BIO 315 Molecular Biology Labs #9 and #10

(Fornari, fall 2006)

(T-groups Nov. 7th; R-groups Nov. 9th): Part I - Cycle Sequencing Reactions: set-up and processing

Part II -

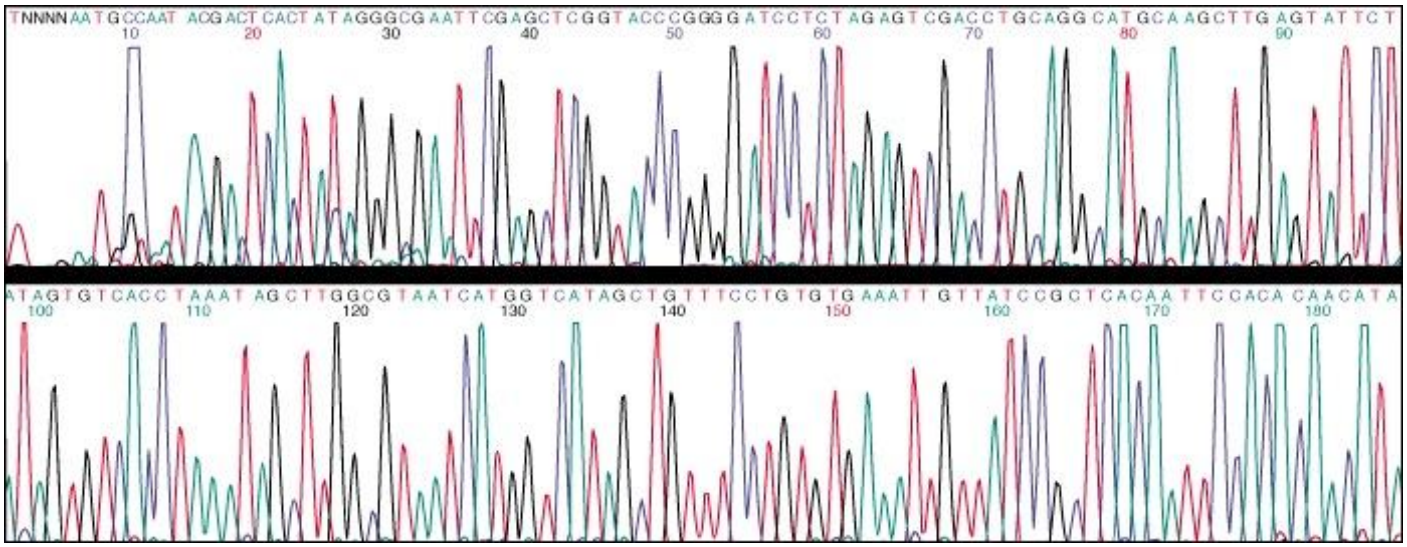
Column purification of Sequencing Reaction products; drying and resuspension in SLS

(Nov. 14th Tues. and Nov. 16th Thur.): Capillary Gel Electrophoresis of Cycle Sequencing Reaction Products in the CEQ 8000 Gene Analyzer (an automated sequencer)

[Lab #11-12: Bioinformatic Analyses and Identification of Sequenced DNA Fragments]

Introduction

The Sequencing experiments consist of two parts spanning 2 lab periods and some time outside of the regularly scheduled lab periods immediately after Part I; this lab series starts with **(Part I)** to perform the cycle sequencing reactions in the PCR machine, and ends with **Lab 11-12** to perform bioinformatic analyses with the BLAST and other programs such as DS-GENE or simpler programs at Sequencing Protocols. The actual DNA sequencing is done by capillary gel electrophoresis of the sequencing reaction products in the Beckman-Coulter CEQ 8000 Gene Analyzer. We will discuss the biochemical and biophysical basis for capillary electrophoresis and integrate these concepts into the biochemistry of Sanger dideoxy sequencing. Other concept areas include dye-terminator sequencing, and fluorescence-based reaction biochemistry.



DNA Electrohperogram 1

Understanding the cycle sequencing technology: Go to this site for a good animation.
http://vector.cshl.org/shockwave/cycseq.html

Lab #9 Part I: CEQ Dye Terminator Cycle Sequencing Reactions

1. The PCR reactions for cycle sequencing will be set up in 0.2 ml thin-walled PCR tubes. **Label** your **tube** (by group# and 'a' or 'b' depending on which plasmid prep is used) on **both the lid and the upper side** of the tube. The heated lid of the PCR machine can sometimes cause labels on the top to become unreadable.

2. Next you need to calculate the volume containing 200 ng DNA of your plasmid prep with its cloned insert. Enter this volume in the table below. The **maximum** volume allowed for your **plasmid template + water is 9.0 ul**. The pCR4-TOPO vector is 3957 bp without an insert. How many fmole DNA are found in 200 ng of plasmid DNA? Does this value change depending on the size of the insert? (See Appendix 1 for a table estimating fmoles DNA from ng DNA).

3. What volume of water do you now need to add to your calculated volume of plasmid DNA prep to equal 9.0 ul? Enter this number into the reaction set-up table.

Fill in the table below with plasmid and water volumes:

DNA Sequencing Experiment BIO 315 Molecular Biology	Tube label →	Grp# rxn-a	Grp# rxn-b		
	DTCS master mix* →	6	6		
	* includes 1.5 ul of M13-F primer + 1.5 of 5X Sequencing Buffer				
Important Note: (1) add plasmid and water together in tube, (2) heat in PCR machine before adding the DTCS master mix (see step #5 next page)	200 ng plasmid template Grp #-rxns a or b →				
	sterile pure water →				
	plasmid + water = 9.0 ul	9	9		
	total volume →	15	15		

*Note: We are using the sequencing primer from the Invitrogen company. This primer is called the Universal M13 forward (-20) sequencing primer and it is 16 bases long with a 56.25% GC content and a T_m of 50 ^{o}C and has the following sequence: **5'[GTAAAACGACGGCCAG]3'**

Now, once your table is complete, follow steps a-c, and then step d:

a. Label tubes as described in above step 1**.**

b. Place plasmid prep for sequencing on ice (briefly spin if you see any condensation around the tube walls).

c. Pipet the correct volumes (see your completed table) of sterile water and plasmid prep into your labeled PCR tube **(total vol. = 9.0 ul).**

d. Super-coiled plasmid (in the CCC topological conformation) DNA templates show higher signal strength and better current stability during sequencing if they are **pre-heated** prior to the cycle sequencing reaction. **This treatment is performed only on the plasmid template and sterile water with no additional reagents present.** After completing steps **a to d** of the set-up protocol, bring your labeled reactions to the thermocylcer for a **pre-heat** treatment. The cycle consists of 1 min at 96 ^{o}C; **return the heated plasmid preps to your cold trays**. The plasmids will be "nicked" into the OC topological conformation, which relaxes their supercoiled state and allows them to be better, more efficient templates during cycle sequencing reactions.

4. Place your pre-heated template **plasmid DNA on ice or in a cold rack and keep them at 0 °C at all times** until centrifuged before going into the thermocycler.

Note: The rest of the reaction components are supplied as a mix. Beckman-Coulter is secretive about the exact composition of the reagents and we know only that the CEQ DTCS- Quick Start "master mix" contains the reaction buffer, dNTP's, dye labeled terminators and DNA polymerase. The dGTP analog, dITP is used to reduce band compressions in the electropherogram and dUTP is used in place of dTTP resulting in better incorporation and improved T pattern. **You will add 6.0 ul** of the master mix to your prepared plasmid/water solution **(9.0 ul of plasmid + water and 6.0 ul MM = 15.0 ul)**.

5. Add the DTCS Quick Start master mix: 6 ul per reaction tube with 9.0 ul nicked plasmid/water.

6. Mix very quickly by flicking **AND IMMEDIATELY RETURN TO ICE OR the cold rack TO KEEP AT 0 °C.**

7. Take ice bucket or cold rack to a refrigerated microfuge in room 219. **Leave the reactions at 0 °C** until all the lab groups' reactions are ready for the thermocycler. When everyone is ready, briefly centrifuge the reactions in the cold centrifuge to collect the liquid at the bottom of the tube, and then place in the thermocycler.

Thermal cycling program:

96°C for 20 sec
50°C for 20 sec
60°C for 4 min

Total run: 35 cycles followed by holding at 4° C

Note: the 4 minute extension time facilitates the sequencing of templates which may have some impurities. This time could be reduced if we knew all our templates were extremely pure. The number of cycles is optimized to give the best value for time spent cycling versus amount of signal generated. Decreasing the number of cycles will decrease the number of extension products generated and therefore decrease the signal. If low signal strength is a problem, a combination of increased primer and increased cycle number may help. The 60 °C extension temperature is used instead of 72 °C because dITP is in the nucleotide mix instead of dGTP.

You must purify your extension products within 24 hours after the reaction. This step requires about 40 minutes if you think about what you must do before coming to the lab.

Lab #9 Part II: Purification, Drying and Resuspension of Extension Products

1. Unincorporated dye terminators must be removed from the sequencing reaction products (all the fragments for separation in the sequencer) before subjecting them to capillary electrophoresis in the CEQ 8000 sequencer. Beckman-Coulter recommends ethanol precipitation, but this can be very tricky until you master the technique. Since you don't have time to master this technique, we are using gel filtration columns to remove the unincorporated dyes. The gel filtration columns are superior for removing unincorporated dye-labeled dNTPS and they are quick and easy to use, but they give us a diminished product recovery compared to ethanol precipitation. However, with due diligence and care you will have sufficient product to yield a good signal during the capillary electrophoresis.

2. The gel filtration columns remove dye terminators, dNTPs, and other low molecular weight molecules from the sequencing reactions. (See **Appendix 2** for a brief introduction to gel filtration)

3. Use this protocol for the Edge BioSystems Performa DTR Gel Filtration Cartridges: **(1)** add 5 ul sterile water to your reaction sample to bring the final volume to 20 ul; **(2)** add 150 ul sterile water to each DTR cartridge, and vortex until the gel is uniformly distributed in its column; **(3)** now follow the Edge Performa instructions attached to this handout. Be sure to label properly so you don't confuse your samples during

centrifugation. Be sure you note where the extension products are recovered so they don't get thrown out. At the end of this procedure, where are the buffer molecules? the dNTPs? the Mg^{+2} ? the DNA polymerase? the sequencing primer? the DNA template?

4. When you finish the gel filtration protocol, your extension products will be in approximately 20 ul volume of an aqueous solution. Before they can be subjected to capillary electrophoresis, the water must be removed and the extension products dissolved in Sample **L**oading **S**olution (**SLS**). The SLS is highly purified formamide.

Drying Extension Products and Dissolution in SLS

Note: SAMPLES CANNOT BE STORED DRY AS THE DYE TERMINATORS ARE UNSTABLE AND WILL DEGRADE.

1. Removing small quantities of water leaving a dried pellet of extension products can be accomplished using a vacuum centrifuge. We will be using the Eppendorf Vacufuge.

2. Place the **open** tubes containing the extension products into the vacuum centrifuge so the tubes are balanced. Set the centrifuge to Function 3 for aqueous solutions following the instructions in the manual.

3. The time it takes to dry the samples depends on the number of samples in the centrifuge and the volume of water to be evaporated. If you are doing only 4 samples it should take about 15 to 30 minutes.

4. **DO NOT OVERDRY SAMPLES.** Stop the centrifuge after15 to 20 min and check to see if your samples are dry. If they are dry, add 40 ul SLS to each tube, **close the lid tightly and vortex well**. It is **very important to completely dissolve** the dried extension products and this may take 5 to 15 minutes.

5. Extension products dissolved in SLS are stable for 1 month at -20 ^{o}C. **Extension products may be stored in SLS at -20 ^{o}C** until ready for sequencing in the CEQ 8000.

Recommended Protocol for use with BigDye™ v3.1

1. **Centrifuge the Performa Gel Filtration Cartridge for 3 minutes at 850 *x g*.**
    - The time and speed of centrifugation are important.
    - The drier the packing (longer centrifugation times and/or higher *g* forces), the longer it takes to recover product and the lower the overall recovery.
    - Conversely, shorter spin times and lower speeds result in elution volumes higher than the input sample volume.
    - See "Notes" for determination of RPM from RCF or visit our website at www.edgebio.com and click on Technical Support.
2. **Transfer the cartridge to the provided 1.5-ml microcentrifuge tube and add the sample to the packed column. Be sure the fluid runs into the gel.**
    - If using a microcentrifuge or other centrifuge which uses a fixed angle rotor, place the sample in the center of the slanted gel bed surface to obtain optimal performance.
3. **Close the cap and centrifuge for 3 minutes at 850 *x g*. Retain eluate.**
    - Up to 4 µl may be lost during sample processing.
    - If the volume loss is greater than 4 µl, this is an indication of an overly dry gel. To optimize recovery of sample, repeat centrifugation.

Recommended Protocol for all other Dye Terminators

1. **Centrifuge the Performa Gel Filtration Cartridge for 2 minutes at 750 x *g*.**
2. **Transfer the cartridge to the provided 1.5-ml microcentrifuge tube and add the sample to the packed column. Be sure the fluid runs into the gel.**
3. **Close the cap and centrifuge for 2 minutes at 750 x *g*. Retain eluate.**

APPENDIX 1: Information from Beckman Coulter, Inc.

2. DNA Template amount:

The amount of template DNA to use in the sequencing reaction depends on the form of the DNA (dsDNA plasmid, ssDNA M13, PCR product, etc.). It is important to accurately quantitate the amount (moles) of DNA when performing the DNA sequencing reaction (see formula and table below for details). The molar ratio of primer to template must be ≥ 40:1. Listed below are the recommended amour of DNA:

dsDNA	0-100 fmol
ssDNA	25-50 fmol
Purified PCR products	25-100 fmol

The following table can be used to estimate DNA concentrations.

Table for estimating the dsDNA concentration.

Size (kilobase pairs)	ng for 25 fmol	ng for 50 fmol	ng for 100 fmol
0.2	3.3	6.5	13
0.3	4.9	9.8	20
0.4	6.5	13	26
0.5	8.1	16	33
1.0	16	33	65
2.0	33	65	130
3.0	50	100	195
4.0	65	130	260
5.0	80	165	325
6.0	100	195	390
8.0	130	260	520
10.0	165	325	650
12.0	195	390	780
14.0	230	455	910
16.0	260	520	1040
18.0	295	585	1170
20.0	325	650	1300
48.5	790	1500*	1500*

For ssDNA, the values (ng) should be divided by 2.
*Use no more than 1.5 µg of template DNA.

APPENDIX 2: Information from Amersham Biosciences (now GE Healthcare) website. For an animation of the gel fitration process: Gel Filtration Animation , then in the left-hand column, click on 'animation' or click on 'basic principles' if the animation is not working properly (pop-up window blocking must be disabled). Also, see 'What is Gel Filtration' at the same site.