Laboratory works for discipline “Genetic engineering and biosafety

*Lab. Work 1-2 Main principles of extraction of DNA.*

 DNA Extraction. Objectives

The objectives of this experiment are to extract DNA from a fruit sample, test the moisture of a soil sample, and perform blood typing and gel electrophoresis. The DNA will be extracted using the basic biochemical techniques for isolating, purifying, and digesting DNA molecules.

Overview

Cellular Biology and Location of DNA

DNA is the blueprint of life and is found in almost all living organisms. These organisms can be as simple as a single-celled bacteria or as complex as a multi-celled human: the human body contains approximately 50 trillion cells. There are two different types of cells: prokaryotes and eukaryotes. An example of prokaryotic organism is bacteria. Prokaryotic cells do not contain a nuclear membrane and so do not have a distinct nucleus. Only eukaryotic cells, which make up plants and animals, will be considered in this lab. Eukaryotic cells have a distinct, membrane-bound nucleus that isolates the DNA from the rest of the cell. The structure of plant cells is different from those of animal cells in structure and cellular contents. Only plant cells will be used in this experiment.



Plant cells are surrounded by a cell wall. It has high mechanical strength and protects the cell. Directly beneath the cell wall lies the plasma membrane (Figure 1), which contains the cytosol. The various cell organelles, including the nucleus, are found within the cytosol. The nucleus houses the DNA in the form of chromatin



Chromatin is the active form of DNA in the cell when it is not preparing for cell division. It is comprised of DNA wrapped around protein particles called histones



DNA Extraction Technique

In this experiment, a goal is to extract the DNA from a fruit sample. Some knowledge of the scientific background behind DNA extraction is needed to do this.

The DNA extraction process is a fairly simple biochemical procedure that can be divided into three major steps: breaking open the cell (lysis), destroying membranes within the cell, and precipitating the DNA out of the solution.

The following sections describe how each step relates to the physical and biochemical properties of DNA.

Cell Lysis (Breaking Open the Cell Wall and Membranes)

Plant cells have a very rigid external structure — the cell wall — which protects it. To get to the DNA, the very first step would be to break open that wall.

The cell wall is the first barrier in that must be broken to extract the DNA molecule inside the cell. It is very rigid and acts as a protector and filter. It is made of cellulose, and is responsible for making wood hard and durable. To destroy the cell wall, a mechanical method is used to break apart the cellulose molecules. In this experiment, the fruit sample is mashed manually.

Destroying Membranes Within the Cell

The cell's plasma membrane is made of phospholipid bilayers; they are made of fat. To disrupt them, that mesh of fat molecules is broken up with soap. The structure of soap is very similar to that of fat and grease.

Precipitating the DNA

When the membrane is successfully disrupted, the DNA is released from the cells into the solution along with protein molecules and other cellular miscellanea.

The DNA molecule is a double-helical polymer consisting of a sugar-phosphate backbone with nitrogenous bases running perpendicular to the backbone. These bases, often represented by letters — A (adenine), G (guanine), C (cytosine), and T (thymine) — are the elementary components making up the coded genetic information (Figure 7). The base sequence acts as the instruction manual of the cell, directing it on how to make proteins and other important molecules that an organism needs to survive and function.

With the cell's contents mixed into a solution, the DNA is separated from the rest; this process is called precipitation. Salt is used because it disrupts the structure of the proteins and carbohydrates found in the solution. Also, the salt provides a favorable environment to extract the DNA by contributing positively charged sodium ions that neutralize the negative charge of DNA. After the addition of salt and soap, the manner by which the DNA is being extracted out of the solution cannot be seen as it is too small to distinguish from the rest of the solution. To aid in precipitating the DNA, alcohol is added since it cannot dissolve DNA. A translucent white substance will begin to form at the top; this is DNA. Once it is thick enough, it can be spooled out. This simple procedure is a rough extraction process that needs further purification before it can be successfully run on a gel for analysis.

 Extracted DNA.

*Lab. Work 3-4. Extraction of DNA from different biological materials.*

Buffer for extraction of DNA from plant materials

Cetyltrimethylammonium-bromide (CTAB) is a non-ionic detergent, which forms insoluble complexes with nucleic acids if the sodium chloride concentration in the solution is around 0.5 M. Polysaccharides, phenolic components and other enzyme-inhibiting impurities from lysates of plant cells can be effectively removed with the CTAB lysis buffer (1).
Contains 2 % CTAB,

20 mM EDTA·Na2·2H2O,

1.4 M NaCl

100 mM Tris, pH 8.

Often times, larger fragments of DNA are cut, or restricted, to extract a particular fragment. This is made possible by the action of restriction enzymes, which are used by bacteria to cut up foreign or enemy DNA. Restriction enzymes are catalytic proteins that recognize specific palindromic DNA sequences and cut the double-stranded DNA at particular sites. The sites that the restriction enzymes recognize are called restriction sites. There are many different types of restriction enzymes. Each type recognizes a different restriction site. In this lab, Lambda DNA, which is a commercially available DNA normally found in a virus called Phage Lambda, will be restricted with the restriction enzyme BamH1.

### Quick and easy method was developed for the extraction of DNA from Legionella pneumophila, which can also be used to extract DNA from a variety of other Gram-negative or Gram-positive bacteria. The method may be performed in 1.5 ml polypropylene tubes, using as little as a single colony, and is suited for the extraction of many samples simultaneously. The procedure uses 6 M NaI as a chaotropic agent and isopropanol to semi-selectively precipitate the DNA. Once the cells have been lysed, the procedure takes approximately 30 min to perform. Without further clean-up, the extracted DNA can be used for restriction endonuclease digestion or polymerase chain reacti Materials and Reagents**.** DNA Extraction

Contaminants such as sugars remain in the DNA collected from the fruit sample. Typically, to properly run it through the electrophoresis gel and get results, it must be sized down considerably and thoroughly rinsed to get rid of the excess. Instead, Lambda DNA is used because it is already prepared and able to run in the electrophoresis gel.

1. Obtain a fruit sample that is about two inches wide and put it in the Ziploc bag provided.
2. Close the bag so there is as little air as possible inside.
3. Mash the sample gently by hans. Be careful not to burst the bag. After about five minutes, the fruit sample will be transformed into a creamy paste. This process is known as homogenization.
4. Prepare the buffer solution while the homogenization of the fruit sample is occurring.
	1. Fill a cup ¼-way with distilled water.
	2. Add one teaspoon of table salt.
	3. Mix the solution until the salt dissolves in the water.
	4. Add two teaspoons of soap.
	5. Stir gently with the spoon so that it does not foam. Keep stirring until the texture of the solution is even.
5. Pour the prepared buffer solution into the Ziploc bag and close it. Make sure that there is no trapped air in the bag.
6. Mix the smashed fruit and the buffer solution gently in the bag. Do it slowly. It is important that it does not foam a lot.
7. Let the mixture sit for about five minutes. If it has foamed, allow the foam to go away during this time. By letting the mixture stay still, the foam will disappear.
8. Filter the solution by using another clear plastic cup. Hold the strainer on top of the empty cup while carefully pouring out the contents of the Ziploc bag. Make sure it does not foam. Pour slowly. Occasionally shake the strainer to make the liquid filter through. There is a lot of debris.
9. Add ice-cold 95% isopropyl alcohol to the filtered solution by pouring the alcohol against the wall of the cup. Do not mix the alcohol with the solution; it should float on top. Alcohol dissolves within water, but it can float if it is poured slowly against the side of the container because it is less dense than water. Pour the alcohol until the total volume reaches ¾ of the cup’s volume. After about one minute, threads of DNA will form into translucent gel-like globs at the interface of the filtered solution and the alcohol.
10. Collect the DNA by spooling it with a paperclip.
11. Split the spooled DNA into two sample containers. Label one S for sugars and the other P for proteins.
12. Return back to step 7.
13. Tris base (Calbiochem-Behring)
14. Proteinase K (Sigma-Aldrich)
15. Phenol\chloroform (1: 1) (EM Science)
16. 200 proof ethanol (Pharmco-AAPER)
17. RNAase (Life Technologies, Invitrogen™)
18. Ethanol
19. SDS
20. EDTA
21. Tryptone
22. Yeast extract
23. NaCl
24. LB medium (see Recipes)
25. TE buffer (see Recipes)
26. Lysis buffer (see Recipes)

# Lab. Work 5-6. Restriction Enzyme Digest Protocol

Often times, larger fragments of DNA are cut, or restricted, to extract a particular fragment. This is made possible by the action of restriction enzymes, which are used by bacteria to cut up foreign or enemy DNA. Restriction enzymes are catalytic proteins that recognize specific palindromic DNA sequences and cut the double-stranded DNA at particular sites. The sites that the restriction enzymes recognize are called restriction sites. There are many different types of restriction enzymes. Each type recognizes a different restriction site. In this lab, Lambda DNA, which is a commercially available DNA normally found in a virus called Phage Lambda, will be restricted with the restriction enzyme BamH1.



Figure 7: Nitrogenous bases of DNA.

|  |
| --- |
| **Figure 8: Restriction enzymes.**  |
| **Enzyme** | **Source** | **Recognition Sequence** | **Cut**  |
| BamH1 | Bacillus amyloliquefaciens  | 5'GGATCC3'CCTAGG | 5'-----G-------CCTAG | GATCC------G-----5' |
| EcoRI | Escherichia coli  | 5'GGATCC3'CCTAGG | 5'-----G-------CCTAG | GATCC------G-----5' |
| HindIII | Haemophilus influenzae  | 5'AAGCTT3'TTCGAA | 5'---A3'---TTCGA | AGCTT---3'A---5' |

Find more protocols and selection guides in the Molecular Biology Guide.

Our restriction enzyme collection has been optimized for digestion using five unique buffers. When digesting DNA using a single enzyme, use the buffer supplied with the enzyme.

## Protocol for DNA Digestion with a single restriction enzyme

1. Add components to a clean tube in the order shown:
     1 µL DNA (concentration 1 µg/µL)
     2 µL 10x buffer
     1 µL restriction enzyme
     16 µL sterile water
2. Incubate the reaction at digestion temperature (usually 37°C) for 1 hour.
3. Stop the digestion by heat inactivation (65°C for 15 minutes) or addition of 10mM final concentration EDTA.
4. The digested DNA is ready for use in research applications.

When using two restriction enzymes at once, first check the enzyme activities in each buffer, using the table on the Restriction Enzyme Buffer Reference. If they both have 100% activity in the same buffer, you can proceed with your double digestion protocol using that buffer. Alternatively, the optimal buffer can be determined from the [chart of common double digestions](https://www.sigmaaldrich.com/technical-documents/articles/biology/restriction-enzyme-double-digest-buffer-guide.html). In some cases, sequential digestion is recommended due to buffer incompatibility (composition or temperature).

## Protocol for DNA Digestion with two restriction enzymes

1. Add components to a clean tube in the order shown:
     1 µL DNA (concentration 1 µg/µL)
     2 µL 10x buffer
     1 µL each restriction enzyme
     15 µL sterile water
2. Incubate the reaction at digestion temperature (usually 37°C) for 1 hour.
3. Stop the digestion by heat inactivation (65°C for 15 minutes) or addition of 10mM final concentration EDTA.
4. The digested DNA is ready for use in research applications.

## Protocol for sequential DNA Digestion

1. Add components to a clean tube in the order shown:
     1 µL DNA (concentration 1 µg/µL)
     2 µL 10x buffer
     1 µL restriction enzyme
     16 µL sterile water
2. Incubate the reaction at digestion temperature (usually 37°C) for 1 hour.
3. Stop the digestion by heat inactivation (65°C for 15 minutes) or addition of 10mM final concentration EDTA.
4. Recover the DNA using a purification kit: re-suspend and dilute the DNA to 1 µg/ µL.
5. Prepare second digestion according to step 1. Continue through step 3.
6. The digested DNA is ready for use in research applications.

|  |  |  |  |
| --- | --- | --- | --- |
| 1. Enzyme
 | Source | Recognition Sequence | Cut  |
| BamH1 | Bacillus amyloliquefaciens  | 5'GGATCC3'CCTAGG | 5'-----G-------CCTAG | GATCC------G-----5' |
| EcoRI | Escherichia coli  | 5'GGATCC3'CCTAGG | 5'-----G-------CCTAG | GATCC------G-----5' |
| HindIII | Haemophilus influenzae  | 5'AAGCTT3'TTCGAA | 5'---A3'---TTCGA | AGCTT---3'A---5' |

### **Preparation of Sample for Restriction**

|  |
| --- |
| **Summary of reagent amounts for DNA restriction.**  |
| **Tube** | **Lambda DNA** | **Restriction Buffer** | **BamH1** | **Distilled H2O**  |  | **Dye**  |
| 1 | 4 µL | 5 µL | 2 µL | 8 µL  |  | 2 µL  |
| Control | 4 µL | 5 µL | 0 µL | 10 µL  |  | 1 µL  |

1. Load the micropipette with a tip and obtain four µL of the lambda DNA using the micropipette.
2. Pipette it into a microcentrifuge tube and dispose of the micropipette tip. Dispose of the tip after each use.
3. Reload the micropipette with another tip and obtain five µL of the restriction buffer. Dispose of the tip.
4. Get another tip and get two µL of BamH1, which is the restriction enzyme used to cut the DNA. Dispose of the tip.
5. Add eight µL of distilled water. After this is done, call the TA.

A TA will oversee the rest of the procedure. As step 6 is occurring, proceed to the DNA extraction portion at step 16.

### ***Restriction of DNA Sample***

1. Place the microcentrifuge tube into the incubator. It should be set to 37 °C. The sample will incubate for 30 minutes.
2. Add two µL of dye. This will show the DNA as it runs through the gel.

Individual Lab Report

Follow the lab report guidelines laid out in the page in the Technical Communication section of this manual. The following discussion points should be addressed in the appropriate section of the lab report:

1. Discuss the structure of a plant cell.
2. Justify the use of salt, soap, and alcohol in the extraction procedure.
3. Explain how to reach the DNA and the barriers that were overcome to get to it.
4. Describe the major techniques used in this lab: DNA Restriction, Gel Electrophoresis, etc.
5. Important properties of DNA directly having an impact on the extraction procedure.
6. Clearly describe the procedural steps the way they were carried out in lab.
7. Describe the steps carried out with the TA.
8. Explain the test results for the Biuret test and Benedict's test.
9. Include appropriate figures to support the observations made.
10. Specify the location in the gel of the DNA sample that belonged to the team.
11. Compare the results with the control group.
12. How would procedure change if meat was used instead of fruit?
13.

### **Lab. Work 7.** Choice of host organism and cloning vector

Although a very large number of host organisms and molecular cloning vectors are in use, the great majority of molecular cloning experiments begin with a laboratory strain of the bacterium *E. coli* (*Escherichia coli*) and a plasmid cloning vector. *E. coli* and plasmid vectors are in common use because they are technically sophisticated, versatile, widely available, and offer rapid growth of recombinant organisms with minimal equipment. If the DNA to be cloned is exceptionally large (hundreds of thousands to millions of base pairs), then a bacterial artificial chromosome or yeast artificial chromosome vector is often chosen.

Specialized applications may call for specialized host-vector systems. For example, if there is need to wish to harvest a particular protein from the recombinant organism, then an expression vector is chosen that contains appropriate signals for transcription and translation in the desired host organism. Alternatively, if replication of the DNA in different species is desired (for example, transfer of DNA from bacteria to plants), then a multiple host range vector (also termed shuttle vector) may be selected. In practice, however, specialized molecular cloning experiments usually begin with cloning into a bacterial plasmid, followed by subcloning into a specialized vector.

Whatever combination of host and vector are used, the vector almost always contains four DNA segments that are critically important to its function and experimental utility:

* DNA *replication origin* is necessary for the vector (and its linked recombinant sequences) to replicate inside the host organism
* one or more unique *restriction endonuclease recognition sites* to serves as sites where foreign DNA may be introduced
* a *selectable genetic marker* gene that can be used to enable the survival of cells that have taken up vector sequences
* a *tag* gene that can be used to screen for cells containing the foreign DNA

**Lab. Work 8-9. Gel Electrophoresis**

The technique of DNA electrophoresis (Figure), will be performed on uncut and cut Lambda DNA, a commercially available DNA on an agarose gel, to visualize the characteristic banding patterns that differentiate between different DNA fragments. Gel electrophoresis is a technique used for separating molecules based on their charge and molecular weight. The sample is loaded in a gel matrix and an electric field is applied across it. The electric field enables the DNA, which is negatively charged to migrate to the end, which is positively charged. Opposites attract and so the negatively charged DNA is attracted to the positive end of the gel. Lighter molecules will migrate to the opposite end of the gel faster than heavier molecules.



Figure. Gel electrophoresis.

The following is a gel after the samples have been run. Each column is referred to as a lane, representing one sample each. The individual bands (Figure 11) contain fragments of DNA that are identical in weight.



Figure 11: DNA banding pattern following gel electrophoresis.

# Materials and Equipment

 

* Fruit sample
* Non-iodized table salt (NaCl)
* Hand soap (clear, unscented)
* 95% isopropyl alcohol (0 °C)
* Distilled water
* Strainer
* Plastic cups
* Ziploc bag
* Lambda DNA
* Dye
* Variable micropipette and tips
* Incubator
* Microcentrifuge tube
* Precast agarose gel
* Electrophoresis system
* Bioimaging system
* DNA sample containers
* Disposable pipets
* Iron Magic Wand
* Iron powder
* Arduino software
* Arduino moisture sensor
* Blood typing kit
	+ Milk, water, vinegar, dye

### **Procedure of Gel Electrophoresis**

1. Prepare the electrophoresis gel when there are 15 minutes left for the incubation.
	1. Plug the power base in.
	2. Open the package containing the gel.
	3. Insert the gel, right edge first, and press firmly at the top and bottom to seat the gel in the base. A snap will be heard when it is in place. A steady red light will appear.
	4. Press and hold either button until the red light turns into a flashing green light. This indicates that the two-minute pre-run of the gel has started. At the end of the pre-run, the flashing green light will change to a flashing red light and the base will rapidly beep.
2. Press and release either button to stop the beeping. One more beep will be heard. The light will change from a flashing red to a steady red light.
3. Remove the comb from the gel by pulling it straight up from both sides and remove any excess fluid using a pipette.
4. Load the samples in 20 µL volumes into the wells. Load 20 µL of distilled water into any remaining empty wells.
5. Press and release the 30-minute button to start the 30-minute electrophoresis run. The light will change to a steady green light.
6. Wait 30 minutes for the run to complete. The light will flash red and there will be a rapid beeping.

While waiting for the gel electrophoresis to complete, proceed to step 28.

1. Press and release either button to stop the beeping and the light will turn to a steady red light.
2. Remove the gel from the base and analyze the results using a UV transilluminator.

 DNA banding pattern following gel electrophoresis.



### **Lab. Work 10-11 Preparations of the DNA insert and vector DNA.**

### **Introduction**

The success and easiness of cloning a DNA fragment into a plasmid vector depends on several factors. Cloning is significantly more successful when there is only *one DNA fragment to be ligated into the plasmid vector.* Compatibility of the ends of the two molecules is extremely essential. Cohesive complementary ends generate more efficient cloning then blunt ends.

However, the highest cloning efficiency is achieved with DNA digested by *two different restriction enzymes*. Ligating templates prepared by this procedure is called directional cloning, because the insert DNA can only be ligated into the vector in a single orientation. This method dramatically decreases background level of non-recombinants. Another popular application is the cloning of PCR products produced by Taq polymerase. This polymerase adds a extra A at the 3'-end of amplified DNA, which assists the cloning of PCR products into a special vector.

### **Preparation of the DNA insert**

1. The DNA is digested with suitable restriction enzymes to produce compatible ends for cloning. To guarantee that the correct restriction fragment is cloned, the DNA insert should be purified on an appropriate percentage agarose gel by gel electrophoresis.

The DNA is then extracted and purified from the agarose gel to enhance ligation efficiency.

1. DNA inserts can be prepared from genomic DNA through *PCR amplification*. Designing appropriate forward and reverse primers is an critical step in this process.

 By designing appropriate flanking primers, a target sequence can be amplified from genomic DNA. Once the PCR reaction has been completed and the target sequence amplified, the products can be run through a 1-2% agarose gel and then run through a gel purification kit to purify the specific sized fragments in question. The DNA eluate can then be sequenced for further confirmation of the correct DNA sequence to be cloned. Then the amplified DNA product can be subjected to restriction digests or ligation.

### **Ligation of plasmid vector and insert DNA**

After the insert DNA and vector have been prepared for ligation, estimate the concentration of each by agarose gel electrophoresis along with molecular weight standards of a known concentration. Different vector:insert DNA ratios can be tested to find the optimal ratio for ligation. Generally, a 1:1 or 1:3 molar ratio works pretty well. Addition of DNA ligase (generally T4 DNA ligase) and ligase buffer containing ATP is required for this ligation step.

Specifically designed vectors can also be purchased where specific polymerase enzymes are already on the entry sites of the vector for easier more efficient cloning of fragments into entry vectors, but at a higher price.

### **Transformation of DNA into bacteria**

Usually this step is subsequent to ligation, transforming the vector-DNA insert into competent cells. The transformed cells are plated on LB plates with selective antibiotics and grown overnight. The vectors with the inserts are screened by various methods: blue/white color screening, antibiotic screening, etc. The correct clones are selected and grown, producing huge amounts of vector with the DNA insert. Plasmid DNA minipreps are then done to obtain these correct vectors of interest from the bacteria.

Transformation methods:

Cells can be transformed with the cloned vectors through electroporating competent cells or through chemically competent heat sensitive cells. Electroporating involves mixing electrocompetent cells with the cloned vectors and then running an electric current through the cells allowing them to take up the DNA. Cells are then shaken at an appropriate growth temperature in growth media to stimulate growth and then plated on antibiotic-selective LB plates to select for successfully transformed cells that took up the antibiotic resistant cloned vectors. Chemically competent cells are similar to electro-competent cells except they take up the cloned vectors through heat shock rather than electrical current

 Gel electrophoresis.

The following is a gel after the samples have been run. Each column is referred to as a lane, representing one sample each. The individual bands (Figure) contain fragments of DNA that are identical in weight.

# Materials and Equipment



**Lab. Work 12-13** BACTERIOPHAGE CLONING VECTORS (λ AND M13) [2.](https://image.slidesharecdn.com/lectut-btn-202-ppt-l4-170428212615/95/lectut-btn202pptl4-bacteriophage-lambda-and-m13-vectors-1-2-638.jpg?cb=1493415228) 2

INRODUCTION •Esther Lederberg found E.coli K12 to be lysogenic for λ phage, which marked the discovery of λ phage as well as phenomenon of lysogeny •This is a temprate phage •It is a lamboid phage that can infect E.coli 3

Characteristic Features of λ phage •Coliphage •Belongs to Siphoviridae family •Nearly 50 nm diameter •Icosahedral head •A flexible tubular protein tail •Connector serves as a site for attachment of preformed head to tail •Cos sites on ends •Has double stranded linear BDNA molecule of MW 31\*10 6 Da •Because of presence of cos sites DNA adopts circular structure when injected into host cell 4

GENE ORGANIZATION Left hand region Genes included are Nu1 through J that encode for head and tail proteins Central region Genes included are J through gam that encode for integration and recombinant functions ( int, xis, red, gam) attP site is an attachment site Right hand region N and Q control lysogeny and lysis ( cI, cII, cIII and cro) O and P for DNA replication S,R and Rz for host cell lysis 7

λ bacteriophage as a cloning vehicle •Large genome size and low insert capacity Head can accommodate certain amount of DNA ( 48.5 kbp) •Presence of multiple sites for a commonly used restriction enzymes •Size diminution and increasing cloning capacity Only 50% of genes of wild type phage are essential for its replication and lysis of host cell 9

•Alteration in the number of cleavage sites for restriction enzymes •Natural selection of modified λ bacteriophage lacking certain restriction sites •Natural selection followed by genetic crossing with λ bacteriophage 10

•Recombinational insertion of regions from other lamboid phages This was done by in vivo genetic recombination that lead to exchange of immunity regions of two phages λ and ф434 •In vitro mutagenesis •In vitro manipulation 11

CLONING IN λ VECTORS Steps are as follows: 1. Restriction digestion of λ vector as well as genomic DNA 2. Ligation of the DNA fragments into λ vector to form recombinants 3. Introduction into host by transfection of competent E. coli host or in vitro packaging of recombinant DNA by addition of packaging extract followed by natural infection of the host 4. Selection and screening of recombinants 12

[.](https://image.slidesharecdn.com/lectut-btn-202-ppt-l4-170428212615/95/lectut-btn202pptl4-bacteriophage-lambda-and-m13-vectors-1-13-638.jpg?cb=1493415228) CHOICE OF λ VECTORS Following considerations influence the selection of vector: 1. Restriction enzymes to be employed 2. The size of the foreign gene to be inserted 3. Type of screening system to be employed 4. Expression of the cloned gene in E. coli 13

Classes of λ vectors 1. Insertional vectors • Vectors containing at least one unique restriction enzyme site for the insertion of the foreign DNA are insertional vectors • Size of insertional vectors should not be below 37kbp and that of foreign DNA should be such that it should not increase the size of vector above 52kbp • E.g. :λ gt10, λ gt11, λ gt18, λ gt23 14

λ phage as insertional vectors 15

2.Replacement or substitution vectors •Substituting the non essential part of vector with gene of interest •Lowest possible size of replacement vector should be 37kbp •E.g.: Charon series, λ EMBL Series, λ gt. λ C, λ GEM 11, λ GEM 12, λ DASH, λ FIX 16

λ phage as substitution vectors 17

INTRODUCTION •Filamentous rod shaped E.coli bacteriophage •Belongs to family Inoviridae •A small phage •Has circular single stranded DNA of 6400 nucleotides in length •During replication inside the host cell M13 is present in double stranded replicative form (RF) •During morphogenesis the DNA is converted into single stranded conformation •Its genome is completely sequenced •Phage particle has dimensions of nearly 900 x 6- 7 nm 19

M13 bacteriophage 20

GENOME MAP OF M13 PHAGE 21

Gene: Product Size: Function: 1 35-40kd NS membrane protein; few copies/cell; interacts with host fip gene product (thioredoxin); required for assembly 2 46kd Site- & strand-specific endonuclease/topoisomerase; ~103 copies/cell; required for replication of RF X (10) 12kd (111aa) N-terminal fragment of gene 2; ~500 molecules/cell; required for replication of RF 3 42kd 5 copies at one end of particle; required for correct morphogenesis of unit-length particles; N-terminal domain binds to F pilus of host cell (receptor) 4 49kd NS membrane protein; few copies/cell; required for assembly 22

Gene: Product Size: Function: 5 10kd (87aa) Major structural protein during replication; ~105 copies/cell; controls expression of g2p; binds to DNA; replaced by g8p during assembly; controls switch from RF replication to progeny (+)stand synthesis 6 12kd (112aa) ~5 copies at same end of particle to g3p; involved in attachment & morphogenesis 7/9 3.5kd ~5 copies at opposite end of particle to g3p/g6p; involved in assembly 8 5kd (50aa) Major coat protein: ~2700-3000 copies/virion 23

Replication cycle of M13 DNA 24

25

M13 bacteriophage as a cloning vector First vectors used – M13mp18 & M13mp19 Advantages – blue/white screening system – genes cloned in pUC18 or pUC19 – can be subcloned to same sites in M13mp equivalent – different directions for multiple cloning sites - both strands of cloned DNA converted to single- stranded form in different vectors 26

Disadvantages – limits to size of cloned DNA (2 kb) – low yield of DNA – cannot amplify phage genome numbers much – phage proteins toxic in high concentrations 27

Types of M13 vectors based on location of cloning sites 1.Cloning into the large intergenic region 2.Cloning into the small intergenic region 3.Cloning into gene X 28

MODIFIED M13 VECTORS Modified M13 vectors have large number of cloning sites Examples: M13mp1, M13mp8, M13mp9, M13mp10, M13mp11, M13mp18, M13mp19 29

Advantages of modified M13 vectors 1. Facilitate directional cloning by using two different enzymes for cleavage 2. Cloning in dual vectors is advantageous 3. Cloned DNA fragments can be moved between M13mp vectors and pUC vectors with great ease 4. The intergenic region of M13 is now standard fixture of most plasmids 5. Single stranded DNA can be directly isolated from M13 phages 30

Bacterial hosts for M13 vector •Only male (F+) bacteria are used for proliferation of M13 phage because it enters the host cell through sex pilli encoded by F factor •Female bacteria can be infected by transfection 31

**Lab. Work 14-15** *M13 phage vectors*

**bacteriophage M13** – filamentous bacteriophage

single-stranded circular DNA genome (6407 bp long)

– packaged inside rod-shaped protein capsid

**M13 life cycle** (Fig. 3.1)

phage particles bind to F pilus

– only infects F+, Hfr, F' cells

single-stranded DNA genome enters cell

designated as “+” strand

“+” strand repaired

– double-stranded **replicative form (RF)**

RF contains “+” and “–” strands

“–” strand is template – for mRNA synthesis

– for production of new “+” strands

– by rolling circle replication

“+” strands are packaged in phage coat protein

– exit cell as phage particle

Important points for cloning vectors

M13 occurs in both single and double stranded forms

RF can be digested with restriction endonucleases

inserts can be cloned in – like plasmid

“+” strands from phage particles

– convenient source of single-stranded DNA

– used for sequencing and site-directed mutagenesis

different sized DNA molecules packaged as phage particle

– (within reason)

– phage with inserts > 2 kb replicated slower

different sized DNA molecules

– produce different size phage particles

M13 does not kill host

– phage particles released without lysing cell membrane

– slows growth of host, produces turbid “plaques”

– really zones of slowed bacterial growth

single-stranded DNA

– collected by growing M13 infected cells in culture

– cultures centrifuged to pellet bacterial cells

– phage remains in supernatant

– until precipitated with Ficoll

DNA extracted from phage by phenol extraction

Production of single-stranded DNA requires:

M13 origin of replication – in DNA molecule

M13 gene products – in cell containing DNA molecule

Phage proteins can be provided in trans by helper phage

– allows “phagemid” vectors to be used

(plasmid + M13 origin of replication)

**cloning vectors**

first vectors used – M13mp18 & M13mp19 (Fig. 3.3)

M13 phage with *lacZ* ' containing multiple cloning site

same gene and cloning site as pUC18 & pUC19

advantages – blue/white screening system

– genes cloned in pUC18 or pUC19

– can be subcloned to same sites in M13mp equivalent

– different directions for multiple cloning sites

–both strands of cloned DNA

– converted to single-stranded form

– in different vectors

disadvantages – limits to size of cloned DNA (2 kb)

– low yield of DNA

– cannot amplify phage genome numbers much

– phage proteins toxic in high concentrations

**phagemid vectors** – plasmid + M13 origin of replication

e.g. pEMBL18& pEMBL19

– pUC18 & pUC19 + M13 origin of replication

maintained in host cell like regular plasmid

– high copy number, lots of copies of cloned DNA

– if M13 helper phage infects cell containing pEMBL

– phage proteins package single-stranded plasmid

– in phage particles – collected like regular phage

Note – helper phage genomes packaged too

– but not as many present as plasmid

– get 100 fold excess of plasmid packaged

helper phage contamination not a problem for sequencing

 – use “universal” primers

– homologous to plasmid sequences

– no cross-reaction with phage sequences

**Improved phagemids**

– e.g. Bluescript (Stratagene product) (Fig. 3.8)

contains: high copy number pMB1 *ori*

ampicillin resistance marker

*lacZ* ' containing multiple cloning site

– in 2 different orientations

– different construct than pUC

– allows different cloning strategies

M13 origin of replication

T7 & T3 phage promoter sequences

– flanking multiple cloning site

– used for *in vitro* RNA synthesis

T7 & T3 phage genomes

– code for unique RNA polymerase enzymes

– recognise different promoters than standard *E. coli*

Bluescript vector with cloned insert

– mixed with: phage RNA polymerase

 radiolabelled NTPs

– get RNA version of cloned DNA

– used as hybridization probe

Note – do not confuse **promoters** with **primers**

**promoters** – needed for RNA synthesis

**primers** – needed for DNA synthesis

– e.g. sequencing