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المرحلة الثالثة

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Lab 1. Bacterial transformation

Making calcium competent cell for bacterial transformation

Overview: There are several ways to prepare competent cells for plasmid DNA transformation. This is the chemical method. Advantages are that it's simple to complete, requires no special equipment and gives good transformation efficiencies. Disadvantages are that the efficiency is somewhat lower (vs. electroporation). In general, it is best to use this when the transformation efficiencies is not the problem, otherwise you might want to use and make the competent cells for electroporation.

Materials:

- Single colony of *E. coli* cells to be transformed.
- Nutrient broth media.
- 0.1 M $CaCl_2$, ice cold.
- Nutrient agar amp plates.
- 42 C° water bath.
- 15% glycerol.

Day one

Streak out bacterial cells (*E.coli)* onto an LB plate without any antibiotic. Grow the bacteria overnight at 37 C°.

Day two:

1. **Autoclave:**
   N.A media or any preferred media (broth and solid).
   100 mM $CaCl_2$.
   100 mM $CaCl_2$ + glycerol.
   Tubes.

2. Chill overnight at 4 C° :
   $CaCl_2$.
   $CaCl_2$+ glycerol.
   Centrifuge rotor.

3. Prepare starter culture of cells
   Select single colony of *E.coli* from fresh N.A and inoculate 5 ml Starter culture of nutrient broth. Grow overnight at 37 C°.

Day three:

1. Inoculate 10 ml of nutrient broth with 1 ml starter culture and incubate at 37 C°. Measure OD_{600} every hour, then every 15-20 minutes when OD_{600} gets above 0.2.

2. When OD_{600} reaches 0.35-0.4, immediately put the cell on ice. Chill the culture for 20-30 minutes.

3. Centrifuge the cells for 10 min at 3300 g.

4. Discard the medium and resuspend the cell pellet in 1 ml 100 mM cold $CaCl_2$.

5. Keep the cells on ice for 30 min.

6. Centrifuge the cells as above.

7. Remove the supernatant, and resuspend the cell pellet in 1ml 100 mM $CaCl_2$ solution plus glycerol.

8. Pipet 0.4-0.5 ml of the cell suspension into sterile 1.5 ml micro-centrifuge tubes. Freeze these tubes on dry ice and then transfer them to -70 C freezer

Bacterial transformation

In molecular biology, transformation is genetic alteration of a cell resulting from the direct uptake, incorporation and expression of exogenous genetic material (exogenous DNA) from its surroundings and taken up through the cell membrane.

Bacterial transformation: natural and artificial

NATURAL TRANSFORMATION:

Transformation occurs naturally in some species of bacteria. For transformation to happen, bacteria must be in a state of competence, which might occur as a time-limited response to environmental conditions such as starvation and cell density. Some species, upon cell death, release their DNA to be taken up by other cells; however, transformation works best with DNA

from closely-related species. These naturally-competent bacteria carry sets of genes that provide the protein machinery to bring DNA across the cell membrane.

ARTIFICIALLY TRANSFORMATION:

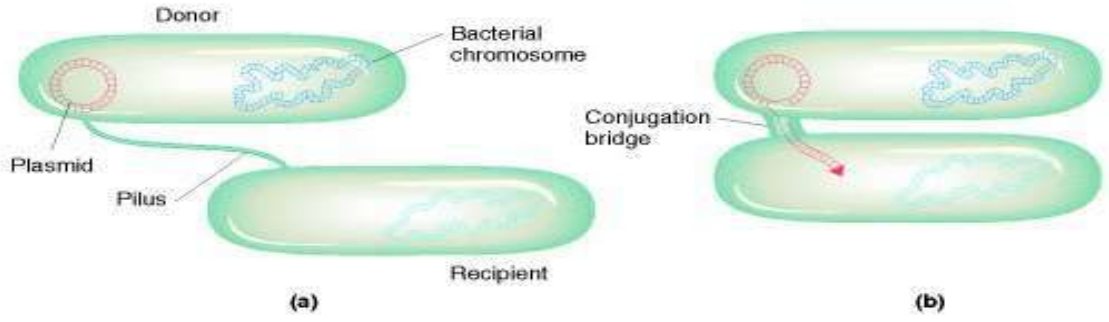
Artificial competence can be induced in laboratory procedures that involve making the cell passively permeable to DNA, by exposing it to conditions that do not normally occur in nature. Typically, the cells are incubated in a solution containing divalent cations; most commonly, calcium chloride solution under cold condition, which is then exposed to a pulse of heat shock.

Standard heat-shock transformation of chemically competent bacteria:

1. Take out chemically competent cells out of -80 C° and thaw on ice.
2. Take agar plate with the desirable antibiotic out of 4 C° to worm up to room temperature.
3. Mix 5µl of 100ng DNA into 50µl thawed competent cells in microcenterfuge or falcon tube and mix gently.
4. Place the mixture of competent cells and DNA on ice for 10-20 min.
5. Place the mixture (transformation tubes) in 42 C° water bath exactly for 45 sec.
6. Place the tubes back on ice for 2 min.
7. Add 250-500µl nutrient broth to each tube.
8. Allow the cell to grow at 37 C° for 45 min.
9. After the 45 min of incubation at 37 C°, take 100 µl of culture and spread it on ampicillin containing nutrient agar plates.
10. Incubate the plates on 37C° for overnight.

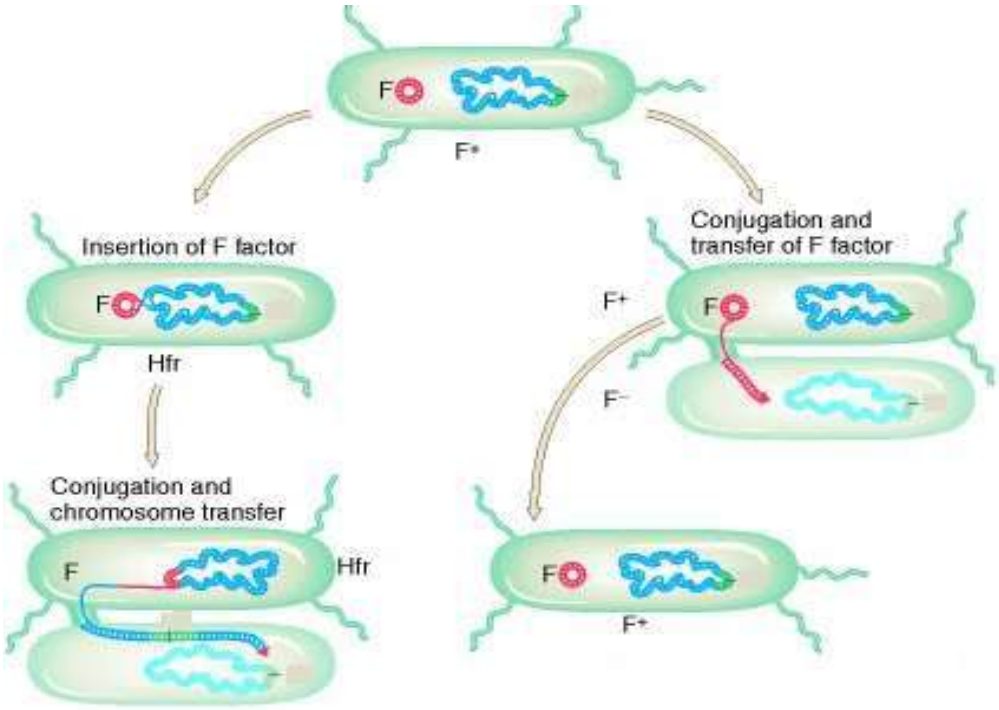
Lab2.Bacterial conjugation.

Conjugation is a mechanism of gene transfer that requires a direct contact between two cells (donor and recipient cells). F^+ cells carry the f plasmid which enabling them to make f pilus and act as a genetic donors. Recipient cells luck the f plasmid are referred to as F^-. The f plasmid encodes for the f pilus, a protein appendage that attaches the donor to the recipient cells. The first step in plasmid transfer is a contact between the donor and the recipient. The f pilus of the donor cell recognizes and binds to a specific receptor on the cell wall of a recipient cell. Then, the plasmid becomes mobilized for transfer when plasmid encoded endonucleases cleaves on strand of the plasmid at a specific nucleotide sequence called the origin of transfer. A single strand of the f plasmid beginning at the origin of transfer and entering the recipient (the f^- cell), a complementary strand to the single strand plasmid remaining in the donor is synthesized by the rolling circle mechanism. Once inside the recipient cell, a complementary strand to the single strand DNA is synthesized. When F^+ and F^- cells are mixed together, eventually all the cells become F+. The genetic material transferred during conjugation often provides the recipient bacterium with some sort of genetic advantage. For instance, in many cases, conjugation serves to transfer plasmids that carry antibiotic resistance genes.



High frequency recombinant cell:

A bacterial cell with plasmid integrated to its chromosome is called Hfr cell. Here the F plasmid of the donor cell is not a free plasmid but it is integrated to the donor bacterial chromosome DNA as an episome. Thus F plasmid together with the bacterial chromosome DNA forms a recombinant DNA called as high frequency recombination DNA or Hfr DNA. Hfr strain can effect high rate of recombinant as some portion of the donor bacterial DNA may also get transferred. Hence they are called Hfr strains.



Bacterial conjugation procedure:

First day

1. Add 200 µl of ampicillin resistant *E.coli* and 200 µl of chloramphenicol resistant *E.coli* to 5 ml of nutrient broth.

2. Incubate the mating cells at 37 C° for overnight to conjugate.

Second day

1. Spread 50 µl of the matting cells on ampicillin containing plate, chloramphenicol containing plate, and ampicillin and chloramphenicol containing plates.
2. Incubate the plates at 37 C° for overnight.

Lab3. Bacterial transduction

Phage transduction is used to move selectable genetic markers from one "donor" strain to another "recipient" strain. Today, phage P1 is commonly used as a transducing agent because it is a generalized transducer (it can package random sections of the host chromosome instead of its own genome) giving rise to "transducing particles". P1vir is a mutant phage that enters the lytic cycle upon infection (ensuring replication and lysis). During the replication and lysis of the phage in a culture of bacteria, a small percentage of the phage particles will contain a genome segment that contains your gene of interest. P1 packages approximately 90 kb of DNA, so you can transduce genes that are linked to a selectable marker. Once a phage population has been generated from a donor host, the phage are used to infect a recipient host. Most of the bacteria are lysed by phage that packaged P1 genomes, but a fraction of the phage inject a genome segment derived from the donor host. Homologous recombination then allows the incoming genomic segment to replace the existing homolgous segment. The infected recipient bacteria are plated on a medium that selects for the genome segment of the donor bacteria (antibiotic resistance, prototrophy, etc.). All of this would not work if the infectivity of the phage could not be controlled. Otherwise, phage released from neighboring cells would infect and lyse the bacteria that had been infected with transducing particles. Someone really smart discovered that phage P1 requires calcium for infectivity. Therefore, you can control P1 infectivity by growing in the presence and absence of calcium. The calcium chelator citrate is usually used because it lowers the concentration of free calcium (by forming Ca-citrate) low enough to prevent P1 infection, but not so low as to starve the cells for calcium.

Transduction protocol

Making the P1 lysate

1. Inoculate 5 ml nutrient broth with colony of donor cell.
2. Grow at 37C to $O.D_{600}$ = 0.3-0.4 (barely turbid).
3. Add 500 µl of 50 mM $CaCl_2$.
4. Add 100 µl P1 virus.

5. Grow at 37 C until lysis occurs.
6. Add 500 µl chloroform.
7. Shake for 5 min.
8. Spin for 10 min at high speed.
9. Add 100 µl chloroform and store the supernatant at 4 C° and in dark.

The P1 transduction

1. Grow overnight of recipient cell.
2. Add 100 µl of 50 mM $CaCl_2$ to Eppendorf tube.
3. Samples are as follow:
    - 200 µl recipient cell with –phage.
    - 200 µl recipient cell with 50 µl phage.
    - - Recipient cell with 500 µl phage.
4. Incubate for 20 min in 37C water bath (This allows adsorption of phage and injection of DNA).
5. Add 100 µl Na-citrate (which will chelate the Ca2+ and Mg2+ required for phage adsorption) and 700 µl nutrient broth.
6. Incubate for 40 min.
7. Plate 50-100 µl on selective media.

Lab4. Isolation of mutant bacteria

Mutation is a heritable change in the nucleotide sequence of DNA. Mutations may be characterized according to either the kind of genotypic change that has occurred or their phenotypic consequences. Mutations can alter the phenotype of a microorganism in several different ways. Morphological mutations change the microorganism's colonial or cellular morphology. Nutritional or biochemical variation may occur in a gene that encodes an enzyme involved in a metabolic pathway of amino acid synthesis. Changes in gene regulation occurs when mutation occur in a gene encoding a transcription factor. Lethal mutations prevent the reproducing capability of the organism, and when expressed, it results in the death of the microorganism.

Mutations often inactivate a biosynthetic pathway of the microorganism, and frequently make a microorganism unable to grow on a medium lacking an adequate supply of the pathway's end product. Based on this principle microorganism are classified as Prototrophic and Auxotrophic. Prototrophic organisms (wild type) have the same nutritional requirements as that of their ancestors. They need only inorganic salts, an organic energy source such as sugar, fat, protein and water to survive and grow. That is, the Prototroph's need only "Minimal medium" for their growth and survival. Auxotrophic mutants are unable to grow without one or more essential nutrients. Auxotrophs are mutant for particular nutrient synthesis pathway enzymes. Such an error is known as an inborn error of metabolism, whether it occurs in a bacterium or a eukaryote. An auxotroph can be grown only on an enriched medium that provides the particular nutrient that the mutant cannot metabolize on its own.

There are two classes of mutations: Spontaneous mutations and induced mutations.

1. **Spontaneous Mutations:**

A mutation without a known cause is called Spontaneous mutations. This occurs at low frequency leading to the chemical instability of purine and pyrimidine bases and also due to low level of metabolic errors, or mistakes during the DNA replication.

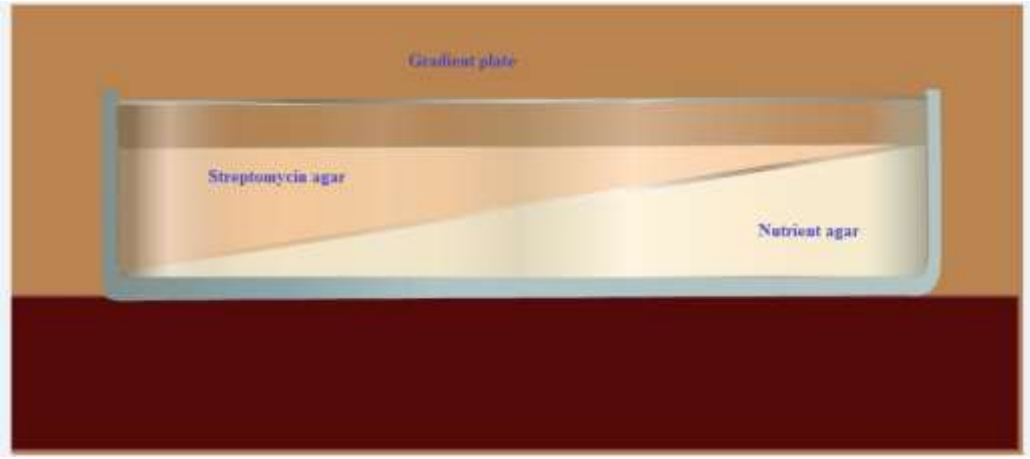
2. **Induced Mutations:**

Mutations that results from exposure of organisms to mutagenic agents such as ionizing irradiation, ultraviolet light or various chemicals that react with nucleic acids. In experimental organisms, researchers often treat them with these mutagens in order to increase the frequency of mutation in them. Generally, chemical mutagens induce point mutations, whereas ionizing radiations gives rise to large chromosomal abnormalities. Point mutations are simple changes in single base-pairs, the substitution of one base-pair for another, or duplication or deletion of single base-pairs. Point mutations occur at a single point on a chromosome. Missense mutation is a type of point mutation, in which a single nucleotide is changed that leads to substitution of a different amino acid and a nonsense mutation, is a point mutation, that changes a normal codon into a stop codon that does not code for an amino acid and arrest peptide synthesis without amino acid insertion resulting in a nonfunctional protein product. Frame shift mutation, is a kind of mutation caused by the addition or deletion of nucleotides which is not a multiple of three so that the codon is read incorrectly during translation. A silent mutation causes base substitution without amino acid substitution and thus has no effect. Such substitutions will not cause any change in their product and cannot be detected without genome sequencing. In any case, the mutation events are often reversible. The subsequent mutations in the nucleotide pair restore the original wild type phenotype. That is, a

gene that has undergone mutation reverses to its original base composition. This is referred to as back-mutation, reverse mutation or reversion. Genetic and biochemical investigations in bacteriology are often initiated by isolation of mutant strains. The spontaneous mutations due to resistance in antibiotics such as Streptomycin are easily detected because they grow in the presence of antibiotic concentrations that inhibit the growth of normal bacteria.

Gradient Plate Technique:

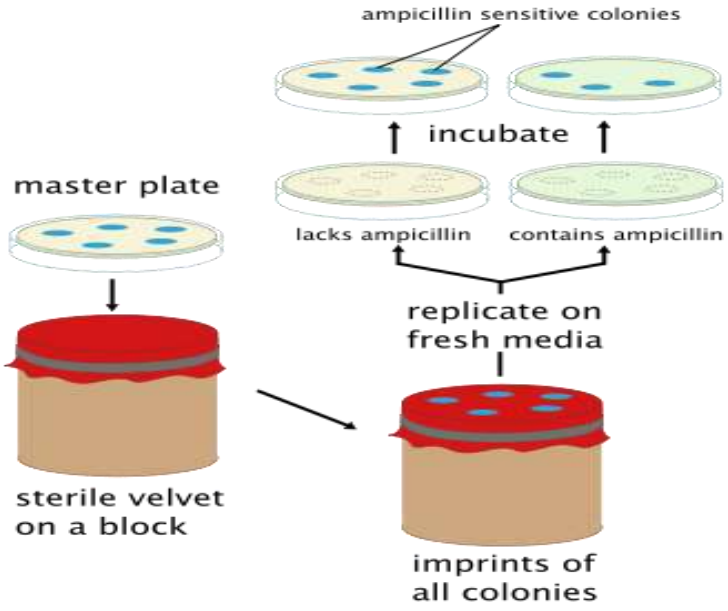
An excellent way to determine the ability of organisms to produce mutants that are resistant to antibiotic is to grow them on a gradient plate of a particular antibiotic. The gradient plate consists of two wedges like layers of media: a bottom layer of plain nutrient agar and top layer of antibiotic with nutrient agar. The antibiotic in the top layer, diffuse into the bottom layer producing a gradient of antibiotic concentration from low to high. A gradient plate is made by using Streptomycin in the medium. E. coli, which is normally sensitive to Streptomycin, will be spread over the surface of the plate and incubated for 24 to 72 hours. After incubation colonies will appear on both the gradients. The colonies develop in the high concentration are resistant to the action of Streptomycin, and are considered as Streptomycin resistant mutants. For isolation of antibiotic resistant of gram negative enteric bacteria, the antibiotics commonly used are Rifampicin, Streptomycin, and Erythromycin etc.



Replica plating method:

If an organism has the ability to produce mutant strains resistant to antibiotics, the nature of mutation, whether it is spontaneous or induced have to be tested. It would be a difficult task to identify a few mutant colonies from a vast population of 100-500 colonies. This can be accomplished by a replica plating technique. The technique was developed by Joshua and Esther Lederberg in 1952 for providing the direct evidence for the existence of pre-existing mutations.

With this method, individual colonies on an agar can be taken up with a stamp covered with velvet and placed onto other culture plates with media of different composition. Some mutant bacteria differ from non- mutants in their ability to grow. Here several colonies are shown in the petri dish of the initial culture. Each of these colonies originated from a single cell. By means of replica plaiting, the colonies are transferred to two new cultures. One culture (right) contains an antibiotic in the culture medium, the other (left) does not. All colonies grow in normal medium, but only those colonies that are antibiotic resistant owing to a mutation grow in the antibiotic- containing medium. In this manner, mutant colonies can be readily identified.



Mutant bacteria identified through an auxotrophic medium:

Here it is shown how different mutants can be distinguished, e.g., after exposure to a mutagenic substance. After a colony has been treated with mutagenic substance, it is first cultivated in normal nutrient medium. Mutants can then be identified by replica plating. The culture with normal medium serves as the control. In one culture with minimal medium, from which a number of substances are absent, two colonies do not grow (auxotrophic mutant Initially, it is known for which of the substances the colonies are auxotrophic. If a different amino acid is added to each of two cultures with minimal medium, e.g., threonine (Thr) to one and arginine (Arg) to the other, it can be observed that one of the mutant colonies grows in the threonine-containing minimal medium but the other does not. He former colony is dependent on the presence of threonine (Thr-), i.e., it is an auxotroph for threonine. The other culture with minimal medium had arginine added. Only here can the other of two mutant colonies, an auxotroph for arginine (Arg-), grow. After the mutant colonies requiring specific condition for growth have been identified, hey can be further characterized. This procedure is relatively simple and makes rapid identification of mutants possible. Many mutant bacteria have been defined by auxotrophism. He wild type cells that do not have special additional growth requirements are called phototrophs.

Lab5.Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA, using a short nucleotide sequence (primer), generating thousands to millions of copies of a particular DNA sequence in a matter of only few hours. Polymerase Chain Reaction was developed in 1984 by the American biochemist, Kary Mullis. Mullis received the Nobel Prize and the Japan Prize for developing PCR in 1993. PCR is a common and often essential technique used in medical and biological research labs for a variety of applications. These include DNA cloning for sequencing, DNA-based phylogeny or functional analysis of genes, detection of infection agents and genetic disease, the study of molecular evolution, medical epidemiology and forensic sciences (used in paternity testing).

The polymerase chain reaction is a powerful technique that has rapidly become one of the most widely used techniques in molecular biology because it is quick, inexpensive and simple. The technique amplifies specific DNA fragments from minute quantities of source DNA material, even when that source DNA is of relatively poor quality. The PCR method is based on the repetitive cycling (usually 20-35) of 3 simple reactions; it varies in temperature of incubation as shown in Figure.

❖ *Denaturation*

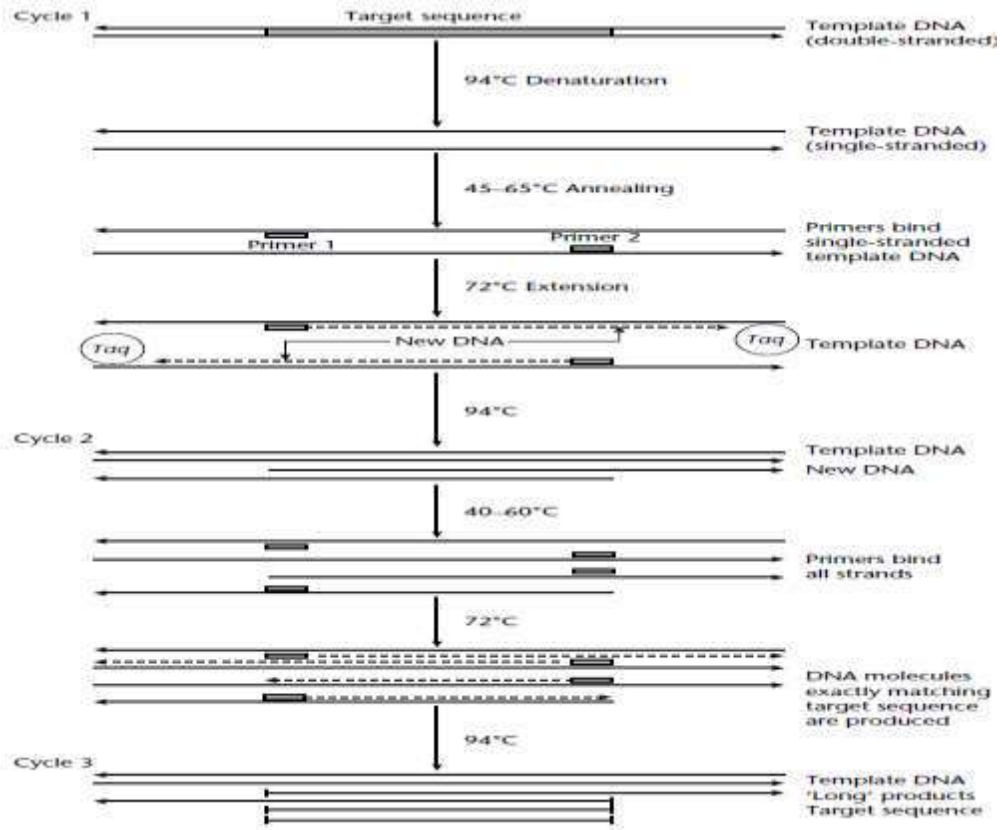
The double-stranded DNA is heated to 94-96°C , breaking down the hydrogen bonds connecting the two DNA strands in order to separate them. Prior to the first cycle, the DNA is often denatured for an extended time to ensure that both the template DNA and the primers have completely separated and are now single strand only. (Time 1-2 minutes up to 5 minutes).

❖ *Annealing*

In order to achieve primer attachment to the single DNA strands , the temperature must be lowered. The temperature of this stage varies depending on the primers length and sequence and is usually 5°C below their melting temperature (45-60°C). A wrong temperature during the annealing step can result in primers not binding to the template DNA at all, or binding at random sites. Time 1-2 minutes.

❖ *Extension*

This is the final step in which the DNA polymerase enzyme binds to the free 3-OH ends of the primers and uses nucleotides (dNTPs) to synthesize a new DNA strand in a 5_ to 3 direction. The optimum temperature for the extension process which is the suitable degree for giving high activity for the enzyme is (72°C) for 2-5 minutes.



General diagram of Polymerase Chain Reaction

PCR technique requirements

❖ Primers

Each PCR requires a pair of oligonucleotide primers. These are short single-stranded DNA molecules (typically 20 bases) obtained by chemical synthesis. Primer sequences are chosen so that they bind by complementary base pairing to opposite DNA strands on either side of the sequence to be amplified. The sequence of primers and their length affect the optimization conditions which bind the primer with the determining region. Because each of the two PCR primers is complementary to a different individual strand of the target sequence duplex, the primer sequences are not related to each other. It is preferable to select primer sequences that do not form duplex.

❖ DNA polymerase

The original method of PCR uses the Klenow fragment of *E. coli* DNA polymerase I. This enzyme, however, denatures at a temperature lower than that required to denature most template duplexes. Thus, in earlier experiments, fresh enzyme had to be added to the reaction after each cycle. The use of heat-resistant DNA polymerase has obviously facilitated the process because the addition of enzymes after every denaturation step is no longer necessary. The first thermostable DNA polymerase used was the ***Taq* DNA polymerase** isolated from the bacterium ***Thermus aquaticus*** living in a hot spring in Yellowstone National Park USA at temperatures close to 85°C. The optimal working temperature of this enzyme is 70 - 80°C. **Ampli*Taq* DNA polymerase** is a genetically modified enzyme expressed by *E. coli.* Since Ampli*Taq* is recombinant, the purity and reproducibility of this enzyme are higher than those of the wild type. This enzyme is very thermostable, with a half-life at 95°C of 35–40 min.

❖ **DNA template**

It contains the region of the DNA fragment to be amplified. A number of simple and rapid procedures for DNA extraction have been developed for particular tissues. The two main concerns about the template are purity and amount. The purity of the DNA sample need not be high. Even relatively degraded DNA preparations can serve as useful templates for generation of moderate-sized PCR products. However, contaminants found in DNA preparations can decrease the efficiency of PCR. Clearly the amount of a template must be sufficient to be able to visualize PCR products using ethidium bromide. Usually 100 ng of genomic DNA is sufficient to detect a PCR product from a single-copy gene.

❖ **Deoxynucleoside triphosphates (dNTPs)**

Nucleotide triphosphates are building block units for DNA, which are four dNTPs (consisting of **dATP**, **dCTP**, **dGTP**, and **dTTP**). The same quantity from these four bases could be added to the reaction mix with enough concentration. The nucleotide concentration is a very important issue in the PCR method. Since these dNTPs affecting the polymerase enzyme activity by chelating free Mg^{+2} ions that act as an enzyme cofactor for DNA polymerase in PCR, high concentration could inhibit the reaction due to the reduction of required ions. Optimum concentration for dNTPs is 200 μ*M*.

Buffers and Salts

This solution is used to regulate the action of the polymerase enzyme and maintain its activity. The optimal PCR buffer concentration, salt concentration, and pH depend on the DNA polymerase in use. The PCR buffer for *Taq* DNA polymerase consists of 50 m*M* KCl and 10 m*M* Tris-HCl, pH 8.3, at room temperature. This buffer provides the ionic strength and buffering capacity needed during the reaction.

Lab6. Design of primers for PCR

Perhaps the most critical parameter for successful PCR is the design of primers. All things being equal, a poorly designed primer can result in a PCR reaction that will not work. The primer sequence determines several things such as the position and length of the product, its melting temperature and ultimately the yield (Innis and Gelfand, 1994). A poorly designed primer can result in little or no product due to nonspecific amplification and/or primer-dimer formation, which can become competitive enough to suppress product formation. This application note is provided to give rules that should be taken into account when designing primers for PCR. More comprehensive coverage of this subject can be found elsewhere (Dieffenbach et al., 1995).

Primer selection

Several variables must be taken into account when designing PCR primers. Among the most critical are:

1. Primer length/ Specificity.
2. Melting temperature (Tm).
3. Primer Annealing Temperature.
4. Primer Secondary Structures
5. Repeats
6. Runs
7. GC clamp

Primer length:

It is generally accepted that the optimal length of PCR primers is 18-22 bp. This length is long enough for adequate specificity and short enough for primers to bind easily to the template at the annealing temperature**.** The longer is the primer, the more inefficient the annealing. With fewer

Templates primed at each step, this can result in a significant decrease in amplified product. The primers should, however, not be too short unless the application specifically requires it. As discussed below, the goal should be to design a primer with an annealing temperature of at least 50°C.

Melting temperature (Tm)

It is important to keep in mind that there are two primers added to a site/target directed PCR reaction. Both of the oligonucleotide primers should be designed so that they have similar melting temperatures. If primers are mismatched in terms of Tm, amplification will be less efficient or may not work at all since the primer with the higher Tm will misprime at lower temperatures and the primer with the lower Tm may not work at higher temperatures. A good working approximation of this value (generally valid for oligos in the 18 - 24 base range) can be calculated using the formula:

$$Tm = 2(A+T) + 4(G+C).$$

Primer length	T_m = 2 (A+T) + 4(G+C)	Primer length	T_m = 2 (A+T) + 4(G+C)
4	12°C	22	66°C
6	18°C	24	72°C
8	24°C	26	78°C

Primer Annealing Temperature

The primer melting temperature is the estimate of the DNA-DNA hybrid stability and critical in determining the annealing temperature. Too high T_a will produce insufficient primer-template hybridization resulting in low PCR product yield. Too low T_a may possibly lead to non-specific

products caused by a high number of base pair mismatches,. Mismatch tolerance is found to have the strongest influence on PCR specificity.

$$T_a = 0.3 \times T_m \text{ (primer)} + 0.7\ T_m \text{ (product)} - 14.9$$

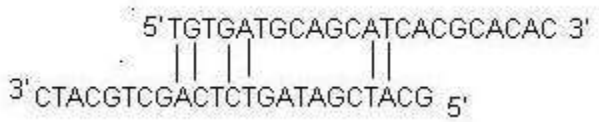
Primer Secondary Structures

Presence of the primer secondary structures produced by intermolecular or intramolecular interactions can lead to poor or no yield of the product. They adversely affect primer template annealing and thus the amplification. They greatly reduce the availability of primers to the reaction.

1. Hairpins: It is formed by intramolecular interaction within the primer and should be avoided.

2. Self-Dimer: A primer self-dimer is formed by intermolecular interactions between the two (same sense) primers, where the primer is homologous to itself. Generally a large amount of primers are used in PCR compared to the amount of target gene. When primers form intermolecular dimers much more readily than hybridizing to target DNA, they reduce the product yield.

3. Cross Dimer: Primer cross dimers are formed by intermolecular interaction between sense and antisense primers, where they are homologous.



Repeats

A repeat is a di-nucleotide occurring many times consecutively and should be avoided because they can misprime. For example: ATATATAT. A maximum number of di-nucleotide repeats acceptable in an oligo is 4 di-nucleotides.

Runs

Primers with long runs of a single base should generally be avoided as they can misprime. For example, AGCGGGGGATGGGG has runs of base 'G' of value 5 and 4. A maximum number of runs accepted is 4bp.

GC Clamp

The presence of G or C bases within the last five bases from the 3' end of primers (GC clamp) helps promote specific binding at the 3' end due to the stronger bonding of G and C bases. More than 3 G's or C's should be avoided in the last 5 bases at the 3' end of the primer