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Measuring Low-Picomolar Apparent Binding Affinities by Minigel Electrophoretic Mobility Shift

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Abstract

Measuring protein/DNA interactions that have apparent binding affinity constants in the low picomolar range presents a unique experimental challenge. To probe the sequence specificity of telomere-binding proteins, our lab has developed an electrophoretic mobility shift assay protocol that allows for the routine measurement of $K_{D,app}$ values in the 1 – 20 pM range. Here, we describe the protocol and highlight the particular considerations that should be made to successfully and reproducibly measure high-affinity interactions between proteins and single-stranded DNA.

Keywords

native gel; EMSA; DNA binding protein; binding assay; high-affinity binding

1. Introduction

Several DNA-binding proteins, including the lactose repressor LacI and some telomere end-binding proteins, interact with their cognate ligands with low to sub-picomolar binding affinities [1, 2]. These interactions are difficult to measure experimentally, primarily due to the extremely small signal that is generated under the concentrations needed to measure K_Ds and the level of accuracy of the concentration of protein. Recent advances in microscale thermophoresis and analytical ultracentrifugation have enabled these equilibrium-based methods to measure high-affinity interactions under certain circumstances ($K_{D,app}$ < 50 pM) [3, 4]. Because such tight interactions usually have relatively slow off-rates, the apparent binding constants for these systems can also be measured with non-equilibrium methods like native electrophoretic mobility shift assays (EMSAs) or double-filter binding assays. As very little specialized equipment is needed for an EMSA, this method accessible to a wide range of laboratories. The literature contains excellent guidance and instruction in optimizing EMSAs for protein-nucleic acid assays [5]. In this protocol, we focus on the

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experimental conditions that are required to measure low- to sub-picomolar binding interactions using EMSAs.

A system with a $K_{D,app}$ in the low pM range requires the use of femtomolar concentrations of ligand, which serves two purposes. First, it allows the assumption that $[P]_{free} = [P]_{total}$, which simplifies the equation used to fit the data and solve for the $K_{D,app}$ [6]. Second, it ensures that the experiment includes conditions in which there is fully free ligand [6]. With some advanced planning in reagent preparation and attention to experimental protocol, it is possible to robustly detect such small amounts of signal using radiolabeled oligonucleotide ligands, allowing for the reproducible and accurate measurement of apparent binding affinities [6].

Here, we describe our laboratory protocol for measuring the binding affinity of telomere end-binding proteins for their cognate ssDNA ligands, which have apparent dissociation constants ($K_{D,app}$) of 2 – 20 pM [7–9]. The most critical step in being able to reliably and consistently measure these tight binding events is to use the freshest radioisotope possible. The binding assay is performed with the hottest radioisotope available, such that the [γ $-^{32}$P]-ATP has a specific activity of >5800 Ci/mmol. As the protein concentration needs to be known accurately, it is essential to use tips and tubes made from a low-retention plastic polymer to avoid non-specific adsorption of the very low concentrations of protein.

2. Materials

2.1 Gel Electrophoresis

1. BioRad Mini-PROTEAN vertical electrophoresis cell or equivalent
2. Intact gel combs, 15-well, 1.5 mm thick
3. Casting stand
4. Glass plates (short and tall, 1.5 mm spacer)
5. Mini-Protean Multi-Casting chamber (optional)
6. 40% polyacrylamide solution (29:1 acrylamide:bisacrylamide)
7. Tetramethylethylenediamine (TEMED)
8. 10% w/v ammonium persulfate (APS)
9. 10× Tris-Borate-EDTA, pH 8.4 (TBE: 890 mM Tris, 890 mM boric acid, 20 mM EDTA)
10. Gel dryer
11. 3 mm Whatman paper
12. Plastic wrap
13. 0.5 mil (12.7 μm) plastic sheets
14. Impact sealer
15. Laboratory Marker (VWR #52877–310 or ThermoFisher #2000)

16. Stir bar
17. Stir plate

2.2 DNA labeling reaction

1. Nuclease-free water
2. Oligonucleotide
3. Illustra MicroSpin G-25 columns (GE Healthcare, #27–5325-01)
4. γ–^{32}P-ATP (Perkin-Elmer, freshest lot possible)
5. T4 polynucleotide ligase (New England BioLabs)
6. 10× T4 reaction buffer (New England BioLabs)

2.3 Binding reaction

1. Appropriate binding buffer
2. Purified recombinant protein
3. Low-retention 96-well plates
4. Low-retention filter tips
5. 12-channel multichannel pipettor
6. Disposable buffer reservoir

2.4 Detection and Analysis

1. Phosphor screen
2. Phosphor imager
3. Gel quantitation software (ImageQuant, ImageJ, or similar)
4. Curve fitting software capable of multivariate fitting (SigmaPlot, Kaleidagraph, or similar)

3. Methods

Carry out all procedures on ice unless otherwise specified or as appropriate for the protein/DNA system of interest.

3.1 Preparation of Native Polyacrylamide Gels

The thicker gel is important for these experiments to increase the signal for tight binding systems; in a 15-well, 1.5 mm minigel, each well can hold ~25–27 μl.

1. If needed, prepare 10× TBE. (*see* Notes 1 and 2)

[1]Be attentive when making both the TBE stock and gel running buffer solutions. Any discrepancies in gels or running buffer could affect the reproducibility of results.

2. Combine 10× TBE, acrylamide, glycerol, and water to create a "gel stock solution". The recipe for one 1.5 mm 5% polyacrylamide gel with 5% glycerol is:

Reagent	Volume	Final
10× TBE	1.0 mL	1X
40% acrylamide (29:1)	1.25 mL	5%
100% glycerol	0.5 mL	5%
milli-Q H_2O	7.25 mL	
10% APS	50 μL	
TEMED	30 μL	

3. Mix well but not too vigorously, being mindful to not over-aerate the solution. This solution can be stored at 4 ºC in the dark for up to 4 months. (*see* Note 3)

4. Prepare the glass gel plates for hand-casting. (*see* Notes 4 and 5)

5. Measure out 10 mL of gel stock per gel to be poured. Add the appropriate amounts of 10% APS and TEMED and mix gently. (*see* Note 6)

6. Gently insert the combs into the plates, being careful to avoid introducing air bubbles. (*see* Note 7).

7. Allow gels to polymerize for 15 – 30 min.

8. While binding reactions are incubating (see Section 3.3.9), set up the gels in the electrophoresis apparatus. It is especially important to set the gels up early if the gels are at room temperature. As this protocol does not use loading dye, it is critical to outline and number each well on the large (spacer) plate to help keep

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2. The boric acid in 10× TBE may precipitate during storage. This can be handled in one of two ways: (1) prepare 5× TBE and adjust all volumes accordingly or (2) use 10× TBE to make ~500 mL of gel stock and use the remaining 10× TBE to make gel running buffer (1× TBE + 5% glycerol).

3. For higher-throughput analyses (5 – 10 gels per week), we recommend preparing at least 500 mL of gel stock. It can be stored in an opaque or amber bottle at 4 ºC for up to four months.

4. The thickness of the 1.5 mm spacers can be challenging when pouring in a side-by-side caster. Heavily used gaskets are often pockmarked, and as a result form a poor seal with the plates that can leak air bubbles into the gel. We recommend reserving a set of four new gaskets just for pouring native gels; alternatively, one could use gel tape. In general, we avoid this problem by using the Multi-Caster to prepare 10 – 12 gels at a time.

5. If more than 8 gels will be needed within 7 – 10 days, the BioRad Mini-PROTEAN III Multi-Caster can easily hold up to 12 sets of 1.5 mm plates.

   i. The dead volume of the Multi-Caster requires about 20 mL more of gel solution than if the gels are poured individually. For example, for 12 gels, the gel solution recipe would be a total volume of 140 mL.

   ii. Before pouring the gel solution, loosely place 9 – 10 of the combs above the gels. When the Multi-Caster has been filled with gel solution, these combs can be pressed into place quickly and the remaining combs inserted.

   iii. Store gels in the fridge with the combs left in place, wrapped in a wet paper towel and sealed in a plastic bag for up to a month.

6. When pouring more than two gels at a time (either in side-by-side casters or using the Multi-Caster), the amounts of APS and TEMED should be reduced so that there is sufficient time to insert all the combs before the gel starts polymerizing. When pouring 12 gels, we typically use the amounts of 10% APS and TEMED for 8 gels.

7. Be very careful when handling the combs. Combs with broken outer sealing tabs prevent proper polymerization of the wells with native gel solution. Store the combs away from the ones for general lab use and treat them with the utmost care.

track of each well during loading. We recommend using a solvent-resistant marker like the VWR-brand Laboratory Marker or ThermoFisher Scientific Laboratory Marking Pen, as the ink in these pens is more resistant to being rinsed off of the plate by the running buffer.

9. Fill the inner chamber with pre-chilled running buffer (1× TBE + 5% glycerol). Fill the outer chamber about 1/3 full with the same cold buffer. (*see* Note 8)

10. Place a stir bar in the outer chamber, and place the assembled apparatus in the cold room or refrigerator. (*see* Note 8)

3.2 Radiolabeling of DNA Oligonucleotide

1. Reconstitute the lyophilized DNA oligonucleotide to approximately 100 μM in nuclease-free water.

2. Determine concentration of the oligonucleotide by absorption spectroscopy, using the appropriate molar extinction coefficient at 260 nm.

3. Dilute to 25 μM with nuclease-free water.

4. On the day of radioisotope delivery (*i.e.* when the radioisotope is >5800 Ci/mmol), carry out the end-labeling reaction per the manufacturer's instructions. We use T4 Polynucleotide Kinase (New England BioLabs, cat. no. M0201), performing the ligation in a 10 μL reaction volume. (*see* Notes 9 and 10)

5. Heat inactivate the DNA ligase by incubating the reaction at 95 ºC for 20 min.

6. During the heat inactivation incubation, prepare the G-25 column:

   a. Briefly vortex to resuspend the resin.

   b. Snap off the bottom tab and slightly loosen the cap.

   c. Insert into 2 mL collection tube.

   d. Centrifuge at 6000×*g* for 10 sec at room temperature. We find it most time-efficient to use a personal-size benchtop centrifuge (*e.g.*, SpectraFuge Mini).

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8. Because the native gels are run at a high voltage (200 V), it is important to carry out electrophoresis in a cold environment to dissipate the heat that is generated from the high voltage. We recommend running the gels in an ambient temperature of 4 ºC with pre-chilled gels and pre-chilled running buffer, as well as ice packs that were frozen at −70 ºC.

9. For detection of sub-picomolar apparent K_Ds, it is *critical* that the specific activity of the [γ–^{32}P]-ATP be as high as possible. We have had the most success using [γ–^{32}P]-ATP that has a specific activity of 6000 Ci/mmol at the time of synthesis and calibration. The order for the [γ–^{32}P]-ATP should be placed with the manufacturer so that the freshest lot ships on the day of synthesis and calibration for next-day delivery. A full set of replicate binding assays are then completed within four days, at which time the [γ–^{32}P] will have decayed to a specific activity of 5590 Ci/mmol when the phosphor screen is imaged. When using < 1 pM of labeled oligonucleotide, it was difficult to reliably measure the signal when the specific activity of the ^{32}P was less than 5400 Ci/mmol at the time of imaging.
We purchase [γ–^{32}P]-ATP radionuclides from Perkin-Elmer with a specific activity of 6000 Ci/mmol at a concentration of 10 mCi/mL (catalog numbers: in a lead-lined container, NEG002Z; in a lead-free container, BLU002Z). Fresh lot schedules can be accessed at their website (http://www.perkinelmer.com/lab-solutions/resources/docs/CAL_2017_Radiochemical_Fresh_Lot_Schedule.pdf)

10. Take appropriate precautions for working with the highly radioactive material during the labeling reaction, as recommended by your institutional safety protocols. For example, our laboratory members wear both a torso badge and a ring badge on the non-dominant hand to thoroughly monitor exposure.

e. Discard flowthrough.

f. Transfer G-25 column to a 1.5 mL microcentrifuge tube.

7. Add 40 μL prechilled nuclease-free water to the heat-inactivated labeling reaction, for a total volume of 50 μL.

8. Apply the entire 50 μL reaction to G25 column and again centrifuge at 6000×*g* for 10 sec at room temperature.

9. Retain flowthrough as the radiolabeled DNA oligonucleotide. Hold on ice for the preparation of the binding reaction as described below, and then store remainder at −20ºC in appropriate storage conditions for radioactivity. (*see* Note 11)

3.3 Binding Reaction

1. Determine protein concentration by A_{280} and appropriate molar extinction coefficient.

2. Prepare the binding buffer using 10× salts stock, BSA, and glycerol. The composition of the binding buffer is critical for accurately and reproducibly measuring binding constants. Parameters that should be considered include pH, buffering agent, salt concentration, and concentration of divalent cations. (*see* Note 12)

3. Using low-retention filter tips, prepare serial dilutions of protein in binding buffer to 2× final concentrations in microcentrifuge tubes. (*see* Notes 13 and 14)

4. Transfer 15 μL of each protein dilution to a 96-well plate on ice.

5. Dilute radiolabeled oligonucleotide to 2× final concentration in a final volume of at least 500 μL, so that the solution is of sufficient volume to fill the base of a disposable buffer reservoir. (*see* Note 15)

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11. Determining the concentration of radiolabeled DNA is challenging, as it would potentially contaminate cuvettes and instrumentation with radioactivity. Therefore, we make the conservative assumption that the G-25 spin column recovers 80% of the total DNA for oligonucleotides up to 20 nucleotides in length.

12. The binding buffer composition should be carefully considered and optimized for each system, because the components in the binding buffer may interact with the complex being studied and alter the complex formation in many ways [5, 6, 11]. For example, protein/DNA complexes may aggregate and be retained in the well (a “well shift”), rather than separating in the polyacrylamide matrix. A well shift prevents the quantitation of the EMSA, as one cannot determine whether the ligand has been specifically bound or is simply aggregating with the protein. The choice of salt concentration and/or buffering agent can minimize the formation of well shifts (Figure 1).
Additionally, we recommend carrying out binding reactions at the same pH as the running buffer, if possible. This ensures that there is as little change as possible as the reactions run in the gel. For conventional TBE, that pH is 8.4. Similarly, the addition of 10% more glycerol than the running buffer to the sample facilitates gel loading. We include glycerol in our binding reactions so that the binding equilibrium is not disturbed by the addition of glycerol immediately prior to loading the gels.

13. The serial dilution of protein should cover at least two orders of magnitude of concentration. The choice of protein concentrations will require optimization. Concentrations should be evenly distributed across the dilution, and the majority of the samples should produce fraction bound values between 0.1 and 0.9. Because the hyperbolic curve fit is dominated by the fraction bound values at high protein concentrations, an increased confidence in the fits can be obtained by using protein concentrations that span three orders of magnitude [6, 11] (Figure 2). For example, for a protein with an apparent binding affinity of 10 pM, we would prepare the following set of 15 protein solutions at 2× final concentration: 0.002 pM, 0.004 pM, 0.02 pM, 0.04 pM, 0.2 pM, 0.4 pM, 2 pM, 4 pM, 20 pM, 40 pM, 200 pM, 400 pM, 2000 pM, 4000 pM, 20000 pM.

14. Low-retention tips are critical for accurate final protein concentrations in the binding reaction. We recommend the low-retention plastic consumables manufactured by Eppendorf and USA Scientific, because these brands use a low-retention plastic polymer, rather than a coating.

15. When considering the amount of radiolabeled ligand to use, the final concentration in the binding reaction must be large enough to detect the signal. Using the conservative assumption described in Note 11, we determined that ~15 attomoles of radiolabeled DNA is

6. Transfer the diluted radiolabeled oligonucleotide to a disposable buffer reservoir.

7. With a multichannel pipettor and low-retention filter tips, add 15 μL of radiolabeled oligonucleotide to each of the wells that contain the 2× protein solutions.

8. Cover the plate with a piece of Parafilm that is cut to the same size as the plate. Ensure a solid seal on each well by pressing firmly in each well with the pads of your fingers.

9. Incubate on ice. Initial experiments should be carried out at several incubation times to determine the appropriate incubation time to ensure that the binding reaction has reached reach equilibrium.

    a. During incubation, prepare the electrophoresis apparatus as described in Section 3.1.8.

3.4 Native Gel Electrophoresis

1. Load the binding reactions directly into the wells of the pre-chilled gel. Up to ~27 μl of a reaction containing 15% glycerol can be loaded into a well of a 15-well, 1.5 mm gel that is prepared in running buffer containing 5% glycerol.

    a. Note that this protocol does not use a loading dye, which would dilute the amount of radiolabeled DNA signal.

    b. To make it easier to load samples and ensure that they are loaded in the correct order, we use a laboratory marker to mark the back of the large spacer plate. We outline the base of each well and then number the wells below the marks.

    c. To make sure the samples sink to the bottom of the well, we take advantage of the difference in refractive index between the running buffer and the sample solution. Schlieren waves will be visible as the binding reaction (containing 15% glycerol) is dispensed into the running buffer (which contains only 5% glycerol).

    d. For higher throughput, we found that multiple wells can be loaded simultaneously using a multichannel pipettor. The micropipettor and type of gel-loading tips should be optimized before proceeding with radiolabeled oligonucleotide. We use a 12-channel multipipettor that does not have O-rings on the tip holders. After samples are aspirated into three tips, the user gently squeezes the outer two tips into the correct geometry for the wells. One should practice extreme caution when handling the sample-containing tips to make sure that the vacuum seal is not broken (*i.e.*, no sample is lost) and to avoid radioactive contamination of gloves, instruments, and surroundings.

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the lower limit for reliable detection. This translates to ~0.5 pM DNA in a 30 μL binding reaction. Additionally, if one wants to use the simplified form of the binding equation, care should be taken to make sure that the final DNA concentration is sufficiently below the K_d of the interaction being measured to allow for the assumption that $[P]_{free} = [P]_{total}$.

2. Place ice packs in the outer chambers of the gel chamber. (*see* Note 16)
3. Fill the outer gel chambers with running buffer.
4. Electrophorese the gels at 200 V for 20 – 30 minutes. The short run time helps with exchange problems and shorter off-rates and the high voltage allows for the short run time. The ice packs keep the gels cool at this high voltage.
    a. If longer run times are required to improve the separation of free and bound oligonucleotide, consider exchanging the ice packs if your protein is sensitive to temperature.
    b. When optimizing run times, be aware that the unbound radiolabeled oligonucleotide may run off the end of the gel and contaminate the running buffer and gel box. The free oligonucleotide can be retained in the gel with a 5 – 10 mm "plug" of 30% polyacrylamide at the bottom of the gel. For reference, in our lab a 12-nt ssDNA will run off a 5% gel prepared as described in ~45 minutes. (*see* Note 17)
5. Promptly disassemble completed gels and place on two layers of 3 mm Whatman filter paper. Check all disassembled buffers and equipment for radioactivity contamination.
6. Place plastic wrap over the gels and dry on a gel dryer for ~25 min per gel.
7. Expose the phosphor screen for two nights when using ≤ 5 pM final concentration of radiolabeled oligonucleotide. We find that exposing the screen for longer times (*i.e.*, three nights) artificially inflates the measured $K_{D,app}$ values due to increased background signal. (*See* Note 18)

3.5 Quantitation and $K_{D,app}$ calculation

The quantitation method is outlined briefly here. Additional details, including how to identify the appropriate background correction, have been published elsewhere [6].

1. Using gel imaging software (ImageQuant, ImageJ, or similar) to quantitate the free and bound DNA bands. (*see* Note 19)
2. Evaluate the sum of counts (bound + free) across all lanes. Total counts per lane should be approximately the same. If the sum of total counts is not equal across

16. To prepare custom-sized ice packs, cut heavy plastic (approx. 0.5 mil thick) in dimensions that will fit in the outer chamber of the gel box. Seal three sides with impact sealer and fill with deionized H_2O. Seal the fourth side while minimizing air bubbles. Freeze flat overnight at < 70 ºC. Between uses, rinse with deionized H_2O and allow to thoroughly dry before re-freezing.
17. When pouring a 30% "plug" to retain free oligonucleotide, the plug layer should be poured and allowed to polymerize before pouring the separating gel. Gels with a plug cannot be poured ahead of time and stored in the fridge, as over time the high acrylamide content of the plug pushes the plates apart and destroys the gel.
18. Phosphor screens should be thoroughly cleaned with phosphor screen cleaner. Screens should be regularly checked for background defects and residual signal by exposing the screen in an empty, clean cassette for two nights, and then scanning on a phosphorimager.
19. The quantitation of the free and bound counts can be carried out multiple ways, depending on the stability of the complex during electrophoresis [6]. Briefly, when protein/DNA complexes were initially bound but dissociated during the short gel run, there may be a smear of signal throughout the lane. Care should be taken to optimize binding and gel conditions to promote complex stability and minimize smearing, including adjusting the composition of the binding buffer and/or the acrylamide concentrations in the gel. However, a small amount of smearing can still be accommodated in quantitation by defining the "bound" counts as the full length of the lane above the free oligonucleotide migration distance. If there are tight, discrete bands for both free and bound DNA, then smaller boxes can be quantitated.

lanes, then it means that some of the labeled ligand was not detected. There are several causes for this, including uneven sample loading or rapid dissociation of the bound complex during electrophoresis or aggregation in the well. This needs to be addressed, as any subsequent curve fitting will have considerable error.

3. Calculate the fraction of ligand bound as the ratio of bound ligand over total ligand (*i.e,* the sum of bound and free ligand).

4. Plot fraction bound as function of total protein concentration.

5. Fit the data to the simplified form of the binding isotherm to solve for $K_{D,app}$:

$$\frac{[PL]}{[L]_T} = S\left(\frac{[P]_T}{[P]_T + K_{D,app}}\right) + 0$$

where $\frac{[PL]}{[L]_T}$ is the fraction of ligand bound; $[P]_T$ is total protein concentration; S is a scalar offset for binding saturation, and O is a background offset, and $K_{D,app}$ is the apparent dissociation constant [10].

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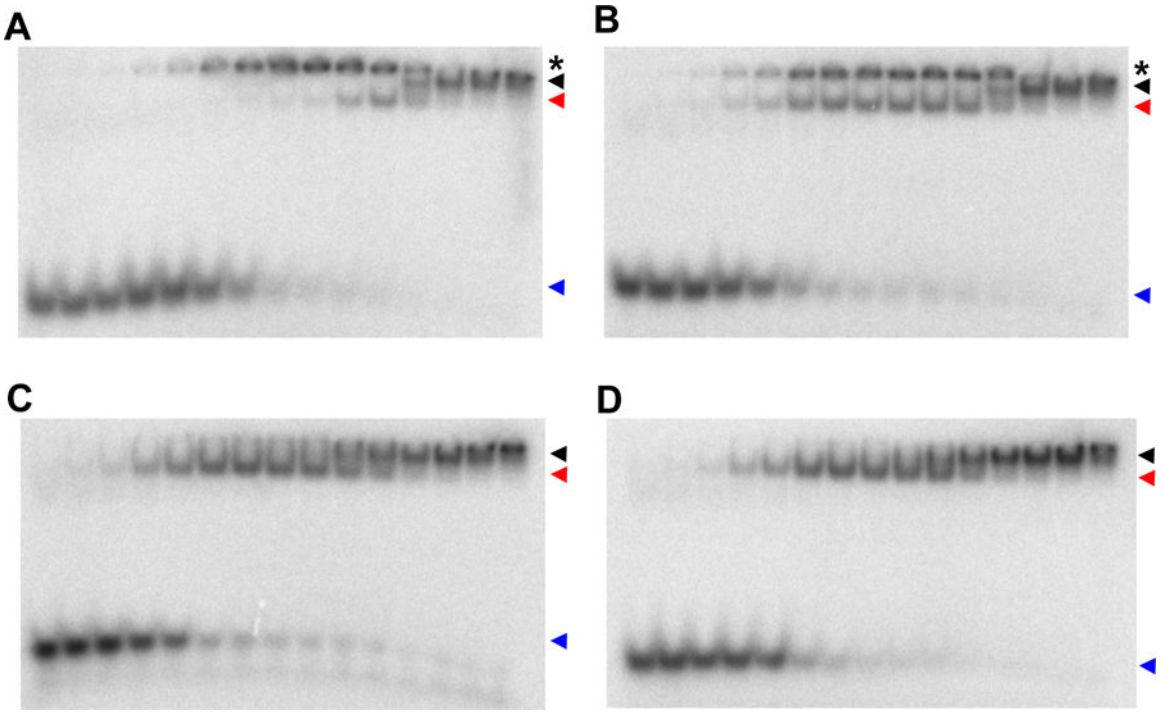

Figure 1. Binding buffer affects protein-DNA complex formation and stability.

S. cerevisiae Cdc13 was incubated with 1 pM oligonucleotide (Tel11, 5'-GTGTGGGTGTG) in one of four buffers at pH 7.5: (A) HEPES, (B) tricine, (C) sodium phosphate, or (D) Tris-HCl. Each binding reaction contained 50 mM buffering agent, pH 7.5, 75 mM KCl, 1 mM DTT, 1 mg/mL BSA, and 15% glycerol. The binding reactions were separated in 6.7% polyacrylamide 1×TBE gels containing 5% glycerol in 1×TBE running buffer containing 5% glycerol. All gels were run at 200 V for 35 minutes at 4 °C. Red and black arrows indicate the monomer and dimer species, respectively; blue arrows indicate free ssDNA; asterisks indicate well-shifted ssDNA. Adapted with permission from Figures 1 and 2 [6], under Creative Commons Attribution NonCommercial 4.0 International License (CC BY-NC 4.0).

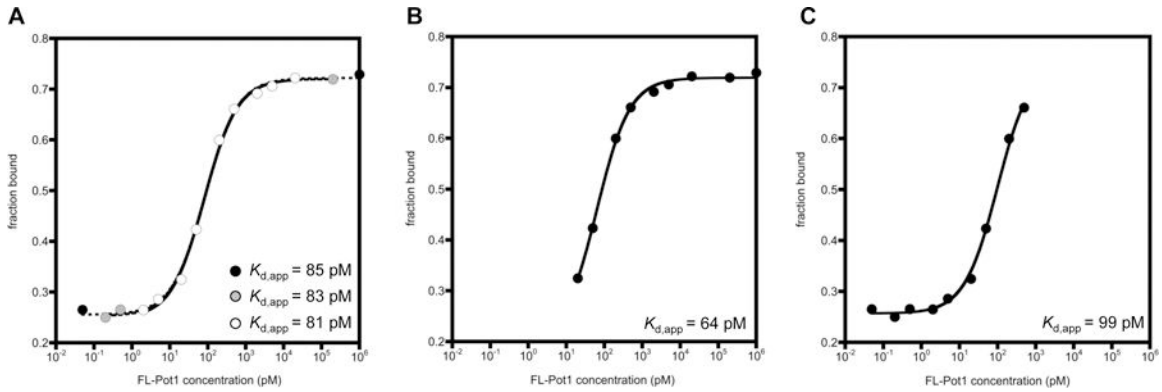

Figure 2. Selection of protein concentrations affects the robustness of the fitted binding constant.

Full-length *S. pombe* Pot1 ("FL-Pot1") was incubated with 1.5 pM oligonucleotide (T3A complemer, 5'-GGATACGGTTACGGT) in binding buffer (20 mM Tris, pH 8.4, 50 mM NaCl, 5 mM DTT, 15% glycerol, 1 mg/mL BSA) before separation in 6.7% polyacrylamide 1×TBE gels with 5% glycerol using 1×TBE running buffer with 5% glycerol. All gels were run at 200 V for 20 minutes at 4 °C. The data were quantitated and fit using three approaches to demonstrate the utility of choosing a wide range of protein concentrations. (A) A range of protein concentrations that is two orders of magnitude above and below the $K_{D,app}$ is needed to obtain a reliable fitted value (open circles). An additional order of magnitude above and below the binding constant (gray circles) increases the fitted value by 2.5%, while a protein range of four orders of magnitude above and below (black circles) increases the fitted value by 5%. (B) Without a baseline of unbound ligand, the fitted value for the $K_{D,app}$ is artificially low. (C) In contrast, lack of binding saturation artificially inflates the fitted $K_{D,app}$ value. Data sets shown in (B) and (C) are subsets of the data shown in (A).