

Session 2

Plasmid Mini-Preparation

Learning Objective:

The goal of this exercise is to become familiar with the procedure for isolating plasmid DNA from bacteria.

Introduction

Our ability to engineer biology depends on our ability to move DNA into and out of cells; today we will focus on moving it *out*. Isolating small DNA (plasmids) from cells is a frequent procedure in molecular biology. Plasmids are typically maintained and stored in live (frozen) cells, for ease of mass production by culturing the cells. To obtain physical DNA as source material for genetic engineering, a suitable quantity of cells must be grown in culture, and the DNA must then be extracted.

Background

Plasmids vs. Genomic DNA

Bacteria have relatively short, circular genomes (e.g. the *E. coli* genome is about five million base pairs and contains about five thousand genes). ***Plasmids*** are much shorter circular pieces of DNA that are not part of the genome, but are still transcribed by the cell, and sometimes propagated to its descendants. Most plasmids are about a few kilobases long. In nature, bacteria use plasmids to swap useful genes with each other, in a process called horizontal gene transfer. Scientists use plasmids, sometimes referred to as ***vectors***, to carry DNA of interest for insertion into cells. Typical plasmid features include: ***origin of replication*** (for copying the plasmid during cell division), ***antibiotic resistance gene*** (to distinguish cells that have taken up the plasmid from those that have not), and ***insertion site*** (place where the plasmid may be cut and genes of interest pasted in).

Overview: Cloning a Gene

Cloning a gene is the process by which a region of DNA, encoding a gene of interest, is put into another setting. Typically when a gene is cloned, the DNA is amplified from a target source and inserted into a vector. The vector is then put into the cell of interest. Cells containing the vector with the cloned gene are selected, isolated, and grown up in culture. At that point, researchers can study or use the cloned gene in whatever ways they want.

In the remainder of this laboratory class, we will go through all of the steps in cloning a gene and characterizing the expression of that gene in *E. coli*. The DNA we will be cloning contains a ***promoter***, ***ribosome binding site (RBS)***, and a gene coding for a ***fluorescent protein***. All three of these parts put together may be referred to as an expression cassette, or (if you're a synthetic biologist) a ***protein generating device***.

The promoter we are using is called pBad, and it becomes active in the presence of *arabinose*, a type of sugar – we say that pBad is **induced** by arabinose. At the end of the semester, we will measure how much fluorescent protein is expressed in response to different amounts of arabinose.

Today we will be taking the first step in our cloning process: isolating vector DNA from cells using a **mini-prep** procedure. At the end of this step, we will have a large quantity of vector DNA, into which we can insert the gene we will clone. Minipreps are explained below in more detail.

After the miniprep is completed, we will obtain a quantity of our DNA of interest by using polymerase chain reaction (**PCR**) to amplify it from source DNA (Session 3). Next, we will do a **restriction digest** on both the vector and PCR DNA (Session 4). This process cuts the DNA and makes it possible to physically join the vector and the insert into a new plasmid. After the DNA has been cut, we will purify the DNA of the proper length using **gel electrophoresis** (Session 5). The segments of DNA will be joined using **ligation** and **transformed** into *E. coli* cells (Session 6). Colonies containing cloned cells will be stored and **sequenced** to verify that we cloned the gene correctly (Session 7 & 8). We will then quantify the expression and function of the cloned gene with different promoter/RBS combinations, and also measure expression of a different gene that can be controlled by adding a certain chemical (Sessions 8 & 9).

Isolating DNA from a Cell

Removing DNA from a cell is a relatively simple process, especially when it comes to small plasmid DNA. The first step in this process is to break open, or *lyse*, the cells. In this step, the cell membrane is disrupted using a detergent and a base, or sometimes through physical means like sonication or pressing. Insoluble cell debris is then removed from the solution containing the soluble DNA. The next step involves purifying the DNA to remove unwanted proteins and salt. This is done by using isopropanol and ethanol to precipitate the DNA to an insoluble form, and by selectively binding the DNA to silica beads. With the DNA firmly bound to the silica, it can be washed to remove impurities. The final step is to *elute* the DNA from the silica beads, re-dissolving it in water or buffer solution. While the exact steps of this procedure vary depending on the location and size of the DNA and the type of cells being used, the basic steps are the same: break open the cells, purify the DNA, and re-suspend the DNA.

Anatomy of a QIAprep Spin Column

A QIAprep spin column has two parts: a blue upper part with a white silica membrane at the bottom, and a clear lower part for collecting waste. The DNA will eventually be bound to the silica membrane, and then recovered off of it by elution at the end. Many steps in the miniprep protocol involve putting a solution into the upper part and centrifuging so the solution flows through to the lower part.

Hazardous Waste Disposal

The reagents in a Qiagen miniprep kit contain hazardous chemicals that can irritate you if they get on your skin or in your eyes. Lab coats, gloves, and safety goggles **MUST** be worn at all times during this lab. If anything gets in your eyes, use the eye-wash immediately.

Waste should be discarded in a liquid waste collection beaker at your station (not the one you put used pipet tips in). At the end of the lab, bring this liquid waste beaker to the instructors' bench where the waste will be collected for disposal. Wash your beaker with soap and water and hang it up to dry.

Session 2: Pre-Laboratory Exercises

Name: _____

Date: _____

- 1) How is DNA isolated from cells?

- 2) What is an enzyme?

- 3) What does a restriction endonuclease do?

- 4) What are “sticky-ends” and why might they be better to use for cloning than “blunt-ends”?

Laboratory Protocol 2

The exercise: In this lab you will use a Qiagen plasmid mini-prep kit to extract plasmids from two strains of *E. coli*. One plasmid is an empty vector (either pSB1AC3 or pSB1AK3); the other contains a fluorescent protein generating device (either pBad-GFP or pBad-RFP), which we will later be inserting into the backbone. Each group will be prepping one backbone and one insert, but you will not find out which until later in the course.

Materials: Cultured bacterial cells containing vector
Cultured bacterial cells containing plasmid with insert
QIAprep buffers P1, P2, N3, PB, PE, EB
QIAprep spin columns
Pipette Tips
1.5 mL microcentrifuge tubes

Equipment: Microcentrifuge

Protocol: Read Before You Begin

This procedure makes heavy use of the tabletop micro-centrifuge. Since we only have two, you should coordinate with the other groups in your half of the room to spin at the same time, so no one has to wait. Make especially sure to do this for the first two spins (3 min and 10 min).

Label your tubes! All tubes should be labeled with their contents and your group color. This is especially important when sharing a centrifuge with other groups, because you won't be able to tell which set of tubes is which unless your group colors are on them. All spins in this protocol will be at 13,000 rpm (~17,900 x gravity).

ALWAYS balance the centrifuge! Pay attention to it while it gets up to speed, and if at any point it begins to vibrate loudly or so much that it moves, HIT THE STOP BUTTON IMMEDIATELY.

Part I – Plasmid Mini-Prep

1. Pellet bacterial cells: take 1.5mL of cultured cells into a 1.5mL tube and centrifuge for 3 min at 13,000 rpm. (If any of your tubes has no pellet or an extremely small pellet compared to others, see an instructor to add more culture to that tube and spin again.)
2. Re-suspend pelleted cells: add 250 μ L Buffer P1 and pipette up and down to break up the pellet. No cell clumps should be visible after re-suspending; be thorough.

3. Lyse cells: add 250 μ L Buffer P2 and mix thoroughly by *gently* inverting the tube 4–6 times; do not rack or vortex. The solution will turn blue. Make sure it is all the way blue, with no clear bits remaining. Go directly to the next step: do not pass Go, do not allow the lysis reaction to proceed for more than 5 min.
4. Neutralize lysis reaction: add 350 μ L Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times. The solution should become cloudy and all trace of blue should vanish, leaving the suspension colorless.
5. Centrifuge for 10 min at 13,000 rpm (coordinate with other groups). A compact white pellet will form, and a clear *supernatant* (un-pelleted liquid part) will remain.
6. Carefully pipet the supernatant from step 5 into the top of the QIAprep spin column. Try not to get any of the white pelleted crud in the column (it is stuff like cell membranes that we don't want in our purified DNA).
7. Centrifuge for 60 s. Discard the flow-through in your liquid waste beaker.
8. First wash: add 500 μ L Buffer PB and centrifuge for 60 s. Discard the flow-through in your liquid waste beaker.
9. Second wash: add 750 μ L Buffer PE and centrifuge for 60 s. Discard the flow-through in your liquid waste beaker.
10. Centrifuge for an additional 1 min to remove residual wash buffer. Discard the flow-through in your liquid waste beaker.
11. Place the blue top of the QIAprep column in a clean 1.5mL tube. To elute DNA, pipet 50 μ L Buffer EB directly onto the white silica membrane of each column. Let it stand for 1 min, and then centrifuge for 1 min. (If the lid of your tube breaks off in the centrifuge, carefully pipet the eluted DNA into a fresh tube and discard the old one.)
12. Bring your liquid waste beaker to the instructors' bench for hazardous waste collection. Wash the beaker with soap and water and hang it up to dry.

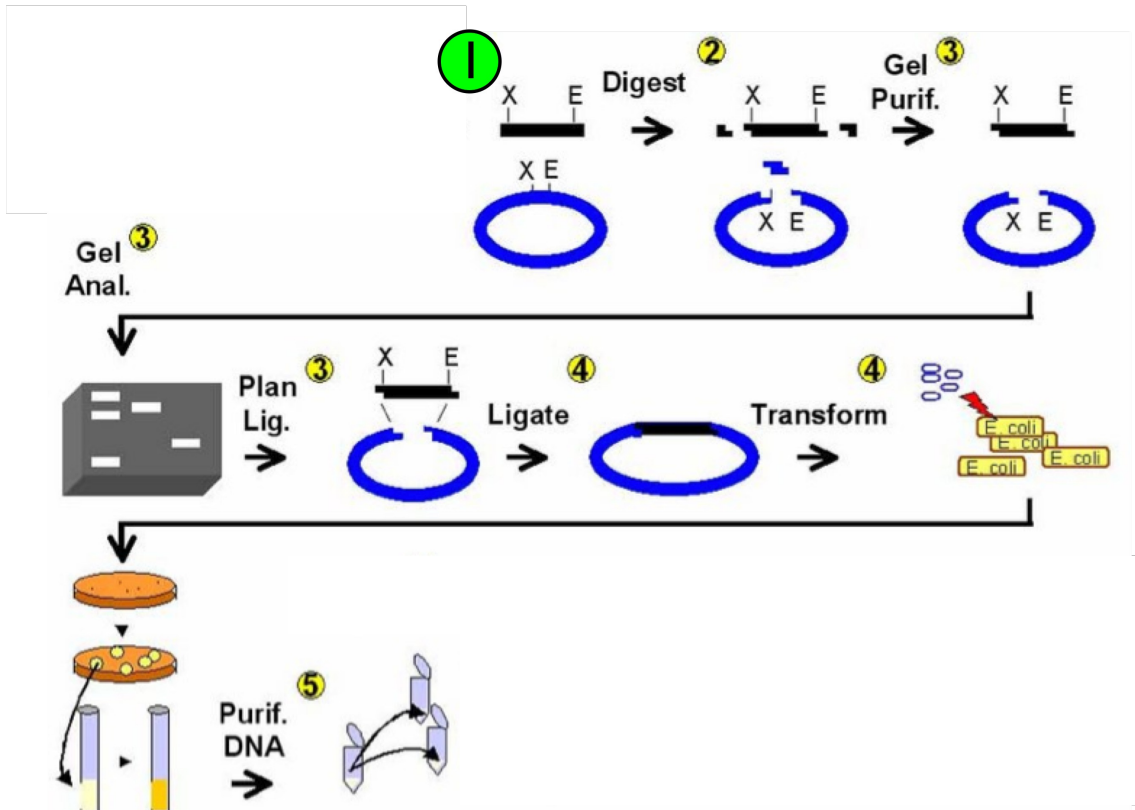
Session 2: Post-Laboratory Exercises

Name: _____

Date: _____

1) Laboratory Project Overview

Here is a schematic of the overall cloning process (adapted from MIT's 20.109 DNA Engineering Module: [http://openwetware.org/wiki/20.109\(F08\):Module_1](http://openwetware.org/wiki/20.109(F08):Module_1)). Please give a brief (1-2 sentence) summary of each of the following steps:



a) Restriction digest

b) Ligation

c) Transformation

2) Go to the New England Biolabs (NEB) website (<http://www.neb.com>). From the homepage, click on “Products” and then “Restriction Endonucleases”. Find the four enzymes that we are going to use in this class (EcoRI, PstI, SpeI, and XbaI).

a) For each enzyme: list the recognition sequence, draw the DNA after the cut has been made (overhang), and describe the inactivation conditions.

b) How is a unit of restriction endonuclease activity defined?

3) Answer the following questions about the sequence below. Refer to the NEB website again if you need help.

5' - ATTAGTCTAGAAATTCGCGACTAGTCAGCA - 3'
3' - TAATCAGATCTTTAAGCGCTGATCAGTCGT - 5'

a) Draw the fragments resulting from XbaI digestion of this sequence.

b) Draw the fragments resulting from SpeI digestion of this sequence.

c) Can any of the fragments from parts a/b be ligated to form a *new* product, different than the original sequence? Draw the new product(s). Do not include potential products from blunt-end ligations.

d) Can the new product(s) be cut by XbaI or SpeI, both, or neither? Why or why not?

4) Where is the plasmid DNA located at each of these points during a miniprep?

a) After pelleting and resuspending cells

b) After adding Buffer N3

c) After moving the supernatant to the QIAprep column and centrifuging

d) After washing with Buffer PE

e) After adding Buffer EB and letting it sit

f) After centrifuging the Buffer EB into a clean 1.5mL tube

References & Additional Reading

Wikipedia

Enzyme: <http://en.wikipedia.org/wiki/Enzyme>

Restriction Endonuclease: http://en.wikipedia.org/wiki/Restriction_endonuclease

Lab project overview: <http://openwetware.org/wiki/SEED/2012/Overview>