

# Yeast RNA Polymerase II Transcription Reconstituted with Purified Proteins

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Protocols are presented for the preparation of a fully defined yeast RNA polymerase II transcription system, consisting of essentially pure TFIIB, -E, -F, and -H, TATA-binding protein, RNA polymerase II, and mediator of transcriptional regulation. This system, comprising 44 polypeptides, is able to initiate transcription at any of a dozen yeast and mammalian promoters thus far tested and responds to a variety of transcriptional activator proteins. © 1997 Academic Press

A set of five essentially pure proteins together with pure RNA polymerase II from the yeast *Saccharomyces cerevisiae* has been shown to transcribe nearly a dozen yeast and mammalian promoters thus far tested (1). These five proteins, commonly referred to as TATA-binding protein (TBP), TFIIB, TFIIE, TFIIF, and TFIIH, were originally resolved from yeast extracts, but three of the proteins, TBP, TFIIB, and TFIIE, are now available in recombinant form. TFIIF can also be expressed from cloned genes in a baculovirus system, but the recombinant protein lacks some functional characteristics of the protein isolated from yeast (N. L. Henry, K. Leuther, and R. D. Kornberg, unpublished observations). With the addition of a multiprotein complex, termed mediator, the reconstituted system not only transcribes a minimal promoter but responds to activator proteins bound upstream of the promoter as well (2). We present here a set of current protocols for the preparation of essentially pure TFIIB, TFIIH, RNA polymerase II, and mediator. The remaining proteins, TBP and TFIIF, are prepared as previously described (3–5).

## 1. TFIIB

*Escherichia coli* strain BL21 (DE3) was transformed with plasmid pET11a containing the coding region for wild-type yeast TFIIB (pET11aSUA7, a gift from M. Hampsey). The transformant was grown to an  $A_{600}$  value of 0.6 in L-broth, and expression of TFIIB was induced with 0.4 mM IPTG for 4 h.  $ZnCl_2$  was added to 0.1 mM at the time of induction. Cells were harvested by centrifugation and resuspended in 10 ml per liter of cell culture of 50 mM Tris, pH 7.6, 50 mM NaCl, 10% glycerol, 0.05 mM  $ZnCl_2$ , 2.5 mM DTT, and protease inhibitors (for all purifications a 100× stock of protease inhibitors contained 100 mM phenylmethylsulfonyl fluoride, 200  $\mu$ M pepstatin, 60  $\mu$ M leupeptin, and 200 mM benzamidine in 100% ethanol). The cell suspension could be frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ . Subsequent steps were performed in the cold.

Cells were disrupted by sonication until the  $A_{600}$  value of a 1:100 dilution of cell suspension in water was reduced to one-fourth of its original value. The sonicate was centrifuged at 12,000 rpm for 45 min. The supernatant was made 0.1% (w/v) in polyethyleneimine, kept for 15 min on ice, and centrifuged as before. The supernatant (about 40 ml from 4 liters of cell culture) was applied to a DE52 (Whatman) column (about 1.2 ml of resin per milliliter of supernatant) equilibrated in 50 mM Tris, pH 7.6, 100 mM NaCl, 10% glycerol, 0.05 mM  $ZnCl_2$ , 1 mM DTT, and protease inhibitors. The column was washed with the same buffer at 1 ml/min, and 10-ml fractions were collected. TFIIB was in the flowthrough.

Fractions containing TFIIB were loaded at 1 ml/

min on a Bio-Rex 70 (Bio-Rad) column (about 1.5 ml of resin per milliliter of TFIIB fraction) equilibrated in 50 mM Hepes, pH 7.6, 100 mM NaCl, 10% glycerol, 0.05 mM ZnCl<sub>2</sub>, 1 mM DTT, and protease inhibitors. After washing with 2 column volumes of the same buffer and 2 column volumes of the same buffer containing 200 mM NaCl, TFIIB was eluted with the same buffer containing 600 mM NaCl, and 5-ml fractions were collected.

Fractions containing TFIIB were loaded on a hydroxyapatite column (about 0.35 g of Bio-Gel HTP (Bio-Rad) HAP or 1 ml of column bed per milliliter of TFIIB fraction) equilibrated in 10 mM sodium phosphate, pH 7.6, 100 mM NaCl, 10% glycerol, 1 mM DTT, and protease inhibitors. After washing with about 2 column volumes of the same buffer, TFIIB was eluted with a linear gradient of 10 to 250 mM sodium phosphate in the same buffer. Elution of TFIIB began at about 160 mM sodium phosphate, and fractions of 2 to 3 ml were collected. In this chromatographic step, full-length TFIIB was resolved from a C-terminal fragment about two-thirds the size arising from an internal translation start site recognized in *E. coli*. Fractions containing full-length TFIIB were concentrated, and the buffer was changed to 50 mM Hepes, pH 7.6, 500 mM NaCl, 1% glycerol, 0.05 mM ZnCl<sub>2</sub>, 2.5 mM DTT with the use of a Centriprep 10 spin filter unit.

Traces of the C-terminal fragment of TFIIB were removed in a final step of gel filtration through Sephacryl S-100 HR (Pharmacia). A column of about 80 ml volume and about 1 cm diameter was equilibrated in 50 mM Hepes, pH 7.6, 500 mM NaCl, 1% glycerol, 0.05 mM ZnCl<sub>2</sub>, 2.5 mM DTT and run in the same buffer. No more than about 3 mg of protein in at most 2 ml was loaded on the column. The flow rate was about 150  $\mu$ l/min, and fractions of 1.5 ml were collected. Peak fractions containing TFIIB (now >98% pure) were precipitated by addition of solid ammonium sulfate. Pellets formed by centrifugation at high speed in a microcentrifuge at 4°C were frozen and stored in liquid nitrogen. Four liters of cell culture yielded about 20 mg of purified TFIIB. Protein was not stored frozen between chromatographic steps, but was kept on ice. Cell lysis and fractionation on DE52 and Bio-Rex 70 can all be carried out in 1 day. Potassium acetate or sodium acetate may be used in place of sodium chloride. TFIIB purification was monitored by SDS-PAGE, and functional activity was determined at the end of purification by gel-shift analysis with TBP, TFIIA, and promoter DNA and by specific transcription assay.

## 2. TFIIE

*E. coli* strain BL21(DE3) was transformed with the double expression vector Dabp28 (referred to as 6his TFA1, TFA2 in Ref. 6). The transformant was grown at 37°C to an A<sub>600</sub> value of 0.5, and expression of TFA1 and TFA2 was induced with 0.4 mM IPTG for 3 h at 30°C. ZnCl<sub>2</sub> was added to 0.1 mM at the time of induction. Cells were harvested by centrifugation and resuspended in 20 ml per liter of culture of 20 mM Tris-Cl, pH 8.0, 100 mM NaCl, 20% glycerol, 0.1% Tween 20, 2 mM imidazole, 5 mM  $\beta$ -mercaptoethanol.

Cells were disrupted by sonication, followed by centrifugation at 18,000 rpm in a Beckman JA-20 rotor for 30 min. The supernatant was applied at 2 to 4 column volumes per hour to a Nickel NTA column (2.5 ml of resin per 20 ml of supernatant) in 20 mM Tris-Cl, pH 8.0, 100 mM sodium chloride, 10% glycerol, 0.1% Tween 20, 2 mM imidazole, 5 mM  $\beta$ -mercaptoethanol. The column was washed with 2 vol of the same buffer, followed by 2 vol of buffer A (100 mM NaCl, 100 mM sodium phosphate, pH 6.3, 10% glycerol, 2 mM imidazole, 0.01% NP-40, 5 mM  $\beta$ -mercaptoethanol), 2 vol of buffer A containing 500 mM NaCl, 2 vol of buffer A containing 5 mM imidazole, 2 vol of buffer A containing 25 mM imidazole, 3 vol of buffer A containing 100 mM imidazole, and 3 volumes of buffer A containing 200 mM imidazole. TFIIE was almost entirely eluted by 100 mM imidazole, as shown by SDS-PAGE. Elution could also be performed with a linear gradient of 20 to 100 mM imidazole.

Fractions containing TFIIE were diluted 1:3 with 20 mM Hepes, pH 7.6, 10% glycerol, 1 mM EDTA, 0.01% NP-40, 1 mM DTT and applied to a Bio-Rex 70 column (2.5 ml per liter of cell culture) at 2 to 4 column volumes per hour. The column was washed with 2 column volumes of buffer B (20 mM Hepes, pH 7.6, 10% glycerol, 1 mM EDTA, 0.01% NP-40, 1 mM DTT, 50 mM NaCl) and eluted successively with a 10-column-volume linear gradient of 50 to 500 mM NaCl in buffer B, 2 column volumes of 500 mM NaCl in buffer B, and 3 column volumes of 1 M NaCl in buffer B. Elution of TFIIE was monitored by SDS-PAGE. Tfa1 eluted early in the gradient, followed by holoTFIIE (Tfa1/Tfa2 complex) and Tfa2.

HoloTFIIE fractions were pooled, diluted with buffer C (20 mM Tris-Cl, pH 7.5, 10% glycerol, 1 mM EDTA, 0.01% NP-40, 1 mM DTT) to a conductivity of 50–100 mM NaCl, and applied to a DEAE column (2.5 ml of resin per liter of cell culture) at 2 to 4 column volumes per hour. The column was washed with 3 column volumes of 50 mM NaCl in buffer C and eluted with a 10-

column-volume linear gradient of 50 to 500 mM NaCl in buffer C, 2 column volumes of 500 mM NaCl in buffer C, and 3 column volumes of 1 M NaCl in buffer C. Purity at this point, judged by SDS-PAGE, was >95%, sufficient for most purposes.

For further purification, DEAE Sephacel (Pharmacia) fractions were pooled and applied to a hydroxyapatite column (0.2 ml per milligram of protein), washed with 2 column volumes of 10 mM potassium phosphate, pH 7.8, in 10% glycerol, 100 mM potassium acetate, 1 mM DTT, 0.01% NP-40, and developed with a 10-column-volume linear gradient of 10 to 300 mM potassium phosphate in the same buffer at 1 to 2 column volumes per hour. TFIIE eluted around 100 mM phosphate and could be concentrated with the use of a Centricon 50 (TFIIE flows through a Centricon 100).

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### 3. TFIH

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A yeast strain expressing a hexahistidine-tagged subunit of TFIH (YPH/TFB1 6His) was grown to mid-log-phase as described (7). Cells were harvested by centrifugation, resuspended, broken with glass beads as described, and centrifuged in Beckman JA-10 rotor at 9000 rpm for 20 min. To the supernatant was added  $\frac{1}{9}$  vol of 5 M KOAc, followed by stirring for 15 min, addition of polyethyleneimine to a final concentration of 0.3%, stirring for another 15 min, and centrifugation in a Beckman 45Ti rotor at 42,000 rpm for 90 min. The supernatant, containing about 50 mg of protein per milliliter, was dialyzed against 10 vol of buffer A-0 (Buffer A contained 20 mM Hepes-KOH, pH 7.6, 1 mM EDTA, 20% glycerol, 1 mM DTT, protease inhibitors; the molarity of potassium acetate is indicated by a hyphen followed by a number) for 4 h, adjusted to the conductivity of buffer A-0.2 by dilution with buffer A-0, and applied to a Bio-Rex 70 column (60 mg of protein per milliliter of resin) equilibrated in A-0.2 at a flow rate of 1.5 column volumes per hour. The column was washed with 1 vol of buffer A-0.2 and 2 vol of buffer A-0.3, and it was eluted with 2 vol of buffer A-0.65. Ten to 15 fractions were collected, and peak protein fractions were pooled, maintaining a final concentration >10 mg/ml. The recovery of protein was 5–10%.

The pooled Bio-Rex 70 fractions were dialyzed against buffer A-0, adjusted to the conductivity of buffer A-0.15 by dilution with buffer A-0, and applied to a P-11 phosphocellulose (Whatman) column (50 mg protein/ml resin) equilibrated with 5 column volumes of buffer A-0.1 at a flow rate of 2 column

volumes per hour. The column was washed with 1 column volume of buffer A-0.1 and 2 column volumes of buffer I-0.2 (Buffer I contained 20 mM Hepes-KOH, pH 7.6, 20% glycerol, 0.01% Nonidet-P40, 0.2% Tween 20, 10 mM imidazole, 5 mM  $\beta$ -mercaptoethanol, protease inhibitors; the molarity of potassium acetate is indicated by a hyphen followed by a number), and it was eluted with 2 column volumes of buffer I-0.6. Ten to 15 fractions were collected, and peak protein fractions were pooled, maintaining a final concentration of >10 mg/ml. The recovery of protein was about 50%.

The pooled P-11 fractions were incubated with Ni<sup>2+</sup>-NTA-agarose (Qiagen) with gentle agitation for 12–14 h at 4°C, and the resin was packed in a small column. The ratio of protein to resin was typically 0.66 g/ml, but a higher ratio could be used, since the amount of resin needed to form a convenient column was much greater than that required for protein binding. The column was washed with buffer I-0.2 at a flow rate of 1.5 column volumes per hour for 6 h. After further washing with buffer J-0.2 (Buffer J contained 20 mM Tris-acetate, pH 7.6, 20% glycerol, 5 mM  $\beta$ -mercaptoethanol, protease inhibitors; the molarity of potassium acetate is indicated by a hyphen followed by a number) containing 20 mM imidazole for 3 h, the column was eluted with buffer J-0.2 containing 200 mM imidazole. TFIH was detected with antisera against Tfb1, Kin28, and Ccl1 and by transcription assays.

The total protein from even a very large amount of cell culture (1–2 kg of cell paste) was small enough after Ni<sup>2+</sup>-agarose chromatography for loading on a 1-ml Mono-Q HPLC column (Pharmacia Q 5/5). The column was equilibrated in buffer B-0.2 (Buffer B contained 20 mM Tris-acetate, pH 7.8, 1 mM EDTA, 20% glycerol, 0.01% Nonidet-P40, 1 mM DTT, protease inhibitors; the molarity of potassium acetate is indicated by a hyphen followed by a number), loaded at a flow rate of 0.5 ml/min, washed with 2 column volumes of buffer B-0.2 and 2.5 column volumes of buffer B-0.5, and developed with a 10-column-volume linear gradient of buffer B-0.5 to buffer B-1.0. TFIH eluted approximately halfway through the gradient. The protein was pure enough for the clear identification of TFIH subunits by SDS-PAGE with silver staining. Further purification could be effected by chromatography on a TSK-Gel Phenyl-5PW HPLC column as described (7).

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### 4. RNA POLYMERASE II

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Yeast RNA polymerase II from logarithmically growing cells is heterogeneous, due to a substoichio-

metric amount of two subunits, Rpb4 and Rpb7 (8). Some polymerase molecules possess these subunits while others lack them completely. In contrast, RNA polymerase II from stationary phase cells appears homogeneous, possessing a full complement of all subunits. Rpb4 and Rpb7 are required for promoter-dependent transcription *in vitro* (9). For studies of transcription initiation with purified proteins, we have therefore isolated polymerase from laboratory-grown cells in late log or early stationary phase, or from commercial yeast (from bakery supplier or brewery) harvested in the stationary phase as well.

Cells were suspended in 0.2 ml per gram of 250 mM Tris-HCl, pH 7.9 (at 4°C), 5 mM EDTA, 50 mM DTT, 50% glycerol, 50 mM ZnCl<sub>2</sub>, 5% DMSO, protease inhibitors and frozen in portions (about 600 ml) to fill three bead beaters (Bio-Spec). A portion was thawed in warm water with constant mixing and placed in a precooled bead beater. The remainder of the bead beater was filled with beads, ensuring that no air was left in the chamber; 1 ml of 100× protease inhibitors was added, and the bead beater chamber was immersed in a salt/ice/water bath. Bead beating was carried out for 15 cycles of 30 s on and 90 s off. The contents of the bead beaters were poured into a 1-liter flask, some buffer A-0 (50 mM Tris-Cl, pH 7.9, 10% glycerol, 1 mM EDTA, 10 mM DTT, 10 mM ZnCl<sub>2</sub>, protease inhibitors; the number after the hyphen indicates the concentration of KCl in millimolar units) was added to reduce the viscosity, and the beads were allowed to settle. The supernatant was decanted, more buffer A was added to the beads, and the process was repeated until the supernatant was clear (or until about 1 liter of buffer A was used). The combined supernatants were centrifuged in a Beckman Ti-45 rotors at 25,000 rpm for 30 min (or in a Beckman JA-20 rotor at 18000 rpm). The supernatant was made 1 M in ammonium sulfate, stirred for 30 min, adjusted to 50% of saturation with ammonium sulfate, stirred for another 30 min, and centrifuged. The pellet was resuspended in 50 mM buffer A to give a conductivity less than that of buffer A-150 and was applied at 1 to 2 column volumes per hour to a column of heparin-Sepharose (300 ml of resin per kilogram of cells) in buffer A-150. The column was washed with 3 vol of buffer A-150 and eluted with buffer A-600. A yellow band that eluted just after the peak of protein could usually be seen migrating down the column. Peak protein fractions were precipitated with 50% ammonium sulfate and frozen at -70°C. Monitoring the elution of polymerase by immunoblot analysis with 8WG16 monoclonal antibody revealed some loss in the heparin flowthrough fraction, for reasons explained elsewhere.

The ammonium sulfate pellet was resuspended in buffer B-0 (50 mM Tris-Cl, pH 7.5, 1 mM EDTA, 10 μM ZnCl<sub>2</sub>, 10 mM DTT, protease inhibitors; the number after the hyphen indicates the concentration of ammonium sulfate, pH 7.5, in millimolar units) to the conductivity of buffer B-400 to B-500, and it was applied to a column of DE52 (5 ml per kilogram of cells) equilibrated overnight in buffer B-500 (without protease inhibitors). The column was washed with buffer B-400, and all protein flowing through the column was collected and applied at 2 to 3 column volumes per hour to an 8WG16 antibody-Sepharose column (5 ml per kilogram of cells of CNBr-activated Sepharose 4B coupled to antibody at 2 mg/ml). The column was washed at 5 column volumes per hour with 10 vol of buffer B-500, warmed to room temperature, washed with 10 vol of buffer B-500, and eluted at 2 column volumes per hour with buffer B-500 containing 50% glycerol. When no more polymerase appeared to be eluting, as judged by SDS-PAGE (usually after 3 column volumes of elution), the column was further eluted with 3 vol of buffer B-500 containing 70% glycerol.

The antibody column eluate was dialyzed to the conductivity of buffer C-60 (50 mM Tris-Cl, pH 7.5, 10% glycerol, 1 mM EDTA, 10 mM DTT, 10 μM ZnCl<sub>2</sub>, protease inhibitors; the number after the hyphen indicates the concentration of ammonium sulfate in millimolar units) and applied to a DEAE 5PW HPLC column (3 ml for 1 kg of cells). The column was washed at 1/3 column volume per minute with 3 column volumes of buffer C-60 and was eluted with a linear gradient of 5 column volumes of buffer C-60 to buffer C-400. Polymerase eluted around buffer C-300.

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## 5. MEDIATOR

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Free mediator was conveniently obtained from commercial yeast (Fleischmann's active dry yeast, collected before drying) without the requirement for displacement from RNA polymerase II holoenzyme, as described for laboratory-grown yeast (2). The active dry yeast (2.6 kg) was suspended in lysis buffer, and the cells were disrupted as described (2) and centrifuged in Beckman JA-10 rotor at 9000 rpm for 20 min. The supernatant was collected, and 1/9 vol of 5 M potassium acetate was added, followed by stirring for 15 min, addition of polyethyleneimine to 0.2% (w/v), stirring for another 30 min, and centrifugation in a Beckman Ti45 rotor at 42,000 rpm for 90 min. The supernatant (containing about 100 g of protein) was diluted with buffer A-0 (25 mM Hepes-

KOH, pH 7.6, 1 mM EDTA, 10% glycerol, 1 mM DTT, protease inhibitors; the number after the hyphen indicates the potassium acetate concentration in molar units) to the conductivity of buffer A-0.15, and one-half of the supernatant was fractionated at a time on a 1-liter column of Bio-Rex 70 (capacity about 50 mg protein/ml) in buffer A-0.15. The column was loaded at 2 column volumes per hour, washed with 1 column volume of buffer A-0.15 and 2 column volumes of buffer A-0.3, and eluted with buffer A-0.6.

Peak protein fractions from Bio-Rex 70 (7.6 g total protein) were pooled, dialyzed against buffer B-0 (25 mM Tris-acetate, pH 7.8, 1 mM EDTA, 10% glycerol, 0.01% NP-40, 1 mM DTT, protease inhibitors; the number after the hyphen indicates the potassium acetate concentration in molar units) for 4 h, diluted with buffer B-0 to the conductivity of buffer B-0.1, and fractionated on two 125-ml DEAE-Sephacel columns (capacity about 30 mg protein/ml) equilibrated in buffer B-0.1. The columns were loaded at 1.5 column volume per hour, washed with 125 ml of buffer B-0.1 and 250 ml buffer B-0.2, and eluted with buffer B-0.55.

Peak protein fractions (770 mg total protein) were pooled, diluted with an equal volume of buffer C (0.01 M potassium phosphate, pH 7.7, 10% glycerol, 0.1 M potassium acetate, 1 mM EDTA, 0.01% NP-40, 0.1 mM CaCl<sub>2</sub>, 1 mM DTT, protease inhibitors), and applied at 1 column volume per hour to a column of hydroxylapatite (80 ml, capacity of 8–10 mg protein/ml) equilibrated in buffer C. The column was washed with 1 column volume of buffer C and developed with a linear gradient (800 ml) of buffer C to buffer D (buffer C with 0.2 M potassium phosphate). The flow rate was 80 ml/h, and 10-ml fractions were collected. Immunoblot analysis with antibodies against core polymerase II and against mediator proteins revealed a peak of polymerase alone followed by a peak containing both polymerase and mediator polypeptides.

Fractions containing mediator polypeptides (105 mg) were pooled, diluted with an equal volume of buffer Q-0 (Buffer Q contained 20 mM Tris-acetate, pH 7.9, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, protease inhibitors; the molarity of potassium acetate is indicated by a hyphen followed by a number), and applied at 1 ml/min to a MonoQ 10/10 HPLC column (Pharmacia) equilibrated in buffer Q-0.15. The column was washed with 40 ml of buffer Q-0.15 and 40 ml of buffer Q-0.5 and was eluted with a linear gradient (112 ml) of buffer Q-0.5 to buffer Q-1.2. The flow rate was 1 ml/min, and 1.2-ml fractions

were collected. Immunoblot analysis revealed a peak of free mediator at about buffer Q-0.6 and holopolymerase II at about buffer Q-0.8.

Peak fractions of free mediator were pooled, dialyzed for 4 h against buffer H-0.1 (0.02 mM potassium phosphate, pH 7.6, 10% glycerol, 0.2 mM EDTA, protease inhibitors; the number after the hyphen indicates the potassium acetate concentration in molar units), diluted with buffer H-0 to the conductivity of buffer H-0.1, and applied at 0.5 ml/min to a TSK-heparin-5-PW HPLC column (3 ml, 75 × 7.5 mm, Supelco) equilibrated in buffer H-0.1. The column was washed with 2 column volumes of buffer H-0.1 and 4 column volumes of buffer H-0.25 and was developed with a linear gradient (33 ml) of buffer H-0.25 to buffer H-0.75. The flow rate was 0.5 ml/min, and 0.75-ml fractions were collected. The peak of mediator was at about buffer H-0.4.

Peak mediator fractions were adjusted to the conductivity of A-0.1, pooled and applied to a MonoS 5/5 HPLC column (Pharmacia) equilibrated in buffer A-0.1. The column was washed with 5 column volumes of buffer A-0.1 and 5 column volumes of buffer A-0.2 and was developed with a linear gradient (12 ml) of buffer A-0.2 to buffer A-1.0. The flow rate was 0.5 ml/min, and 0.35-ml fractions were collected. The mediator peak was centered at about buffer A-0.5.

A portion (0.25 ml) of the peak fraction of mediator was applied to a Bio-Sil SEC 400 HPLC column (Bio-Rad) equilibrated in buffer A-0.5. The flow rate was 0.25 ml/min, and 0.35-ml fractions were collected.

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