they are not essential for the bacterium but benefit the survival of the organism. There are three general classes for plasmids which can be advantageous for host cell: i. Virulence plasmids encoding toxin genes, ii. Drug-resistance plasmids that confer resistance to antibiotics and iii. Plasmids encode gene required for bacterial conjugation.

- PAUSE AND THINK ➔ How plasmid could contribute to drug resistance?

Plasmids have become used in different applications including molecular cloning, gene therapy, drug production and making a large amount of proteins. Plasmids are widely used as **vectors** in molecular cloning, serving to drive the replication of recombinant DNA sequences within host organisms (It is used to provide a "vehicle" in which to insert a desired DNA fragment). In the laboratory, the modified plasmids (recombinant DNA) are usually reintroduced into a host cell for replication via process called *transformation*.

- PAUSE AND THINK ➔ Is there another method than molecular cloning used for DNA amplification?

Generally, plasmid vectors should contain three important parts: origin of replication (Ori), antibiotic resistance gene and cloning site. The Ori is a DNA sequence which allows initiation of replication of the plasmid by cellular enzymes. Antibiotic resistance gene allows for selection of plasmid-containing bacteria. Cloning site is a short segment of DNA which contains several restriction sites allowing for the easy insertion of DNA (A place to insert foreign DNAs).

Isolation of plasmid DNA from bacterial cells is an essential step for many molecular biology procedures. In general, plasmid purification involved three steps: 1. Growth of the bacterial culture, 2. Harvesting and lysis of bacteria and 3. Purification of plasmid DNA. Depending upon nutritional status, bacteria exhibit different growth patterns which include:

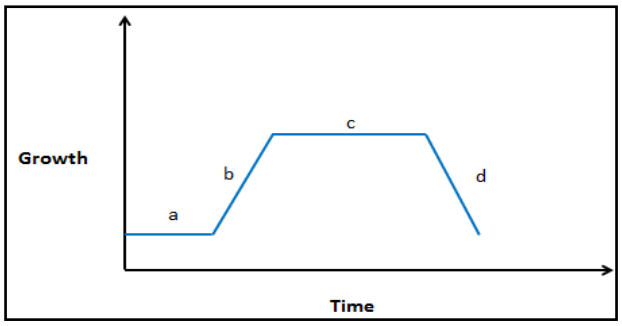

Figure.1. Bacterial culture growth curve.

a) **Lag phase:** in this phase bacteria adapt themselves to growth conditions and synthesis its own DNA, RNA and proteins.
b) **Log phase:** it is exponential phase, the bacterial cells divide and the production of new cells is proportion to increased time.
c) **Stationary phase:** the growth rate slows as nutrients become limited, waste products accumulate and the rate of cell division equals the rate of death.
d) **Death phase:** due to continuous accumulation of toxic metabolites and the lack of nutrients, death occurs of the bacteria.

- PAUSE AND THINK ➔ In which phase should the bacteria take for plasmid isolation?

In the second step, the bacteria are recovered by centrifugation and lysed by any one of many methods, including treatment with detergents, alkali, organic solvents, and heat. The choice among these methods depends on three factors: the size of plasmid, the bacterial strain and the technique used to subsequently purify the plasmid DNA.

The plasmid purification procedures, unlike the procedures for purification of genomic DNA, should involve removal of not only protein but also another major impurity - bacterial chromosomal DNA. There are basic methods of plasmid preparation: chemical base lysis methods, and application of affinity matrixes for plasmid or proteins.

Principle:

In the alkaline lysis method, cells are lysed and DNA denatured by SDS and alkaline pH. The SDS will lyse the bacterial cell membrane and denature the proteins. While the alkaline pH will denature the genomic DNA and the proteins too. Neutralization of the solution results in a fast reannealing of covalently closed plasmid DNA due to the interconnection of both single-stranded DNA circles. Much more complex bacterial chromosomal DNA cannot reanneal in this short time and forms a large, insoluble DNA network, largely due to interstrand reassociation at multiple sites along the long linear molecules. At the next step of the procedure, lowering the temperature results in precipitation of protein-SDS complexes. Subsequently both complexes, DNA and protein, are removed by centrifugation leaving native plasmid molecules in the supernatant.

Alkaline lysis purification method performing steps:

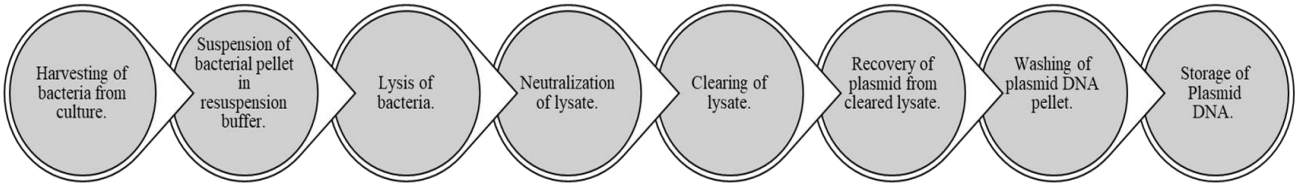

Figure.2. A typical steps of isolation procedure by alkaline lysis method.

Materials:

Chemical

LB medium, Ethylene diamine tetra acetate (EDTA), NaOH, Tris-HCl, glucose, potassium acetate, acetic acid, Sodium dodecyl sulphate (SDS), NaCl, Tryptone, Yeast extract, Tris-Cl, Ethanol.

Preparation of solutions

1) LB medium

To 950 ml of deionize H_2O add 10g Tryptone, 5g yeast extract, and 10g NaCl. Shake until the solution dissolve. Adjust pH to 7.0 with 5N NaCl. Adjust the volume to 1L with deionize H_2O. Sterilize by autoclave for 20 minutes at 15 psi on liquid cycle.

2) Alkaline lysis solution I

50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0), deionized water.

3) Alkaline lysis solution II

0.2 N NaOH, 1% (w/v) SDS,deionized water.

4) Alkaline lysis solution III

5 M potassium acetate, acetic acid, deionized water.

Equipment and Glassware

Microfuge centrifuge, electronic balance, microcentrifuge tube, centrifuge tube, Pasteur pipette, micropipette, tips.

Protocol:

1. Centrifuge the bacterial samples at 4 °C, maximum speed for 5 minutes, using microcentrifuge device.
2. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.
3. Resuspend the bacterial pellet in 100 µl of ice cold alkaline lysis solution I then vortex vigorously.
4. Add 200 µl of freshly prepared alkaline lysis solution II to the bacterial suspension. Invert the tube rapidly 5 times. Store the tube on ice for 1 min.

5. Add 150 µl ice cold alkaline lysis solution III to the microcentrifuge tube. Invert the tube 3-5 times, then incubate the tube on ice for 3-5 minutes.
6. Centrifuge the bacterial lysate at maximum speed for 3 minutes.
7. Transfer the supernatant to a new labelled microcentrifuge tube.
8. To the tube, add 2 volumes of 95% ethanol.
9. Vortex and allow the tube to stand at room temperature for 2 minutes.
10. Centrifuge at maximum speed for 5 minutes.
11. Remove the supernatant by gentle aspiration.
12. Stand the tube in an inverted position over a paper towel to allow all fluid to drain away.
13. Add 20 µl of 70% ethanol, then invert the closed tube several times.
14. Centrifuge at maximum speed for 5 minutes.
15. Remove the supernatant by gentle aspiration.
16. Remove any beads of ethanol from the sides of the tube. Leave tube open at room temperature until residual ethanol has evaporated.
17. Dissolve the pellet in 25-50 µl sterile water or TE buffer and vortex the solution gently for few seconds.
18. The plasmid DNA can be stored at -20 °C.

Results:

- Concentration of plasmid DNA (ng/µl) = _____
- Plasmid purity: A_{260}/A_{280} = _____

References:

1. Clark D, Pazdernik N. (2013)Molecular biology. Elsevier Inc.
2. Surzycki S. (2000) Basic techniques in molecular biology. Springer.
3. Cox M, Doudna J, O'Donnell M. (2012) Molecular Biology genes to proteins.
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Experiment (2): Competent Cells Formation and Transformation of Competent Cells with recombinant plasmid DNA

Aims:

- Making a competent cells using calcium chloride method.
- Transformation of the competent cells with recombinant plasmid DNA using chemical transformation method.

Introduction:

Molecular cloning is an important tool to understand the structure, function and regulation of individual genes and their products. It is a cell-based technique used to create copies of certain DNA fragments using a vector carrying the DNA of interest, which eventually inserted to a host cell (usually bacteria) and self-replicate. The cell-based DNA cloning involve four steps:

1. *Construction of recombinant DNA molecules:* which is the insertion of DNA fragment into a cloning vector (e.g. plasmid). This step is facilitated by cutting the target DNA and replicon molecules with specific restriction endonucleases before joining the different DNA fragments using the enzyme DNA ligase.

- PAUSE AND THINK ➔ What are different important features that the cloning vectors should have?

2. *Transformation:* which is the introducing of the recombinant DNA into bacterial cells (the host) and in which the recombinant DNA amplified using bacterial DNA replication machinery.
3. *Selective propagation of cell clones:* which is the plating of clones on selection media to allow screening.
4. *Isolation of recombinant DNA.*

Bacteria are able to take up DNA from their environment (exogenous DNA) in three ways: *conjugation, transformation, and transduction*. Only transformation is the direct uptake of DNA, since conjugation requires cell-cell contact via a sex pilus and transduction requires a bacteriophage intermediary to transfer DNA from one cell to another. For a bacterial cell to take up DNA from its surroundings, it must be in a special physiological state called **competence**. It defines as the ability of the cell to undergo transformation (the ability of a cell to take the DNA from the environment). There are two classes of competent cells: *Natural competence:* a genetically specified ability of bacteria that is occur under natural condition, and *artificial competence:* when cells in laboratory cultures are treated to be permeable to DNA.

Transformation is a very basic technique that is used on a daily basis in a molecular biological laboratory. In 1971-1973 recombinant DNA technology was developed as gene cloning to be its major application. Transformation can be done using different methods including: electroporation (or Electropermeabilization) and less the less efficient method called Chemical transformation.

Transformation efficiency is a quantitative value that describes how effective you were at getting plasmid DNA into your competent cells. The number represents how many cells were transformed per microgram (µg) of plasmid DNA used. This calculation requires two values: the number of cells that were successfully transformed and the amount of plasmid DNA used for the transformation.

Principle:

Introduction of recombinant plasmid into cells is achieved by the transformation of competent cells. In the chemical transformation method the competent cells are prepared by treating the cell with a divalent cation like calcium chloride solution which help the cells to take up the DNA plasmid by increasing the bacterial cells membranes permeability [renders them competent to take up DNA]. Once the cells are made competent, the plasmid DNA is mixed with the cells. The competent cells are then subjected to heat shock, which allows the DNA to enter the cells. The cells are then plated onto a LB agar plate containing appropriate antibiotic to be able to count the transformed colonies only (which they are colonies containing transformed cells containing the recombinant DNA), each colony on an antibiotic plate presents a single transformation event. The recombinant plasmid can be amplified as well.

Materials:

Chemical

$CaCl_2$, LB medium, NaCl, Tryptone, Yeast extract**,** Ampicillin

Preparation of solutions

1) LB medium

To 950 ml of deionize H_2O add 10g Tryptone, 5g yeast extract, and 10g NaCl. Shake until the solution dissolve. Adjust pH to 7.0 with 5N NaCl. Adjust the volume to 1L with deionize H_2O. Sterilize by autoclave for 20 minutes at 15 psi on liquid cycle.

2) 50 M $CaCl_2$ solution:

Dissolve 7.4g of CaCl2.2H2O in d.H2O, make up the volume up to 1L by d.H2O. Autoclave the solution then store it at 4°C

3) Ampicillin:

Dissolve ampicillin sodium salt in AnalaR water to a concentration of 100 mg/ml. Filter the solution through a 0.2µm filter. It should be added to a final concentration of 100 µg/ml. Store at 20 °C**.**

Equipment and Glassware

Microfuge centrifuge, electronic balance, water bath, roller, plate, incubator, microcentrifuge tube, centrifuge tube, Pasteur pipette, micropipette, tips.

Protocol:

A) Competent cells formation:

1. Centrifuge 10-15 ml of bacterial sample for 7 minutes at 3000 rpm at 4°C. allow centrifuge to decelerate without break.
2. Discard the supernatant and resuspend each pellet gently in 10 ml ice cold $CaCl_2$ solution.
3. Centrifuge the cells for 5 minutes at 2500 rpm at 4 °C.
4. Discard the supernatant and resuspend each pellet in 10 ml ice cold $CaCl_2$ solution.
5. Keep resuspended cells on ice for 10 minutes.
6. Centrifuge the cells 5 minutes at 2500 rpm at 4 °C.
7. Discard the supernatant and resuspend each pellet in 2 ml ice cold $CaCl_2$ solution.

B) Transformation of competent cells with DNA:

1. Transfer 100 ng of plasmid DNA in a volume of 25 µl into clean microcentrifuge tube and place it on ice.
2. Rapidly add 100 µl of competent cells immediately into microcentrifuge tubes containing the plasmid DNA. Gently swirl tubes to mix, then place them on ice for 10 minutes.
3. Store the remaining competent cells at -80 °C.
   Note: Competent cells should be used immediately after thawing. Remaining cells should be discarded rather than refroze it.
4. Using the thermomixer, heat shock the cells by placing the tubes into a 42 °C for 45 sec.
5. Add 1 ml LB medium (without antibiotic).
6. Place each tube on the thermomixer at 250 rpm for 20-60 minutes at 37°C.
7. Plate aliquot of transformation culture on LB/Ampicillin or other appropriate antibiotic containing plates.
8. When plates are dry, incubate 24 to 48 hours at 37 °C.
9. A negative control should also be included that contains cells with no added plasmid DNA.
10. Calculate the transformation efficiency.

Results:

- **Transformation efficiency calculation:**

Count white colonies as recombinant transformants and test for insert. Calculate the transformation efficiency in terms of the number of colony-forming units (CFU) per microgram of transforming DNA as follows:

$$\text{Transformation efficiency} = \frac{\text{Total number of colonies}}{\text{Amount of DNA plated [µg]}}$$

$$= \_\_\_\_\_ \text{ CFU/µg}$$

References:

1. Campbell N, Reece JB. (2005) Biology. 7th edition. Pearson.
2. Cohen S.N., Chang A.C.Y., and Hsu L. 1972. Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of Escherichia coli by R-factor DNA.Proc. Natl. Acad.Sci. 69: 2110-2114.
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Experiment (3): Extraction and Determination of Bacterial Proteins

Aims:

- Extraction of total bacterial proteins.
- Determination of bacterial proteins using biuret method.

Introduction:

A bacterial protein is a protein which is either part of the bacterium structure or produced by bacterium as a part of its life cycle. Research on bacterial proteins has been performed with the goal of learning more about specific proteins and their function which impact human health. A bacterial protein can be toxic, causing illness or death in an organism which has been infected including humans. In addition, the information which has been gained form studding bacterial proteins can be extrapolated to gather more data about the proteins associated with larger organisms.

Furthermore, bacteria can produce foreign proteins from introduced genes, using their own gene expression machinery. Scientists routinely clone the gene that encodes 'their' protein and express large amounts of it in bacteria. Many medicines and drugs – particularly hormones – are proteins. These include insulin (for treating diabetes), erythropoietin (for treating anaemia), growth hormone (for treating growth disorders) and others. Today, bacteria (and other organisms) are used routinely as biological 'factories' to produce protein medicines in large amounts by cloning the desired genes.

Principle:

Isolation of bacterial proteins involves several steps: 1. Growth and induction of bacterial cultures, 2. Lysis of cells in a suitable buffer containing a detergent, 3. DNase and RNase treatment for the removal of the nucleic acids, 4. Determine the protein concentration using suitable method and 4. Passage of the extract through an affinity resin and finally elution of proteins.

In this lab determination of total bacterial proteins will done using biuret method. Biuret method is based on copper ions binding to peptide bonds of protein under alkaline condition to give a violet (purple) color which has maximum absorbance at 540 nm. The intensity of the color resulting from the (Cu+protein) complex is linearly proportional to the concentration of protein present in the solution.

Materials:

Chemical
LB medium, Distilled water, BSA stock solution (3 g/l), Biuret reagent, and Lysis buffer
Preparation of lysis buffer
Containing the following: 140mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4
Equipment and Glassware
Microfuge centrifuge, electronic balance, water bath, spectrophotometer, microcentrifuge tube, centrifuge tube, Pasteur pipette, micropipette, tips, pipette 5ml, test tubes, plastic cuvettes.

Protocol:

A) Extraction and isolation of bacterial proteins:

1. Centrifuge the bacterial sample (6 ml of overnight culture) for 5 minutes at 3000 rpm at 4 °C.
2. Resuspend the pellet in 1 ml lysis buffer.
3. Sonicate for 30–60 s in ice bucket until the cells are completely disrupted.
4. Transfer the resuspended sample to microcentrifuge tube, then spin 5 min at 13000 rpm at 4°C.
5. Separate soluble proteins (supernatant) from insoluble proteins (pellet). Use supernatant (soluble proteins) for next step.
6. Resuspend the pellet in another 1 ml lysis buffer and use supernatant for next step. (Insoluble proteins).

B) Determination of total bacterial proteins concentration:

1. Set up 10 test tubes as following:

Test tube	Distilled water [µl]	Stock BSA solution (35 g/l) [µl]	Sample [µl]	Protein concentration [g/l]
Blank	250	-	-	
A	200	50	-	
B	150	100	-	
C	100	150	-	
D	50	200	-	
E	-	250	-	
F (Unknown soluble proteins)	-	-	250	?
F'	125	-	125	?
G (Unknown insoluble proteins)			250	?
G'	125		125	?

2. Incubate all the tubes in water bath at 37°C for 5 minutes.
3. To each tube, add 1000 µl of Biuret reagent. Mix well and allow standing for 5-10 minutes in the 37 °C water bath.
4. Measure the absorbance of solutions at 540 nm.

5. Plot standard curve for absorbance against BSA concentration using results for solutions (A-E).
6. From the standard curve, estimate the concentration of proteins presents in your samples.

Results:

Test tube	Protein concentration [g/l]	Absorbance at 540 nm
Blank		
A		
B		
C		
D		
E		
F	_____	
F'	_____	
G	_____	
G'	_____	

References:

1. https://www.sciencelearn.org.nz/resources/1959-producing-foreign-proteins-in-bacteria
2. Todar K. "Bacterial Protein Toxins." Online Textbook of Bacteriology. University of Wisconsin, 2011.

Experiment (4): Western Blot

Aims:

- To understand how proteins (antigens) can be analysed using antibodies raised against these proteins by Immunoblotting technique.
- To perform the steps of western-blot technique to detect the specific protein.

Introduction:

Immunoassay is a test that uses the highly specific and selective antigen-antibody reactions forming antibody and antigen complexes [immuno-complexes] as a means of generating measurable results. Western blot (also called protein immunoblot) is a widely used immunoassay technique, used to identify proteins specific proteins [antigens] in a sample of tissue homogenate or extract, based on their ability [the antigens] to bind to antibodies resulting in colour indicate the presence of this specific protein. [1,2]

Western blot has various applications for research use such as protein expression level, epitope mapping and to detect the phosphorylation signal and structure domain analysis.[3]

- PAUSE AND THINK ➔ Is there other reactions with high specificity than antigen-antibody reactions?

Principle:

The mixture of proteins is separated based on molecular weight, and thus by type, through SDS-PAGE. These results are then electro-transferred to a nitrocellulose polyvinylidenedifluoride (PVDF), or nylon membrane producing a band for each protein. The transferred protein is detected by incubating the gel with specific primary antibody to the protein of interest, secondary antibody labelled with an enzyme which target the primary antibody, and substrate which in the end you will get coloured product. Alkaline phosphatase (AP) and horseradish peroxidase (HRP) are the two enzymes used most extensively as labels for protein detection. The colour indicates the presence of the protein of interest. The thickness of the band corresponds to the amount of protein present; thus doing a standard can indicate the amount of protein present. [4]

Thus, the molecular weight and amount of the desired protein can be characterized from a complex mixture of proteins by western blotting.

Western blot performing steps:

The technique uses three elements to accomplish this task: (1) separation by size using SDS-PAGE, (2) transfer to a solid support (electro-blotting), and (3) marking target protein using a proper primary and secondary antibody to visualize.

- **1st phase (SDS-PAGE):** A protein sample is subjected to polyacrylamide gel electrophoresis. The separation of the sample can be confirmed by: 1.Replica of the gel and stain it as usual [with Coomassie brilliant blue R-250] , 2.prestained marker and 3.Ponceau S.

- **2nd phase (Electro-blotting):** After that the gel is placed over a sheet of nitrocellulose, the protein in the gel is electrophoretically transferred to the nitrocellulose membrane. The transfer can be done by wet method or semi-wet method. This done by Creating a transfer sandwich: filter papers-gel-nitrocellulose membrane-filter papers. The filter papers, gel and nitrocellulose membrane will be soaked in transfer buffer. Because the samples in the gel are negatively charged, the applied electric current will facilitate their transferring to nitrocellulose membrane, the samples will move toward the anode. Also, the capillary action has its effect in the movement of the samples from the gel to the nitrocellulose membrane.

- **3rd phase (Marking target protein to visualize):** The nitrocellulose is then soaked in blocking buffer to block the nonspecific binding of the proteins. The nitrocellulose is then incubated with the specific primary antibody for the protein of interest. After that the nitrocellulose will washed and then incubated with a second antibody, which is specific for the primary antibody. The second antibody will typically have a covalently attached enzyme which, when provided with a chromogenic substrate, will cause a color reaction (detection step) by converting a colorless substrate to a colored product. Several substrates can be converted to colored precipitate "product" by (AP) and (HRP) enzymes. As the precipitate accumulate on the membrane, a visible band develops.

Material

Transfer buffer: (25mM Tris, 190 mM glycine, 20% methanol, 0.1% SDS)
Adjust the pH to 8.3

Block buffer: (10% milk with 0.5% Tween 20) **Or** 5% BSA (with fluorescent system)

Washing buffer (TBST): (25mM Tris, 0.15M NaCl, 0.05% Tween-20)
Adjust the pH to 7.5

10X PBS: (NaCl, KCl, Na_2HPO_4, KH_2PO_4)
Adjust the pH to 7.4

Protocol

I. Crude extraction of protein from animal tissue

1. Weight the sample, wash it with normal saline, and cut it into small pieces.
2. For each 1g of the sample add 5ml of the extraction buffer (0.1 M Tris-HCl, pH 7.4) and homogenized it in the blender.
3. Transfer the homogenized tissue/buffer mixture into centrifuge tubes.
4. Centrifuge your homogenate for 15 minutes at 3000 xg.
5. Measure the volume of the supernatant.

II. Separation of the protein by SDS-PAGE

1. Prepare 12% of separating gel and 7% of stacking gel.
2. Mix the sample with 10X loading buffer (1:1), then heat them at boiling for 5-10 min.
3. Load 20ul of prepared samples into wells and in different well load 10ul of pre-stained protein marker
4. Run at 120 volts for 60 – 90 minutes

According to: http://www.assay-protocol.com/molecular-biology/electrophoresis/denaturing-page.html

III. Electro-blotting of separated protein (Semidry blotter)

1. Wet the filter papers with transfer buffer.
2. The prewetted nitrocellulose / PVDF membrane is put on top the filter. (i.e PVDF membrane need to be activated with 100% methanol)
3. The gel is put on top of the membrane.
4. Ensure that no air bubbles are anywhere in this stack of membranes.
5. Then wetted filter papers should be placed on top of the gel.
6. Again, remove any bubbles.
7. Put it onto the apparatus.
8. apply a continuous voltage of 25V for 15-20 minutes

IV. Visualization of target protein

1. Block the membrane for 2 hrs at room temperature or overnight at 4°C using blocking buffer.
2. Wash the membrane in three times using TBST, 5 min each. (Optional)
3. Incubate the membrane with 1:1000 dilutions of primary antibody, prepared in 1XPBS for 2 hrs at room temperature or overnight incubation at 4°C.
4. Wash the membrane in three times using TBST, 5 min each.
5. Incubate the membrane with the 1:10000 dilution of secondary antibody, prepared in 1XPBS at room temperature for 1 hr.
6. Wash the membrane in three times using TBST, 5 min each.
7. Put the membrane in 1XPBS and visualize it in ODYSSET CLx device.

Supporting materials:

- Performing western blot: http://www.youtube.com/watch?v=VgAuZ6dBOfs
- Ponceau S staining: http://www.youtube.com/watch?v=Jj_37cDsO7o

References:

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Experiment (5): Enzyme-Linked Immunosorbent Assay (ELISA)

Aim:

- To understand the principle of different types of ELISA.

Introduction:

Immunoassay is a test that uses the highly specific and selective antigen-antibody reactions forming antibody and antigen complexes [immuno-complexes] as a means of generating measurable results. Antigen (Ag) is a substance that when introduced into the body stimulates the production of an *antibody*. Antigens include toxins, bacteria, foreign blood cells, and the cells of transplanted organs. Antibodies (Ab) are large Y-shaped glycoproteins. They are produced by the immune system in response to foreign objects (*antigen*) to identify and neutralize them. Each antibody recognizes a specific antigen (not normally found in the body).[1,2,3]

- PAUSE AND THINK ➔ What called the specific part of the antigen that is recognized by the antibody?

ELISA (enzyme-linked immunosorbent assay) is a biochemical plate-based assay technique designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones. ELISAs are typically performed in 96-well (or 384-well) polystyrene microtiter plates, where antibody or antigen of interest is immobilized. [4] In qualitative ELISA format, the results provide a positive or negative result for a sample whereas in quantitative ELISA, the optical density or florescent units of the sample is interpolated into a standard curve (obtained from serial dilutions of a standard). [5]

ELISA can be used in the field of medicine, food industry and in toxicology labs to evaluate the presence of a specific Ag or Ab in a sample. Different applications include Screening donated blood for evidence of viral contamination and measuring hormones level.[6]

- PAUSE AND THINK ➔ Can we use ELISA to detect autoimmune diseases? How?

Basic Principle:

The basic principle of ELISA is, to detect a specific antibody- antigen reaction by assessing the conjugated enzyme activity which can convert a colourless substrate to a measurable coloured product, indicating the presence of the antibody - antigen [Ab-Ag] binding. The detection enzyme can be linked directly to the primary antibody or introduced through a secondary antibody that recognizes the primary antibody. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction. [4]

ELISA format:

ELISAs can be performed with a number of modifications to the basic procedure:

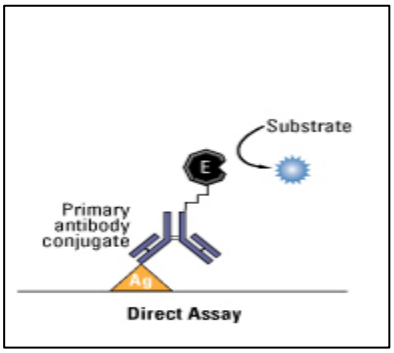
(**1**) Direct ELISA. (**2**) Indirect ELISA. (**3**) Sandwich ELISA. (**4**) Competitive ELISA.

1. Direct ELISA:

This type considered the simplest type of ELISA. It is used to detect the presence and the concentration of specific antigen in the sample.

- **Principle:**

Immobilization of the antigen of interest can be accomplished by direct adsorption or fixation to the assay plate. The antigen is then directly detected by an antibody conjugated with an enzyme. By adding, the enzyme's substrate, the enzyme will convert colourless substrate to coloured product. The colour produced is proportional the amount of the antigen of interest.



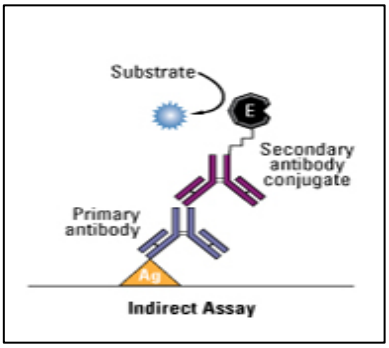
- PAUSE AND THINK ➔ Why this format called "direct ELISA" ?

2. Indirect ELISA:

Is used to detect the presence and the concentration of specific antigen or antibody.

- **Principle:**

This method differs than direct ELISA, in that one more labelled secondary antibody is added in the reaction. The antigen is first captured by primary antibody (which can be the interest), then a secondary enzyme conjugated antibody is added which recognizes the primary antibody. The color or the signal produced as a result of addition of substrate is proportional to antigens/antibodies in the sample.



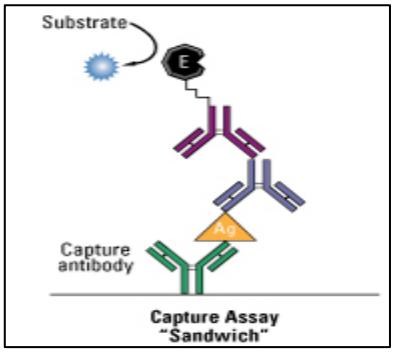
- PAUSE AND THINK ➔ Why this format called "Indirect ELISA" ?

3. Sandwich ELISA:

The most powerful ELISA assay format is the sandwich assay. In sandwich ELISA the antigen in indirectly captured by antibody immobilized in the microtiter plate. This type of capture assay is called a “sandwich” assay because the analyte to be measured is bound between two primary antibodies – the capture antibody and the detection antibody. It is used to detect the presence and the concentration of specific antigen in the sample. The sandwich format is used because it is sensitive and robust. (4)

➢ **Principle:**

The sandwich ELISA quantify/detect antigens between two layers of antibodies (i.e. capture and detection antibody just like a sandwich). The antigen to be measured must contain at least two antigenic epitopes since at least two antibodies bind to the antigen. The colour or the signal produced as a result of addition of substrate is proportional to antigen concentration.



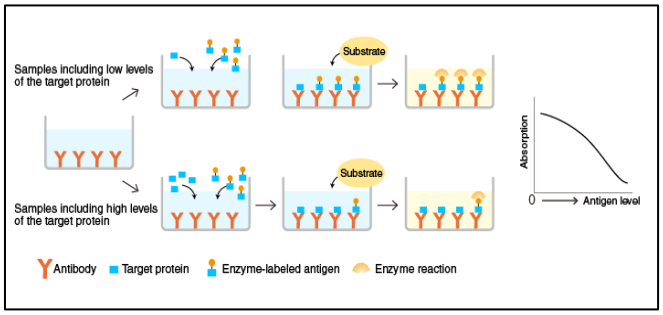
- PAUSE AND THINK ➔ What is the main difference between direct/indirect ELISA and sandwich ELISA?

4. Competitive ELISA:

Is a strategy that is commonly used when the antigen is small and has only one epitope, or antibody binding site. It measures the amount of antigen in a sample. One variation of this method consists of labelling purified antigen instead of the antibody.

➢ **Principle:**

In this type of ELISA, another version of your antigen of interest is labelled instead of the antibody. Unlabelled antigen "your interest" and the "labelled antigen" compete for binding to the capture antibody (antibody that fixed in the plate). The colour or the signal produced as a result of addition of substrate is inversely proportional to antigens of interest in the sample. (4)



- PAUSE AND THINK ➔ What are the differences between this format and standard previous format?

Material & Protocol

According to Ultra Sensitive Mouse Insulin ELISA Kit (Cat# 90080)

Supporting materials:

- Overview of ELISA and supporting videos: https://www.thermofisher.com/sa/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-elisa.html

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Experiment (6): Kirby-Bauer test

Aim:

- To test the ability of antimicrobial agents to inhibit the growth of microorganisms using Kirby-Bauer test method.

Introduction:

A true antibiotic is an antimicrobial chemical produced by microorganisms against other microorganisms. In addition, many drugs are now completely synthetic or the natural drug is manipulated to change its structure somewhat, the latter called semi-synthetics. Bacteria respond in different ways to antibiotics and chemosynthetic drugs, even within the same species. (1)

- PAUSE AND THINK ➔ What could be the cause of the different response of different bacterial species or even bacteria from the same specie toward the antibiotic? How?

An important task is the performance of antimicrobial susceptibility testing of significant bacterial isolates. The goals of testing are to detect possible drug resistance in common pathogens and to assure susceptibility to drugs of choice for particular infections. Different automated and manual methods have been developed in order to screen the antimicrobial susceptibility. (2)

The Kirby-Bauer test (also called the disc diffusion test or zone of inhibition test) is a standard test for antibiotic susceptibility that has been used for years. First developed in the 1950s, it was refined and by W. Kirby and A. Bauer. It has been superseded in clinical labs by automated tests. However, the K-B is still used in some labs or used with certain bacteria that automation does not work well with.

This test is used to determine the resistance or sensitivity of aerobes or facultative anaerobes to specific chemicals, which can then be used by the clinician for treatment of patients with bacterial infections. It tests the ability of antimicrobial agents to inhibit the growth of microorganisms over an 18-24 hour period of contact. The presence or absence of an inhibitory area (zone of inhibition) around the disc identifies the bacterial sensitivity to the drug. (1)

The advantages of the disk method are the test simplicity that does not require any special equipment, least costly of all susceptibility methods and flexibility in selection of disks for testing. The disadvantages of the disk test are the lack of mechanization or automation of the test. Although not all fastidious or slow growing bacteria can be accurately tested by this method, the disk test has been standardized for testing *streptococci, Haemophilus influenzae, and N. meningitidis* through use of specialized media, incubation conditions, and specific zone size interpretive criteria. (3)

Principle:

The activity of the antimicrobial drug is evaluated by the ability of the antibiotic disks with certain concentration in inhibition of the microbial growth. If substantial antimicrobial activity is present, then a zone of inhibition appears around the test product. The zone of inhibition is simply the area on the agar plate that remains free from microbial growth. The diameter of the zone of inhibition is usually related to the level of antimicrobial activity present in the sample or product (a larger zone of inhibition usually means that the antimicrobial is more potent). (4)

Performing steps:

1. The test is performed by applying a bacterial inoculum of approximately $1–2\times10^8$ CFU/mL to the surface of a large (150 mm diameter) Mueller-Hinton agar plate.
2. Up to 12 commercially-prepared, fixed concentration, paper antibiotic disks are placed on the inoculated agar surface (Figure 1).
3. Plates are incubated for 16–24 h at 35°C prior to determination of results.
4. The zones of growth inhibition around each of the antibiotic disks are measured to the nearest millimetre (The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium).

References:

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Experiment (7): Restriction Fragment Length Polymorphism

Aim:

- To get familiar with the RFLP technique.

Introduction:

Restriction endonucleases have come to play a key role in all aspects of molecular biology. These enzymes recognise certain DNA sequences, usually 4–6 bp in length, and cleave them in a defined manner.

PAUSE AND THINK ➔ How restriction enzyme cut the DNA?

Individuality in humans and other species derives from their genetic polymorphism; homologous human chromosomes differ in sequence, on average, every ~1250 bp. These genetic differences create and eliminate restriction sites.
Restriction fragment length polymorphism (RFLP) is an inherited difference in the **pattern of restriction**, which is type of polymorphism that results from variation in the DNA sequence (i.e. Genetic polymorphism is defined as the inherited genetic differences among individuals in over 1% of normal population). Some of the sequence changes affect recognition sites for restriction enzymes, resulting in variation in the size of DNA fragments produced by digestion with a particular restriction enzyme. RFLPs may disrupt the function of the gene or may have no biologic consequences depending on they occurrences at known gene loci and/or in sequences that have no known function.

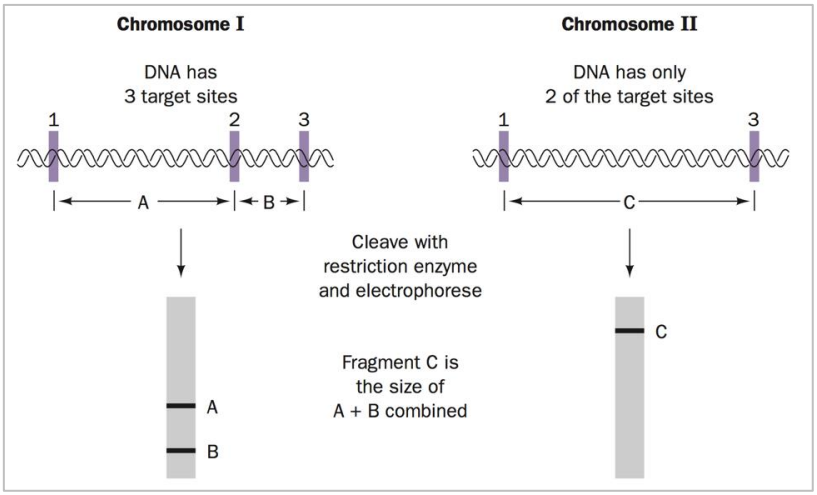


Figure 1: RFLP. A mutational change that affect a RS in a DNA segment alters the number and sizes of its restriction fragments.

RFLP Technique

Restriction fragment length polymorphism (RFLP) is a standard laboratory technique that used to analyse the DNA of genes, it was invented in 1984 by the English scientist Alec Jeffreys during research into hereditary diseases. RFLP analysis allows individuals to be identified based on unique patterns of restriction enzyme cutting in specific regions of DNA. it was an important early tool in genome mapping, localization of genes for genetic disorders, determination of risk for disease, and paternity testing.

Principle:

Restriction endonucleases are enzymes that cut lengthy DNA into short pieces. Each restriction endonuclease targets different nucleotide sequences in a DNA strand and therefore cuts at different sites. The distance between the cleavage sites of a certain restriction endonuclease differs between individuals. Hence, the length of the DNA fragments produced by a restriction endonuclease will differ across both individual organisms and species.

RFLP Workflow:

1st DNA Extraction.
2nd Perform PCR for the region of interest.
3rd DNA Fragmentation by RE.
4th Gel Electrophoresis.
5th Visualization of Bands.

RFLP in molecular analysis and its Applications:

An extensive RFLP map of the human genome has been constructed. This is proving useful in the human genome sequencing project and is an important component of the effort to understand various single-gene and multigenic diseases.

- RFLP in different molecular analysis of disease state:

It is a useful diagnostic tools in genetic diseases where the RFLP can result from single-base changes such in sickle cell disease and in phenylketonuria. Moreover, in thalassemias the RFLP result from deletions or insertions of DNA into a restriction fragment.

- Applications of RFLP:

- To determine or confirm the source of a DNA sample such as in **paternity tests** or **criminal investigations.**
- In genetic mapping to determine recombination rates that show the genetic distance between the loci.
- To identify a carrier of a disease-causing mutation in a family.
- To determine the status of genetic diseases such as Cystic Fibrosis in an individual.

References:

Saraswathy, N., & Ramalingam, P. (2011). Genome mapping. Concepts and Techniques in Genomics and Proteomics, 77–93. doi:10.1533/9781908818058.77

Experiment (8): Quantitative reverse transcription PCR (RT-qPCR)

Aim:

- To have a basic knowledge about using RT-qPCR to evaluate the gene expression.

Introduction:

The polymerase chain reaction (PCR) is one of the most powerful technologies in molecular biology. Using PCR, specific sequences can be amplified. In such traditional PCR (endpoint), detection of the amplicon is performed at the end of the reaction after the last PCR cycle, using gel electrophoresis. In real-time quantitative PCR, a fluorescent reporter dyes used to combine the amplification and detection steps of the PCR reaction. The assay relies on measuring the increase in fluorescent signal, which is proportional to the amount of DNA produced during each PCR cycle, and thus gives a quantitative information on the starting quantity of the amplified target. The reaction is characterized/identified by the PCR cycle at which fluorescence first rises above a defined or threshold background fluorescence, a parameter known as the threshold cycle (Ct).

Generally, RT-qPCR is a combination of three steps: (i) the reverse transcriptase (RT)-dependent conversion of RNA into cDNA, (ii) the amplification of the cDNA using the PCR and (iii) the detection and quantification of amplification products in real time.

PAUSE AND THINK ➔ What are the reaction components of RT-qPCR ?

RT-qPCR applications

The most common application of real-time PCR is the quantification of mRNA (gene expression). However, qualitative detection could be used.

RT-qPCR types:

The RT-qPCR assay can be performed either as a one-tube single RT and PCR enzyme method or a separate RT and PCR enzyme technique using one or a two tubes. **In One-step qRT-PCR,** combines the first-strand cDNA synthesis reaction and real-time PCR reaction in the same tube, simplifying reaction setup and reducing the possibility of contamination. **In Two-step RT-qPCR,** the reverse transcription of RNA into cDNA is done using a reverse transcriptase (RT).

RT-qPCR quantification methods:

Absolute quantification: describes a real-time PCR experiment in which samples of known quantity are serially diluted and then amplified to generate a standard curve. Unknown samples are then quantified by comparison with this curve.

Relative quantification: in which the expression of a gene of interest in one sample (i.e., treated) is compared to expression of the same gene in another sample (i.e., untreated). The results are expressed as fold change (increase or decrease). A normalizer gene (such as β-actin) is used as a control for experimental variability in this type of quantification.

RT-qPCR chemistries (detection systems):

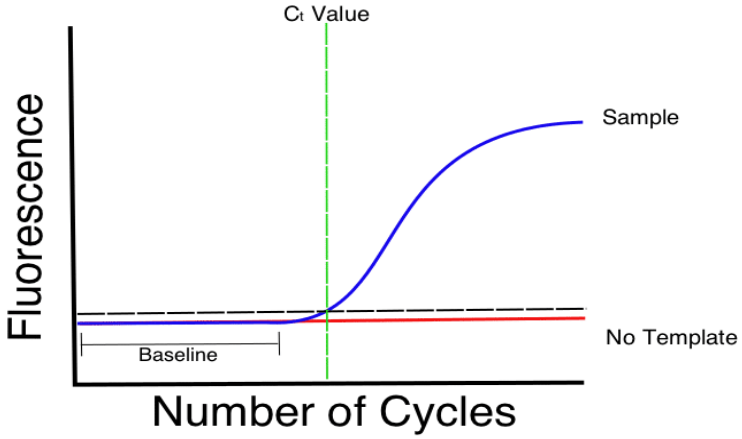
I. Principle of SYBR-green based assay:

The double-steands DNA- intercalation agent (DNA-binding dyes) such as SYBR Green 1. The SYBR Green 1 is only fluorescing when intercalated into dsDNA. The intensity of the fluorescence signal is therefore dependent on the quantity of dsDNA present in the reaction. The main disadvantage of this method is that it is not specific since the dye binds to all dsDNAs formed during the PCR reaction (i.e., nonspecific PCR products and primer-dimers).

II. Principle of fluorogenic 5' nuclease assay (TaqMan® probe assay):

A probe is used in the real-time quantitative TaqMan assay. The probe is a specific sequence which has a fluorescent reporter dye linked to its 5′ end and a non-fluorescent quencher at its 3′ end. The TaqMan probe anneals downstream the target sequence from one of the primer sites and is cleaved by the 5′ nuclease activity of the Taq polymerase during the PCR extension phase. Whilst the probe is intact, the quencher absorbs the fluorescence emitted by the reporter dye. Cleavage of the probe by Taq polymerase during PCR will cause the separation of the reporter and quencher dyes, thereby allowing the reporter's fluorescent signal to be liberated. With each cycle additional reporter dye molecules are cleaved from their respective probes, leading to an increase in fluorescence intensity proportional to the amount of amplicon produced.

RT-qPCR amplification curve:



Used terms:

1. **Baseline**: The baseline of the real-time PCR reaction refers to the little change in fluorescent signal during the initial cycles of PCR (background or the "noise" of the reaction).
2. **Threshold:** The threshold of the real-time PCR reaction is the level of signal that reflects a statistically significant increase over the calculated baseline signal.
3. **C_t (threshold cycle):** The threshold cycle (Ct) is the cycle number at which the fluorescent signal of the reaction crosses the threshold.

Steps of evaluation gene expression by RT-qPCR:

Step 1: RNA Extraction from Tissue/ Cell line or blood

According to the manufacturer's instruction of RNeasy® Mini Kit (Qiagen, DE).

Step 2: Reverse transcription to convert RNA to cDNA

According to the manufacturer's instruction of High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, US).

Step 3: Determination of cDNA using real time PCR

According to Power SYBR® Green PCR Master Mix and RT-PCR Protocol (PN 4367218) (Applied Biosystems, US).

Step 4: Data analysis

References:

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