

## Quantitative nonisotopic nitrocellulose filter binding assays: bacterial manganese superoxide dismutase–DNA interactions

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### Abstract

Nitrocellulose filter binding assays (NCFBAs) have been used for many years to qualitatively and quantitatively determine protein–nucleic acid affinities. While this technique can be robust thermodynamically and fairly simple to perform, the requirement of radiolabeled nucleic acids (typically  $^{32}\text{P}$ ) has several major drawbacks. Some disadvantages are the short half-life of  $^{32}\text{P}$ , the inherent safety concerns, and the cost of working with radioisotopes. Another drawback is that over time the beta emissions cause fragmentation of the nucleic acids. We have modified standard NCFBAs by developing a quantitative nonisotopic chemiluminescent method using biotin-labeled DNA and a dual-filter format. The biotin tag is detected on both nitrocellulose and positively charged nylon membranes by conventional streptavidin-conjugated alkaline phosphatase recognition systems. Quantitation of the photon emissions is simplified by use of a cooled charge-coupled device camera, although exposure to X-ray film and quantitation by densitometry could also be employed. The binding affinity of bacterial manganese superoxide dismutase to nonsequence-specific DNA has been quantitated previously by standard NCFBAs. We have replicated parts of a published binding study using identical solution conditions and the nonisotopic method that we have developed. We provide quantitative agreement between the isotopic and the nonisotopic methods.

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The family of superoxide dismutases (SODs)<sup>1</sup> provides important protection from oxidation of cellular components by catalyzing the conversion of the biologically damaging superoxide radical to hydrogen peroxide (Eq. (1)) [1].



While the dismutation of superoxide is the most well known function of the superoxide dismutase family, it has been found that the manganese isoform of SOD (MnSOD) in *Escherichia coli* can bind nonspecifically to DNA [2]. A prominent positively charged region

formed at the dimer interface has been postulated to comprise the DNA-binding domain [2]. Thus, the binding of MnSOD to DNA may provide valuable genomic protection against oxidative attack in bacterial cells [3]. The binding affinity of *E. coli* MnSOD protein for non-specific DNA has been determined by quantitative radioisotope-based nitrocellulose filter binding assays (NCFBAs) [2]. We have replicated these data using a nonisotopic NCFBA technique. The most important modification from the standard procedure is the replacement of radioactively labeled DNA with biotinylated DNA that can be detected by a streptavidin-linked chemiluminescent system. In simple terms, the assay consists of separating protein, DNA, and protein–DNA complexes by vacuum filtration through a double-membrane filter, similar to that used by Wong and Lohman [4]. The top layer is nitrocellulose, which binds protein and protein–DNA complexes, and the bottom

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<sup>1</sup> Abbreviations used: SOD, superoxide dismutase; NCFBA, nitrocellulose filter binding assay; BSA, bovine serum albumin; CCD, charge-coupled device.

layer is positively charged nylon which binds free DNA. We have shown sensitivity limits down to the low-femto-gram level of DNA using similar chemiluminescent techniques in our laboratory [5]. Using this technique, we show quantitative agreement with previously published work that relied upon radiolabeled DNA [2].

## Materials and methods

### *Buffers, proteins, and oligodeoxynucleotides*

Solutions were made with reagent-grade chemicals using sterile deionized water. The basic binding buffer is Buffer B: 25 mM phosphate, pH 7.2, 2 mM Mg<sub>2</sub>Cl<sub>2</sub>, 3% glycerol, 75 mM KCl. The binding buffer contains bovine serum albumin (50 µg/ml), which we will term Buffer B (+BSA). Bovine serum albumin was purchased from Sigma (Cat. No. A-7906). Manganese superoxide dismutase protein was purchased from Sigma (Cat. No. S-5639) and its concentration was determined by UV absorbance using a molar absorptivity value of  $8.66 \times 10^{-4} \text{ M}^{-1} \text{ cm}^{-1}$  on a dimer basis [6]. MnSOD purity was also verified by SDS-PAGE analysis (data not shown). The purified 34 mer DNA of the same sequence as that used in Steinman et al. [2] was purchased from Invitrogen Life Technologies. The DNA concentration was determined spectrophotometrically in 100 mM Tris-HCl, pH 7.5/1 M NaCl/10 mM EDTA/DEPC-treated water. The sequence of the sense strand used is 5'-biotin-CTT CTC TAG CTG AAT AAC CGG AAG TAA CTC ATC G.

### *Nitrocellulose and nylon filters*

The nitrocellulose filters were Protran Pure Nitrocellulose Transfer and Immobilization Membrane (pore size = 0.2 µm). Each filter was precut to fit the desired number of lanes needed for the BIO-DOT SF (Bio-Rad) vacuum apparatus. To reduce retention of free DNA, nitrocellulose filters were presoaked for 10 min in 0.4 M KOH followed by three 5-min washes in distilled water to bring the pH to neutral [4]. Nitrocellulose filters were then equilibrated in Buffer B (-BSA) at 4 °C for a minimum of 1 h before use. The nylon membranes (MagnaGraph, Micron Separations) were cut to size and presoaked in Buffer B (-BSA) for 1 h.

### *Titration procedure*

Binding assays were performed with constant [DNA] and varying [MnSOD] in Buffer B (+BSA). Each binding reaction was mixed in a single well of a sterile low-protein-binding polystyrene 96-well assay plate. Each component of the reaction was mixed in

the wells and then the reactions were incubated at 20 °C for 30 min.

Immediately before filtering each set of samples, the wells of the slot-blot apparatus are flushed with 200 µl of Buffer B (-BSA) at 20 °C using an eight-channel pipettor to make the step more rapid (to hopefully obtain a better “snapshot” of the representative molecular interactions present in the solution) and vacuum filtered (house vacuum, ~20 psi). Care is taken during all vacuum steps to avoid overdrying the membrane. The 30-µl reaction samples are loaded into the wells individually with a single-channel micropipettor and then vacuum filtered. Immediately after filtering each set of samples, the wells of the slot-blot apparatus are washed once with 200 µl of Buffer B (-BSA) at 20 °C using an eight-channel pipettor. The nitrocellulose is then removed from the apparatus and placed in a heated vacuum desiccator (Precision Scientific) for 20 min at 80 °C under constant vacuum (house vacuum, ~20 psi), although baking for up to 45 min showed no difference in results (data not shown). Overbaking (>1 h) resulted in browning and fragmentation of the nitrocellulose membrane (data not shown).

A special note concerning the reason that BSA was included only in the reaction mixtures and not in the membrane wash solutions should be mentioned. It was determined that the inclusion of BSA in the wash buffer had no effect on positively charged nylon membranes, but the nitrocellulose membrane was “hyperblocked” in that the BSA-containing blocking buffer did not block the areas of the membrane as well as the sample well areas, leading to “negative” data (data not shown).

### *Chemiluminescent detection of biotinylated DNA on nylon and nitrocellulose membranes*

The nylon membranes are treated as described previously to visualize and quantitate the biotinylated DNA [5]. The nitrocellulose membrane was washed and blocked as follows. First, the nitrocellulose is blocked using Buffer TBST (+BSA) (10 mM Tris-Cl, pH 8, 100 mM NaCl, 0.1% Tween 20 containing 1% BSA) as the blocking agent for 30 min. The block is discarded and replaced with an identical block buffer containing 40 µl of AvidX streptavidin (PE Biosystems) (or 1 µl of streptavidin-alkaline phosphatase conjugate; Ambion) for 30 min. Block buffer is next added to the nitrocellulose for 10 min as an initial rinse of the membrane. This is followed by three 5-min washes each with the TBST before two 2-min washes with assay buffer. The assay buffer that we use contains 5% solution of Nitroblock (Ambion) in 1× Ambion Brightstar assay buffer. Then, the substrate, CDPSTAR (Ambion), was used to react with the alkaline phosphatase moiety of the membrane-bound AvidX for 5 min before a digital picture was taken with a GDS-8000 cooled CCD camera

(UVP, Palo Alto, CA). Exposure times ranged from 4 to 10 min for quantitative data collection, although band intensity was typically visible within 1 or 2 min (data not shown). If no cooled CCD camera is available, one could, alternatively, expose the membrane to X-ray film and quantitate by densitometry.

## Results

### Preliminary experiments

The signal monitored in these NCFBA experiments originates from the biotinylated DNA. However, one must first determine whether the protein contains any biotin contamination due to copurified biotin-containing proteins (free biotin is not efficiently retained on either the nitrocellulose or the positively charged nylon membranes under the conditions used in this study (data not shown)). The easiest way to determine this is to perform one reaction that contains no DNA but contains the same protein concentration in corresponding DNA–protein-binding assays. We have determined that for the *E. coli* MnSOD preparation that we used, the intrinsic signal originating from the protein is less than 1% of the signal from the corresponding biotinylated DNA–MnSOD complexes retained on the nitrocellulose (Fig. 1). Therefore, we have neglected the signal originating from our protein in our analyses. If there were substantial background originating from our protein, we would have to run an extra lane containing identical increments of protein as in the DNA-binding assays to subtract the background values at each point. Incidentally, the nylon filter contained no measurable signal for free MnSOD in the absence of DNA (data not shown).

### Comparison of nonisotopic to isotopically labeled DNA results

Since the goal of the study was to determine whether chemiluminescent NCFBAs could replicate radiolabeled NCFBAs, we chose to use the same reaction conditions as indicated in Steinman et al. [2]. Typical raw nitrocellulose membrane data are shown in Fig. 2A. As expected, Fig. 2A shows a strong qualitative increase in the [DNA] retained by the nitrocellulose as [MnSOD] increases. Also as expected, Fig. 2B shows a strong qualitative decrease in the [DNA] passed through to the positively charged nylon membrane as [MnSOD] increases.

By generating integrated optical density values from images such as that found in Fig. 2A from the nitrocellulose membrane, the amount of DNA retained on the nitrocellulose is determined by subtracting the intensity measured in the DNA-only well as a background. The nonspecific background binding of DNA was routinely

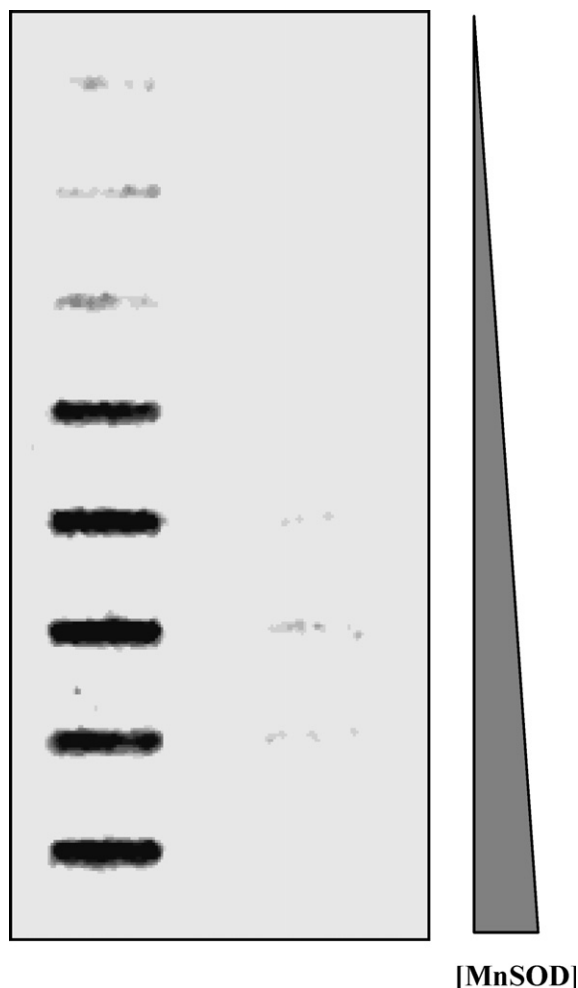


Fig. 1. Representative chemiluminescent NCFBA binding experiment in the presence and absence of DNA. These data are photoinverted from the raw picture of the nitrocellulose membrane. The two columns represent duplicate wells containing increasing [MnSOD] (from 0 to 62.3  $\mu$ M) down each column as indicated. The left column contains  $6.7 \times 10^{-10}$  M DNA (20 fmol) in each reaction, while the right column contains no DNA.

less than 5% of the maximum signal on the nitrocellulose (data not shown). These data are then plotted as intensity vs  $\log$  [MnSOD] and fit to Eq. (2) (a single-binding-site model),

Fraction of DNA on nitrocellulose

$$= [L_t - (v/K_a(1 - v))]/D_t, \quad (2)$$

where  $v$  = [bound protein]/[total DNA],  $L_t$  = [total protein],  $D_t$  = [total DNA], and  $K_a$  is the equilibrium association constant for the complex. The ratio of [bound protein]/[total protein] is determined from the ratio of intensity at each protein concentration divided by the intensity at saturation. By generating integrated optical density values from images such as that found in Fig. 2B for the [free DNA] from the nylon membrane, these data were fit to the complement of Eq. (2) (i.e., fraction of

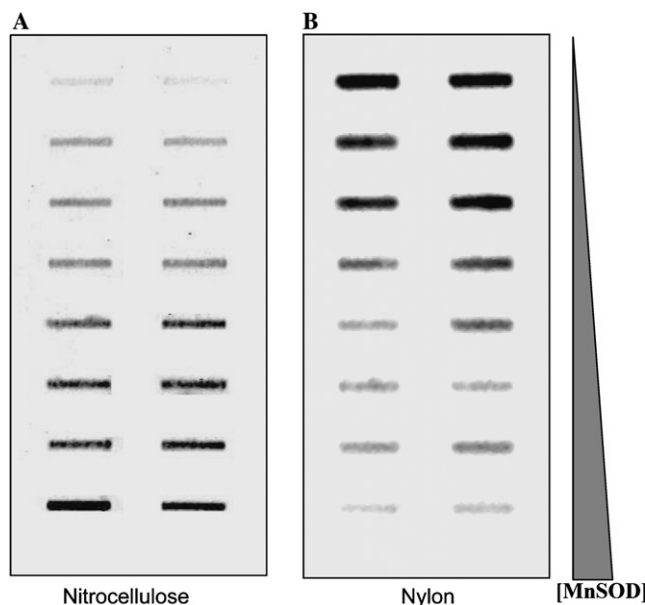


Fig. 2. Representative double-filter chemiluminescent NCFBA binding experiment. These data are photoinverted from the raw picture of the nitrocellulose (A) and positively charged nylon (B) membranes, as indicated. The two columns on each membrane represent duplicate wells that each contain  $6.7 \times 10^{-10}$  M DNA with increasing [MnSOD] (from 0 to  $62.3 \mu\text{M}$ ) down each column as indicated.

DNA on nylon = 1 – fraction of DNA on nitrocellulose). All data were normalized to the free-DNA well intensity. Fig. 3 shows representative data for determination of  $K_a$  for MnSOD–DNA interactions, normalized to maximum retention efficiency of the nitrocellulose mem-

brane. The maximum retention efficiency appears to be nearly 100% for *E. coli*–MnSOD interactions based on the amount of DNA passed through to the nylon at high [MnSOD] (see Figs. 2B and 3).

Note that quantitation of both membranes results in data that are best fit by an association constant,  $K_a$ , of approximately  $3 \times 10^5 \text{ M}^{-1}$ , which is identical to that obtained using traditional single-filter NCFBAs [3]. The excellent fit to a one-to-one binding complex for *E. coli* MnSOD to a 34 mer duplex DNA molecule was determined by Steinman et al. [2] by use of various-length oligonucleotides.

## Discussion

### Potential artifacts

The background retention of biotinylated contaminants in the protein must be carefully examined. If a small amount of biotinylated protein contamination is present, one can resolve this issue by subtracting the signal originating from the free protein from that of the protein–DNA complex. Alternatively, if this amount is small and linear with respect to the amount of protein present, this may not be necessary since the binding isotherm is empirically fit to a maximal signal anyway. Therefore the normalized plots would be identical.

A substantial artifact could arise if one uses only a single nitrocellulose filter without a second nylon filter. For instance, one may observe an increase in apparent

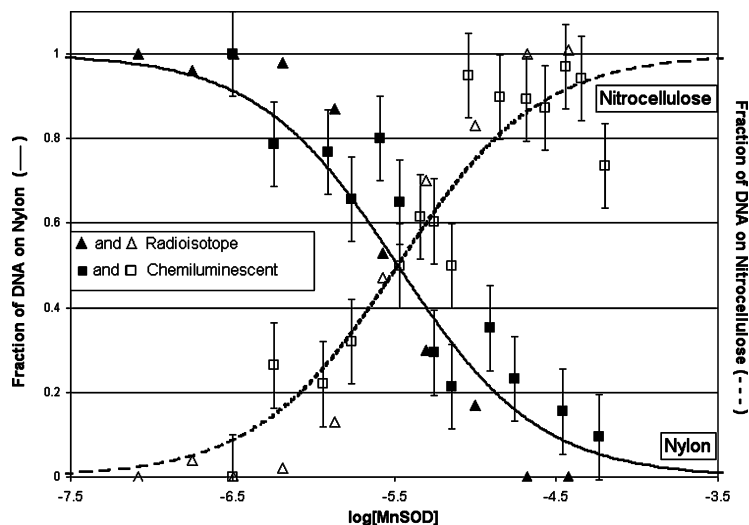


Fig. 3. Quantitation of the nitrocellulose and positively charged nylon membranes from chemiluminescent NCFBA binding experiments. This shows the binding isotherm for MnSOD binding to 34 mer duplex DNA in Binding Buffer B (+BSA) at  $20^\circ\text{C}$ . Each reaction contained  $6.7 \times 10^{-10}$  M DNA and [MnSOD] ranged from 0 to  $62.3 \mu\text{M}$ . Each point represents the average of four determinations. The best-fit lines to Eq. (2) corresponds to  $K_a = 3 \times 10^5 \text{ M}^{-1}$ . The “Radioactive” data are interpolated points from Fig. 2 of Steinman et al. [2]. Each point represents the average of four determinations. The “Radioisotope” data for the positively charged nylon membrane are predicted values based on their published values of DNA retained on the nitrocellulose in Fig. 3 of Steinman et al. [2].

signal from the nitrocellulose with increasing [protein] even when there is no bona fide protein–DNA complex formation. This could be due to biotinylated contaminants in the protein stock. This can be easily detected by performing a lane of free-protein-only reactions as outlined above (Fig. 1). Also, in this case of artifactual binding, a nylon membrane would show no decrease in the signal as [protein] increases.

The legitimacy of data derived from DNA- or RNA-binding proteins obtained in the crude state must be assessed in the same manner as data from traditional radiolabeled NCFBAs. For proteins that have high affinity and specificity for a nucleic acid sequence/structure, inclusion of a small amount of nonspecific competitor nucleic acid (e.g., poly(dI).(dC) or calf thymus DNA) would limit interference by other nonspecific nucleic-acid-binding proteins. However, as is the case with bacterial MnSOD, when the binding is nonspecific, other nonspecific nucleic-acid-binding proteins would likely interfere. For instance, at substantially higher concentrations (>0.5 mg/ml), BSA binds to the oligomer DNA used in this study (data not shown). Therefore, nonspecific nucleic acid–protein interactions would likely not be amenable to study by either this or the radioactive version of the NCFBA technique if the proteins were in a crude lysate.

#### Comparison with published literature

We conclude that nonisotopic NCFBAs provide an equally rapid, sensitive, quantitative, and far safer alternative to radiolabeled NCFBAs. A drawback to the nonisotopic method is the inability to assess both nitrocellulose and nylon membranes simultaneously. By that we mean that the signal intensity from each membrane could be added together to obtain total loading intensity when using radiolabeled DNA. However, the intensity of the chemiluminescent signal is not identical for both nitrocellulose and positively charged membranes. Despite that limitation, when each membrane is quantitated and the data are fit to an appropriate binding model, we obtain the same association constant.

Another nonisotopic modification to the standard radioisotope NCFBA technique has been developed by Snapyan et al. [7] with some success. In that method, protein samples are robotically spotted (similar to a gene array) onto nitrocellulose, blocked with nonspecific competitor DNA, probed with fluorescently labeled specific DNA, and visualized using an infrared imaging system. This method has the advantage of being radioisotope-free and appears to be well suited to the study of proteins that bind DNA in a high-affinity sequence-specific manner. However, the cost of the robotic arrayer unit and infrared imaging system would make that method far more costly than the one pro-

posed in this work (especially if the chemiluminescent signal were obtained by exposure to X-ray film and quantitated by densitometry). Also, it is not known whether that method is quantitatively reliable or better suited to qualitative or relative quantitation studies.

#### Additional utility

The use of biotinylated RNA in these nonisotopic NCFBAs appears to be a straightforward extension of the technique with little or no alteration. Also, although we have not tested this experimentally, it would appear to be feasible to amend this technique to the quantitative study of protein–lipid or protein–carbohydrate interactions. This would be true only in cases where the lipid or carbohydrate could be tagged with a biotin moiety. Most likely, the binding of proteins to polysaccharides would be most amenable to study by this method.

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#### References

- [1] J.M. Gutteridge, B. Halliwell, Free radicals and antioxidants in the year 2000. A historical look to the future, *Ann. N. Y. Acad. Sci.* 899 (2000) 136–147.
- [2] H.M. Steinman, L. Weinstein, M. Brenowitz, The manganese superoxide dismutase of *Escherichia coli* K-12 associates with DNA, *J. Biol. Chem.* 269 (1994) 28629–28634.
- [3] K.A. Hopkin, M.A. Papazian, H.M. Steinman, Functional differences between manganese and iron superoxide dismutases in *Escherichia coli* K-12, *J. Biol. Chem.* 267 (1992) 24253–24258.
- [4] I. Wong, T.M. Lohman, A double-filter method for nitrocellulose-filter binding: application to protein–nucleic acid interactions, *Proc. Natl. Acad. Sci. USA* 90 (1993) 5428–5432.
- [5] J.T. Rodgers, P. Patel, J.L. Hennes, S.L. Bolognia, D.P. Mascotti, Use of biotin-labeled RNA for protein purification and agarose-based chemiluminescent electromobility shift assays, *Anal. Biochem.* 277 (2000) 254–259.
- [6] W.F. Beyer Jr., J.A. Reynolds, I. Fridovich, Differences between the manganese- and the iron-containing superoxide dismutases of *Escherichia coli* detected through sedimentation equilibrium, hydrodynamic, and spectroscopic studies, *Biochemistry* 28 (1989) 4403–4409.
- [7] M. Snapyan, M. Lecocq, L. Guevel, M.-C. Arnaud, A. Ghochikyan, V. Sakanyan, Dissecting DNA–protein and protein–protein interactions involved in bacterial transcriptional regulation by a sensitive protein array method combining a near-infrared fluorescence detection, *Proteomics* 3 (2003) 647–657.