Determining Functional Aptamer-Protein Interaction by Biolayer Interferometry

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Short single-stranded nucleic acids called aptamers are widely being explored as recognition molecules of high affinity and specificity for binding a wide range of target molecules, particularly protein targets. In biolayer interferometry (BLI), a simple Dip-and-Read approach in which the aptamer-coated biosensors are dipped into microplate wells is used to study the interactions between an aptamer and its target protein. Here we describe the protocol for the analysis of the interaction between a well-characterized anti-thrombin RNA aptamer with thrombin (Basic Protocol). We also report on the protocol for the affinity screening of a panel of anti-thrombin RNA aptamers with a single phosphorodithioate (PS2) modification, whereby the position of the modification along the RNA backbone is varied systematically (Support Protocol). The PS2 modification uses two sulfur atoms to replace two non-bridging oxygen atoms at an internucleotide phosphodiester backbone linkage. The PS2-modified RNAs are nuclease resistant and several in vitro and in vivo assays have demonstrated their biological activity. For example, combining the PS2 with the 2′-OMe modification affords increased loading of modified small interfering RNA (siRNA) duplexes into the RNA-induced silencing complex (RISC) as well as enhanced gene-silencing antitumor activity.

Keywords: aptamer • nucleic acid-protein interactions • phosphorodithioate • biolayer interferometry • affinity

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INTRODUCTION

Nucleic acid-protein interactions are very important for many biological processes (Guille and Kneale, 1997; Draper, 1999; Gosai et al., 2015; Jankowsky and Harris, 2015). Direct measurement of nucleic acid-protein interactions plays an important role in designing specific nucleic acids such as aptamers; the design of nucleic acid binding proteins has also been an important goal for biotechnology (Ellington and Szostak, 1990; Tuerk and Gold, 1990). Traditionally, assaying specific nucleic acid-protein formation in vitro often involves the use of specific labeling or modification of the components of the complex to provide a unique signal that can be used to assess the affinity of the interaction (Ceglarek and Revzin, 1989; Fried, 1989; Murphy et al., 2001). Biolayer interferometry (BLI) is a biosensor-based technique that doesn't need radio or fluorescent labeling of the compounds of a complex provided that one of the partners is immobilized on a solid support. Unlike standard endpoint assays such as the electrophoretic mobility shift assay (EMSA; Fried, 1989; Yang et al., 1999), BLI enables monitoring of binding

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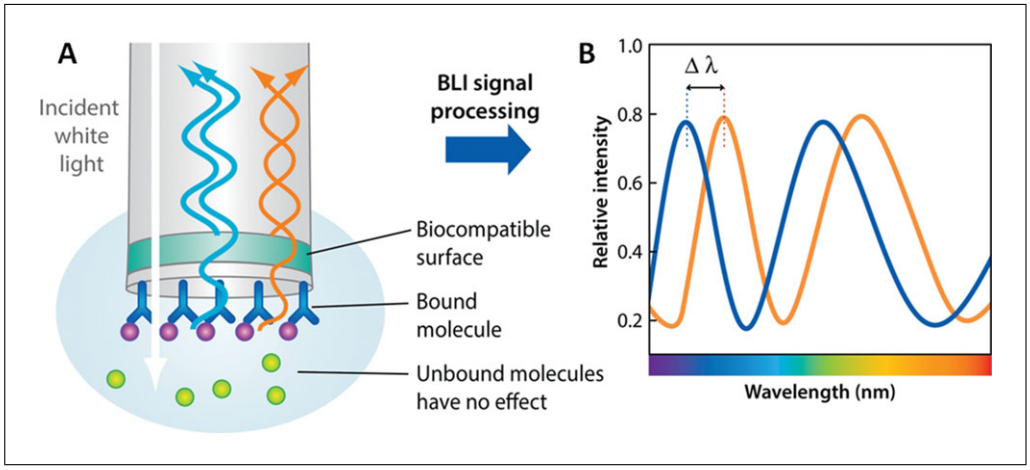

Figure 7.25.1 The principle of biolayer interferometry (BLI). (**A**) Schematic drawing of the light interference on a biomolecule-bound layer and an internal reference layer of a fiber-optic biosensor. (**B**) The wavelength shift of the interference pattern caused by the target binding is measured in real time (provided by Dr. Sriram Kumaraswamy and Dr. Gemma Milan from FortéBIO, a division of Pall Life Sciences).

interactions in real time and is generally applicable to the study of any molecules that express affinity for one another, including proteins, nucleic acids, and carbohydrates. The wealth of information provided by BLI includes qualitative data such as binding specificity, quantitative data such as stoichiometry, dissociation constants (K_D), and kinetics parameters.

Principle of Detection

The principle of BLI detection relies on the optical analytical technique that is sensitive to the interference between waves of light. In BLI, white light is directed down the fiber-optic biosensor towards the two interfaces separated by a thin layer at the tip of the fiber: The ligand-coated layer of the surface of the tip and an internal reference layer (Fig. 7.25.1A). Light is reflected from both layers and the reflected beam interference is detected via a charge-coupled device (CCD) array detector. When the tip of a biosensor is dipped into a 96-well microplate containing a sample analyte such as protein, the protein binds to immobilized aptamer on the biosensor surface. As more proteins bind to the surface of the biosensor, the interference pattern of the reflected light changes more significantly, creating a wavelength shift (nm) that is reported in real time on a sensorgram (Fig. 7.25.1B). Monitoring the interference pattern in real time provides kinetic data on molecular interactions.

The protocols described herein include a binding kinetic assay for studying functional aptamer-protein interactions (see Basic Protocol) and for affinity screening of modified aptamers (see Support Protocol).

BASIC PROTOCOL

ANALYSIS OF FUNCTIONAL APTAMER-PROTEIN INTERACTIONS

This protocol describes the setup and step-by-step procedures for studying functional aptamer-protein interactions. In this protocol, aptamers are immobilized on the BLI biosensor tip surface on the FortéBIO Octet RED96 system (Fig. 7.25.2) equipped with a Data Acquisition and Data Analysis 7.0 workstation. The protocol can easily be adapted to other Octet systems such as Octet RED384. All assays used to develop this protocol were performed with streptavidin-coated biosensors (FortéBIO, cat. no. 18-5020) that were designed to efficiently capture the biotin-labeled aptamers, owing to the extremely high affinity of the biotin-streptavidin interaction (association constant 10^{15} M^{-1}). In

Figure 7.25.2 The Octet RED96 system.

addition, the interaction between streptavidin and biotin is non-covalent, but is rapid, stable, and essentially irreversible.

Here we describe how to perform kinetic characterization of the interaction between the anti-thrombin RNA aptamer and thrombin (White et al., 2001) using the BLI-based Octet RED96 system. The crystal structure of this aptamer in complex with thrombin has been determined (Long et al., 2008).

Materials

Water, molecular biology grade (DNase, RNase, and Protease are not detectable; Phenix Research Products, cat. no. W-8001-1000)
1× assay buffer (20 mM HEPES-KOH, 125 mM NaCl, 2 mM $CaCl_2$, and 0.05% Tween 20, pH 7.4)
10× PBS (Sigma-Aldrich, cat. no. P5493-1 liter)
Streptavidin (SA) Dip and Read biosensors (FortéBIO, a division of Pall Life Sciences, cat. no. 18-5020)
Thrombin (Haematologic Technologies, cat. no. HCT-BFPRCK)
Anti-thrombin aptamer sequence:
5′-biotinTEG-GGGAACAAAGCUGAAGUACUUACCC-3′ (all U and C are 2′-F-modified nucleosides, synthesized at AM Biotechnologies)

96-well black microtiter plate (only the black plate is suitable;
Greiner Bio One, cat. no. 655209)
Disposable reagent reservoirs (Vistalab Technologies, cat. no. 3054-1002)
50 to 300 μL BioPette Plus Autoclavable Multichannel pipets, 8 channels (Labnet, manufacturer number P4808-300 and P4808-10)
Octet RED96 system with Data Acquisition and Analysis software (see Fig. 7.25.2; FortéBIO, a division of Pall Life Sciences)

1. Ready instrument for BLI assay.

   Turn on the instrument at least 1 hr in advance to allow the lamp to warm up. This is necessary to minimize noise and drift in optical signal during the experiment.

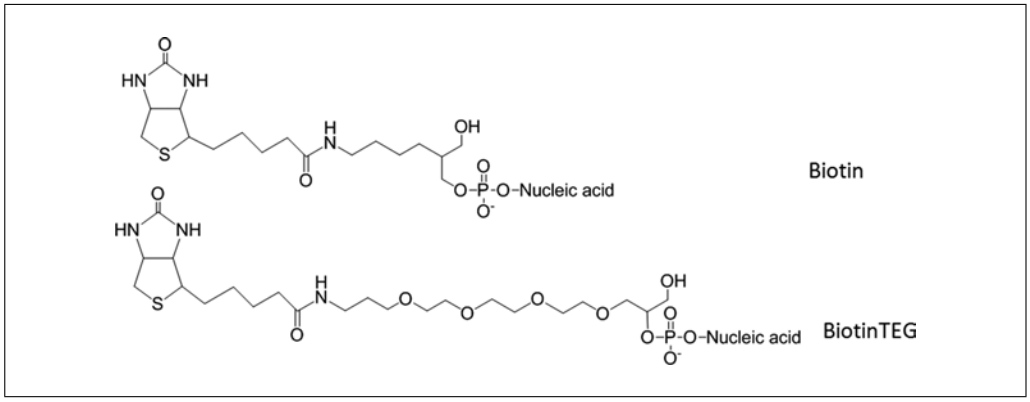

Figure 7.25.3 The structures of biotin and BiotinTEG.

2. Prepare 1× assay buffer(s) for studying the aptamer-protein interaction by mixing the following:

   1 mL 1 M HEPES-KOH, pH 7.4
   750 μL 5 M NaCl
   150 μL 1 M $CaCl_2$
   25 μL Tween 20
   48.1 mL water
   50 mL total volume.

   In general, the buffer should be used throughout the assay. The buffer for the aptamer-thrombin interaction contains 20 mM HEPES-KOH, 125 mM NaCl, 2 mM $CaCl_2$, and 0.05% Tween 20, pH 7.4.

3. Prepare biotinylated aptamer stock solution.

   Aptamer molecules are readily biotinylated in the course of oligonucleotide synthesis by the phosphoramidite method using commercial biotin phosphoramidite. Following standard deprotection and desalting, the conjugate obtained without further purification can be directly used for preparing the aptamer stock solution. The typical concentration of the aptamer stock solution should be around 10 μM.

   Although the biotin moiety (Fig. 7.25.3) can be incorporated at the terminus (generally at the 5′ terminus) of an aptamer, we have found that the long arm chain, covalently attached to biotin such as BiotinTEG (Glen Research, cat. no. 10-1955) that is depicted in Figure 7.25.3, can improve binding kinetics and increase binding capacity at the biosensor surface.

Determine proper aptamer concentration for optimal loading of aptamer on the streptavidin biosensors

The 96-well plate format used on the Octet platform allows for rapid testing of several experimental parameters at once, minimizing time spent on assay development. To determine the proper concentration of aptamer in the loading step, several concentrations of aptamer are loaded onto the biosensor. An association step is performed for each aptamer concentration using a high concentration of thrombin target such as 10 to 20 μM. A zero concentration of aptamer biosensor should also be run as a control for determining whether the thrombin binds non-specifically to the biosensor.

4. Design plate map diagram (Fig. 7.25.4).

5. Prepare sensor tray.

   Based on the map diagram shown in Figure 7.25.4, we will run eight independent assays including a control assay in one experiment.

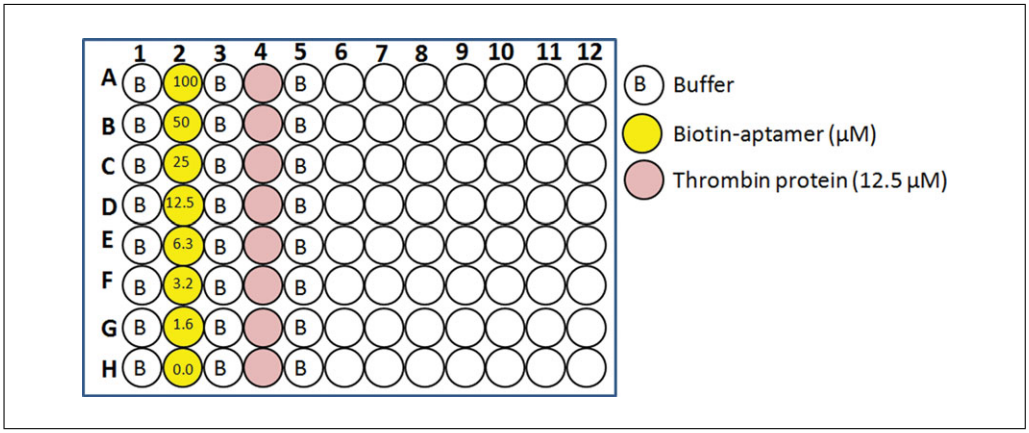

Figure 7.25.4 Plate map diagram for optimization of aptamer loading on the streptavidin biosensors.

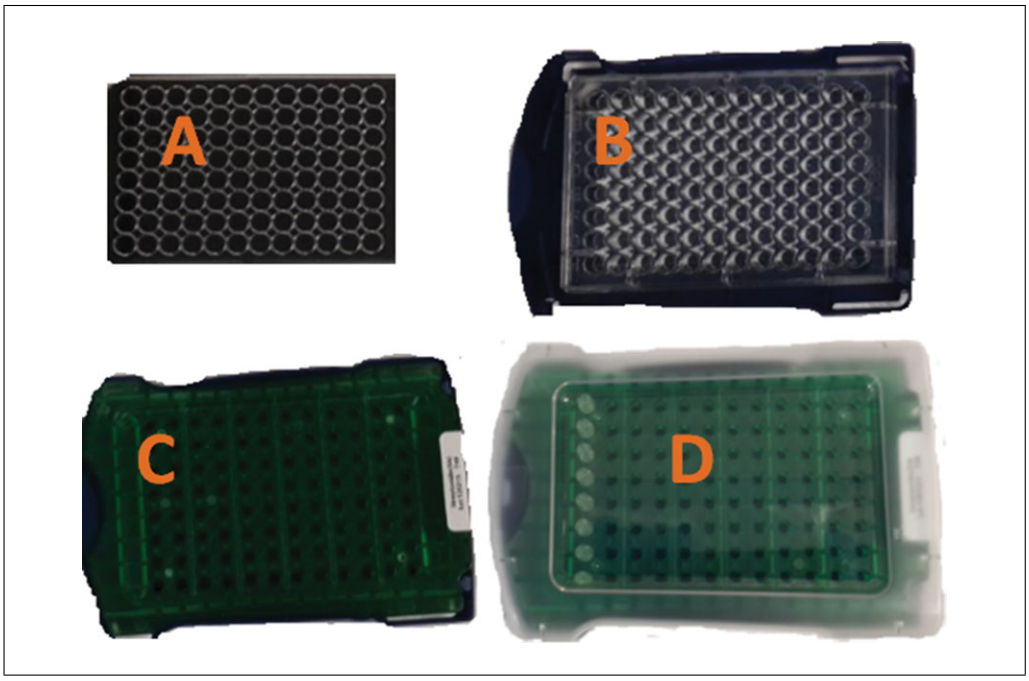

Figure 7.25.5 Flowchart to show how to pre-wet the BLI biosensors. BLI, biolayer interferometry.

Pipet 200 μL per well of 1× assay buffer into wells of Column 1 of a 96-well black flat-bottom microplate (Fig. 7.25.5A).

The buffer used for hydration should be the same as that used throughout the assay.

Insert hydration plate into biosensor tray (Fig. 7.25.5B). Align biosensor rack over the hydration plate (Fig. 7.25.5C) and load streptavidin biosensors into wells (Fig. 7.25.5D), taking care not to scrape or touch the tips of the biosensors. Hydrate all biosensors at least 10 min.

6. Prepare sample tray: Pipet 200 μL per well 1× assay buffer into Columns 1, 3, and 5 as shown in Figure 7.25.4.

A multichannel pipet is recommended for transferring the buffer volume.

i. Dilute biotinylated anti-thrombin aptamer to 100 nM in 1× assay buffer (400 μL). Heat solution to 90°C 5 min, then slowly cool to room temperature over ~2 hr. Perform dilutions of biotinylated aptamer in 1× assay buffer to

Table 7.25.1 Experiment Settings for Determining the Aptamer Loading Concentration

Assay step number	Step data name	Assay time (sec)	Flow (RPM)	Sample plate column
1	Baseline	60	1000	1
2	Loading	300	1000	2
3	Baseline2	100	1000	3
4	Association	300	1000	4
5	Dissociation	300	1000	5

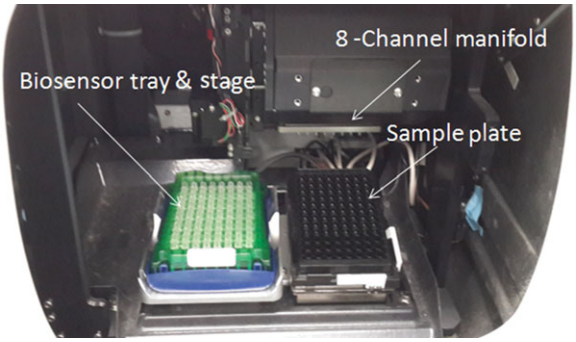

Figure 7.25.6 The internal layout of the Octet System consists of a biosensor stage (left position) and a sample microplate stage (right position). The eight-channel optical manifold can acquire up to eight biosensors for parallel processing.

yield aptamer concentrations of 50, 25, 12.5, 6.3, 3.2, 1.6, and 0.0 in 200 μL. Pipet each dilution into each well of Column 2 of sample plate depicted in Figure 7.25.4.

ii. Prepare thrombin protein to a 12.5 nM concentration in 1.6 mL assay buffer (for example, if your stock thrombin protein concentration is 1000 nM, add 20 μL [20 μL = (12.5 × 1600)/1000] stock solution into 1.6 mL assay buffer). Pipet 200 μL per well into each well of Column 4 of the sample plate depicted in Figure 7.25.4, except for the last well, which contains only binding buffer.

iii. Place sample plate on the sample plate stage inside the Octet system with well A1 toward the back right corner (Fig. 7.25.6, right position). Place biosensor hydration assembly on the biosensor stage (Fig. 7.25.6, left position). Make sure both tray and sample plate are securely in place.

7. Set up assay in the instrument software.

For details, refer to the Octet Data Acquisition User Guide. Table 7.25.1 shows an example of the settings for a kinetic assay, which can be used for optimization of aptamer loading. Steps consist of baseline (equilibration), loading (immobilize the biotinylated aptamer on the biosensor containing the streptavidin [SA]), baseline 2 (remove unbound aptamer from the biosensor and to assess drift caused by non-specific binding, aggregation, or buffer effects), association (the binding interaction of thrombin to the immobilized aptamer is measured), and dissociation (the biosensor is dipped into buffer solution that does not contain thrombin).

8. Run assay.

An example of the sensorgram output for the anti-thrombin aptamer loading determination is shown in Figure 7.25.7. The shape of individual loading curves can be seen.

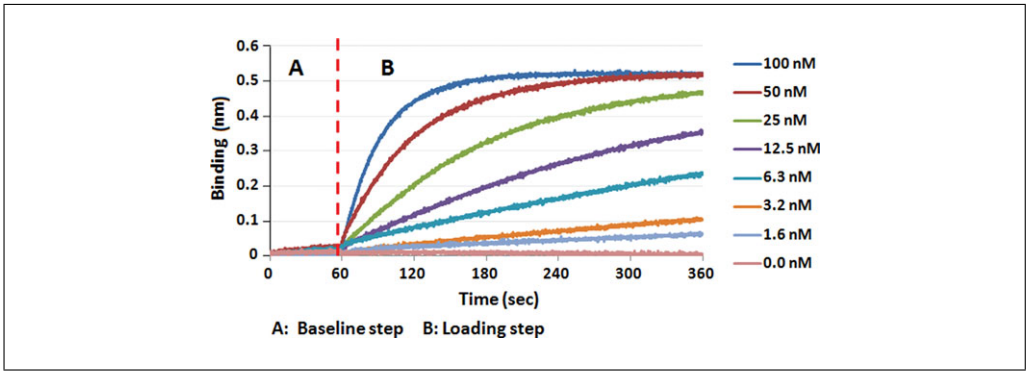

Figure 7.25.7 Sensorgram traces showing the baseline and loading steps of the anti-thrombin aptamer at 8 concentrations.

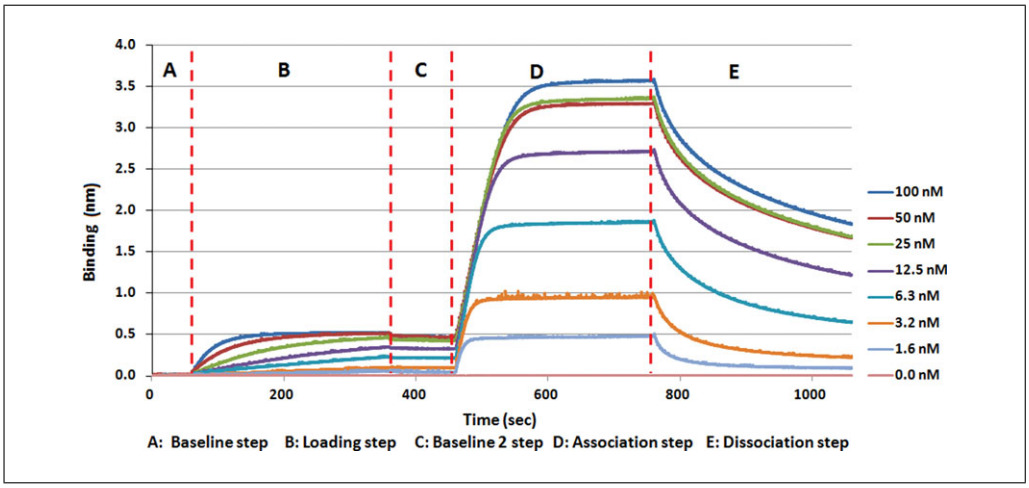

Figure 7.25.8 Sensorgram traces showing all the steps using anti-thrombin aptamer at 8 concentrations.

9. In the Octet Data Analysis software, process data.

   Figure 7.25.8 shows the raw data trace for determining the anti-thrombin aptamer concentrations for a loading experiment. The relative signal of the analyte thrombin binding at each corresponding aptamer concentration can be clearly differentiated. The concentration of the biotinylated aptamer for a binding kinetics experiment can be selected based on the analysis of Figures 7.25.7 and 7.25.8. The loading concentration to be selected for an assay should be the lowest concentration of aptamer at which an acceptable signal in the thrombin protein association step is generated. In the loading step, higher concentrations of aptamer such as 100 nM quickly saturate the biosensor (Fig. 7.25.8). In this example, the 25 nM aptamer loading concentration is appropriate. In addition, the loading time is estimated to be ~100 sec. The 300 sec association time is too long for a 25 nM aptamer concentration.

10. Determine non-specific binding of thrombin to the biosensor.

    From Figure 7.25.8, the non-aptamer loaded sensor shows a flat response even in the presence of thrombin, suggesting that non-specific binding of thrombin to the biosensor is negligible.

11. Set up second experiment to confirm aptamer loading concentration and thrombin binding concentration.

    All the assay parameters are the same, except for the assay times shown in Table 7.25.2. Figure 7.25.9 shows the sensorgram output for all the assays based on the raw data.

Table 7.25.2 Experimental Settings for Verifying Loading Concentration Assays

Assay step number	Step data name	Assay time (sec)	Flow (RPM)	Sample plate column
1	Baseline	60	1000	1
2	Loading	100	1000	2
3	Baseline2	100	1000	3
4	Association	100	1000	4
5	Dissociation	300	1000	5

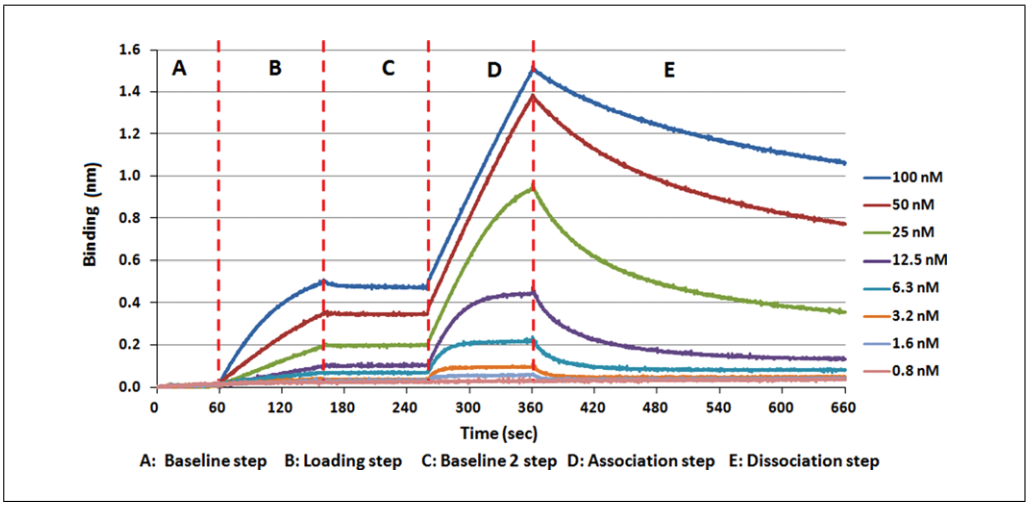

Figure 7.25.9 Sensorgram traces showing all the steps using aptamer at 8 concentrations.

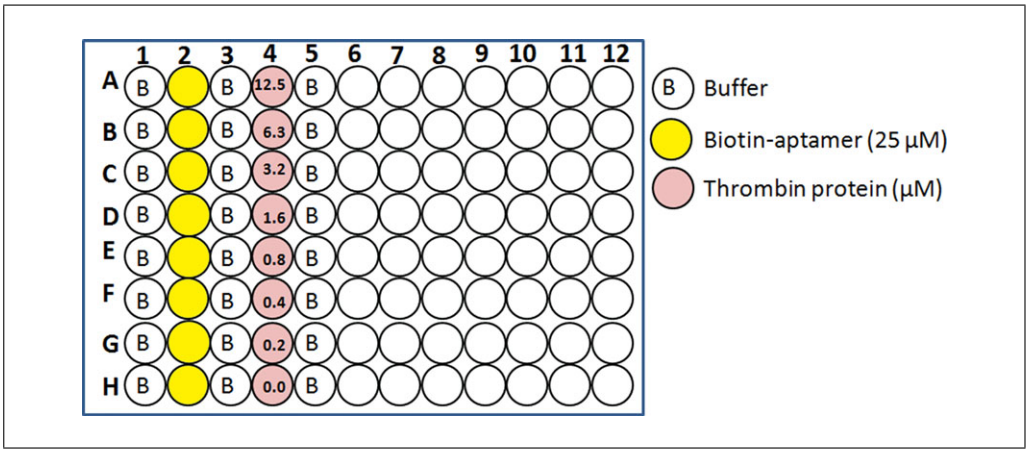

Figure 7.25.10 Plate map diagram for aptamer-protein kinetics experiment.

Run aptamer-thrombin kinetics experiment

12. Design plate map diagram (Fig. 7.25.10).
13. Pre-wet biosensors as described in step 5.
14. Prepare sample tray:
    i. Pipet 200 µL per well of 1× assay buffer into each well of Columns 1, 3, and 5 as shown in Figure 7.25.10. Use multichannel pipet to transfer buffer volume.
    ii. Dilute biotinylated anti-thrombin aptamer to 25 nM in 1× assay buffer (1600 µL). Heat solution to 90°C 5 min, then slowly cool to room temperature

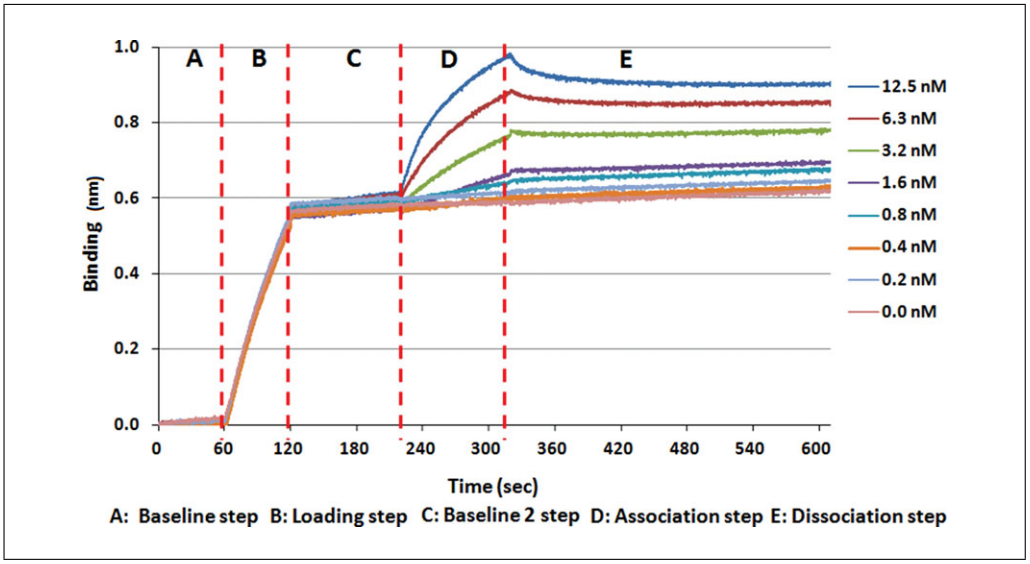

Figure 7.25.11 Sensorgram traces showing all the steps at 8 concentrations of thrombin.

over ~2 hr. Pipet 200 µL into each well of Column 2 of sample plate as shown in Figure 7.25.10.

iii. Dilute thrombin protein to a 12.5 nM concentration in 1× assay buffer (400 µL). Perform dilutions of thrombin in 1× assay buffer to yield concentrations of 6.3, 3.2, 1.6, 0.8, 0.4, 0.2, and 0.0 nM. Pipet 200 µL per well into each well of Column 4 of sample plate as shown in Figure 7.25.10.

15. Place sample plate on the sample plate stage inside the Octet system with well A1 toward the back right corner (see Fig. 7.25.6). Place biosensor hydration assembly on the biosensor stage (see Fig. 7.25.6). Make sure both tray and sample plate are securely in place.

16. Set up assays in the instrument software based on Table 7.25.2.

17. Run assay.

Process kinetic data

18. In the Octet Data Analysis software, load raw data.

Figure 7.25.11 shows raw sensorgram output of the eight assays.

19. Select parameters for data processing; the following parameters are generally recommended for a standard aptamer-protein interaction:

i. Subtract reference well. Well 4H in the sample tray contains a 0.0 concentration of thrombin protein. Subtract all data of assay 1 through 7 from data of assay 8.
ii. *y*-axis alignment (select baseline 95 to 99.8 sec).
iii. Use inter-step correction and Savitzky-Golay filtering.

20. Process data.

Figure 7.25.12 shows aligned association and dissociation steps.

21. Analyze data: In the analysis tab, select 1:1 model for curve fitting.

According to the literature reports, aptamer binding to thrombin has been demonstrated to have 1:1 stoichiometry. Figure 7.25.13 shows the real and fitted traces of aligned association and dissociation.

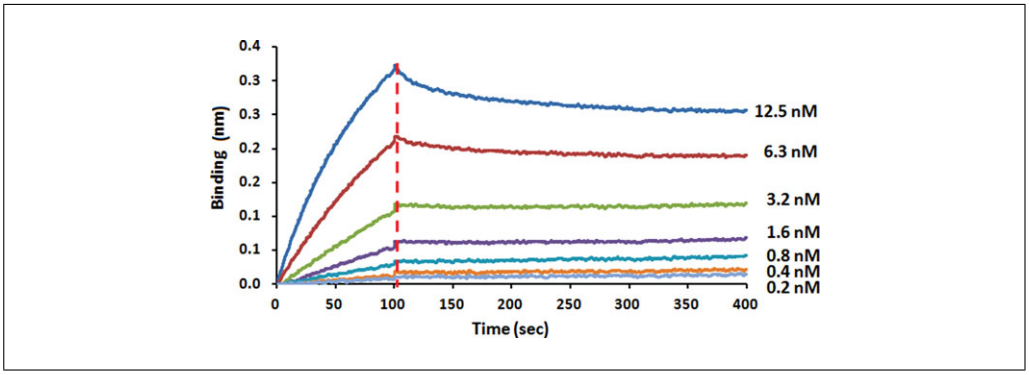

Figure 7.25.12 Processed data for aptamer binding to thrombin (only aligned association and dissociation steps are shown).

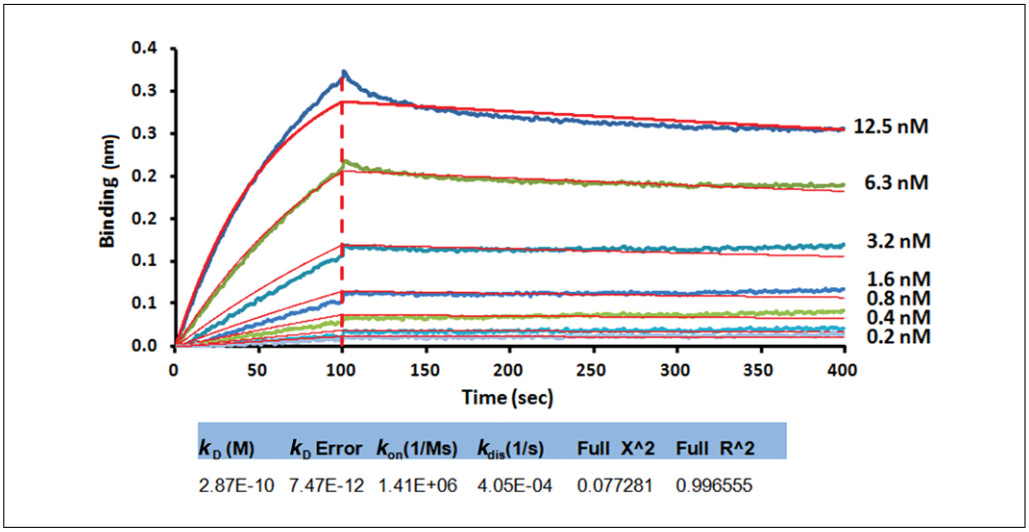

Figure 7.25.13 Analyzed data for aptamer binding to thrombin using a 1:1 binding model. Curve fit overlays are shown as red thin lines over traces.

SUPPORT PROTOCOL

AFFINITY SCREENING OF A LARGE NUMBER OF MODIFIED APTAMERS

Aptamers are isolated from a vast library consisting of some 10^{12} to 10^{14} randomized oligonucleotide sequences. Typically hundreds of aptamer sequences are produced initially with varying affinities for their respective protein target. Comparative sequence analysis is therefore necessary to identify the winning aptamer sequence. In order to develop new aptamer therapeutics or diagnostics, the selected aptamer sequence needs to be optimized. The ability to characterize mutations or substitutions in the optimization of the lead aptamer candidates is essential for improving affinity and/or other properties such as improved nuclease resistance. The Octet System provides a solution for rapidly screening and identifying binding interactions between aptamer-protein pairs. After completion of the assay, the association rate, dissociation rate, and affinity (K_a, K_d, and K_D, respectively) of aptamer-protein interactions can be determined. This support protocol describes how to screen a large number of phosphorodithioate (PS2) modified aptamers based on the anti-thrombin aptamer studied above. For screening purposes or qualitative analyses, measuring binding curves for a single thrombin concentration is often sufficient. Here, a dilution series of four thrombin concentrations is measured in terms of association.

Additional Materials *(also see Basic Protocol)*

20 PS2-anti-thrombin aptamers (shown in Table 7.25.3; synthesized and characterized at AM Biotechnologies)

Table 7.25.3 Anti-Thrombin Aptamer Sequences with PS2 Modification Positions

Number	PS2-modified aptamer sequences with 5′-BiotinTEG
1	5′-GGGAACAAAGCUGAAGUACUUACCC-3′
2	5′-G$_{PS2}$GGAACAAAGCUGAAGUACUUACCCT-3′
3	5′-GG$_{PS2}$GAACAAAGCUGAAGUACUUACCCT-3′
4	5′-GGG$_{PS2}$AACAAAGCUGAAGUACUUACCCT-3′
5	5′-GGGA$_{PS2}$ACAAAGCUGAAGUACUUACCCT-3′
6	5′-GGGAA$_{PS2}$CAAAGCUGAAGUACUUACCCT-3′
7	5′-GGGAAC$_{PS2}$AAAGCUGAAGUACUUACCCT-3′
8	5′-GGGAACA$_{PS2}$AAGCUGAAGUACUUACCCT-3′
9	5′-GGGAACAA$_{PS2}$AGCUGAAGUACUUACCCT-3′
10	5′-GGGAACAAA$_{PS2}$GCUGAAGUACUUACCCT-3′
11	5′-GGGAACAAAG$_{PS2}$CUGAAGUACUUACCCT-3′
12	5′-GGGAACAAAGC$_{PS2}$UGAAGUACUUACCCT-3′
13	5′-GGGAACAAAGCU$_{PS2}$GAAGUACUUACCCT-3′
14	5′-GGGAACAAAGCUG$_{PS2}$AAGUACUUACCCT-3′
15	5′-GGGAACAAAGCUGA$_{PS2}$AGUACUUACCCT-3′
16	5′-GGGAACAAAGCUGAA$_{PS2}$GUACUUACCCT-3′
17	5′-GGGAACAAAGCUGAAG$_{PS2}$UACUUACCCT-3′
18	5′-GGGAACAAAGCUGAAGU$_{PS2}$ACUUACCCT-3′
19	5′-GGGAACAAAGCUGAAGUA$_{PS2}$CUUACCCT-3′
20	5′-GGGAACAAAGCUGAAGUAC$_{PS2}$UUACCCT-3′

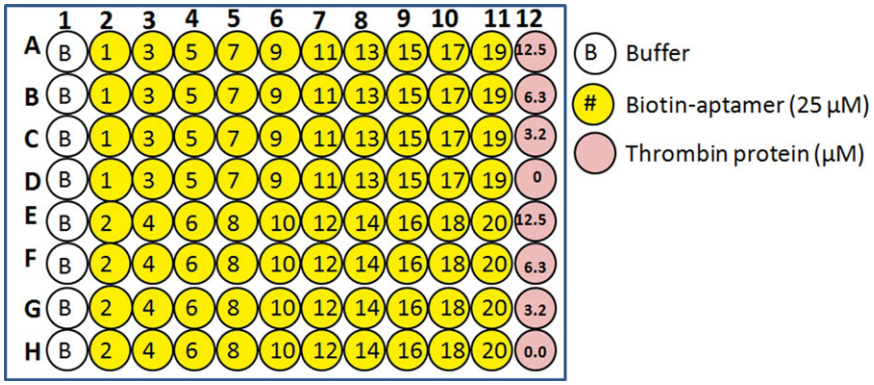

Figure 7.25.14 Plate map diagram for 20 PS2-aptamer affinity screenings.

1. Design plate map diagram (Fig. 7.25.14) for affinity screening.
2. Pre-wet biosensors (see Basic Protocol, step 5).
3. Prepare sample tray (see Basic Protocol, step 6).
4. Place sample plate on the sample plate stage and place biosensor tray on the biosensor stage (see Figure 7.25.6).
5. Set up assays in the instrument (refer to Table 7.25.2).
6. Run assay.

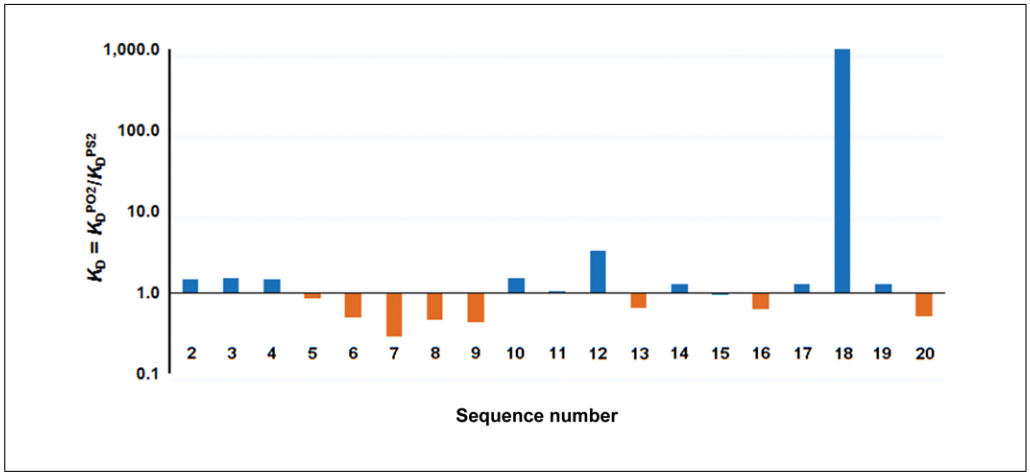

Figure 7.25.15 The relative binding affinities determined by BLI involving a library of RNAs with single-PS2 substitutions based on the native aptamer (1) against thrombin. Blue bars represent enhanced binding, and orange bars represent decreased binding. The numbering given on the *x* axis is described in Table 7.25.3. BLI, biolayer interferometry.

7. Process kinetic data.

Kinetic characterization of each aptamer by BLI allowed affinity ranking of PS2-aptamers with respect to their native counterpart 1. Figure 7.25.15 shows the relative K_D of each variant plotted against the position of each PS2 substitution. For most positions, a single PS2 substitution had only a relatively small effect on protein binding. However, the aptamer with PS2 substituting the phosphate between U17 and A18 (aptamer 18) that resulted in an ~1000-fold enhanced binding affinity ($K_D^{PS2} \approx 2.0$ pM) relative to the native aptamer (aptamer 1; $K_D^{PO2} \approx 2000$ pM).

COMMENTARY

Background Information

The study of nucleic acid-protein interactions is currently one of the most rapidly growing areas of molecular biology. Functional nucleic acid molecules such as aptamers (Tuerk and Gold, 1990; Osborne and Ellington, 1997; Keefe et al., 2010) show a broad range of applications in therapeutics, biosensor, diagnostics, and research. Quantitative determination of aptamer binding affinities can be established by a number of techniques such as the filter binding assay (Woodbury and von Hippel, 1983) and the gel-shift assay (Fried, 1989), which often involve radio-labeling of nucleic acids or aptamers. Recent advances in bioanalytical methods include the ability to develop label-free bioanalytical methods such as surface plasmon resonance (SPR; McDonnell, 2001) and acoustic measurements (Cooper and Whalen, 2005; Godber et al., 2005) for the characterization and analysis of biomolecular interactions. These systems have their limitations since many of them use microfluidics that can become clogged when working with samples that contain cells or particles (Concepcion et al., 2009). Biolayer interferometry (BLI) has been designed to minimize these limitations, while maintaining the ability to accurately measure biomolecular interactions in real time, without the need for labeling. BLI measured in real time provides the ability to monitor binding specificity, rates of association and dissociation, or concentration with precision and accuracy.

Most aptamers derived from large random libraries are capable of binding protein molecules with high affinity and specificity (Ellington and Szostak, 1990; Tuerk and Gold, 1990). Nevertheless, the spectrum of aptamer applications can be broadened by chemically modifying their structure. The phosphate backbone of nucleic acid plays a crucial role in nucleic acid-protein interactions (Varani, 1997; Draper, 1999). Phosphate groups are typically well exposed on the surface of nucleic acids and thus readily available for contacts to binding partners. PS2-DNAs are capable of binding proteins with higher affinities than their native phosphate counterparts as observed by several research labs (Marshall and Caruthers, 1993; Tonkinson et al., 1994; Yang et al., 1999, 2011; Volk et al., 2002; Yang and Mierzejewski, 2010; Zandarashvili et al., 2015). In the case of RNA, the improved

gene silencing activities in vitro and in vivo (Yang et al., 2012; Wu et al., 2014) as a result of introducing two PS2 linkages at the 3′ end of sense strand siRNAs were suggested to be a consequence of the higher affinity of PS2-RNA for Ago2 protein, caused by a hydrophobic effect (Pallan et al., 2014). Invoking a hydrophobic effect that drastically increases the binding affinity between a protein and a modified RNA is the focus of current investigations (Abeydeera et al., 2016).

Critical Parameters and Troubleshooting

The density of immobilized aptamer on the biosensor surface is critical for obtaining high-quality kinetic data. The immobilization of a saturated amount of aptamers on the biosensor surface is not recommended. An excess of aptamer bound to the biosensor can lead to data artifacts due to crowding, steric hindrance, and possible aggregation on the surface. Over-saturation of the biosensor may also promote non-specific interaction. On the other hand, if the loading density of the immobilized aptamer is too low, the signal in the protein binding association step may be too low to detect. Ideally, the loading curve in the loading step should show a gradual increase in signal and should not be allowed to reach saturation (see Figure 7.25.7, aptamer concentration 100 nM). In the association step, running the association step for a long time should be avoided as weaker and non-specific interactions could occur. Conversely, the dissociation step should be run long enough to observe decay in the binding response, particularly for a high-affinity binding interaction.

Non-specific binding is often observed in any assay under any conditions because biological molecules tend to interact with surfaces. Although the biocompatible layer on the biosensors greatly mitigates non-specific binding, some consideration must be given to minimize buffer effects. Generally, pre-wetting the biosensor for at least 10 min in the assay buffer will greatly reduce non-specific binding. Be sure to use the same buffer for baseline, association, and dissociation steps during the assay. When non-specific binding occurs, proper assay optimization and buffer selection can minimize the effects. In most assays measuring nucleic acid-protein interactions, blocking agents such as BSA (up to 1% to 2%) and/or non-ionic detergents such as Tween 20 (up to 0.09%) can be added.

Always include a reference sample (protein concentration = 0) with every kinetics experiment to allow for subtraction of background signal and assay drift. The reference biosensor is used to subtract non-specific binding of protein to the biosensor.

Anticipated Results

Using the protocols presented here, steps to set up an aptamer-protein kinetics assay are simple and straightforward using the Octet RED96 system. In addition, for screening purposes or qualitative analyses, measuring binding curves for a single protein concentration is often sufficient. However, when reliable, accurate kinetic constants are required, a dilution series of at least four protein concentrations (see Figure 7.25.14) should be measured in the association step.

Time Considerations

The amount of time required for testing one single aptamer or several aptamers for a single target assay varies little when using the Octet RED96 instrument. Generally, a reliable binding affinity characterization (such as K_a, K_d, K_D) can be achieved within 1 day if no optimization is needed, such as loading concentration and trouble-shooting the non-specific binding.

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