Digital Immunoassay Protocol for Solid-State Nanopore Sensing

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Method Article

Keywords: nanopore, immunoassay, protein, digital detection, diagnostics, thyroid stimulating hormone

Posted Date: February 10th, 2022

DOI: <https://doi.org/10.21203/rs.3.pex-1591/v1>

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Abstract

The ability to digitally count single molecules enables accurate and precise determination of the concentration of a disease biomarker. Owing to their intrinsic single-molecule sensitivity and fully electronic detection capability, solid-state nanopores show great promise for this task toward point-of-care diagnostic applications. Here, we describe the protocols for implementing a magnetic bead-based immunoassay strategy coupled with digital detection and downstream solid-state nanopore electrical readout. The digital scheme employs DNA nanostructures, as proxy labels for the presence (“1”) or absence (“0”) of the target protein. We provide step-by-step protocols for assembling and purifying DNA nanostructures; Preparing magnetic beads decorated with capture antibodies; Conjugating secondary detection antibodies to bind the DNA label; Functionalizing gold nanoparticles; and Running the full assay where thyroid stimulating hormone (TSH) from human serum samples is quantified down to the femtomolar range. The protocols and assay scheme presented herein are easily generalized to the quantification of a wide range of target proteins, by selecting the appropriate antibody pair.

Introduction

Reagents

- SiN_x membranes (Norcada, NBPX5004Z)
- SYLGARD 184 Silicone Elastomer Kit (Dow Chemical Company, 4019862) Potassium chloride (Fisher Scientific, P217-500)
- Lithium Chloride (Fisher Scientific, L121-500)
- Lithium Hydroxide Anhydrous (Fisher Scientific, L128-500)
- HEPES (Fisher Scientific, BP310100)
- Agarose (Fisher Scientific, BP160500)
- Mini-PROTEAN TBE precast gels (Bio-rad, 4565013)
- Mini-PROTEAN TGX gels (Bio-rad, 456-1093)
- NoLimits 2kbp dsDNA fragments (ThermoFisher Scientific, SM1701)
- GeneRuler 1kbp plus DNA ladder (ThermoFisher Scientific, SM1331)
- DNA Gel Loading Dye 6X (ThermoFisher Scientific, R0611)
- Carboxylated paramagnetic beads 2.7 μm (Quanterix, 103612)

- EDC (ThermoFisher Scientific, A35391)
- Sample/detector diluent (Quanterix, 101359)
- Wash buffer 1 (Quanterix, 100486)
- Mouse monoclonal anti human TSH beta subunit capture antibody (Fitzgerald Industries International, 10C-CR2151M4)
- Anti-human TSH beta subunit detection antibody (Maine Biotechnology Services, MAB130P)
- Streptavidin Conjugation Kit (Abcam, ab102921)
- Recombinant TSH (rTSH) (BiosPacific, J11030)
- Serum samples (BioIVT, BRH1391829 – BRH1391838)
- DTT (Dithiothreitol) (ThermoFisher Scientific, A39255)
- Ultramer/HPLC purified DNA oligos with modifications (Integrated DNA technologies)
- Standard 30 nm gold nanoparticles (Cytodiagnosics, G-30-20)
- Tween-20 (Sigma-Aldrich, P1379-100ML)
- Bovine serum albumin (Proliant Biologicals, 68100-100G)
- Bead Wash Buffer (Quanterix, 101355)
- Bead Blocking Buffer (Quanterix, 101356)
- Bead Diluent (Quanterix, 100458)
- Bead Conjugation Buffer (Quanterix, 101357)
- 6x Loading Buffer (ThermoFisher Scientific, R0611)
- 5x TAEMg pH 8 (200 mM Tris, 100 mM Acetic acid, 10 mM EDTA, and 62.5 mM MgCl₂)
- 10x TBE buffer (ThermoFisher Scientific, V36401)
- 10x Tris/Glycine Buffer (Bio-Rad, 1610734)

Equipment

- 3W LED flashlight (LIGHTFE, UV301D)

- 3 kDa Amicon Ultra-0.5 Centrifugal Filter Unit (Millipore Sigma, UFC500396)
- 100 kDa Amicon Ultra-0.5 Centrifugal Filter Unit (Millipore Sigma, UFC510096)
- D-Tube Dialyzer Maxi, MWCO 6-8 kDa (Millipore, 71509-3)
- Illustra NAP-5 columns (GE Healthcare, 17185601)
- Multi-Functional Tube Rotator (VWR, PTR-35).
- Gel imaging workstation (Azure biosystems, C150).
- MiniAmp Plus Thermal Cycler (Applied Biosystems, A37834).
- VWR Mini Centrifuge (VWR, 76269-064)

Procedure

DNA Nanostructure Assembly

1. Add 12 μL of each oligo (stock: 100 μM) for P1 (Star oligo 1 – 12, Extension oligo A – E, F, and G) and P2 (Star oligo 1 – 12, Extension oligo A – E, and F') in separate tubes, 72 μL of 5x TAEMg buffer and deionized water to reach a total reaction volume of 360 μL with a final concentration of 3.3 μM for each oligo in 1x TAEMg¹ (40 mM Tris, 20 mM acetic acid, 2 mM EDTA, and 12.5 mM magnesium acetate, pH 8).
2. Vortex the samples, divide and transfer each sample into 4 PCR tubes, and place the samples in a thermal cycler such as the MiniAmp Plus Thermal Cycler (Applied Biosystems, A37834).
3. Incubate samples at 95 °C for 5 minutes then cool to 90 °C. Ramp down from 90 °C to 60 °C at 0.4 °C/min, 60 °C to 26 °C at 0.03 °C /min, then snap cooled to 4 °C indefinitely.
4. Prepare 50 mL 0.5x TBE in an Erlenmeyer flask, weigh out and add 1 g of agarose to the 0.5x TBE buffer to make 2 % gel, and microwave for 15-30 seconds at a time for approximately 2 minutes and 30 seconds until the agarose is completely dissolved. Let the agarose cool, add 5 μL of 10,000x GelRed to agarose solution, and gently mix to avoid creating bubbles. Pour the agarose solution into the gel tray, insert comb, and let agarose solidify for 20 minutes. Note: the volume of agarose and gel stain needed depends on the size of the gel plate used. For this protocol a gel tray with the following dimensions (10 cm x 7 cm) was used.
5. Pool the DNA nanostructure probes from the four PCR tubes after the assembly, mix 0.5 μL of each DNA nanostructure probe with 9.5 μL of deionized water and 2 μL of 6x Loading buffer (ThermoFisher Scientific, R0611) for gel characterization. Load the samples on 2 % agarose gel, run the gel at 70 V for

approximately 1 hour, and visualize on Gel imaging workstation (Azure biosystems, C150). The remaining probe can be stored at 4 °C.

DNA Nanostructure Purification

1. Take 90 μL of assembled probe (P1/P2), add 14 μL of 5x TAEMg, 15 μL of 10x GelRed, 25 μL of 6x Loading Buffer (ThermoFisher Scientific, R0611), and 26 μL of deionized water. Vortex the samples and load into wells of 5% Mini-PROTEAN TBE polyacrylamide pre-cast gels (BioRad, 4565013). Run the gel in 0.5x TBE buffer at 100 V on ice for 3 hours.
2. Take the gels out of cassettes and visualize using gel imager (Azure biosystems, C150) UV plate.
3. Identify the band of interest and excise with a razor blade.
4. Place the excised gel slices (containing products of interest) in custom extraction apparatus or D-Tube Dialyzer Maxi (MWCO 6-8 kDa, Millipore, 71509-3) tubes, and apply 100 V in 1x TBE buffer to electrophoretically extract the DNA samples out of the gel slices.
5. Take the supernatant (0.5 mL to 3 mL) from the extraction tubes, and perform buffer exchange into 1x TAEMg using 100 kDa Amicon Ultra-0.5 Centrifugal Filter Units (Millipore Sigma, UFC510096). This should concentrate the sample into $\sim$ (35 μL).

Quantification of Nanoprobes

6. Quantify the purified probes by taking 2 μL of the sample and performing nucleic acid concentration measurements using a UV-Visible spectrophotometer for small sample volumes such as the Take3 micro-volume plate accessory of the EPOC 2 spectrophotometer (BioTek, BTEPOCH2).
7. Take equimolar concentrations of purified DNA nanostructure probes, where the probe concentrations are fixed at either 15 nM or 20 nM, and add various amounts of junction strands, ranging from 0.2 nM (0.01:1 junction:probe) to 400 nM (20:1 junction:probe) for dumbbell assembly.
8. Incubate for up to two days (48 h) at room temperature.
9. Confirm the quality of dumbbell formation on 2% agarose gel as shown in Figure 1.

Nanopore Characterization Measurements

10. Take 4 μL of the assembled dumbbell samples, add 35.5 μL of 3.6 M LiCl and 0.5 μL 2kbp dsDNA fragments (ThermoFisher Scientific, SM1701).
11. Vortex the sample and load it into the nanopore flow cell (cis side)^{2,3}.

12. Apply -100 mV and sense on a nanopore for 10 - 30 minutes, anticipated results are shown in Figure 2.

Paramagnetic Bead Ab Conjugation

1. Add 234 μg of primary antibody (Fitzgerald Industries International, 10C-CR2151M4) to 100 kDa Amicon Ultra-0.5 Centrifugal Filter Unit (Millipore Sigma, UFC510096).
2. Add Bead Conjugation Buffer (Quanterix, 101357) to the filter unit to reach a final volume of 500 μL .
3. Centrifuge at 14,000 g for 5 minutes, discard flow-through, and wash the samples by adding 450 μL Bead Conjugation Buffer.
4. Repeat the 450 μL washes three times.
5. Invert the filter unit membrane and centrifuge into a new tube at 1,000 g for 2 minutes.
6. Rinse the filter unit membrane by adding another 50 μL of Bead Conjugation Buffer, centrifuge at 1,000 g for 2 minutes into the same tube.
7. Collect the sample and measure the protein concentration using the Take3 micro-volume plate accessory of the EPOCH 2 spectrophotometer (BioTek, BTEPOCH2).
8. Add enough Bead Conjugation Buffer to dilute the sample to 0.3 mg/mL.
9. Store the antibody sample on ice.
10. Take 300 μL (8.5×10^8) of carboxylated paramagnetic beads sized 2.7 μm (Quanterix, 103612) and resuspend by vortexing.
11. Briefly centrifuge the sample using the pulse spin setting of a small 8 sample rotor bench top centrifuge and place solution on magnet for > 1 minute, remove supernatant.
12. Add 600 μL of the Bead Wash Buffer (Quanterix, 101355), and vortex to resuspend beads.
13. Repeat 600 μL washes three times using Bead Wash Buffer ensuring to vortex the beads each time, then remove the buffer after the samples have been placed on the magnet for >1 min.
14. Add 600 μL of Bead Conjugation Buffer (Quanterix, 101357), vortex to resuspend beads, then use the magnet to retain the beads and remove the buffer.
15. Repeat 600 μL washes three times using Bead Conjugation Buffer (Quanterix, 101357) ensuring to vortex the beads each time, then remove the buffer after the samples have been placed on the magnet for >1 min.

16. Add 570 μL of the Bead Conjugation Buffer and store on ice.
17. Add 100 μL of the Bead Conjugation Buffer to 1 mg vial of EDC (ThermoFisher Scientific, A35391), close lid and vortex.
18. Add 30 μL of the EDAC to the washed paramagnetic beads and incubate on shaker (VWR, PTR-35) for 30 minutes to allow for bead activation.
19. Pulse spin using tabletop centrifuge (VWR, 76269-064), and wash activated paramagnetic beads with 600 μL Bead Conjugation Buffer (Quanterix, 101357), then place on magnet for >1 min and remove supernatant.
20. Add prepared buffer exchanged primary antibody to activated paramagnetic beads.
21. Incubate for 2 hours on shaker.
22. Pulse spin, place paramagnetic bead/primary antibody solution on magnet, collect supernatant and label as "supernatant".
23. Perform one wash with 600 μL Bead Wash Buffer (Quanterix, 101355), collect supernatant and label as "wash 1".
24. Measure protein concentrations of supernatant and wash 1 using EPOCH 2 spectrophotometer (BioTek, BTEPOCH2). Compare values to total amount of antibody loaded to estimate total antibody coated and percentage antibody coated.
25. Perform another wash with Bead Wash Buffer (Quanterix, 101355), then discard the supernatant.
26. Add 600 μL of Bead Blocking Buffer (Quanterix, 101356) and incubate for 30 minutes on Multi-Functional Tube Rotator (VWR, PTR-35) at 5 rpm to keep particles in suspension.
27. Perform one wash using 600 μL of Bead Wash Buffer (Quanterix, 101355).
28. Perform two more washes using 600 μL of Bead Diluent (Quanterix, 100458), resuspend in 600 μL Bead Diluent and store at 4 °C.

Secondary Antibody Streptavidin Conjugation

1. Take 62.5 μL (100 μg) of secondary antibody (Maine Biotechnology Services, MAB130P) and add 37.5 μL of 1x PBS to have secondary antibody at 1 $\mu\text{g}/\mu\text{L}$ in 100 μL .
2. Add 10 μL of Modifier reagent (Abcam, ab102921) to 100 μL of secondary antibody.

3. Add the above 100 μL sample to lyophilized Streptavidin Mix (Abcam, ab102921), pipette up and down to have material fully dissolved.
4. Incubate at room temperature for 3 hours.
5. Add 10 μL of Quencher (Abcam, ab102921) to the reaction.
6. Incubate at room temperature for 30 minutes.
7. Confirm the quality of the conjugation using native gel, Mini-PROTEAN TGX gels (Bio-rad, 456-1093) in 1x Tris/Glycine Buffer at 100 V
8. Store at 4 °C.

Assay Procedure

1. Take 72 μL (7.2×10^7) of prepared bead-capture antibody conjugates, add Sample Diluent (Quanterix, 101359) to a total volume of 500 μL .
2. Place the tube on the magnet, and remove supernatant.
3. Perform two more 500 μL washes.
4. Add various amounts (0.8 fM to 1.2 nM) of recombinant TSH (rTSH) (BiosPacific, J11030) and bring the volume up to 500 μL using Sample Diluent or diluted human serum sample.
5. Incubate for 1 hour at room temperature on Multi-Functional Tube Rotator (VWR, PTR-35) at 5 rpm.
6. Place the tube on the magnet for >1 min, and remove the supernatant.
7. Add 500 μL of Wash Buffer 1 (Quanterix, 100486), and incubate for 5 minutes on Multi-Functional Tube Rotator (VWR, PTR-35) at 5 rpm.
8. Place the tube on the magnet for >1 min, and remove the supernatant.
9. Add 500 μL of Wash Buffer 1 (Quanterix, 100486), and incubate for 10 minutes on Multi-Functional Tube Rotator (VWR, PTR-35) at 5 rpm.
10. Place the tube on the magnet for >1 min, and remove the supernatant.
11. Add 500 μL of Wash Buffer 1 (Quanterix, 100486), and incubate for 15 minutes on Multi-Functional Tube Rotator (VWR, PTR-35) at 5 rpm.
12. Place the tube on the magnet for >1 min, and remove the supernatant.

13. Resuspend in 500 μL of Sample Diluent (Quanterix, 101359) containing 6 nM secondary antibody-streptavidin conjugate. OPTION 1: for the amplification assay, add 10 μL of AuNP amplification complex to a final volume of 50 μL in Sample Diluent (Quanterix, 101359).
14. Incubate for 1 hour on Multi-Functional Tube Rotator (VWR, PTR-35) at 5 rpm at room temperature.
15. Perform three 30 sec washes with 1x Wash Buffer 1 (Quanterix, 100486), and resuspend in 500 μL of Sample Diluent (Quanterix, 101359). OPTION 1: for the amplification assay, skip this step.
16. Add 12 nM of biotinylated ssDNA junction strand. OPTION 1: for the amplification assay, skip this step.
17. Incubate for 15 min at room temperature on Multi-Functional Tube Rotator (VWR, PTR-35) at 5 rpm. OPTION 1: for the amplification assay, skip this step.
18. Perform three 30 sec washes with 1x Wash Buffer 1 (Quanterix, 100486), and resuspend in 500 μL of 1x TAEMg buffer containing 0.025 % Tween-20. OPTION 1: for the amplification assay, resuspend in 20 μL of 1x PBS containing 0.025 % Tween-20.
19. Apply UV using a 3W LED flashlight (LIGHTFE, UV301D) at a distance of 1 cm for 20 minutes to release the ssDNA junction strand. OPTION 1: for the amplification assay, release the junction strand by incubation for 45 minutes in 0.2 M DTT (ThermoFisher Scientific, A39255) and 0.3 M NaH_2PO_4 buffer in a final volume of 30 μL .
20. Place the samples on the magnet for >1 min and transfer the supernatant to 3 kDa Amicon Ultra-0.5 Centrifugal Filter Unit (Millipore Sigma, UFC500396).
21. Perform buffer exchange and concentrate the ssDNA junction strands into 35 μL of 1x TAEMg containing 0.025 % Tween-20.
22. Add the purified shooting star probes at either 15 or 20 nM final concentrations.
23. Incubate at room temperature for up to 2 days for dumbbell assembly.

OPTION 1: Gold Nanoparticle Amplification Scheme

DTT Reduction

1. Take thiolated ssDNA oligos and perform three washes of 1x PBS using 3 kDa Amicon Ultra-0.5 Centrifugal Filter Unit (Millipore Sigma, UFC500396) to have final concentration of 10 μ M in 1x PBS.
2. Prepare 0.3 M NaH₂PO₄ containing 0.2 M DTT solution (ThermoFisher Scientific, A39255).
3. Mix 250 μ L of the buffer exchanged DNA oligo and 250 μ L of 0.3 M NaH₂PO₄ and 0.2 M DTT.
4. Incubate at room temperature for 2 hours (unless otherwise stated incubations were done at room temperature on the benchtop.).
5. Take a NAP-5 column (GE Healthcare, 17185601), remove all caps and let storage buffer flow through.
6. Equilibrate the NAP-5 column with 10 mL deionized water.
7. Add the reduced DNA sample to the column and allow sample to fully enter membrane.
8. Place column on top of a new tube.
9. Add 1 mL deionized water to elute purified sample.
10. Reduce the volume to 200 μ L using 3 kDa Amicon Ultra-0.5 Centrifugal Filter Unit (Millipore Sigma, UFC500396), confirm and adjust the final concentration of the sample to 25 μ M with deionized water.
11. Split the sample into 50 μ L aliquots and store at -20 °C.

Preparation of Amplification complex. (AuNP Functionalization with antibody and ssDNA oligos)

1. Take 960 μ L of 30 nm AuNP (Cytodiagnostics, G-30-20), add 40 μ L 0.1 M borate buffer to adjust the solution to pH 9.
2. Add 4 μ g of secondary antibody (Maine Biotechnology Services, MAB130P) to a 1.7 mL tube, and add the adjusted AuNP solution to the tube with the secondary antibody^{4,5}.
3. Incubate at room temperature for 30 minutes.
4. Add 25 μ L of reduced ssDNA oligos and incubate for 1 hour.
5. Salt age to a final concentration of 0.15 M NaCl by adding 6 additions of 1 M NaCl every 30 min.
6. Incubate at 4 °C overnight (optional pause point).
7. Add 50 μ L of 10 % BSA and incubate for 5 minutes.
8. Centrifuge at 4,500 g for 15 minutes. Discard the supernatant.

9. Add 950 μL of 1x PBS containing 0.025 % v/v Tween-20, resuspend by gentle shaking or vortexing.
10. Repeat steps 8 – 9 two times.
11. Add 150 μL of 1x PBS containing 0.025 % v/v Tween-20.
12. Store at 4 °C.

Troubleshooting

Time Taken

- Paramagnetic bead preparation: 5 hours
- DNA nanostructure assembly: overnight
- DNA nanostructure purification: 5 hours
- Secondary antibody streptavidin conjugation: 4 hours
- DTT reduction: 3 hours
- Amplification complex preparation: 5 hours
- Assay workflow: 4 hours
- Dumbbell formation: up to 2 days
- Nanopore sensing: 10 – 20 minutes per sample

Anticipated Results

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Figures

Figure 1

Agarose gel (2%) of shooting star probes P-1 and P-2 assemblies and purification. Lane 1: GeneRuler 1kb Plus DNA Ladder (ThermoFisher Scientific, SM1331), from bottom 75, 200, 300, 400, 500, 700, 1000, 1500, 2000, 3000, 4000, 5000, 7000, 10000, and 20000 bp, lane 2: gel extracted P-1, lane 3: gel extracted P-2, lane 4: crude P-1 and lane 5: crude P-2.

Figure 2

Dose response and time dependence of dumbbell assembly. (a) Gel electrophoresis (2% agarose) of dumbbells formation and remaining shooting star probes as junction concentration increases. (b) Comparison of dose response with junction concentration ranging from 0.2 nM to 400 nM (junction to shooting star probes ratio of 0.01:1 to 20:1) between nanopore sensing and gel band densitometry.