Purification of DNA-binding proteins using tandem DNA-affinity column

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The development of DNA-affinity chromatography greatly facilitated purification of sequence-specific DNA-binding proteins (1). Here we describe a tandem DNA-affinity column that allows fast purification to homogeneity of sequence-specific DNA-binding proteins. We also describe a simple method for the synthesis and maintenance of DNA-affinity resins which enables the resins to be used repeatedly over extended periods of time with remarkable reliability.

The first step in the purification scheme is an anion exchange chromatography. Fractions from the anion exchange column containing the sequence-specific DNA-binding protein are pooled, diluted in 50–100 ml binding buffer (25 mM NaCl, 5 mM Tris, 1.0 mM EDTA, 0.05% NP-40, pH = 7.5) and run by gravity at 0.5–1.0 ml/min through a tandem DNA-affinity column which consists of 3–6 DNA-affinity modules connected in series. Each module contains 1–2 ml of DNA-affinity resins in 10 ml Poly-Prep Chromatography columns (Bio-Rad Laboratories, Richmond, CA) overlaid with 4–6 ml buffer. The top 3–4 column modules contain DNA-affinity resin made up of agarose beads conjugated with double-stranded oligonucleotides containing DNA motif of interest with a mutation that eliminates sequence-specific binding. The bottom 1–2 modules contain beads with double stranded oligonucleotide containing the wild-type motif that specifically binds the protein to be purified. After running the load through the DNA-affinity column, modules containing the wild type DNA-resin are disconnected from the top modules containing the mutated DNA-resin. Sequence-specific DNA-binding proteins are simply eluted with buffer containing an appropriate salt concentration (0.2–1.0 M NaCl) after the module is washed with low salt buffer (25–200 mM NaCl).

The top modules containing the DNA-resin with the mutated motif adsorb proteins that bind to DNA non-specifically. The number of mutated DNA-affinity modules is adjusted to ensure that little or no protein is left to bind to the bottom module containing the mutated DNA motif. Thus, with the right number of the top mutated DNA-affinity modules the sequence-specific DNA-binding proteins are isolated from the non-specific DNA-binding proteins, and are allowed to flow and bind to the bottom module containing the wild type DNA-affinity resin. The number of mutated modules required to remove non-specific DNA-binding proteins, for a given total amount of protein in the load, are easily determined by gel-electrophoresis and staining of eluates from each module after the column is first used.

After each use, all modules are washed separately first with 10 ml binding buffer containing 1% Sarkosyl (*N*-lauroylsarcosine sodium salt) and, 1.0 M NaCl, and then washed again with 10 ml binding buffer containing 1.0 M NaCl. DNA-beads can be kept at 4°C in the same modules suspended in binding buffer containing 1.0 M NaCl. Introduction of the Sarkosyl wash allows repeated use (30–40 times) of the same DNA-affinity column modules over months without any loss of specificity or efficiency.

To synthesize DNA-affinity resins we developed a simple method using biotinylated oligonucleotides and streptavidin agarose beads. To couple synthetic oligonucleotides to agarose beads, one of the strands is labelled at the 5' end with biotin using DMT-biotin-C6-PA per the manufacturers direction (Cambridge Research Biochemicals, Wilmington, DE). The biotinylated single stranded oligonucleotides are then annealed to the complementary unmodified strands. The biotinylated double-stranded DNA is simply coupled to streptavidin agarose beads (BRL, Bethesda, MD) by mixing 50–100 µg DNA per 500 µl beads in 1.0 ml binding buffer for one hour at 25°C. DNA-agarose beads are then washed 2–3 times with binding buffer and are stored at 4°C. These DNA-affinity beads can be stored for months without loss of function.

We have applied the tandem DNA-affinity column to purify 65 kD κ B-binding phosphoprotein (2). Figure 1 shows purification of this protein using DNA-affinity modules that have been used repeatedly in our laboratory for over a year. Anion exchange was used as a first purification step. This is one way to separate different proteins that bind specifically to the κ B motif (3–6). This first step also decreases the amount of protein that is loaded onto the tandem DNA-affinity column and consequently fewer number of modules containing the mutated DNA are needed for complete adsorption of non-specific DNA-binding proteins. Nuclear protein extracts purified on DEAE–Sephacel were reappplied to the tandem DNA-affinity column. Staining of the eluates from the top to the bottom of the κ B-mutated DNA modules series shows progressively fewer protein bands; essentially no protein could be detected from the last κ B-Mutated module (Figure 1, lanes 2–4). Eluate from the wild-type κ B column shows only one major protein band of approximately 65 kD (Figure 1, lane 5). Amino acid sequences of the cyanogen bromide digests reveals that the 65 kD band is a novel protein (7).

In addition to being efficient, reproducible and rapid, the tandem DNA-affinity method can be applied for simultaneous purification of two or more proteins each specifically recognized

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by a different DNA sequence. This is achieved by connecting to the bottom of the column a series of tandem-connected modules each containing a different specific protein-binding DNA motif. Individual sequence-specific DNA-binding proteins are then recovered by elution from each DNA-specific module as described above.

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Figure 1. Purification of 65 kD χ B-binding protein using tandem DNA-affinity column. Nuclear protein extracts (10 mg) from EL-4 6.1 C10 thymoma (2) were first run over a DEAE–Sephacel column (10 ml resin volume). After washing with a column buffer containing 100 mM NaCl, proteins were eluted with 30 ml of elution buffer containing 200 mM NaCl. The DEAE–Sephacel eluate was diluted with 50 ml binding buffer and run by gravity over the tandem DNA-affinity column. The DNA-affinity column modules were disconnected, washed with 20 ml of 175 mM NaCl binding buffer, and proteins were eluted from each module with 4 ml of elution buffer containing 500 mM NaCl. An aliquot of each eluate (5% of the total amount eluted) was separated by SDS–PAGE and gels were stained with Fast Stain (Zoion Research, Allston, MA). χ B^{m1}, χ B^{m2} and χ B^{m3} (lanes 2–4) proteins eluted from the top first, second and third module containing the mutated χ B sequence 5'-CAGAGCTCACTTTCCGAGAGGAAGCT-3', mutated bases of the χ B motif are underlined) and from the wild-type module, χ B^w (5'-CAGAGGGGACTTTCCGAGAGGAAGCT-3', χ B motif shown in bold) (lane 5) after it was disconnected from the outflow of the χ B^{m3} module. Molecular weight standards (in kD) were run in lane 1.