

Session 7

Glycerol Stocks & Sequencing Clones

Learning Objective:

In this lab you will prepare several of your clones for DNA sequencing and make glycerol stock cultures as a stable and uniform starting point for future experiments.

Introduction

In order to interpret the results of biological experiments, you must know precisely what DNA you are working with. DNA sequencing technology allows experimenters to verify that a plasmid has been constructed correctly, without error at any step of the cloning process. In order to reproduce an experiment in the future, it is useful to keep stocks of microbial strains cryogenically frozen, to be used as a consistent starting material. Together, these two methods allow biologists to ensure their experiments are consistent and their bacterial strains are free of random errors or mutations.

Background:

DNA Sequencing

In addition to being a useful tool for studies of biological evolution, phylogeny, microbial ecology, forensics, and healthcare, DNA sequencing provides a way to verify the correct construction of cloning products. There are numerous technologies still emerging in the field of DNA sequencing. New methods in DNA sequencing include increasing the throughput of existing methods (such as Emulsion PCR) or using “sequencing by synthesis” methods (such as Pyrosequencing and Reversible Terminator methods). However, chain-termination methods are still the most common and cheapest methods for sequencing.

Sanger sequencing is one of the earliest sequencing technologies and still the most widely used. This method is a chain-termination method which closely resembles PCR; a template DNA strand binds a known primer and is amplified by a polymerase. However, unlike PCR, only one primer is used for sequencing (no reverse primer is needed) and none of the chains reach full length. The polymerase is provided with a mixture of dNTPs and ***dideoxynucleotide triphosphates (ddNTPs)***, which are randomly incorporated into the growing DNA strand. When a ddNTP is incorporated, it prevents any more bases from being added to the new strand (because it is missing an oxygen atom that the new base would link to – hence “di-deoxy”). Thus, DNA strands of varying lengths are produced.

After elongation, these DNA strands are separated using a specialized electrophoresis technique. The final bases of each strand, the ddNTPs, each have a fluorescent marker attached – one color for each of A, G, C, and T. Thus, adjacent bands in the gel

correspond to adjacent bases in the template DNA, and the sequence of colors can be read along the length of the gel to give the complete sequence. (The use of fluorescent markers is a modern adaptation; the classical Sanger technique used four different reactions, each containing one of ddA, ddG, ddT, or ddC, and these four reactions were run in side-by-side lanes on a gel.)

Glycerol Stocks: Preserving the Cloned Strain

An amazing feature of cells is the ability to remain viable after long periods of time at or below -70°C . At these low temperatures, all cellular activity essentially stops. As a result, properly prepared cells can be kept frozen essentially forever, with little loss of viability. Biologists exploit this to keep “archives” of important cell lines, growing new cultures from a few frozen cells whenever needed.

However, cells cannot simply be cooled to very low temperatures. The water on the inside and outside of the cell expands upon freezing, forming ice crystals that can crush or burst the cell. To avoid this problem, *cryoprotectant* chemicals are added to the samples to inhibit the formation of ice crystals. In microbiology, the two most commonly used cryoprotectants are dimethyl sulfoxide (DMSO) and *glycerol*. The choice of solution depends on the type of cells being frozen. Glycerol solutions (about 10-20% by volume) are suitable for *E. coli*, and are what we will use for making frozen stocks of our strains.

Laboratory Protocol

The exercise: You will pick monoclonal colonies from your transformations. You will be amplifying the DNA from these clones by rolling amplification to prepare it for Sanger DNA sequencing; this will be done directly from the colonies without isolating the plasmid DNA. You will also grow culture of these same colonies to be grown and cryogenically frozen for later use.

Materials: Monoclonal Colonies from Transformation

- 4 PCR Tubes
- 4 Falcon Culture Tubes
- 4 Cryo Tubes
- Sterile Toothpicks
- 5 mL Disposable Serological Pipette
- Templphi Sample Buffer
- Templphi Reaction Buffer
- Templphi Polymerase Enzyme
- Sterile LB Culture Media
- Ampicillin
- Sterile 20% Glycerol

Equipment: Heat block
-80° C freezer

Protocol:

1. Observe the negative control plate. You should not see any colonies on the negative control plate! If you do, this means one of several things; you need to check stocks for contamination and improve sterile technique.
2. Next, check the positive control plate. You should see numerous blue colonies. If you have no colonies at all this means something went wrong with your transformation. There was likely problem with how the cells were handled, the control vector added, or the actual technique for the heat shock transformation.
3. Observe the remaining plates for colonies. Ideally, there will be plenty of colonies to choose from, but not so many that they start to touch each other. The colonies should be blue in color because they are expressing a Beta-galactosidase gene which catalyzes a reaction to produce a blue compound. If the colonies are not blue, they still may be the correct construct just expressing the enzyme at too low of a concentration for visible detection.

4. Put 2.5 uL sample buffer into each of four PCR tubes. Label the tubes with numbers 1-4 and your initials.
5. Put 3 mL LB into four falcon culture tubes. Add 3 uL ampicillin to each tube. Label the tubes with the name of the construct and your initials.
6. Very carefully, pick a colony from each of the plates with a sterile tooth pick into one of the PCR tubes. Just touch the colony gently, do not break the surface of the agar. Then, move the tooth pick to the proper falcon culture tube. If you do not have four successful transformations, ask your instructor for an additional colony to sequence.
7. Place the falcon culture tubes into the incubator at 37C
8. Heat the PCR tubes to 95C for 3 minutes to break open cells and separate DNA.
9. Add 2.5 uL of the reaction buffer and 0.1 uL of the enzyme (Hint: use a master mix) to each of the PCR tubes
10. Incubate the PCR tubes at 30C overnight
11. When the LB cultures are turbid (cloudy) pipette 0.7 mL of each culture into sterile cryo tubes.
12. Add 0.7 mL sterile 20% glycerol to each cryo tube and freeze the tubes at -80C.

Session 7: Post-Laboratory Exercises

Name: _____

Date: _____

1) **Cloning: Complete (Essentially...)**

You have essentially completed the necessary steps for cloning a gene (you should remember, though, that verifying whether your product is correct, via sequencing or another method, is a crucial step in the cloning process). Referring back to the previous sessions, draw a schematic of the different steps in the cloning process. Give the name of each technique and draw the DNA pieces present at each step. (Hint: do not blindly copy the cloning diagram that appeared in previous homeworks... it is not the clearest representation of exactly what we did.)

2) DNA Sequencing

In this session, you prepared your cloning products for sequencing. In the next session you will analyze your sequencing results.

- a) Modern Sanger sequencing methods, with different color fluorescently labeled nucleotides, require only one reaction and use automated DNA sequencing machines. This is the method we will use in this class.

Visit the course wiki (<http://openwetware.org/wiki/SEED/2012>), go to the Schedule tab, and open the Day 7 page to view a sample of raw DNA sequencing data. Suppose that, as we scan along the electrophoresis gel, we get the chromatogram shown on that page. Write out the sequence of the corresponding DNA. (Green=A, Blue=C, Black=G, Red=T)

- b) Using the previous DNA sequence, figure out what gene this sequence is likely from. To do this, you will use BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>). BLAST is essentially a DNA sequence search engine. Under “Basic BLAST”, click on “nucleotide blast”. Enter your sequence in the large box at the top. For the database option, choose “Others”. Select “nucleotide collection (nr/nt)” from the drop-down menu, and hit the blue BLAST button at the bottom. Can you figure out what gene this sequence corresponds to? (Hint: This is a very common gene, appearing in many species' natural genomes and in many artificial plasmids. Look for a blue “G” icon in the right-hand column, which is a link to the Gene database.)

References & Additional Reading

Wikipedia:

DNA Sequencing: http://en.wikipedia.org/wiki/DNA_sequencing