**Yeast Fluctuation Lab 4**

**Sequencing Part II: Purifying PCR products, gel electrophoretic analysis, and submitting purified PCR products for chain-termination sequencing**

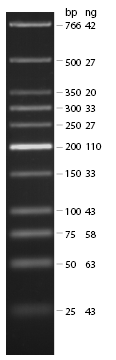
**Instructions**

*Materials for students*

* Your PCR products from <YeastFluctuationLab3.docx> step 13 (24 color-coded 1.5-ml microtubes in deepfreeze)
* Qiagen Buffer PB (yellow-colored binding buffer; 12 lavender 1.5-ml microtubes)
* Qiagen spin columns in their 2-ml collection tubes (24 columns with color-coded labels)



* Microcentrifuge
* Qiagen Buffer PE (washing buffer; 12 yellow 2.2-ml microtubes)
* Tubes for the final purified PCR products (24 empty color-coded 1.5-ml microtubes)
* Qiagen Buffer EB (elution buffer) (12 orange 500-µl microtubes)
* Tubes for electrophoresis samples (24 color-coded 500-µl microtubes, each containing 19 µl loading dye)
* A 500-µl microtube containing at least 90 µl DNA markers of known sizes and amounts (size range 766 to 25 bp; nominally 389 ng total DNA per 14-µl sample, including 32.7 ng of the 766-bp marker)



* A 1.7% MetaPhor agarose gel with 30 wells
* A gel electrophoresis unit
* A DC power supply
* A transilluminator for visualizing DNA bands stained with Sybr Green
* Micotubes containing 8 µl of 3-µM forward or backward sequencing primer (48 1.5-ml microtubes; contents are pale yellow)

*Instructions*

1. Each student will purify one or two of the PCR products on a QIAquick spin column as follows:

* Into the 1.5-ml microtube with the PCR product in 100 µl pipette 500 μl QIAGEN buffer PB (yellow solution in lavender 1.5-ml microtube; one tube per student); the color should remain yellow (you may not be able to see the color very well through the colored microtube walls though)
* Close the cap of the microtube and vortex it to mix the contents
* Pipette all 600 μl of the diluted PCR product into the correspondingly labeled spin column
* Microfuge the spin column for 1 min
* Discard the flow-through from the collection tube; shake out any excess flow-through; replace the column in the collection tube

NOTE: When diluted in the PB binding buffer, the PCR product DNA binds to a filter at the bottom of the spin column, while other components of the PCR reaction mixture, including the enzyme, the unused primers, unused dNTPs, and buffer components, pass through the filter.

* Into the spin column pipette 750 μl QIAGEN buffer PE (in yellow 2.2-ml microtube; one tube per student)
* Microfuge the spin column for 1 min
* Discard the flow-through from the collection tube; shake out any excess flow-through; replace the column in the collection tube
* Microfuge again for 1 min to drive out all residual PE
* Shake out any residual flow-through

NOTE: The previous five substeps wash the filter at the bottom of the spin column to remove residual unbound components, while leaving the PCR product DNA bound to the filter; the extra centrifugation to remove residual PE wash buffer is important, since any residual wash buffer can interfere with subsequent use of the purified PCR product (including sequencing).

* Wipe off the outside of the now-empty spin column and place it into the correspond empty purified PCR product 1.5-ml microtube; discard the 2-ml collection tube
* Pipette 100 μl QIAGEN buffer EB (in yellow 500-µl microtube; one tube per student) into each spin column; EB is an elution buffer that releases the PCR product DNA from the filter at the bottom of the spin column
* Allow to stand for 1 min
* Microfuge 1 min
* Discard the labeled spin column



After use in the next step, the 1.5-ml microtubes containing the purified PCR products will be stored in the deepfreeze

2. Pipette portions of the purified PCR product from the previous step into three microtubes as follows:

* 8 µl into the corresponding special 1.5-ml microtube for sequencing with “forward” primer (labeled on cap; already contains 8 µl of 3-µM primer); contents of tube should change color from pale yellow to red
* 8 µl into the corresponding special 1.5-ml microtube for sequencing with “backward” primer (labeled on cap; already contains 8 µl of 3-µM primer); contents of tube should change color from pale yellow to red
* 2 µl into the corresponding 500-µl microtube for electrophoresis sample (already contains 19 µl blue loading dye)

Vortex all three microtubes; the 1.5-ml microtubes will be submitted to the DNA Core facility for sequencing (the sequencing data will be edited in Yeast Fluctuation Lab 5); meanwhile, the 500-µl microtubes with the electrophoresis samples will be used at step 4.

NOTE: In the next two steps, the purified PCR products will be analyzed by agarose gel electrophoresis. Read the Wikipedia article on that method: <http://en.wikipedia.org/wiki/Agarose_gel_electrophoresis>.

3. I will have set up a 1.7% Metaphor agarose gel with 30 wells; load the gel with 14-µl portions of DNA markers (*Materials*) and of the electrophoresis samples step 2 in the following pattern using the loading method below:

Ladder step 4

Ladder step 4

Colony R1 previous step

Colony R2 previous step

Colony R3 previous step

Colony R4 previous step

Colony R5 previous step

Colony R6 previous step

Colony R7 previous step

Colony R8 previous step

Ladder step 4

Colony B1 previous step

Colony B2 previous step

Colony B3 previous step

Colony B4 previous step → direction of migration

Colony B5 previous step

Colony B6 previous step

Colony B7 previous step

Colony B8 previous step

Ladder step 4

Colony G1 previous step

Colony G2 previous step

Colony G3 previous step

Colony G4 previous step

Colony G5 previous step

Colony G6 previous step

Colony G7 previous step

Colony G8 previous step

Ladder step 4

×

**Loading method:**

* Make sure the pipetter is dialed to 14 µl
* Put a tip on the pipetter
* Press the pipetter plunger all the way to position tube, and hold it there
* Immerse tip in the proper blue electrophoresis sample and slowly allow the plunger to return to its highest position; this will overfill the tip to more than 14 µl
* Carefully place the tip of the tip slightly inside the proper well; it’s best to steady the tip with your other hand braced on the bench or the edge of the gel apparatus
* Slowly press the plunger down to the first position (not the second), thus delivering 14 µl of blue sample into the well and leaving a few µl still in the tip; **don’t release the plunger, and don’t press it down to the second position**; the sample is denser than water, so it will sink into the well
* Without releasing the plunger (i.e., keeping it in the first position), gently withdraw the pipette tip out of the well; only when the tip is completely out of the gel buffer should you release the plunger; discard the tip

Connect the power supply and run the gels at 120 volts for 90 min.

4. The DNA bands in the gel are invisible: they absorb UV light but no visible light. In order to make the DNA bands visible, I will stain the gel in the evening with a fluorescent dye called Sybr Green. When illuminated from below through a blue filter (I will demonstrate the so-called *transilluminator* in class), the dye absorbs photons of the illuminating light (these are called excitation photons) and re-emits fluorescence photons of a slightly higher wavelength (these are called emission photons). The emission photons, but not the excitation photons, are able to pass through the orange filter through which the gel is viewed and photographed. Conversely, the blue filter allows excitation photons to pass, but blocks photons with the wavelength of the emission photons. Thus only Sybr Green fluorescence, not the illumination, is visible though the orange filter. The fluorescence emission from Sybr Green itself is very weak. However, Sybr Green binds strongly to DNA, and when it does so its fluorescence emission increases dramatically. The DNA bands become so fluorescent that they’re visible through the orange filter as bright green bands against a much duller background. The size of the PCR products can be estimated by comparing their migration with those of the nearby DNA marker bands, whose sizes are known exactly.

Below is a picture of the gel after staining. All samples but G6 had a single intense fluorescent band co-migrating with the 766-bp marker fragment. Since the expected size of the PCR product is 763 bp, and since the electrophoretic gel can’t resolve DNA molecules differing in length by only a few bp, these results indicate that all but one of the PCR amplifications (from colony G6) and subsequent purifications worked as expected.

What happened to the DNA from mutant colony G6? One explanation is that there was some procedural error in its processing. More likely, however, this mutant clone had a complex mutation that simultaneously conferred canavanine resistance and prevented PCR amplification. A large deletion removing all or part of the “red” gene along with one or both of the flanking PCR amplification sites would yield this result. Evidently, however, most of the canavinine resistance mutations are simpler events that don’t prevent PCR amplification. Our sequence results will reveal the exact nature of the resistance mutation in those remaining 23 canavanine-resistant colonies.

