EMSAs using fluorescent probes

1. Materials

1. Protein stocks (10-50 µM concentration): POT1/TPP1(87-337) and POT1/TPP1(87-544)/GFP-TIN2. Expressed in insect cells and purified by affinity chromatography, heparin (for POT1/TPP1/GFP-TIN2 only), and gel filtration. Stored at -80°C in 20 mM HEPES-KOH pH 7.5, 300 mM NaCl, 0.1 mM TCEP, 0.05% Tween-20, 5% v/v glycerol.
2. Cy5.5 DNAs: 5'Cy5.5GGTTAGGGTTAG3' (telomeric) and 5'Cy5.5GGTTAGCGTTAG3' (mutant), 100 nmol ordered with HPLC purification from Integrated DNA technologies
3. Unlabeled DNAs: 5'CATCAATAGGGTTCATCCTAGGGTTGTACTG3' (G-strand) and 5'CAGTACAACCCTAGGATGAACCCTATT3' (C-strand), 50 nmol ordered with HPLC purification from Thermo-Fisher.
4. 100 mM dNTP set (Thermo R0181 or similar, store at -20°C).
5. Sulfo-Cyanine5.5 dUTP (Lumiprobe 2071-50nmol, resuspended in 50 µL water for 1 mM, store at -20°C)
6. Klenow Exo- (5 U/µL, New England Biolabs M0212S)
7. Desalting columns (Bio-Rad 7326227)
8. ELGA Purelab Ultra 18.2 MΩ-cm ultrapure water or equivalent for all solutions.
9. 15 mL conical tubes.
10. 1.5 mL microcentrifuge tubes.
11. 200 µL thin-walled PCR tubes.
12. Refrigerated microcentrifuge capable of 20,000 x g force (e.g. Eppendorf 5424r)
13. 1 M HEPES-KOH pH 7.5 (filtered through 0.2 µm, stored at 4°C).
14. 5 M NaCl (filtered through 0.2 µm, stored at room temp).
15. 0.5 M $MgCl_2$ (filtered through 0.2 µm, stored at room temp).
16. 10 % (v/v) Tween-20 (filtered through 0.2 µm, stored at room temp).
17. 2.5 M KCl (filtered through 0.2 µm, stored at room temp).
18. 0.5 M Ethylenediaminetetraacetic acid pH 8.0 (EDTA. Filtered through 0.2 µm, stored at room temp).
19. 0.5 M Tris (2-carboxyethyl) phosphine hydrochloride (TCEP. Adjust pH to 7.5 with NaOH before use. Soltec Bioventures 51805-45-9 or similar).
20. 80% v/v glycerol (filtered through 0.2 µm, stored at room temp).
21. 10 mg/mL bovine serum albumin (BSA, New England Biolabs B9000S or similar)

22. 10x Buffer 2 (New England Biolabs)
23. Annealing Buffer: 10 mM HEPES-KOH pH 7.5, 50 mM NaCl, 0.1 mM EDTA.
24. Binding Buffer: 20 mM HEPES-KOH pH 7.5, 150 mM KCl, 0.5 mM TCEP, 1 mM $MgCl_2$, 0.05 mg/mL BSA, 0.05% v/v Tween-20, 8% v/v glycerol)
25. 1 M Tris-borate (For 1 L dissolve 121 g Tris base and 61.8 g boric acid in 800 mL water, adjust to 1 L, filter through 0.2 µm, and stored at room temp).
26. NanoDrop 1000 Spectrophotometer (Thermo Scientific) or equivalent.
27. Thermocycler with hot lid (e.g. Bio-Rad C1000 Touch, 1851148)
28. Power supply (e.g. Bio-Rad PowerPac basic, 1645050EDU)
29. XCell SureLock Mini-Cell electrophoresis system (Thermo-Fisher EI0001)
30. Novex 4-20% polyacrylamide TBE gels (Thermo-Fisher EC62252BOX).
31. SeaKem LE Agarose (Lonza 50004)
32. Agarose gel tank with casting apparatus, gel trays, and thin combs (e.g. Bio-Rad 1704489EDU, 1704422EDU, 1704416EDU, 1704447EDU)
33. Amersham Typhoon Biomolecular imager equipped with 685 nm laser and near-IR filter (GE Healthcare).

2. Methods

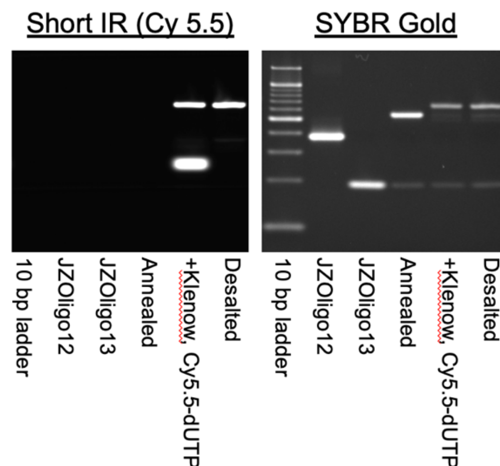
2.1.1. Preparation of ssDNA substrates

1. Dissolve DNA pellets (they should be blue) in annealing buffer to approximately 100 µM (e.g. if the tube says 9.5 nmol, dissolve in 95 µL buffer).
2. Measure UV-Vis of a 1:10 dilution to calculate their concentrations (e_{685} = 209,000 $M^{-1}cm^{-1}$).
3. Prepare 1 µM stocks by diluting in water. Aliquot 50-200 µL and store all DNA solutions in the dark at -20ºC until use.

2.1.2. Preparation of dsDNA substrate

1. Dissolve DNA pellets in Annealing Buffer to approximately 100 µM.
2. Measure UV-vis of a 1:10 dilution to calculate their concentrations (e_{260} = 300,100 $M^{-1}cm^{-1}$ and 263,300 $mol^{-1}cm^{-1}$ for the G-strand and for the C-strand, respectively). **Optional: make a sample for gel analysis by adding 10 pmol to 10 µL Annealing Buffer containing 10% v/v glycerol.**

3. Mix 750 pmol G-strand with 900 pmol C-strand in a thin-walled PCR tube. Adjust volume to 25 µL with annealing buffer.
4. In a thermocycler with a hot lid, heat to 98°C for 2 min, then cool rapidly to 12°C and hold. **Optional: make a sample for gel analysis by adding 0.5 µL to 9.5 µL Annealing Buffer containing 10% v/v glycerol.**
5. During this time, prepare a dNTP mix by adding equal volumes of 1:100 diluted 100 mM dATP/dCTP/dGTP and 1 mM Cy5.5dUTP (final concentrations = 250 µM each). Store this mix at -20°C for later use.
6. To this add (in this order) 8 µL 250 µM dTNP mix (final conc = 40 µM), 5 µL 10x NEB buffer 2, 11 µL H_2O, 1 µL Klenow exo- and mix well
7. Incubate at 37°C for 45 min.
8. During this time, equilibrate a desalting column in Annealing Buffer according to the manufacturer's instructions.
9. When the fill-in reaction has finished, use the desalting column to exchange the reaction into Annealing Buffer. **Optional: make samples from before and after desalting for gel analysis by adding 1 µL to 9 µL Annealing Buffer containing 10% v/v glycerol.**
10. Attain concentrations by measuring UV-vis spectrum (e_{685} = 209,000 $M^{-1}cm^{-1}$).
11. Aliquot, flash freeze, and at -20°C for later use.
12. **Optional: Run gel of the samples at each step of the reaction. For this I load 2 µL sample/well and image first with the near-IR setting of a Typhoon scanner and second with SYBR Gold staining (Thermo-Fisher S11494) and ethidium bromide setting of an Alpha Imager (Alpha Innotec). Below is a representative example of such an experiment:**

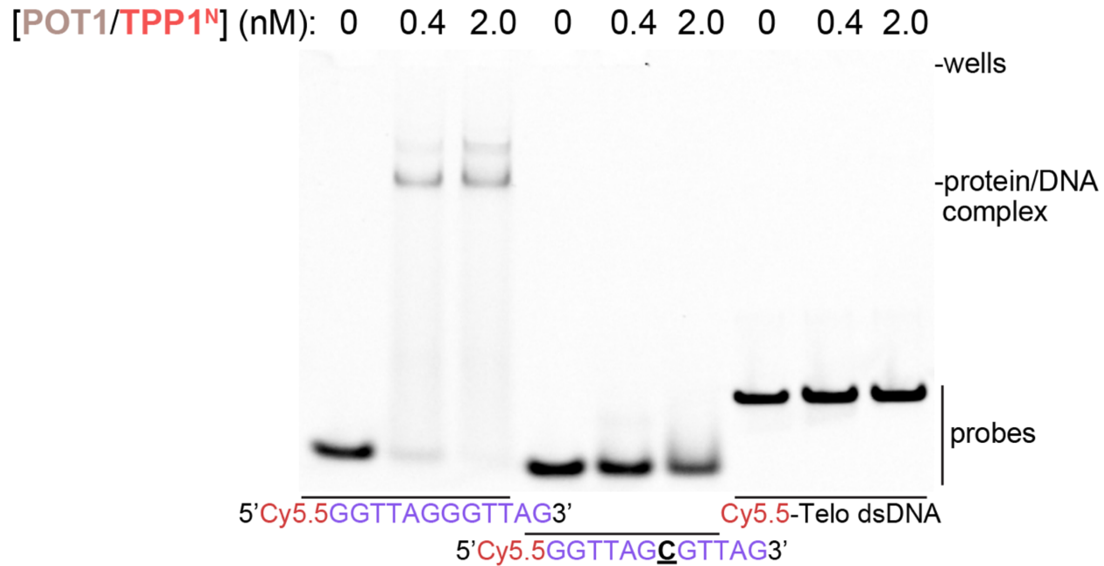


EMSAs using fluorescent probes

2.1.3. POT1/TPP1^N Electrophoretic mobility shift assay (EMSA)

1. Perform all steps on ice and with cold buffers unless otherwise noted.
2. Prepare 500 mL of cold 100 mM Tris-Borate by adding 50 mL 5x TBE to 450 mL water and cooling in slushy ice for at least 30 min.
3. Thaw an aliquot of purified POT1/TPP1(87-337).
4. Centrifuge protein stock at 20,000 x g and 4°C for 1 min.
5. Measure the concentration of the supernatant by UV-vis (e_{280} = 95,230 $M^{-1}cm^{-1}$ from Expasy protparam).
6. Prepare 1 µM protein stock by diluting in Binding Buffer
7. Thaw aliquots of 1 µM Cy5.5-DNA.
8. Make 0.5 nM (2x) substrate stocks by adding 0.5 µL DNA to 1 mL Binding Buffer.
9. Dilute the 1 µM protein mix to 4 nM final concentration by adding 1 µL to 249 µL Binding Buffer.
10. Make a 1:5 dilution by adding 20 µL 4 nM protein to 80 µL binding buffer. This will give a concentration series of 0, 0.4, and 2 nM final concentrations.
11. Initiate DNA binding by adding 10 µL protein to 10 µL 0.5 nM DNA in separate 1.5 mL Eppendorf tubes. Include a 0 nM protein point by adding Binding Buffer instead of protein.
12. Incubate at room temperature for 30 minutes.
13. Prepare a 12-well 4-20% polyacrylamide TBE gel by removing the tape and well-guard, inserting it into the gel-running apparatus, filling the reservoir with 500 mL cold 100 mM Tris-Borate, and blowing out the unpolymerized acrylamide in the wells with a P200.
14. Run the gel at 50 V limiting while loading.
15. Load 2.5 µL sample/lane using gel-loading tips and run at room temperature, 200 V limiting, for 30 minutes.
16. Image gel using the near-IR setting of a Typhoon scanner (600V).
17. Adjust levels and convert to 8-bit with ImageJ image processing software (NIH).

EMSAs using fluorescent probes

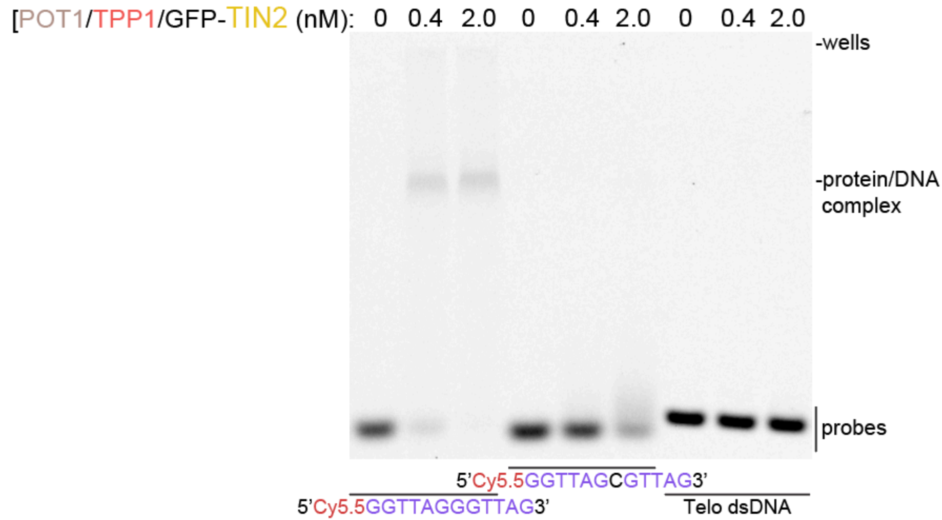


Adapted from Figure 1 of Zinder JC *et al*., *bioRxiv* **2022**

2.1.4. POT1/TPP1/TIN2 EMSA

1. Perform all steps on ice and with cold buffers unless otherwise noted.
2. Prepare 1 L of cold 100 mM Tris-Borate by adding 100 mL 5x TBE to 900 mL water and cooling in slushy ice for at least 30 min.
3. Make a 0.6% agarose gel by boiling 0.45 g agarose in 75 mL 100 mM Tris-Borate for 2 min and pouring into a gel tray with comb in a casting device. Let cool to room temp and solidify for at least 1 hr before removing the comb.
4. Thaw an aliquot of purified POT1/TPP1/GFP-TIN2.
5. Centrifuge protein stock at 20,000 x g and 4°C for 1 min.
6. Measure the concentration of the supernatant by UV-vis (for eGFP e_{488} = 53,000 $M^{-1}cm^{-1}$).
7. Prepare 1 µM protein stock by diluting in Binding Buffer
8. Thaw aliquots of 1 µM Cy5.5-ssDNA.
9. Make 0.5 nM (2x) substrate stocks by adding 0.5 µL DNA to 1 mL Binding Buffer.
10. Dilute the 1 µM protein mix to 4 nM by adding 1 µL to 249 µL Binding Buffer.
11. Make a 1:5 dilution by adding 20 µL 4 nM protein to 80 µL Binding Buffer.
12. Initiate the binding reaction by adding 10 µL protein to 10 µL 0.5 nM DNA in separate 1.5 mL Eppendorf tubes. Include a 0 nM protein point by adding Binding Buffer instead of protein.
13. Incubate at room temperature for 30 minutes.
14. Place the agarose gel into the tank with ~800 mL cold 100 mM Tris-Borate

15. Run the gel at 100 V limiting while loading.
16. Load 6 µL sample/lane using gel-loading tips and run at room temperature, 200 V limiting, for 30 minutes.
17. Image gel using the near-IR setting of a Typhoon scanner (600V).
18. Adjust levels and convert to 8-bit with ImageJ image processing software (NIH).



Adapted from Figure 2 – Figure Supplement 1 of Zinder JC *et al.*, *bioRxiv* **2022**

3. Notes

1. This strategy originally came from a suggestion by Dr. Logan Myler in our lab, who had previously used Atto647 (another near-IR fluor) conjugated to streptavidin to detect biotinylated proteins on membranes with a Typhoon scanner. I have not directly compared Atto647 to Cy5.5 probes but this may be worth investigating.
2. I have successful performed EMSAs with these substrates at concentrations of 0.1 nM, though the background was significantly higher
3. Using substrate depletion to infer fraction bound, I have been able to reproduce literature values (Nandakumar J *et al.*, *PNAS* **2009**) for the POT1/TPP1^N K_D (unpublished, see below. Quantification performed in ImageJ). The behavior of the bound species isn't reproducible, to my frustration, so I have not yet tried to publish any quantitative EMSAs using these substrates.