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7.13 Experimental Microbial Genetics

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Bootcamp Day 4 – Partial digestion of *Pseudomonas aeruginosa* PA14 genomic DNA and Gel Electrophoresis

A. PARTIAL DIGESTION OF *PSEUDOMONAS AERUGINOSA* PA14 GENOMIC DNA

Today's goal is to isolate fragments of PA14 genomic DNA of the appropriate sizes to clone into a library. You will be making two libraries: one with fragments ranging in size from 2kb-4kb, and one with fragments of size 4kb-8kb. In order to create these fragments, you will do a partial digestion using the restriction enzyme BfuC1.

Your tasks today will be to determine the optimal conditions for the restriction digestion in order to yield fragments of the appropriate size, and then to run a scaled-up digestion using those conditions to create a large amount of those fragments. Later you will purify the correctly sized fragments and clone them into the vector pMQ64 that you linearized and dephosphorylated last week.

In order to determine the appropriate conditions for the partial digestion, you will use a set amount of PA14 genomic DNA and vary the concentration of enzyme used to digest it.

Before you begin, collect the following reagents from the TA's:

PA14 genomic DNA of a known concentration

NEB Buffer 4 (10x stock)

BSA, 10 mg/mL (100x stock)

Diluent B

BfuC1 restriction enzyme

Stop solution

Keep your reagents and mixes on ice at all times. Remember, restriction enzymes are heat sensitive!

Pilot digestion to determine appropriate conditions:

1. Create a master mix of everything for the digestion **except the enzyme**. This includes:
 - PA14 genomic DNA: 20ug (you will have to calculate the volume based on the concentration of DNA you isolated last week)
 - NEB buffer 4 to a final concentration of 1X
 - BSA to a final concentration of 0.1 mg/ml
 - Water (enough to bring the total volume to 220uL)

Component	Volume to add
PA14 Genomic DNA	
dH ₂ O	
NEB Buffer 4 (10X)	
BSA (100x)	
Total Volume	220 ul

After adding all the components, vortex and centrifuge briefly. Aliquot 20uL **into the bottom** of each of 10 tubes, labeled 1-10. (you should have about 20 ul of master mix left over). These 10 tubes will be used for the digestions.

2. Create serial dilutions of enzyme BfuC1.

- Aliquot 15uL Diluent B into the bottom of each of 9 tubes labeled 1-9. **DO NOT GET THIS SET OF TUBES CONFUSED WITH THE FIRST SET OF TUBES!!!** It would be a good idea to label this set of tubes with a different color pen.
- Add 15uL of BfuC1 to the diluent in Tube #1. Mix thoroughly by pipetting up and down several times.
When taking out enzyme, do not plunge the pipette tip farther than absolutely necessary. The enzyme is preserved in glycerol which is very sticky and viscous and sticks to the pipette tip. Because of this, the farther you put your pipette tip into the enzyme mix, the more likely you are adding MORE enzyme than you intend.
- Transfer 15uL of this mix into the diluent in Tube #2. Again, mix thoroughly by pipetting up and down.
- Transfer 15uL of this mix into the diluent in Tube #3, and continue diluting the enzyme this way all the way to Tube #9.
- The BfuC1 stock concentration is 4 units per uL. Make a note of the concentration in each of your dilutions. Again, be careful not to mix up your tubes!

Tube	BfuC1 stock	1	2	3	4	5	6	7	8	9
Enzyme concentration	4U/ul									

***** Do step (#3) as quickly as possible, and keep your digestions on ice as much as possible!***** If you hold the tube excessively in your warm little mitt, the enzyme will be digesting your DNA!

3. Add 1uL of the appropriate enzyme dilution to each of your aliquots of master mix. Add the enzyme directly into the liquid that is already in the tube, **not** to the side of the tube.

NOTE: Don't use the first dilution (2 units/uL) for the first digestion—instead, use dilution #2 for digestions #1 and #2, but add 4uL of stop solution to digestion #1 immediately after adding enzyme. (Think: why is this an important control?)

For digestion #10, use 1uL of water instead of enzyme. (Why is this an important control?)

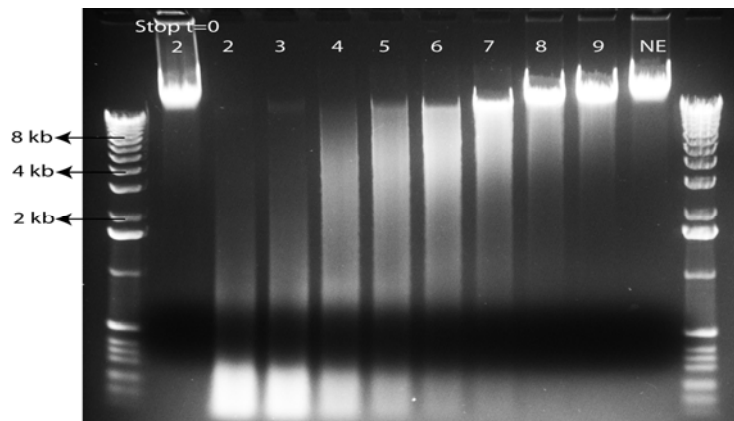
4. Vortex the digestion tubes **briefly** two at a time, and return them immediately to ice.

5. Set a timer for 30 minutes, and get a rack that will hold all 10 of your reaction tubes.

Keep the reactions on ice as you walk to the 37°C room and once there transfer the tubes quickly from the ice to the rack. Start your timer.

6. After 30 minutes, quickly transfer your tubes back to ice, and add 4uL stop solution to each tube. Mix well. The reaction is now quenched. Relax and hold the reaction in your warm little mitt as long as your heart desires. (Think: How does the stop solution quench the reaction?)

7. Load the medium sized analytical gel you prepared last Fri. with your reactions and with 10 ul of 1 kb DNA ladder in the lanes flanking them. Load the entire reaction and run the gel (see more details below in the Agarose Gel Electrophoresis and DNA Band Excision section). Visualize the gel and determine which conditions were best for creating 2kb-4kb fragments, and 4kb-8kb fragments. Your gel should look similar to the gel below. What reaction would you choose to upscale to isolate large amounts of 2-4kb fragments? 4-8 kb?



B. SCALED-UP PARTIAL DIGESTION TO OBTAIN CORRECTLY SIZED FRAGMENTS:

1. Create two mixes, one to create 2-4kb fragments, and one to create 4-8kb fragments.

Each mix should contain:

- 20ug genomic DNA (How much is this compared to each pilot reaction?)
- NEB buffer 4 to a final concentration of 1X
- BSA to a final concentration of 0.1 mg/ml
- An appropriate volume of the appropriate tube of diluted BfuC1 (How much do you need? Remember, this time you are digesting more DNA)
- Water (enough to bring the total volume to 200uL)

2-4 kb digest

Component	Volume to add
PA14 Genomic DNA	
dH ₂ O	
NEB Buffer 4 (10X)	
BSA (10x)	

Total Volume	200 ul
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4-8 kb digest

Component	Volume to add
PA14 Genomic DNA	
dH ₂ O	
NEB Buffer 4 (10X)	
BSA (10x)	

Total Volume	200 ul
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Mix well, and incubate at 37C for 30 minutes. Stop the reaction by returning it to ice and promptly adding 40uL stop solution.

2. Load a small 1% agarose gel with as much of the each of the two reactions as you can fit (without it spilling over into other wells!). Load the two reactions into separate wells of course! Also, add an appropriate amount of 6x loading dye to your BamHI linearized, dephosphorylated, pMQ64 from last week, and load it all in a well on the same gel. Again load 10ul of 1 kb DNA ladder in the lanes that flank your samples. Run the gel at about 120V, visualize it, and carefully cut out and put into a tube the pieces of gel with appropriately sized fragments for gel extraction (see Agarose Gel Electrophoresis and DNA Band Excision Protocols below for the details on how to do this properly!!).
3. Leave tubes with chunks of gel at -20C.

C. AGAROSE GEL ELECTROPHORESIS

How it works:

Agarose gel electrophoresis is a simple and highly effective method for separating, identifying, and purifying 0.5 to 25 kb DNA fragments. Voltage applied at the ends of an agarose gel generates an electric field with a strength defined by the length of the gel and the potential difference at the ends (V/cm). DNA molecules exposed to this electric field migrate toward the anode (positive end) due to the negatively charged phosphates along the DNA backbone. The migration velocity is limited by the frictional force imposed by the gel matrix. While charge and/or size can affect the rate at which macromolecules will pass through the gel, the charge to mass ratio is the same for DNA molecules of different lengths. It is the size of the DNA, therefore, that determines the rate at which it passes through the gel, thereby allowing an effective separation of DNA fragment-length mixtures by electrophoresis. To visualize the DNA, the gel is treated with ethidium bromide. This dye intercalates between the stacked bases of nucleic acids and fluoresces red-orange (560 nm) when illuminated with UV light (260 to 360 nm).

Reagents and materials:

- **50x TAE buffer** (agarose gel running buffer)
- **Ethidium bromide 10 mg/ml (20,000x stock)** - *wear gloves when handling EtBr*. Since it is a DNA-intercalating molecule, EtBr is a mutagen! Dispose of all tips etc in labeled receptacle in fume hood.
- **6X Gel loading buffer**

1. After adding loading dye to your DNA samples to a final concentration of 1x, load the samples into the wells of the gel. Remember to also load a molecular weight marker!!! See end of this protocol for 1 kb and 100 bp molecular weight marker patterns.
2. Run gel with an overlay of 1x TAE (about 5-8 mm deep above the gel) at 100-120V.
3. When the gel has run far enough, so that your bands have clearly separated and you can tell what size they are, take a picture of the gel.
The bromphenol blue in the loading dye will migrate with an apparent molecular weight of 500 bp and the xylene cyanol of 2 kb.

D. DNA BAND EXCISION:

1. Weigh as many Eppendorf or 15 ml Falcon tubes (depending on the size of the gel piece you expect to cut out, for bands >~400 mg use a Falcon tube) as you will have pieces to cut out—this will allow you to determine the mass of gel you cut, which is necessary to know for the DNA purification procedure.
2. Take the gel to the dark room and the UV-light transilluminator to visualize the DNA bands. In order to minimize damage to the DNA, use a long-wave UV light for as short a time as possible. ***Make sure to wear a face shield mask during this procedure since the UV light will burn your retina and/or your skin.*** Excise the desired band using a razor blade. Minimize the amount of extra, non-DNA containing agarose that you cut out. Place the band in an eppendorf tube (or 15 ml Falcon tube if it is >400 mg).
3. Take a picture of the gel without the band in it to be sure the band is successfully recovered.
4. Weigh each of the tubes with the pieces of gel in them, and calculate how much gel is in each one. For now, freeze the gel fragments at -20°C. Make sure your tubes are well labeled!