

Texas A&M University-Corpus Christi
CHEM4402 Biochemistry II Laboratory

Laboratory 7: Plasmid DNA Isolation and Restriction Enzyme digestion

Note* - Please bring your laptop and an electronic copy of the *formatted* GFP sequence you obtained for GFP from our second lab, *database searching*,

Over the past few weeks we have been constructing a recombinant DNA molecule from a plasmid DNA and our GFP PCR amplification product. Today we will build on last week's transformation experiment and actually isolate the GFP-containing plasmid DNA from its *E.coli* host. To do this, a single positive (white) colony is selected from the LB-agar-ampicillin plate and placed in a nutrient media (Luria Broth, or simply "LB") with an antibiotic (ampicillin) to prevent growth of any non-transformed bacterial cells that find their way into the solution. The inoculated media is then allowed to grow overnight while shaking at 37°C. Shaking helps to provide oxygen to the cells and prevents them from clumping together and settling. 37°C is the optimal temperature for cell growth. A single overnight incubation is sufficient for an inoculation of *E.coli* cells to increase in number a billion-fold. This quantity of cells will provide milligram quantities of DNA, enough for experiments using PCR, restriction enzymes, sequencing or other techniques.

To complete the isolation of plasmid DNA from overnight cultures in a single day, your instructor has already selected a representative positive (white) clone and performed an overnight incubation to allow enough cells to grow. All groups will be isolating plasmid DNA from this culture. To analyze the isolated plasmid DNA, we will perform a restriction enzyme digest of the DNA, followed next week by electrophoresis on agarose gels for analysis. Restriction enzymes are DNA endonucleases; they cut DNA molecules at specific sequence target sites. By comparing the fragments generated from an enzyme digest to those expected from known nucleotide sequence data, a research scientist can verify whether their plasmid DNA preparation does indeed harbor the cloned DNA product of interest. To determine what fragments should be produced from our digest, we will be submitting our DNA sequence to a web-based program that performs a "virtual" restriction enzyme digest. We will examine our sequence for particular restriction enzyme recognition sites (Csp45I, Eco RI) and estimate the size of fragments that would be produced in an actual digestion.

To begin, we will isolate our GFP-containing plasmid DNA using a DNA purification kit containing all the necessary materials and reagents. These include: a **cell resuspension solution**, to re-solubilize the *E.coli* cells after isolation from the cell media via centrifugation, a **cell lysis solution** (NaOH and detergent) to break up the cellular lipid membranes and release the DNA, a **neutralization solution** (Guanidine hydrochloride and acetate buffer), to lower the pH of the lysed cell solution and denature proteins, a **spin filter**, to bind and separate the plasmid DNA from other cellular components (including chromosomal DNA), two **wash solutions** (containing varying amounts of ethanol, potassium acetate and TRIS hydrochloride) which remove salts, detergents and cellular debris. The plasmid DNA is finally released from the filter using an **elution buffer**, which keeps the DNA in a high pH (~8.0) solution (DNA is more stable at pH > 7.5), which contains the chelating agent ethylenediaminetetraacetic acid (EDTA). Chelating agents such as EDTA sequester small ions such as **Mg²⁺**, which are essential for activity in many enzymes, particularly DNAses. These enzymes could destroy our DNA if not inactivated.

Plasmid DNA Isolation

Materials

2 x 1.5 ml microcentrifuge tubes	
1.5 ml of transformed E.coli culture	1 spin column/2 ml collection tube combo
250 ul Cell Resuspension solution (P1)	250 ul Cell Lysis solution (P2)
350 ul Neutralization solution (N)	500 ul Wash Solution 1 (PB)
750 ul Wash Solution 2 (PE)	50 ul Elution Buffer (EB)

Procedure

1. Transfer **1.5 ml of overnight culture** (provided by instructor) to a microcentrifuge tube. **Centrifuge for 3 minutes at 6800 x g.** Remove as much as possible of the supernatant (liquid layer on top) to an ethanol waste beaker. Repeat, using same microfuge tube.
2. Add **250 ul of Cell Resuspension Solution (P1)**. Strip tube across microcentrifuge tube rack (see instructor) until cell pellet is completely resuspended.
3. Add **250 ul of Cell Lysis solution (P2)**. **Mix by gently inverting the tube 4-6 times .**
4. Add **350 ul of Neutralization solution (N)**. **Mix by gently inverting tube 4-6 times.** **Centrifuge** for 10 minutes at maximum speed. Be sure to balance your tube with another group's sample. Perform step 1 of the Restriction Enzyme Digest procedure while you are waiting.
5. Transfer **supernatant** (liquid layer, ~800 ul) **from step 4 to a spin filter/collection tube** by decanting or using a p-1000 pipet. Avoid transferring any of the white precipitate.
6. Centrifuge the supernatant for 1 minute at maximum speed.
7. Remove spin filter, **discard liquid in bottom of tube** and reinsert the filter into the 2 ml collection tube.
8. Add 500 ul of **wash solution 1 (PB)** to spin filter. Centrifuge for 1 min. at max speed.
9. Remove the spin filter, **discard liquid** and reinsert the filter.
10. Add **750 ul of wash solution 2 (PE)** to spin filter. Centrifuge for 1 minute at max speed.
11. **Discard liquid.** Centrifuge for an additional 1 minute to remove residual buffer.
12. Transfer spin column to a clean 1.5 ml microcentrifuge tube. Add **50 ul of elution buffer (EB) directly to the top of the filter membrane.** Let stand for 1 min.
13. **Centrifuge** for 1 min. at max speed. Discard filter. **Plasmid DNA is in liquid.**
14. Mark your initials on your sample. Proceed to the restriction enzyme digest procedure.

Restriction Enzyme Digest of Plasmid DNA

Materials

3 x 1.5 ml microcentrifuge tubes	Plasmid DNA prep
Eco RI restriction enzyme	Csp45I restriction enzyme (instructor)
Restriction enzyme buffer	sterile H ₂ O

Procedure

1. Take 3 microcentrifuge tubes and label with your initials. Label one 'E' for an Eco RI digest, one 'EC' for an Eco RI/ Csp45I "double digest" and one "C" for a Csp45I digest.
2. Add **10 ul** of your plasmid DNA prep to each tube. **KEEP REMAINING PORTION FOR DNA SEQUENCING EXPERIMENT TO BE PERFORMED NEXT WEEK.**
3. Add **6.5 ul** of sterile H₂O to tubes "E" and "C" and **5 ul** of sterile H₂O to tube "EC".
4. Add **2 ul** of restriction enzyme buffer to each tube.
5. Add **1.5 ul** of EcoRI restriction enzyme to tubes "E" and "EC".
6. Your instructor will add **1.5 ul** of restriction enzyme Csp45I to tubes "EC" and "C".
7. Mix each tube gently by tapping with your finger. Centrifuge *briefly* to bring tube contents to bottom
8. Place all samples in the 37°C water bath for 60 minutes. While DNA is digesting, proceed to the "virtual" restriction digest exercise.
9. Remove your samples from the water bath. Give them, and your remaining plasmid DNA prep (appropriately identified), to your instructor for storage until next week.

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READING ASSIGNMENT: Lehninger 9.1-9.2 (DNA cloning)

1. Go the web site “restriction mapper” (<http://www.restrictionmapper.org/>). Copy and paste the formatted sequence you obtained for GFP from the *database searching* lab to the “**Paste Sequence Here**” box (do not copy and paste any other information, such as the genbank ID, your name, etc.). Enter the name of your sequence in the “**Name Your Sequence**” text box.
2. Select “**Csp45I**” from the list of restriction enzymes in the “**Select Individual Enzymes**” text box. You do not need to worry about any of the other parameters, they are already set the correct way by default. Press the “**Virtual Digest**” button. The program will return a copy of a “virtual digest” of your sequence, showing the fragments generated and their length. Note the length of the fragments generated and the position where the enzyme cuts the sequence. Pay careful attention to the fragment on top. Is this the 5’ or 3’ fragment that is generated from the digest? Perform the exercise again selecting “**EcoRI**” as the restriction enzyme.
3. What size fragments should be produced when our PCR product is cut with Csp45I? What about EcoRI (1 pt).
4. What are the DNA sequences (restriction sites) recognized by the enzymes EcoRI and Csp45I? (2 pt)
5. Return to your formatted GFP sequence. Highlight the site where Csp45I makes its cut. Save, print and turn in a copy of this sequence next week. (5 pt).
6. Read the section on “Restriction Endonucleases” in your text. What is the normal function of a restriction endonuclease in a bacterial cell? (2 pt)
7. Why isn’t the bacterial cell’s own DNA cut to shreds? (2 pt)
8. How long are the DNA sequences typically recognized by restriction enzymes? (1 pt)
9. What is meant by the terms “sticky” or “blunt” when used to describe the types of ends produced by the action of various restriction enzymes? (2 pt)
10. Laboratory Performance (2 pt).