CHROMATOGRAPHY THROUGH SEPHADEX G-50

This technique, which employs gel filtration to separate high-molecular-weight DNA from smaller molecules, is used most often to segregate DNA that has been labeled by nick-translation or by filling in of recessed 3′ ends from unincorporated, labeled deoxynucleotide triphosphates. However, it is also used at several stages during the synthesis of double-stranded cDNA, during addition of linkers to blunt-ended DNA, and, in general, whenever it is necessary to change the composition of the buffer in which DNA is dissolved.

Two methods are available: conventional column chromatography and centrifugation through Sephadex G-50 packed in disposable syringes.

Preparation of Sephadex G-50

Slowly add 30 g of Sephadex G-50 (medium) to 250 ml of TE (pH 8.0) in a 500-ml beaker or bottle. Make sure the powder is well dispersed. Let stand overnight at room temperature, or heat at 65°C for 1-2 hours, or autoclave for 15 minutes at 15 lb/in^2 on liquid cycle. Allow to cool to room temperature.

Decant the supernatant and replace with an equal volume of TE (pH 8.0). Store at 4°C in a screw-capped bottle.

Column Chromatography

1. Prepare a Sephadex G-50 column in a disposable 5-ml borosilicate glass pipette plugged with sterile glass wool. Wash the column with several column volumes of TE (pH 8.0).

2. Apply the DNA sample (in a volume of 200 μl or less) to the column. Wash the tube with approximately 100 μl of TE (pH 8.0) and load the washings onto the column. Connect a reservoir of TE (pH 8.0) to the column so that the flow rate is about 0.5 ml/min.

3. Collect 12-15 fractions (0.5 ml) into Eppendorf tubes. If the DNA is labeled with ^{32}P, measure the radioactivity in each of the tubes, by using either a hand-held minimonitor or by Cerenkov counting in a liquid scintillation counter.

   The DNA will be excluded from the Sephadex gel and will be found in the void volume (usually ~30% of the total column volume). The leading peak of radioactivity therefore consists of nucleotides incorporated into DNA, while the trailing peak consists of unincorporated [^{32}P]dNTPs.

4. Pool the radioactive fractions in the leading peak and store at −20°C.
   Instead of collecting individual fractions, it is possible with practice to follow the progress of the incorporated and unincorporated [^{32}P]dNTPs down the column using a hand-held minimonitor. The leading peak should be collected into a sterile polypropylene tube as it elutes from the column. The bottom of the column should then be clamped off and the buffer reservoir disconnected. The column should be discarded into the radioactive waste.

Caution

Columns should be run behind lucite screens to shield personnel from exposure to radioactivity.

Note

Column chromatography can be used with a variety of matrixes (Sephadex G-75, G-100, Sepharose CL-4B, etc.) to separate DNA from small oligonucleotides or to fractionate DNA crudely by size (see page 226). The matrixes used for particular purposes are indicated at appropriate places in the text.

Solution for Sephadex

25 ml 2M Tris·Cl (pH 8.0)	20 mM
25 ml 500 mM EDTA	5 mM
7.5 ml 5M NaCl	150 mM
192.5 ml dH_2O	
250 ml Total	

Slowly add 15g Sephadex G-50 (from Sigma 9048-71-9), Autoclave

Spun-column Procedure

This method is useful when several preparations of DNA are labeled simultaneously or when it is necessary to change the buffer in which DNA is dissolved.

1. Plug the bottom of a 1-ml disposable syringe with a small amount of sterile glass wool. In the syringe, prepare a column (0.9-ml bed volume) of Sephadex G-50 equilibrated in TE (pH 8.0), containing 0.1 M NaCl (STE).
2. Insert the syringe into a glass centrifuge tube, as shown in Figure A.2. Centrifuge at 1600*g* for 4 minutes in a bench centrifuge. Do not be alarmed by the appearance of the column. Usually the Sephadex packs down during centrifugation. Continue to add Sephadex until the packed column volume is 0.9 ml.
3. Add 0.1 ml of STE and recentrifuge at exactly the same speed and for exactly the same time as before.
4. Repeat step 3.
5. Apply the DNA sample to the column in a total volume of 0.1 ml (use STE to make up the volume).
6. Recentrifuge at exactly the same speed and for exactly the same time as before, collecting the 100 μl of effluent from the syringe in a decapped Eppendorf tube (see Fig. A.2)

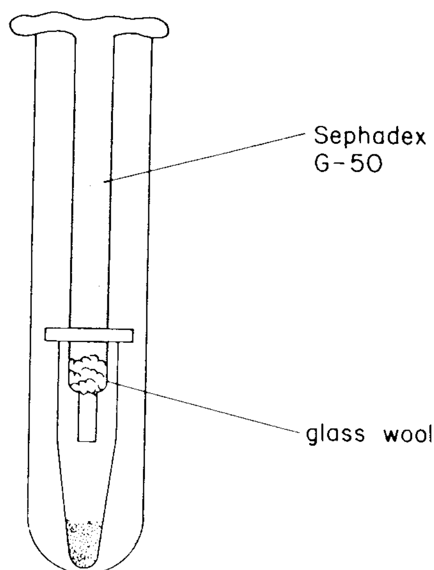

Figure A.2

7. The unincorporated [^{32}P]dNTPs remain in the syringe, which should be carefully discarded. The labeled DNA is collected from the decapped Eppendorf tube.

Note

i. If the spun column is being used to change the buffer, the column should be washed 4–6 times (step 3) with the desired buffer in order to equilibrate the Sephadex G-50.

ii. Spun-column chromatography can also be carried out with Sepharose CL-4B. However, not all matrixes are suitable for this purpose. DEAE-Sephacel forms an impermeable lump during centrifugation; and the larger grades of Sephadex (G-100 and up) cannot be used because the beads are crushed by centrifugation.