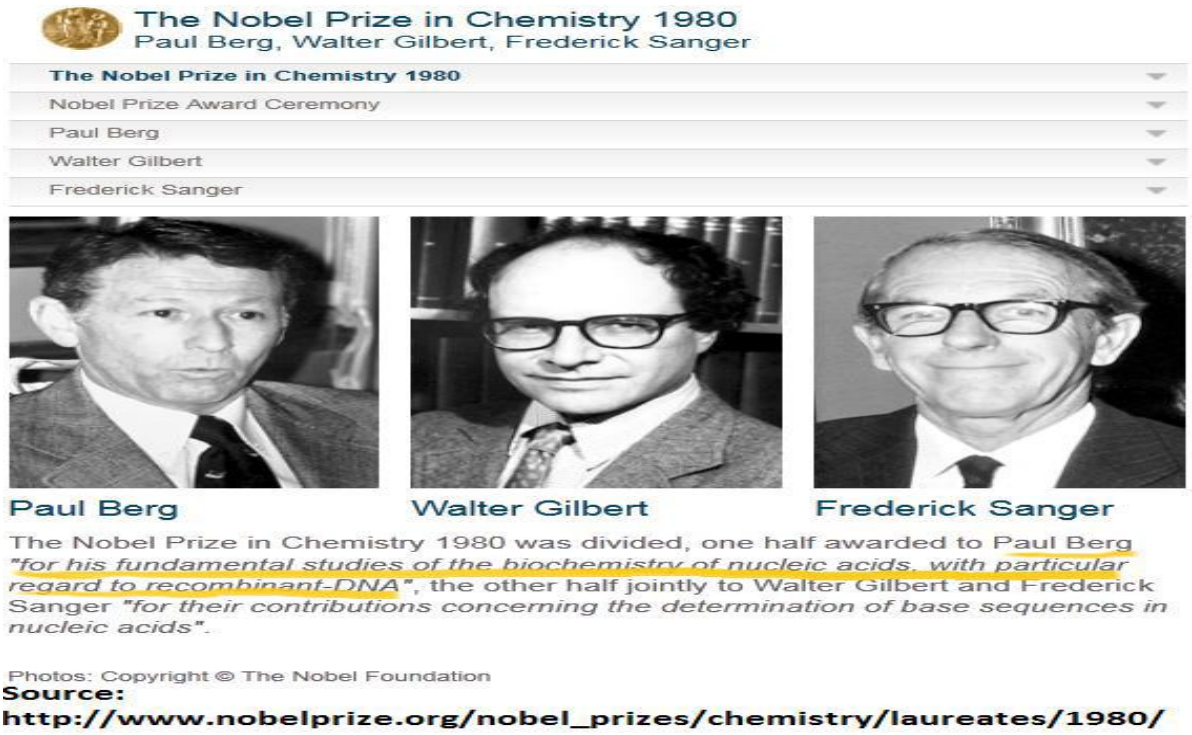
Basics of DNA Cloning

DNA cloning is process of making several identical copy of a gene or gene fragment.

DNA fragment from an organism is cleaved or amplified and inserted in a DNA carrier called vector. Vectors are generally double stranded closed circular DNA which has origin of replication through which they can replicate in the host system. Vectors also have a selectable marker (generally antibiotics resistance gene) for screening of recombinant colonies. Vector with desired DNA insert is called **recombinant DNA**. This can be transferred to suitable host system (generally ***E. coli***) where it finds machinery for replication and makes several copies of it (may also express protein). This process is largely based on the work of Paul Berg, Herbert W. Boyer and Stanley N. Cohen although many other scientists have also made important contributions. Paul Berg in 1972, isolated a gene from a human cancer-causing monkey virus (SV40) using a restriction enzyme and joined this virus DNA with a molecule of DNA from the bacterial virus lambda using an enzyme called **DNA ligase**. This way the first recombinant DNA molecule was created. Later on, in 1980, Paul Berg shared Nobel prize in Chemistry for the work . A simplified concept of cloning is given in Fig. 1.



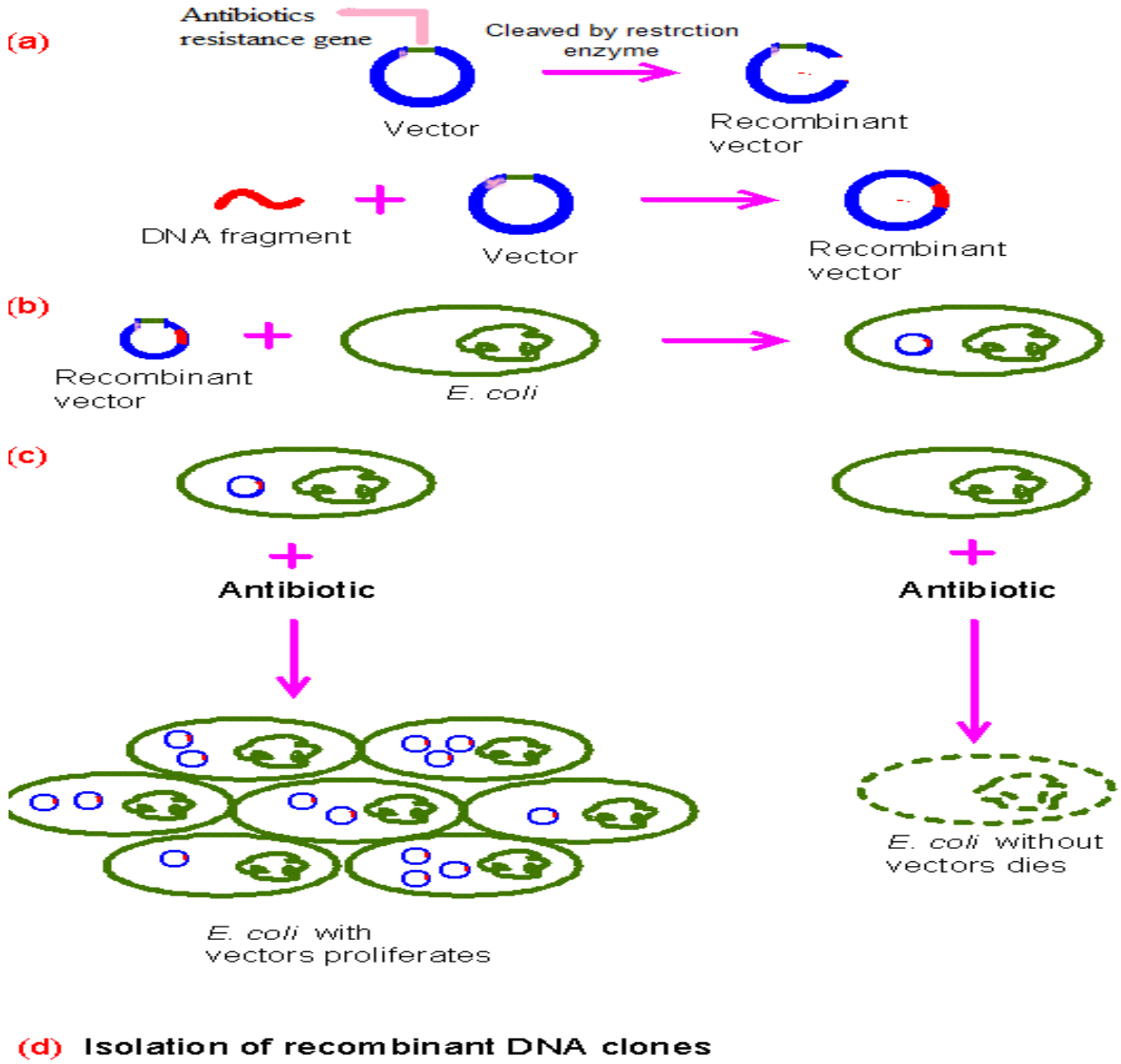
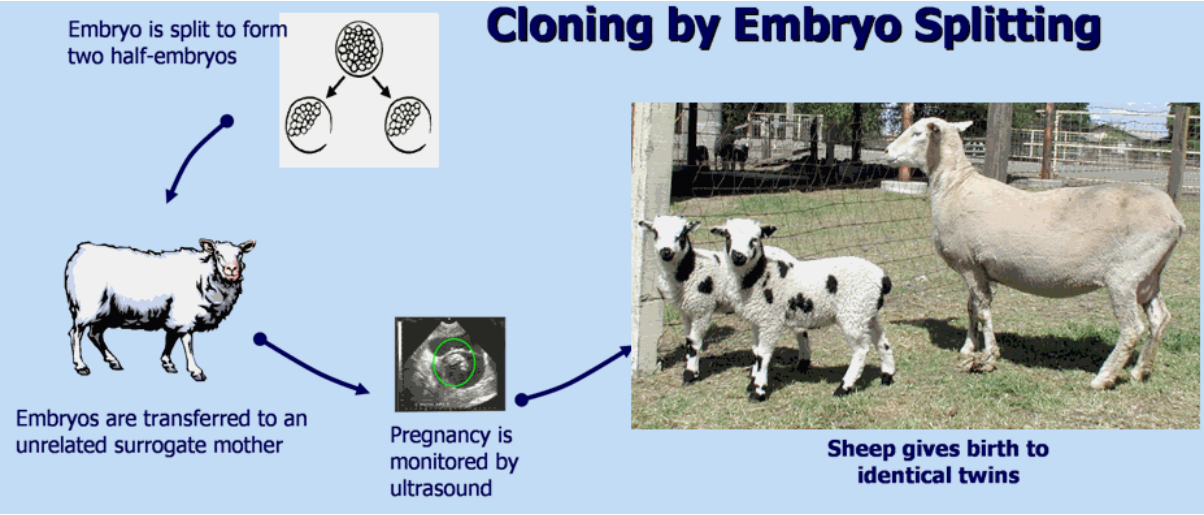


Figure 1: A simpler concept of cloning.(هذا الرسم للاطلاع فقط)

Cloning is a **natural process** in biology where genetically identical individuals are produced by asexually reproducing organisms such as bacteria, insects or plants. In **biotechnology**, the process of producing multiple identical copies of DNA fragments (molecular cloning), cells (cell cloning), or organisms is referred to as **cloning**. A clone has an exact genetic imprint as that of the original cell, tissue or organism.

There are different types of cloning technologies used for various purposes besides producing the genetic copy of an organism. Basically the cloning technology can be divided into **three** types as **reproductive cloning**, **therapeutic cloning** and **recombinant DNA technology** or **DNA cloning**.

Reproductive cloning is a technology used to generate a twin of an animal that is genetically same as another currently or previously existing animal. The best example for reproductive cloning is **Dolly**, the first cloned sheep.



Therapeutic cloning which is also known as "**embryo cloning**," is production of human embryos for use in research and treatment of diseases. The aim of this technique is not human cloning, but rather to harvest stem cells that are used for research studies and to treat diseases.

The last and most widely used cloning technique in biotechnology is **recombinant DNA technology**. In Biotechnology the gene is the cornerstone of most molecular biology studies. The study of genes can be facilitated by isolation and amplification of gene of interest. Cloning is one method used for isolation and amplification of gene of interest. The gene is cloned by inserting it into another DNA molecule which acts as **vehicle** or **vector** that will replicate in living cells. As the two DNA molecules of different origin are combined, the resulting DNA is known as **recombinant DNA molecule**.

The term "**gene cloning**," "**DNA cloning**," "**molecular cloning**," and "**recombinant DNA technology**" all refer to same technique: Insertion of DNA fragment of interest from one organism into a vector which is a self- replicating genetic element inside a living cell. Gene cloning processes include removal of DNA from the cell, carrying out the DNA manipulations in test vial (tube) and, transformation of constructed DNA molecule back into the cells.

The first step in cloning is to prepare large amount of the vector and chromosomal DNAs. To carry the gene or the desired DNA fragment to the cell there is a need of a vector molecule. All cloning vectors are carrier DNA

molecules. These **carrier molecules** host few common features in general such as; all vectors are [1]self replicating in the cell, they[2] contain a number of unique restriction enzyme cleaving sites that are present only once in the vector, they carry the selectable marker gene which is useful in selection of clone (usually an antibiotic resistance gene that is absent in the host cell) and, they can be very easily isolated from host cell. Depending on the purpose of cloning there are many vectors available. For use in the bacterial host *E. coli* system a greatest variety of cloning vectors have been developed. **Thus, the first thing in cloning that a molecular biologist requires is to grow pure culture and isolate the cloning vector from the cells.**

Cloning vectors

The most commonly used cloning vectors include plasmids and bacteriophages (phage λ) beside all the other available vectors (Table 1). The cloning vectors are limited to the size of insert that they can carry. Depending on the size and the application of the insert the suitable vector is selected. The different types of vectors available for cloning are plasmids, bacteriophages, bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs) and mammalian artificial chromosomes (MACs).

1- Plasmids*:* Plasmids are extra chromosomal circular double stranded DNA replicating elements present in bacterial cells. Plasmids show the size ranging from 5.0 kb to 400 kb. Plasmids are inserted into bacterial calls by a process called transformation. Plasmids can accommodate an insert size of upto **10 kb** DNA fragment. Generally plasmid vectors carry a marker gene which is mostly a gene for antibiotic resistance; thereby making any cell that contains the plasmid will grow in presence of the selectable corresponding antibiotic supplied in the media.

2- Bacteriophage*:* The viruses that infect bacteria are called bacteriophage. These are intracellular obligate parasites that multiply inside bacterial cell by making use of some or all of the host enzymes. Bacteriophages have a very high significant mechanism for delivering its genome into bacterial cell. Hence it can be used as a cloning vector to deliver larger DNA segments. Most of the bacteriophage genome is non-essential and can be replaced with foreign DNA. Using bacteriophage as a vector, a DNA fragment of size up to **20 kb** can be transformed.

3- Cosmids

Both λ phage and *E. coli* plasmid vectors are useful for cloning only relatively small DNA fragments. However, several other vectors have been developed for cloning larger fragments of DNA. **One common method for cloning large fragments makes use of elements of both plasmid and λ-phage cloning**. In this method, called **cosmid** cloning, recombinant plasmids containing inserted fragments up to a length of 45 kb can be efficiently introduced into *E. coli* cells. A **cosmid** vector is produced by inserting the **cos sequence** from **λ-phage DNA** into a small ***E. coli* plasmid vector** about **5** kb long. Cosmid vectors contain all the essential components found in plasmids. The cosmid can incorporate foreign DNA inserts that are between **35** and **45** kb in length. Such recombinant molecules can be packaged and used to transform *E. coli*. Since the injected DNA does not encode any λ-phage proteins, no viral particles form in infected cells and likewise the cells are not killed. Rather, the injected DNA circularizes, forming in each host cell a large plasmid containing the cosmid vector and the inserted DNA fragment.

Cells containing cosmid molecules can be selected using antibiotics as described for ordinary plasmid cloning. A recently developed approach similar to cosmid cloning makes use of larger *E. coli* viruses such as bacteriophage P1. Recombinant plasmids containing DNA fragments of up to ≈100 kb can be packaged *in vitro* with the P1 system.

4- Bacterial artificial chromosomes (BACs): Bacterial artificial chromosomes (BACs) are simple plasmid which is designed to clone very large DNA fragments ranging in size from 75 to 300 kb. BACs basically have marker like sights such as antibiotic resistance genes and a very stable origin of replication (ori) that promotes the distribution of plasmid after bacterial cell division and maintaining the plasmid copy number to one or two per cell. BACs are basically used in sequencing the genome of organisms in genome projects (example: BACs were used in human genome project). Several hundred thousand base pair DNA fragments can be cloned using BACs.

5- Yeast artificial chromosomes (YACs): YACs are yeast expression vectors. A very large DNA fragments whose sizes ranging from 100 kb to 3000 kb can be cloned using YACs. Mostly YACs are used for cloning very large DNA fragments and for the physical mapping of complex genomes. YACs have an advantage over BACs in expressing eukaryotic proteins that require post

translational modifications. But, YACs are known to produce chimeric effects which make them less stable compared to BACs.

6- Human artificial chromosomes (HACs): Human artificial chromosomes (HACs) or mammalian artificial chromosomes (MACs) are still under development. HACs are microchromosomes that can act as a new chromosome in a population of human cells. HACs range in size from 6 to 10 Mb that carry new genes introduced by human researchers. HACs can be used as vectors in transfer of new genes, studying their expression and mammalian chromosomal function can also be elucidated using these microchrosomes in mammalian system. Different types of vectors are summarized in Table 1.

Table 1: Different types of vector with their properties.(للاطلاع فقط)

Vector	**Basis**	**Size limit of insert**	**Major application**
Plasmid	Naturally occurring multi copy plasmids	≤ 10 kb	Subcloning and gene manipulation, cDNA cloning and expression studies.
Phage	Bacteriophage λ	10- 20 kb	Genomic DNA cloning, cDNA and expression libraries.
Cosmid	Plasmid containing a bacteriophage λ *cos* site	35- 45 kb	Genomic library construction.
BACs	*Escherichia coli* F factor plasmid	75- 300 kb	Analysis of large genomes.
YACs	Saccharomyces cervisiae centromere, telomere, and autonomously replicating sequence	100- 3000 kb	Analysis of large genomes, YAC transgenic mice.
MACs	Mammalian centromere, telomere, and origin of replication	4- 10 Mb	Still in budding stage for use in animal biotechnology and human gene therapy

Major steps of DNA Cloning

Molecular cloning using a plasmid vector involves **five** major steps .

Step 1: **Isolation of DNA (gene of interest and vector):**

The first initial step in cloning a DNA fragment is to isolate foreign DNA containing gene of interest and bacterial plasmid. If the sequence of the gene of interest is known it is isolated by PCR amplification using gene specific primers which include restriction sites selected from the multiple cloning site of the plasmid selected for cloning. When the sequence of the gene is not known degenerate primers are used for PCR amplification. Most of the time people generate genomic DNA library and screen for the gene using southern hybridization technique. According to the result of southern hybridization, the DNA is sequenced and the gene was confirmed by BLAST analysis. Now the gene is amplified by PCR and cloned. There are many plasmids available commercially for cloning.

Step 2: Treatment of plasmid and foreign DNA with the same restriction enzyme and ligation. (Figure 1).

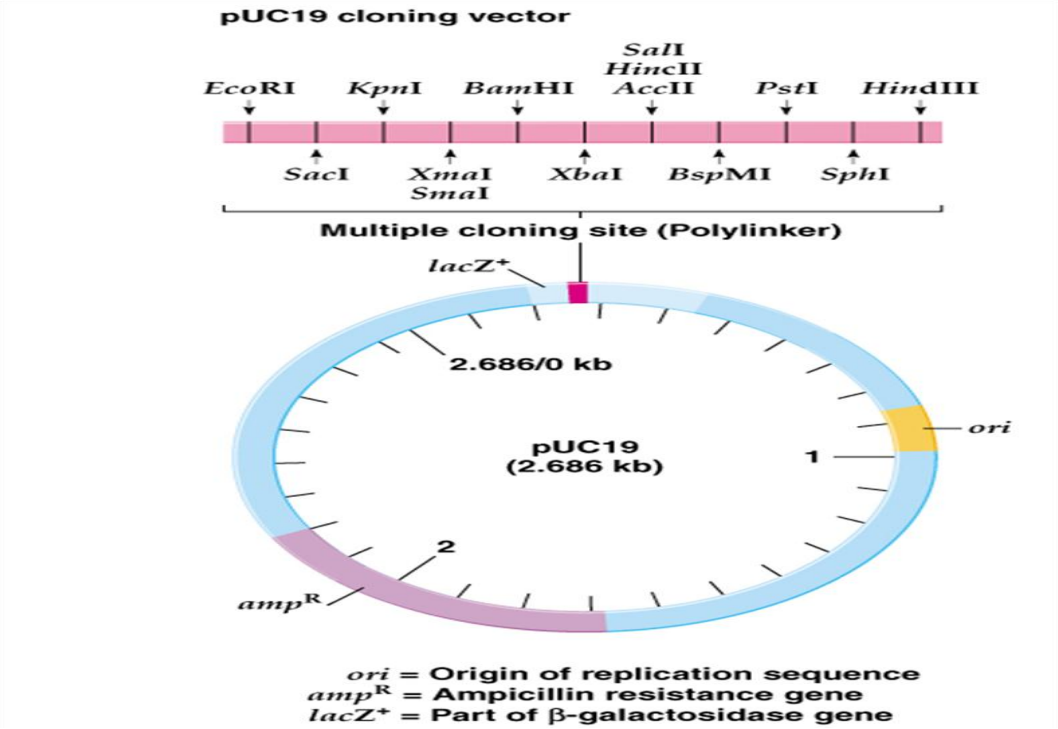


Figure 1: Plasmid pUC 19, The commonly used plasmid pUC19 (“puck 19”) is a small plasmid with the essential elements for a vector: An origin of DNA replication A dominant selectable marked (resistance to an antibiotic, ampicillin) And a cloning site, usually a polylinker with recognition sites for numerous restriction enzymes.

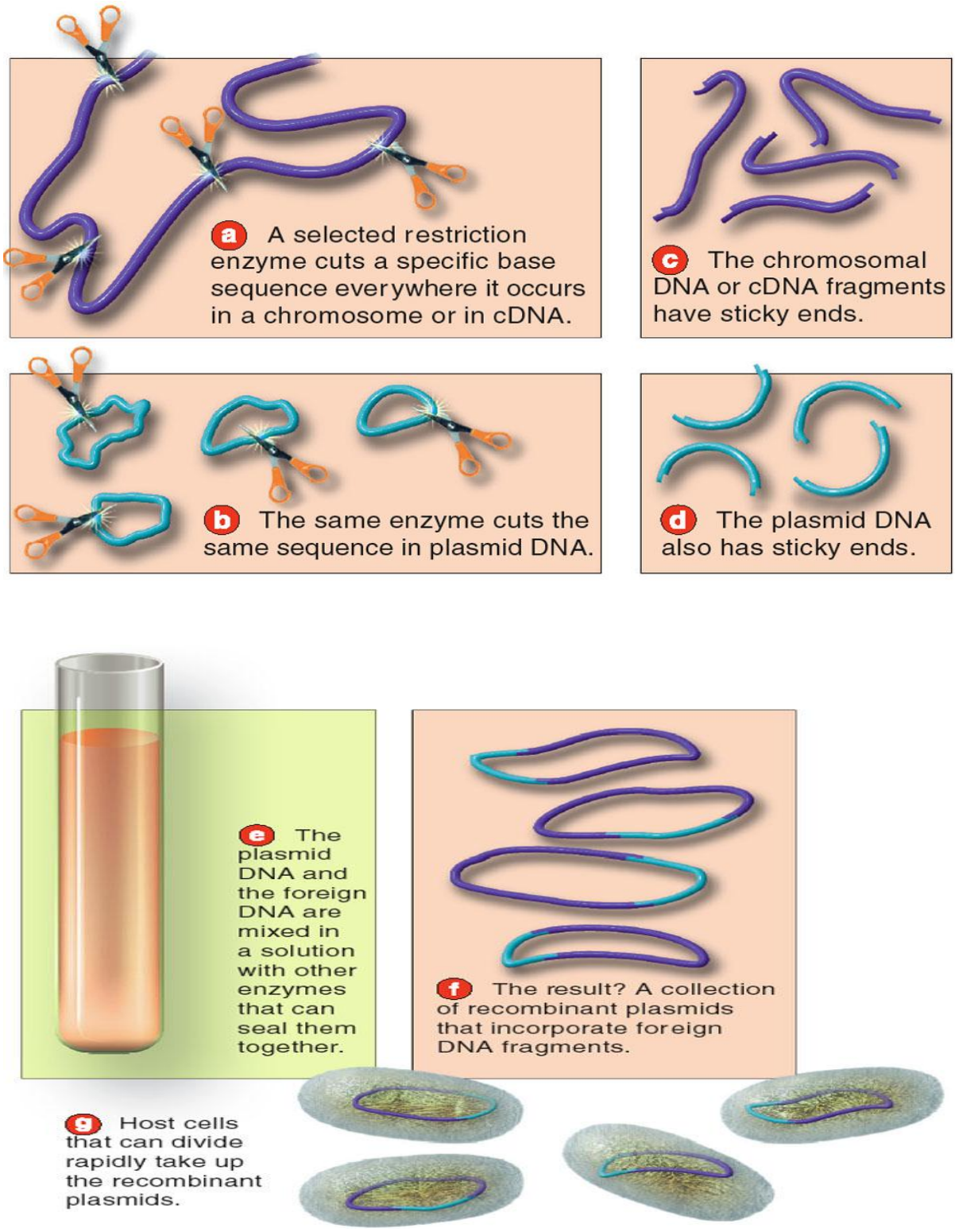


Figure 2: Treatment of plasmid and foreign DNA with the same restriction enzyme and ligation.
(الرسم ليس للحفظ لكن الخطوات مطلوبة)

Step 3: Transformation: transfer of recombinant plasmid DNA to a suitable host:

There are basically two general methods for transforming bacteria:

The first is a **chemical method** utilizing CaCl2 and heat shock to promote DNA entry into cells.The traditional method to prepare cells for transformation process is to incubate the cells in a concentrated calcium salt solution to neutralize the negative charge of membrane (due to salicylic acid), so that the negatively charged DNA molecules can come close to bacterial membrane and during heat shock can easily enter in the cells. These "competent" cells are then mixed with ligation product to allow entry of the DNA into the bacterial cell (Figure 3).

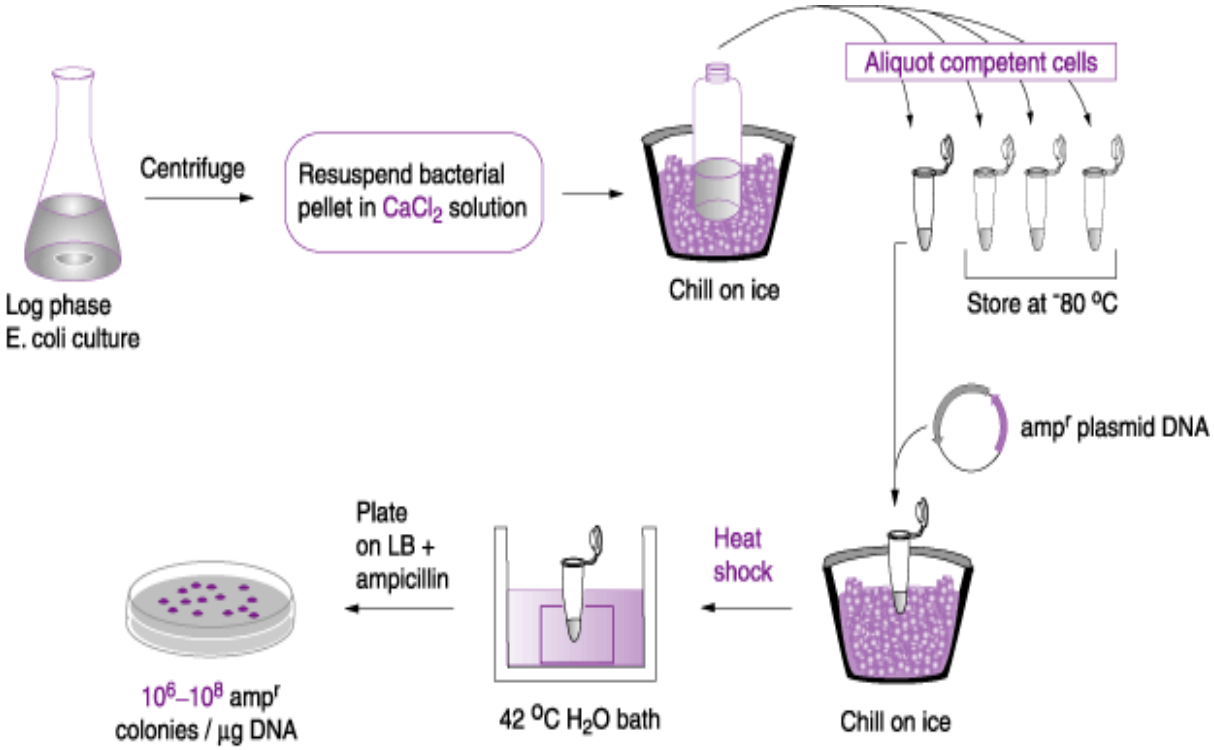


Figure 3: Chemical transformation with calcium chloride
(الرسم ليس للحفظ لكن خطوات الطريقة مطلوبة)

A second method is called **electroporation** based on a short pulse of electric charge to facilitate DNA uptake. Electroporation method is an alternative mode of transformation used to drive DNA (comparatively larger size) into cells by a strong electric current. This method is not very common due to less percentage of survival of transformed cells (Figure 4).

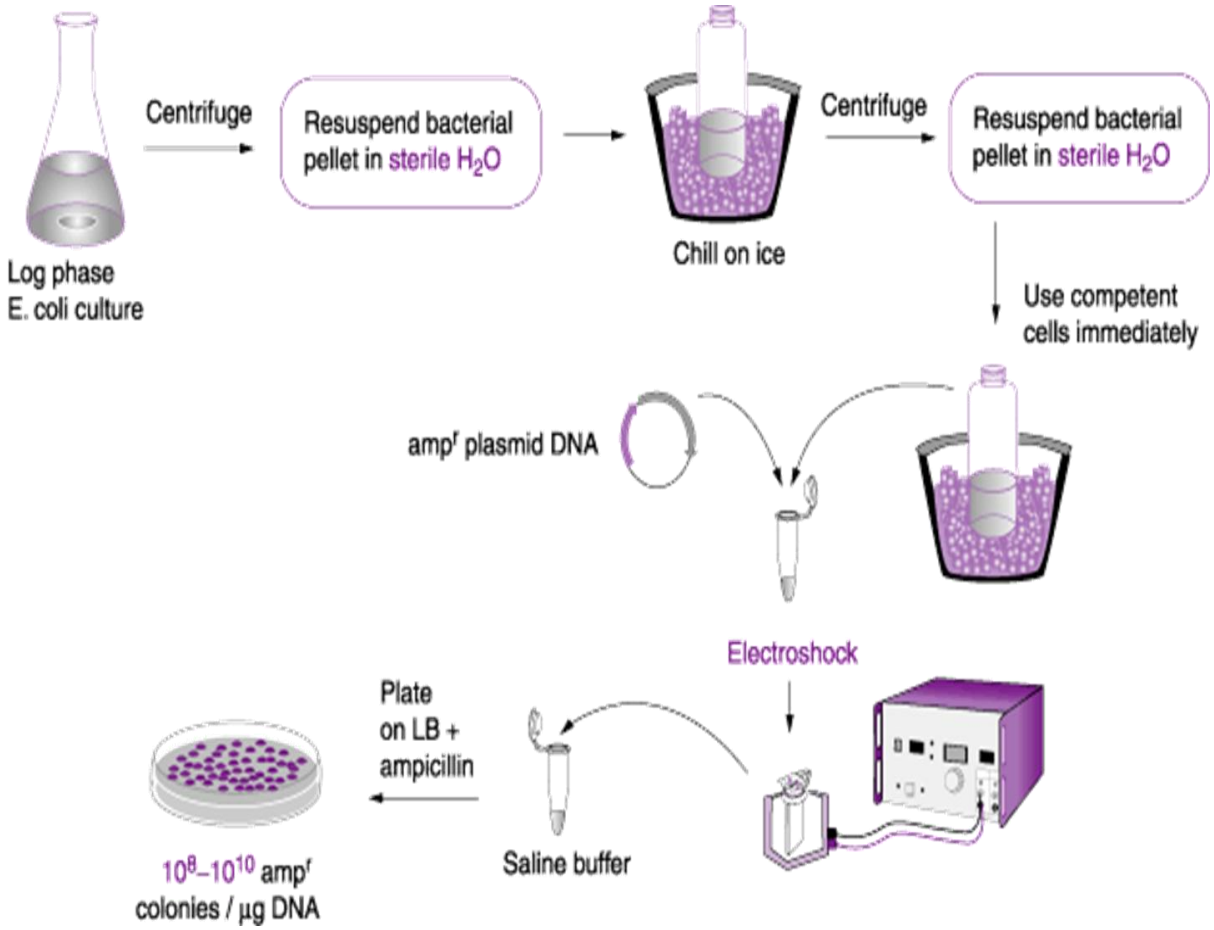


Figure 4: Transformation by electroformation.
(الرسم ليس للحفظ لكن خطوات الطريقة مطلوبة)

Step 4: Screening for transformed cells:

To avoid the growth of the untransformed bacterial cells, plasmid vectors are engineered with selectable marker gene for resistance to the antibiotics (Table 1). The media in which the transformed bacterial cells are grown is supplied with that antibiotic whose resistance gene is present in the plasmid. Due to this only transformed cells show antibiotic resistance will grow in the media supplied with antibiotic and untransformed cells cannot grow as they do not carry antibiotic resistance gene. Transformed bacterial cells may contain either recombinant plasmid DNA (vector containing foreign DNA insert) or non-recombinant plasmid DNA (self ligated vector only). Both type of transformed bacterial cells will show antibiotic resistance and grow on the agar media plate.

Table 1: Some commonly used antibiotics and antibiotic resistance genes.(للاطلاع فقط)

antibiotic	**Mode of action**	**Resistance gene**

Kanamycin	Inactivates translation by interfering with ribosome function	Neomycin or aminoglycoside phosphotransferase (*neo*r) gene product inactivates kanamycin by phosphorylation
Ampicillin	Inhibits bacterial cell wall synthesis by disrupting peptidoglycan cross-linking	β-Lactamase (*amp*r) gene product is secreted and hydrolyzes ampicillin
Tetracycline	Inhibits binding of aminoacyl tRNA to the 30S ribosomal subunit	*tet*r gene product is membrane bound and prevents tetracycline accumulation by an efflux mechanism

Blue-white screening or "*lac* selection" (also called α-complementation) can be used to distinguish between recombinant transformants and non- recombinant transformants. Bacterial colonies are allowed to grow on selective media containing antibiotic and X-gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside), a colorless chromogenic compound. Not all plasmid vectors are engineered for "*lac* selection"; the plasmid that are engineered for blue-white screening carry a MCS site in between gene that encodes for amino acids for enzyme β-galactosidase which cleaves β-glycosidic bond in D- lactose. X-gal mimic D-lactose and β-galactosidase enzyme acts on X- gal and produces a blue color complex (**Figure 5**).

A successful ligation of the desired gene disrupts the *lac Z* gene, hence no functional β-galactosidase is produced resulting in white colonies. Hence successful recombinant transformed colonies can be easily identified by its white coloration from unsuccessful blue ones. pUC19, pBluescript, pGem-T are few example of cloning vectors used for this test and it also requires the use of specific *E. coli* host strains such as DH5α which carries the mutant *lacZΔM15* genes.

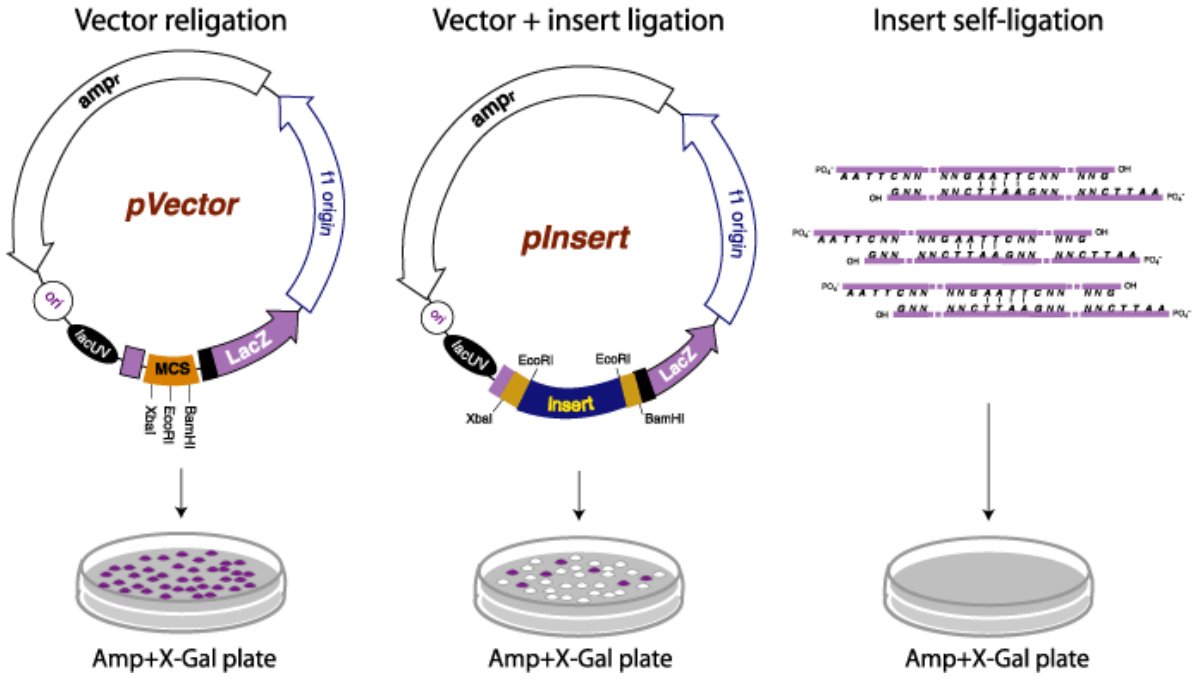


Figure 5: Growth on agar plates. Blue colonies represent Ampicillin-resistant bacteria that contain pVector and express a functional alpha fragment from an intact LacZ alpha coding sequence. White colonies represent Ampicillin-resistant bacteria that contain pInsert and do not produce LacZ alpha fragment.

Explanation of the colony selection: finding the rare bacterium with recombinant DNA
Only *E. coli* cells with resistant plasmids grow on antibiotic medium
Only plasmids with functional lacZ gene can grow on Xgal
lacZ(+) = blue colonies
lacZ functional = polylinker intact = nothing inserted, no clone
lacZ(-) = white colonies polylinker disrupted = successful insertion & recombination.

Step 5: Amplification and purification of recombinant plasmid DNA

The final step in DNA cloning is the isolation of the cloned recombinant DNA. A positive colony containing recombinant plasmid is identified and it is aseptically transferred to liquid medium and cell are allowed to grow exponentially overnight. A fully grown culture contains trillions of identical cells, which is harvested for the isolation of the plasmid DNA. The plasmid DNA is purified from harvested bacterial cell lysates. The purified plasmid DNA is dissolved in an appropriate buffer solution and can be used for further confirmation of the clone by restriction digestion and sequencing the plasmid DNA.

Additional information about The BLAST Sequence Analysis Tool

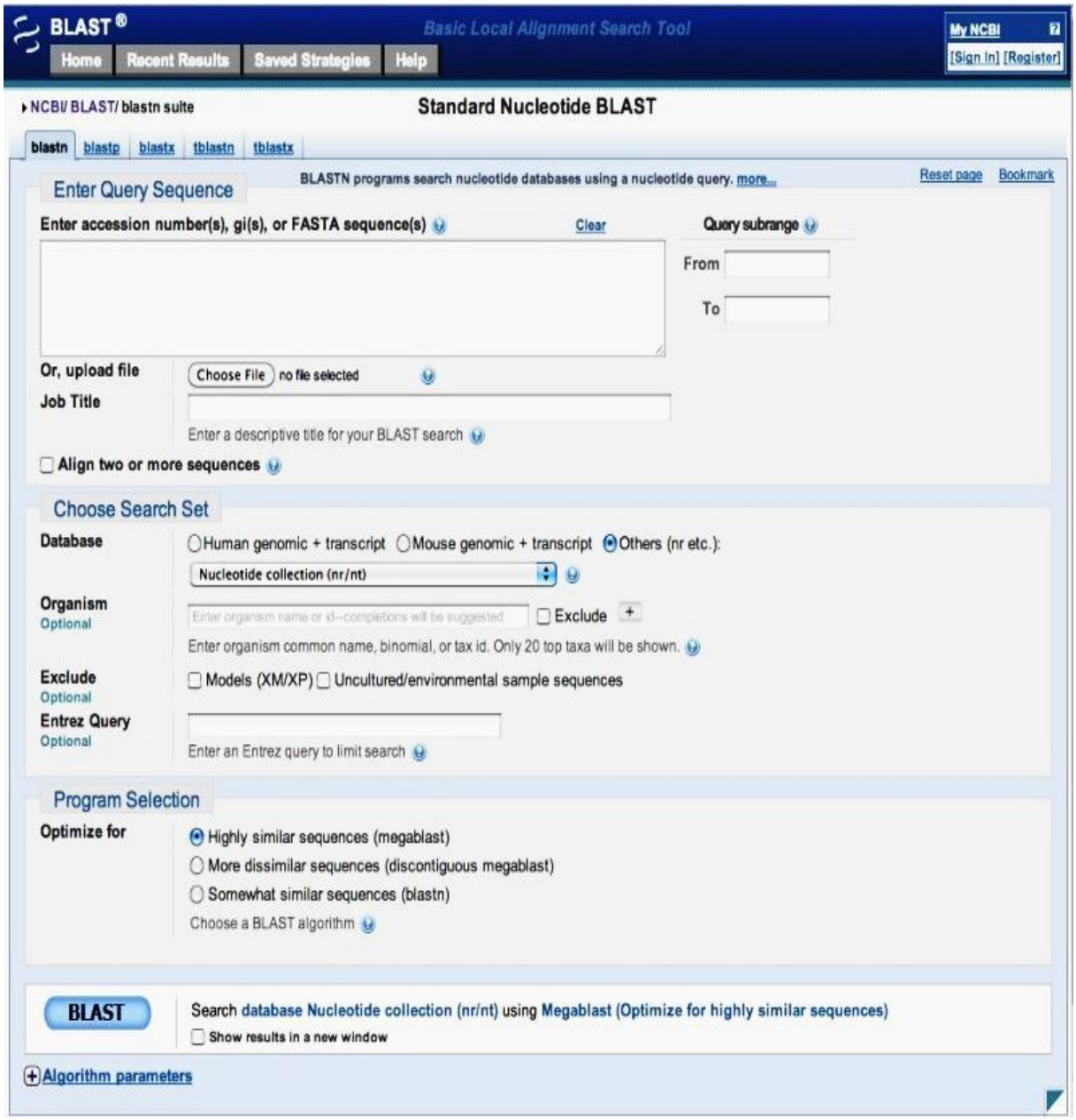
(للاطلاع فقط تعريف البرنامج مطلوب)

BLAST (**B**asic **L**ocal **A**lignment **S**earch **T**ool) is one of the most widely used bioinformatics programs for sequence searching. BLAST is an algorithm for comparing primary biological sequence information, such as the amino-acid sequences of proteins or the nucleotides of DNA sequences. A BLAST search enables a researcher to compare a query sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold.

Different types of BLASTs are available according to the query sequences. For example, following the discovery of a previously unknown gene in the mouse, a scientist will typically perform a BLAST search of the human genome to see if humans carry a similar gene; BLAST will identify sequences in the human genome that resemble the mouse gene based on similarity of sequence. The BLAST algorithm and program were designed by Stephen Altschul, Warren Gish, Webb Miller, Eugene Myers, and David J. Lipman at the National Institutes of Health and was published in the Journal of Molecular Biology in 1990 .

If we sequence a DNA clone, the first bioinformatics analysis is a similarity search against a nucleotide database. The most widely used similarity search program accessible on the internet is BLAST, which will be described here and will be used by the students during the laboratory practice. The BLAST program is available online at several servers including the one at NCBI: http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Note: NCBI (**N**ational **C**enter for **B**iotechnology **I**nformation) is part of the United States National Library of Medicine , a branch of the National Institutes of Health (NIH). The NCBI is located in Bethesda, Maryland and was founded in 1988 through legislation sponsored by Senator Claude Pepper.


BLAST®
Basic Local Alignment Search Tool
Home
Recent Results
Saved Strategies
Help
My NCBI
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NCBI/ BLAST/ blastn suite
Standard Nucleotide BLAST
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Job Title
Enter a descriptive title for your BLAST search
Align two or more sequences
Choose Search Set
Database
Human genomic + transcript
Mouse genomic + transcript
Others (nr etc.):
Nucleotide collection (nr/nt)
Organism
Optional
Enter organism name or id--completions will be suggested
Exclude
Enter organism common name, binomial, or tax id. Only 20 top taxa will be shown.
Exclude
Optional
Models (XM/XP)
Uncultured/environmental sample sequences
Entrez Query
Optional
Enter an Entrez query to limit search
Program Selection
Optimize for
Highly similar sequences (megablast)
More dissimilar sequences (discontiguous megablast)
Somewhat similar sequences (blastn)
Choose a BLAST algorithm
BLAST
Search database Nucleotide collection (nr/nt) using Megablast (Optimize for highly similar sequences)
Show results in a new window
Algorithm parameters

Problems in DNA Cloning:

Q1) A scientist isolates a human DNA repair gene and inserts it into a plasmid. The plasmid is inserted into a bacterial cell, so more plasmid can be produced. After isolating the plasmid from the bacterial cells, the scientist inserts the plasmid DNA into a human tissue culture cell using a virus. Which of the following statements is true?

- This experiment did not involve the use of vectors.
- One vector was used in this experiment.
- Two different vectors were used in this experiment.
- Three different vectors were used in this experiment.
- Four different vectors were used in this experiment.

Q2) A scientist isolates a human DNA repair gene and inserts it into a plasmid. The plasmid is inserted into a bacterial cell, so more plasmid can be produced. After isolating the plasmid from the bacterial cells, the scientist inserts the plasmid DNA into a human tissue culture cell using a virus. Which of the following statements is true?

- This experiment did not involve the use of hosts.
- One host was used in this experiment.
- Two different hosts were used in this experiment.
- Three different hosts were used in this experiment.
- Four different hosts were used in this experiment.

Q3) Can we reproduce Extinct animals very long ago such as dinosaurs be using DNA Cloning techniques? Explain this?