DNA Composition Analysis by Nuclease Digestion and HPLC

Adapted from: S. M. Wietstock, *J. Chem. Educ.*, 1995, **72**, 950-2.

The object of this lab is to determine the percent composition of nucleotide in two samples of DNA. The DNA will be hydrolyzed into nucleotides by the enzyme nuclease P1. This particular nuclease hydrolyzes single-stranded DNA, but does not appreciably hydrolyze double-stranded DNA. The DNA will be made single-stranded by heating it. The percent of each of the four nucleotides in the hydrolyzed solution will be determined using HPLC (high pressure liquid chromatography).

**Solution Preparations:**

HPLC Solutions:

2 L of 0.010 *M* potassium phosphate buffer (pH 7.2) (the HPLC eluant):

Add 1.76 g anhydrous K2HPO4 and 1.35 g anhydrous KH2PO4 to a 2 L volumetric flask. Fill to the mark with deionized water. Invert to mix.

100 mL standard solution of nucleotides, 0.0002 *M* in each:

Add 0.0078 g of “T”, 0.0068 g of “A”, 0.0070 g of “C”, or 0.0069 g of “G” to a 100 mL volumetric flask. Dilute to the mark with deionized water. Mix well to ensure that everything is dissolved. Store in refrigerator.

DNA:

1 mL 1 mg/mL DNA from calf thymus:

Fisher: Trevigen Inc. sheared (fragment size: 80–500 bp) calf thymus DNA, 5 x 1 mL, 10 mg/ml, 50-948-284, $114.30. Dilute 100 µL to 1 mL with deionized water in a 1.5 mL plastic centrifuge tube. Label the tube “calf DNA”.

1 mL 1 mg/mL DNA from salmon sperm:

Fisher: Trevigen Inc. sheared (fragment size: 80–500 bp) calf thymus DNA, 5 x 1 mL, 10 mg/ml, 50-948-286, $114.30. Dilute 100 µL to 1 mL with deionized water in a 1.5 mL plastic centrifuge tube. Label the tube “salmon sperm DNA”.

Nuclease P1

100 mL of 0.050 *M* sodium acetate buffer (pH 5.3) containing 5 × 10-4 *M* ZnCl2:

Dilute 0.133 g of NaCH3CO2·3H2O(s) and 225 µL of conc. acetic acid to 100 mL. Add 0.0068 g ZnCl2, or 0.0143 g of ZnSO4·7H2O.

1 mL 5 units/mL nuclease P1:

Into a new vial of nuclease P1 (350 units per vial), inject 1 mL of deionized water with a syringe. Ensure the contents completely dissolve. Withdraw about 14.3 µL with a gc syringe. That’s about 5.0 units. Dilute to 1 mL with the sodium acetate buffer containing Zn ion in a 1.5 mL plastic centrifuge tube.

Into a new vial of nuclease P1 (350 units per vial), inject 1 mL of deionized water with a syringe. Ensure the contents completely dissolve. Withdraw about 14.3 µL with a gc syringe. That’s about 5.0 units. Dilute to 1 mL with the sodium acetate buffer containing Zn ion in a 1.5 mL plastic centrifuge tube.

*Denature the DNA*

Two samples of DNA will be used by the entire class. Each sample needs to be denatured, which is to say that this step should not be done by everyone. Start water boiling in a small beaker. Place 0.25 mL of a DNA sample in a small, glass test tube. Stopper gently with a clean, rubber stopper. Place the sample in the boiling water, immersing so that the contents are slightly below the water level in the beaker. After 10 minutes, transfer the test tube to a small beaker containing ice. This slows down recombination of the separated strands. For best results, this step should be done immediately before the digestion.

*DNA Digestion*

Pick a DNA sample. Transfer 10 µL of the sample, and 10 µL of the P1 nuclease to a 1.5 mL plastic centrifuge tube. Seal the lid, making certain it is truly tight. Label the tube with your initials. Incubate the sample by placing the tube in a water bath a 50°C. Leave it there for 30 min to 1 hr. The mixture may be stored at 4°C for later analysis, or may be analyzed immediately, once it reaches room temperature.

*HPLC Analysis*

The HPLC detector should be set at 260 nm. The flow rate should be about 1.2 mL/min. Before injecting a DNA sample, flush out contamination from the earlier injection. To do this, the injector should be in the INJECT position. Use the needle port cleaner to flush out the needle port with about 1 mL of mobile phase. Now, turn to LOAD. Insert the syringe into the needle port. You will feel tightness during the last 2-3 mm of travel as the needle passes through the needle seal, and then stops against the injector’s surface. Inject 40 or 50 µL of sample. Leave the syringe in and turn to INJECT. Remove the syringe.

*Software Notes*

To start a run, go to “Control/Single Run”. It will indicate when to inject the sample. If a run is stopped by clicking on “Stop Run”, a new window (a cancel window) appears behind the current window. This window must be closed before the run will end. To print the results, go to the top-level “Reports” menu, and select the “area %” option.

A sample of each of the standards must also be run. The software will provide the area under the peak for each nucleotide.

*Analysis*

The area under the peak for each of the standards may be represented as As, Ts, Cs, and Gs. The area under the peaks for the “unknown” DNA samples may be represented as Au, Tu, Cu, and Gu. The percent C+G in the sample may be calculated using the following equation.

$$\% C+G=\frac{\frac{C\_{u}}{C\_{s}}+\frac{G\_{u}}{G\_{s}}}{\frac{C\_{u}}{C\_{s}}+\frac{G\_{u}}{G\_{s}}+\frac{A\_{u}}{A\_{s}}+\frac{T\_{u}}{T\_{s}}}$$

(This formula assumes that the standards of A, T, C, and G are all at the same concentration.)

*Waste*

Rinse the sample tubes and dispose of them in the garbage.

*Note:* HPLC retention times are very dependent on temperature; changes in the room temperature will change the retention times, so frequent calibration is needed. Also, the composition of standards appears to change with time. This is presumably due to hydrolysis of phosphate from the nucleotides. To reduce this, keep the standard and samples refrigerated.