

# [Lambda genome shotgun cloning](https://assignbuster.com/lambda-genome-shotgun-cloning/)

A genomic library is a set of recombinant clones that contains the entire DNA of an organism. The “ shotgun cloning” method was performed to construct a genomic library of the bacteriophage lambda (). The restriction endonuclease, PstI, was used to cleave the Escherichia coli pUC19 vector and the DNA. Agarose gel electrophoresis was carried out to prove that PstI digested the DNA successfully. The pUC19 were treated with alkaline phosphatase to minimize self-ligation and were ligated to the fragments. The enzyme DNA ligase was used to join the fragments. Competent E. coli DH5β± were transformed with the ligated DNA. This resulted in recombinant DNA plasmids. The pUC19 had a marker gene, lacZ, which allowed for blue-white screening. Recombinant plasmids appeared white. Using agarose gel electrophoresis of PstI fragmented white colonies it was noted that one fragment was successfully cloned. These results were further validated using a Southern blot. Gel electrophoresis of EcoRI and MluI fragmented DNA and a HindIII marker was carried out to be used in the preparation of the Southern blot. A gene probe from a white colony was also prepared via random priming and was then hybridised to the Southern Blot. The size of the gene was found to be 805bp. The exact identity of the gene was established with the use of the Protein Blast. The gene fragment identified was part of gene B which codes for Portal protein B, a protein involved in the assembly of a capsid component of the bacteriophage.

The total number of extant DNA-containing bacteriophages is enormous. They are probably the most numerically abundant group of similar organisms in the biosphere [1]. Less than twenty-five years after these organisms were first described in 1915 [2], scientists started taking advantage of their ease to be biochemically and genetically manipulated in order to describe central concepts in the science of molecular biology. Their contribution to molecular biology is therefore momentously important. Furthermore, recent research has indicated that phage studies will have significant future influence on genetics, global ecology and bacterial pathogenicity.

Phages are viruses that infect bacteria, each being specific to a bacterial host. Coliphages are the phages whose host-range is restricted to the Escherichia coli bacterium. One of the most extensively studied coliphages is the bacteriophage.

Bacteriophage is an example of a temperate phage, meaning it can grow via two distinct cycles, lytic or lysogenic. Lytic growth results in phage production

Abbreviations: BLAST, Basic Local Alignment Research Tool; β²-gal, β²-galactosidase; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl-1-thio- β ²-D-galactoside; LB, Luria broth; X-Gal, 5-bromo-4-chloro-3-indolyl- β ²-D-galactoside.

During lysogeny, on the other hand, the bacterium manages to maintain the phage genome in its quiescent state. This cycle entails the recombination of the phage genome into its host DNA and the repression of most of the phage genes [3].

The genome of the bacteriophage is a linear double-stranded DNA molecule which is 48, 514 base pairs in length. This chromosome is large enough to encode 46 average sized genes [4]. The genome is quite compact with almost very little non-coding DNA. Its structure has been vastly studied and a fairly complete gene map was created by Echols and Murialdo [5], while Sanger et al. used dideoxy chain termination along with random “ shotgun” cloning to determine the exact nucleotide sequence [6].

What is interesting about this chromosome is that all correlated functions are clustered together. For instance, the genes which encode for capsid proteins are all grouped together and found next to a cluster of genes encoding for the tail. Similarly, the replication genes, the insertion excision system and the repressor-operator control complex are all found in similar functional ‘ modules’.

This type of gene clustering is perhaps necessary for three reasons. First, the regulation of the growth of the phage is simplified as only a small number of regulatory proteins and sites is needed to control the great number of genes whose products will work together. Second, genes which encode for proteins that must recognize each other are rarely separated by recombination. Last, recombination of the “ modules” results in the evolution of new phage species. These three purposes are further enabled by the close proximity of genes to the target sites on the DNA. Moreover, as proteins are formed near their activity sites, protein activity is kinetically increased. This is due to the fact that proteins do not need to diffuse through many different nonspecific interactions in order to reach their target site [5].

In addition, each 5′ end of the linear molecule has twelve unpaired bases which form single-stranded ends. These ends are complementary, and can cohere to each other to form linear multimers or circular molecules, coined ‘ Hershey’ circles after their discoverer. They are termed cos (cohesive) sequences, and are formed during bacteriophage packaging by the staggered cleavage of the DNA at cos sites. Upon infection of the host, the linear DNA will circularize by annealing of these cos sites [7].

Integration of into its host’s chromosome occurs by recombination involving unique sequences on the phage (attP) and the host chromosome (attB). These sequences have an identical region, consisting of 15 bp, called the core sequence. Recombination is catalyzed by the phage int-encoded product which specifically recognizes the attP sequence. This mechanism is called site-specific recombination as it can occur only at the specific attachment sites [3].

Briefly, in our experiment to produce a genomic library of the phage a restriction endonuclease digest was used to cleave the DNA into 27 manageable size fragments. The DNA fragments were then ligated into the vector pUC19 from E. coli. These recombinant plasmids were used to transform E. coli cells, which were afterwards plated. A random gene fragment was then selected from the plated colonies and identified. The main identification method depended on the use of the restriction enzyme site map of the genome, most recently described by Daniels et al. who precisely located 41 different restriction enzyme target sites relative to one another [8]. The entire procedure and results will be explored in further detail in this report.

## Materials and methods

Plating bacteria

A set of nine premade 1. 1% agar plates consisting of LB and100 µg/mL ampicillin were provided. The LB was produced following the original recipe as indicated by Brent and Elbing [9]. A different solution was pipetted to the centre of each plate. A sterile glass spreader was used to spread the solutions over the entire surface of the plate. The concentrations and volumes of the solutions used were exactly as described in pages 24 and 25 of the research project instructions [10]. Briefly, both IPTG and the chromogenic substrate X-Gal were pipetted at equal volumes to all plates while SOC buffer was added to only four plates. The solutions to be plated contained uncut pUC19, PstI cut, phosphatased and religated pUC19 or recombinant pUC19 with inserts. Both the uncut and the religated pUC19 plasmids served as controls. These solutions were each added to three different plates at different dilutions. The plates were then incubated in an inverted position at 37°C overnight. The protocol followed allowed the identification of the recombinants by β±-complementation. Either blue or white colonies occurred which were observed and counted a week later.

Restriction Enzyme Digestion

Four separate restriction digests were set up. First, a sample of 1. 5µg DNA was digested with 10 units of the restriction endonuclease Pst1. This should have been done in the presence of a 1x restriction enzyme buffer, but was mistakenly done in the presence of a 10x restriction enzyme buffer. In the second digestion a sample of pUC19 DNA was diluted with 34 µL sterile distilled water and digested with 5 units of Pst1, in the presence of a 1x restriction enzyme buffer. A volume of 2 µL of Alkaline Phosphatase was then added to this sample. Alkaline phosphatase removes the phosphate groups from the 5′ terminal and thus minimizes self-ligation of the plasmids. The sample was then incubated at 85°C for 15 minutes in order to heat-inactivate the alkaline phosphatase. In addition, two separate samples of 17µg DNA were each digested with 5 units of either EcoR1 or Mlu1, in the presence of 1x restriction enzyme buffer. All samples were then centrifuged and incubated at 37°C for 30 minutes. Finally, the restriction enzymes used in the lab were kept on ice throughout the experiment in order to extend their life and were stored at -20°C.

Gel Electrophoresis

Standard 0. 8% agarose gel electrophoresis was performed three times as described in Maniatis et al. [11]. In all three cases 5 µL of loading buffer was added to 10 µL of the samples to be loaded. The loading buffer contained Coomassie Blue dye which is easily observed on the gel during loading and glycerol which makes the samples heavier than water enabling them to sink to bottom of the wells. In the first electrophoresis, PstI cut pUC19 and PstI cut DNA were loaded with uncut pUC19 and DNA and a standard size molecular marker. This gel was used to test if the PstI successfully cleaved the DNA. In the second case, a gel was prepared to be used in the Southern blot. The MluI and EcoRI cut samples were loaded with 20 µL of digoxigenin-11-dUTP labelled, HindIII cut DNA marker. Contamination occurred in the EcoRI cut DNA. The pipette tip which was used to load the MluI was mistakenly not changed. Last, a gel was loaded with two samples of PstI cleaved white colonies; a PstI cleaved blue colony and a standard size molecular marker. This gel was used to estimate the length of the insert DNA.

DNA Ligation and Transformation

Two ligations were performed using the enzyme T4 DNA ligase. In the first sample 1 unit of ligase was added to 8µL of PstI cut and phosphatased pUC19, 2 µL of 10mM ATP and 9µL of ligase buffer. The pUC19 was religated. In the second sample 1 unit of ligase was again added to 8µL of PstI cut and phosphatased pUC19 and 2 µL of 10mM ATP. However, 7µL of ligase buffer was used and 2µL of PstI cut DNA added. Therefore, recombination occurred and the fragments joined to the cleaved pUC19. The two samples were then treated and stored according to page 16 and 17 of the research project instructions [10]. E. coli cells, competent for DNA uptake, were pre-prepared by the method of CaCl2 treatment [12] and supplied in a frozen suspension. A volume of 200 µL of these E. coli DH5β± cells was added to each of the prepared ligation solutions and to a sample of 0. 1µg uncut pUC19. The three samples were prepared as the research project instructions indicated on page 25 [10]. This resulted in transformation, that is the uptake and incorporation, of the DH5β± cells with recombinant, self-ligated or uncut pUC19 plasmids.

Southern blot

Southern blot analysis was performed according to Ausubel et al. [13]. In brief, the EcoRI and MluI fragmented DNA and the HindIII marker from the pre-prepared electrophoresis gel were blotted to a Nylon membrane. The blot was incubated with a white colony probe, labelled via the random priming method. During this method, isolated DNA from a white colony was first denatured, that is separated into single strands, by exposure for 10 minutes to 100°C followed by rapid cooling in ice water. A volume of 2 µL of DIG-High prime solution then labelled the single-stranded DNA. The sample was incubated at 37°C for an hour and the reaction was halted by the addition of 1 µL of 0. 2M EDTA. A commercially prepared Dig Easy Hyb solution was used for both the pre-hybridisation and hybridisation steps of the filter. The blot was pre-treated so that any non-specific protein binding sites would be blocked. The probe was incubated with the blot and as it has complementary sequences on the blot, it hybridised to them. The last step was to colour develop the blot. The last five steps were carried out exactly as described on pages 38-40 of the research project instructions [10].