Chapter 19
Surface Plasmon Resonance Analysis of Interactions Between Replicase Proteins of Tomato Bushy Stunt Virus

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Abstract Replication of the viral RNA genome performed by the viral replicase is the central process during the viral infection cycle (Nagy and Pogany, see earlier chapter four). Most RNA viruses assign one or more proteins translated from their own genomes for assembling the viral replicase complex, which consists of the viral RNA, viral proteins, and several subverted host proteins embedded in cellular membranes. Understanding the various biochemical activities of the replication proteins can lead to target identification for human intervention to control viral infections or the damage to the host cells. The replicase proteins of tomato bushy stunt virus (TBSV) are selected as model system to study the dynamics of interactions between viral replicase proteins using surface plasmon resonance (SPR) analysis. The SPR assay provides real-time protein interaction data by measuring the change in refractive index at the surface of the sensor chip due to the change in mass resulting from the interaction between the immobilized protein and the protein that is being passed over the immobilized chip surface. SPR-based biosensor BIAcore X was used to carry out TBSV replicase protein interaction studies.

Keywords Tomato bushy stunt virus; Replicase proteins; Protein–protein interaction; BIAcore X; Surface plasmon resonance; Protein interaction kinetics

1 Introduction

RNA viruses code for one or more replicase proteins, which are necessary for the replication of their genetic material. The viral replicase is a multisubunit enzyme consisting of virus-coded proteins, including the RNA-dependent RNA polymerase (RdRp), and host proteins (1–3). The viral subunits of the replicase complex usually contain multiple functional domains distributed among them. The interactions between the viral replication proteins are one of the important forces in the assembly and function of the viral replicase complex (4–6). Therefore, identification of domains involved in such interactions and the kinetics of interactions assume greater practical significance for devising viral control strategies.

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Surface Plasmon Resonance (SPR)-based optical biosensors are now widely recognized and are well established tools to obtain quantitative as well as qualitative data on interactions between biological macromolecules (7–9). The kinetics and energetics of interaction data is collected in real time with nanomolar quantities of proteins and it obviates the need for labeling the interacting partners. Although there are several biosensors available in the market, BIAcore instruments are the most widely used.

The principle behind this method is SPR, which measures changes in the refractive index on the sensor chip surface when interactions between the two molecules (one fixed on to the chip surface and the other passed over the immobilized chip surface in a buffer) occur. The change in the refractive index is then plotted in a graph, called sensogram, with time on the *X*-axis and resonance units on the *Y*-axis. The data from the sensogram can be used to extract the association and dissociation constants of protein complexes.

In this chapter, we describe the use of BIAcore X instrument to study the interactions between replicase proteins of tomato bushy stunt virus (TBSV). TBSV is a small, messenger-sense, single-stranded RNA virus (10). The recombinant TBSV replicase proteins p33 and p92 were expressed in *E. coli* as C-terminal fusion to maltose binding protein (MBP). Affinity purification of MBP-p33 and MBP-p92 was done using amylose-resin chromatography. The data collected from the SPR assay were analyzed using BIAevaluation provided by the manufacturer.

2 Materials

2.1 pH Scouting

1. Purified recombinant MBP-p33C protein. We used the C-terminal half of p33, which is soluble when expressed in *E. coli* (6, 11). The protein solution was centrifuged for 15 min at 14,000 *g* to remove any particulate matter before using in BIAcore X instrument to avoid clogging of the microfluidic flow circuit.
2. CM-5 biosensor chip (BIAcore, Piscataway, NJ).
3. Immobilization buffer: sodium acetate, 10 mM, pH 4.0, 4.5, 5.0, 6.0. All buffers and solutions used in BIAcore X instrument are degassed and filtered using 0.22 μm filter. Alternatively, a set of sodium acetate buffers can be purchased from BIAcore Inc, NJ.
4. Running buffer: 10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% Tween-20. This buffer is also available from BIAcore Inc, NJ, as HBS-EP.
5. Wash solution: 50 mM NaOH.

2.2 Protein Immobilization on Sensor Chip

1. Affinity purified recombinant MBP-p33C and MBP proteins.
2. Snake skin dialysis membrane (Pierce).

3. Sodium acetate, 10 mM, pH 5.0.
4. CM-5 sensor chip (BIAcore, Piscataway, NJ).
5. Amine coupling kit (BIAcore Inc, NJ). It includes 115 mg *N*-hydroxy-succinimide (NHS), 750 mg 1-ethyl-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), and 10.5 ml 1 M ethanolamine–HCl pH 8.5. Stock solutions of 0.1 M and 0.4 M NHS and EDC, respectively, are prepared by resuspending the salt in sterile water and stored in single-use aliquots at −20 °C.
6. BIA normalizing solution from BIAcore Inc, NJ. This solution contains 70% (w/w) glycerol.
7. Running buffer: 10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.05% Tween-20.
8. Wash solution: 50 mM NaOH.

2.3 Interaction Analysis

1. Affinity purified recombinant MBP-p33C and MBP proteins.
2. Running buffer: 10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.05% Tween-20. This buffer can also be purchased from BIAcore Inc, NJ.
3. Regeneration buffer: 50 mM NaOH.

2.4 Data Analysis

1. BIAevaluation 4.1 software from BIAcore Inc, NJ.

3 Methods

There are four major steps in BIAcore-based SPR experiments suitable for analysis of protein–protein interactions: (1) obtaining pure protein samples, (2) immobilizing one of the binding partners on the chip surface, (3) running the other protein (or the same protein if self-interaction is the goal of the study) over the immobilized surface of the chip to obtain the original data, and (4) data analysis. The second and third steps involve the core BIAcore X instrument and a computer (PC), which operates the BIAcore X control software. Users are required to inject samples and buffers to the injection port on the instrument and run the software. The data analysis is done using BIAevaluation software 4.1 provided with the instrument. The analyzed data can be exported as spreadsheet and nicely presentable graphs can be made using Microsoft Excel software.

3.1 Familiarize with BIAcore X Instrument and the Software

Before starting the actual SPR assay, it is very important to first get familiarized with the basic operations of the BIAcore system, especially docking/undocking of sensor chip, sample loading, BIAcore X control software, and BIAevaluation software. The "BIAcore X getting started" kit from BIAcore Inc, NJ, is also very helpful for the first time user for training in the basic procedures involved in using the instrument. The kit includes all the buffers, reagents, and proteins.

3.2 Preparation of Protein Samples for the SPR Assay

1. TBSV replicase proteins, namely p33, p92, and their truncated versions, were expressed in *E. coli* as MBP fusion and purified using amylose resin affinity chromatography (6, 11).
2. The concentration of proteins is quantified using Bio-Rad protein assay reagent.
3. The purity of the protein samples were tested in SDS-polyacrylamide gel electrophoresis (SDS-PAGE). If there are multiple proteins in the sample, the accuracy of the kinetic data obtained with such protein samples may not be correct.
4. To keep the buffer composition of the protein solution as close to buffers used in SPR assay as possible, the proteins are dialyzed against BIAcore assay buffers. The proteins to be fixed on sensor chip are dialyzed against 10 mM sodium acetate buffer pH 5.0 and the proteins to be passed over the chip surface are dialyzed against running buffer in a snake skin dialysis membrane – 10 kDa molecular weight cut off (Pierce). Alternatively, the protein samples can be diluted in appropriate SPR assay buffers.

3.3 pH Scouting to Determine Appropriate Immobilization pH

pH scouting is referred to the experimental procedure of finding the appropriate immobilization buffer pH and is performed on new CM-5 chip without modifying its surface. The level of protein immobilization is affected by the electrostatic attraction of proteins to the chip surface and this attraction is referred to as preconcentration. The carboxymethylated dextran matrix of CM-5 chip is negatively charged at pH values above approximately 3.5. Therefore, the pH of the immobilization buffer should be above 3.5 and below the isoelectric point of the protein – in our case 5.5 for MBP-p33C to achieve efficient immobilization.

1. The CM-5 sensor chip should be equilibrated at room temperature 30 min before use.
2. Switch on the BIAcore X instrument and the PC and run the BIAcore X control software.
3. The buffer inlet tube is inserted into priming buffer and an empty beaker is placed under waste outlet to collect the liquid waste.

4. A "Dock" dialogue box will appear when the software is started and there is no chip in the instrument. To dock the sensor chip, open the chip port cover, pull the slider, place the chip in the slider and push the slider into the instrument, close the port cover, and click "Dock" in the software.
5. Prime the instrument using running buffer three times. A "Prime" dialogue box from the software guides users to complete this step.
6. When new chip is docked, it is important to normalize the signal response using BIAnormalizing solution. Choose Tools > Working Tools > Normalize and inject 100 µl BIAnormalizing solution.
7. Dilute the MBP-p33C protein to 1 µM in immobilization buffers at different pH levels, such as pH 4.0, 4.5, 5.0, and 6.0.
8. Start a sensogram by choosing Run > Run Sensogram. In the "Flowcell" dialogue box, choose single "Detection mode" and FC1 (Flow cell 1) "Flow Path" and set the flow rate at 10 µl min^{-1}.
9. Inject 10 µl of 1 µM MBP-p33 protein diluted in immobilization buffer pH 4.0, following sample injection procedures recommended by the manufacturer.
10. After the baseline of the sensogram is stabilized, repeat the injections with MBP-p33 diluted in immobilization buffer pH 4.5. Follow this step for MBP-p33 diluted in buffers at pH 5.0 and 6.0
11. After all the sample applications, inject 10 µl wash solution (50 mM NaOH) to clean the sample loop off the protein samples, stop data collection by selecting Run > Stop Sensogram, and save the data.
12. Compare the difference in electrostatic attraction between different buffers as shown in the sensogram in Fig. 1. Buffers at pH 4.5 and 5.0 facilitate highest preconcentration of MBP-p33C protein on chip surface. Since amine coupling is more efficient with uncharged amino groups on the protein at higher pH values and the theoretical pI of MBP-p33C is 5.5, we chose pH 5.0 for our immobilization procedure.

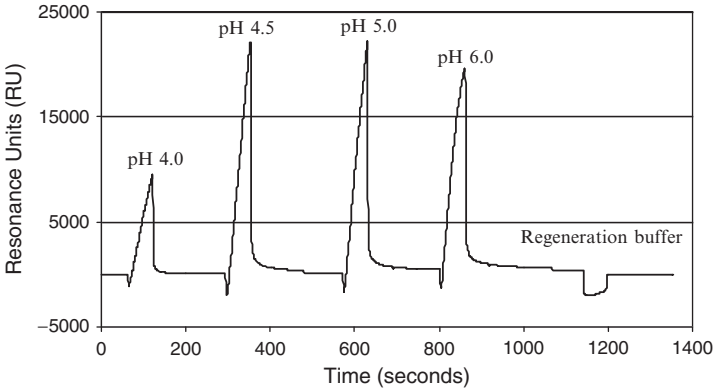


Fig. 1 The sensogram shows the effect of the pH of the immobilization buffer on electrostatic attraction of MBP-p33C to the chip surface

3.4 Immobilization of MBP-p33C on CM-5 Sensor Chip

Immobilization is performed using amine coupling kit from BIAcore following the manufacturer's protocols. An "Amine Coupling" surface preparation procedures wizard guides the user through the immobilization procedure with instructions for every step. BIAcore X divides the chip surface into two flow cells, each of which can be used to fix different proteins. We immobilized 8,000 RU MBP-p33C in Flow cell 1 (Fc1) and 8,000 RU MBP in Fc2 for qualitative (yes/no) binding analysis.

1. Thaw EDC and NHS stock solutions on ice and place ready-to-use ethanolamine–HCl on ice.
2. Choose Surface preparation procedures from "Tools" menu and select "Amine Coupling".
3. In the "Flow Cell" box, select "single" detection mode and "Fc1" flow path. Set the flow rate to 5 µl min^{-1}.
4. Mix NHS and EDC to 1:1 and inject 35 µl (7 min). This step activates the dextran matrix to give reactive esters.
5. Inject 35 µl of 1 µM MBP-p33C diluted in immobilization buffer pH 5.0.
6. Inject 35 µl ethanolamine–HCl to deactivate any remaining active esters.
7. Inject 5 µl of regeneration buffer (50 mM NaOH) to condition the immobilized chip surface. Stop the sensogram and save the data.
8. Using View > Baseline and View > Reference Line commands in BIAcore X control software determine the level of MBP-p33C bound to the chip (Fig. 2). This level is used to calculate the theoretical maximum binding capacity (R_{max}) of immobilized chip surface.
9. Repeat step 2–8 to prepare control surface in Flow cell 2 with MBP. This control surface is used to filter out noises in the experiment that result from nonspecific binding of proteins to chip surface or bulk effect due to differences in running buffer and protein solution.
10. For kinetic studies, we immobilized 250 RU of MBP-p33C in FC 1 and MBP in FC2 by following the steps 2–9 (12).

3.5 Binding Analysis

The chip prepared with one of the binding partner immobilized on its surface can be used for up to 100 times in binding assays. We prepared p33C protein chip and tested several mutant proteins of p33 and p92 proteins for their interaction with p33C. Here we describe the self-interaction between p33C molecules.

3.5.1 Qualitative Binding Analysis

1. Dock the CM-5 chip immobilized with 8,000 RU MBP-p33C in Flow cell 1 and 8,000 RU MBP in Flow cell 2 (6).

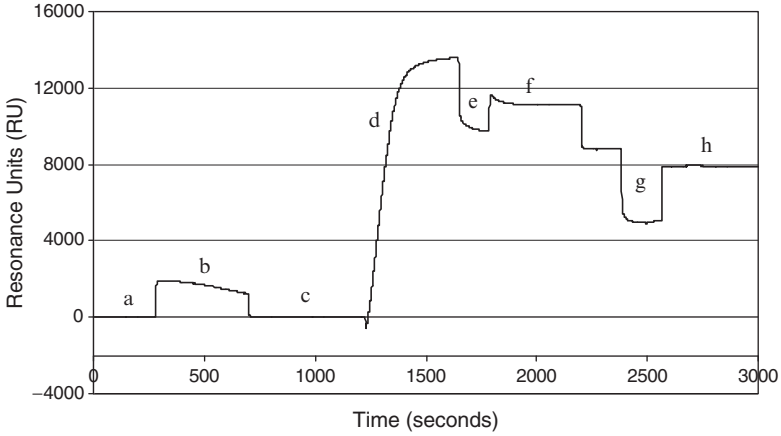


Fig. 2 Sensogram showing MBP-p33C immobilization. (a) Starting baseline, (b) EDC/NHS activation of dextran matrix, (c) baseline after surface activation, (d) covalent coupling of MBP-p33C to dextran matrix, (e) loosely bound MBP-p33C is washed away by immobilization buffer, (f) deactivation by ethanolamine, (g) surface conditioning by regeneration buffer (50 mM NaOH), (h) actual immobilization level of MBP-p33C, which is 8,000 RU in this case

2. Prime the system with running buffer as guided by the "Prime" wizard in the software.
3. Start a sensogram from BIAcore X control software and in the "Flowcell" dialogue box choose multichannel detection mode, flow path Fc1–2 and Fc2 (MBP) as reference cell.
4. Set the flow rate at 20 µl min^{-1}.
5. Dilute MBP-p33C to 1 µM in the running buffer.
6. Inject 60 µl (3 min) of 1 µM MBP-p33C in the running buffer over the p33C chip surface. The protein flows through both Fc1 (coupled with p33C) and Fc2 (coupled with MBP) and a sensogram for each flow cell is generated in real time.
7. After 3 min of protein injection, allow the running buffer to pass for additional 3 min to record the dissociation phase of protein interactions.
8. Inject 10 µl of regeneration buffer to remove any bound proteins and bring the chip surface back to base level for the next binding assay.
9. Stop the sensogram and save the data. Undock the sensor chip from the instrument and store it in refrigerator.
10. Open the data file in BIAevaluation software, select both sensogram Fc1 and Fc2 to display graph, remove any air spikes and the regeneration phase data set to highlight only the association and dissociation phases of the interactions in the graph as shown in Fig. 3a,b.
11. Zero baseline on *Y*-axis using "Y-transform" menu.
12. Subtract the experimental data (MBP-p33C) from reference surface (MBP) using Y-transform menu. Any nonspecific binding of p33C to MBP or dextran matrix chip surface or bulk effect resulting from differences in running buffer and protein solution is canceled out by this subtraction based on data from the reference surface.

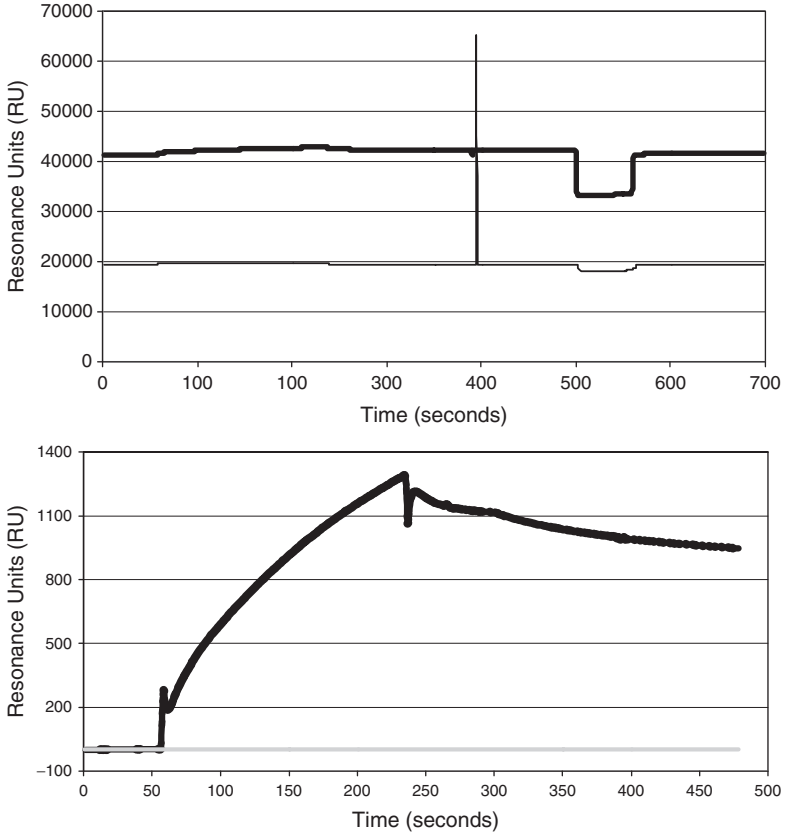


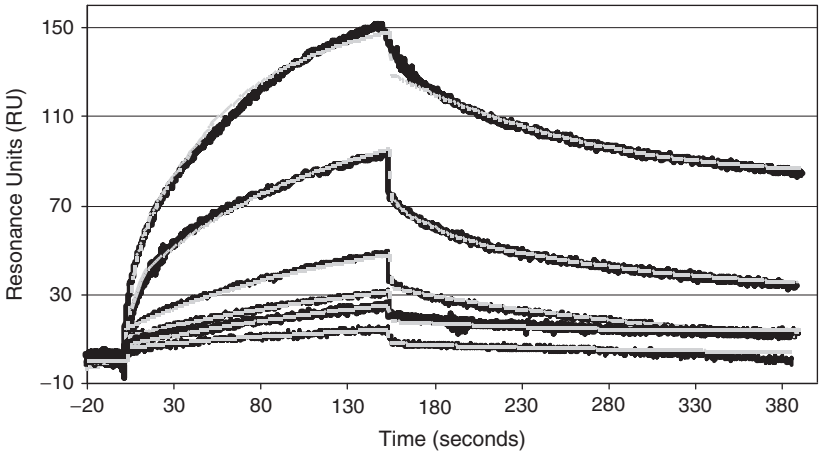
Fig. 3 (**a**) Sensogram as generated in real time by BIAcore X control software. The darker line represents data from p33C-coupled surface (Fc1) and lighter line represents data from MBP-coupled reference surface (Fc2). Note that the air spike in Fc2 and also the regeneration phase burry the most important data showing association and dissociation phases of the binding curve. (**b**) Sensogram after processing by using BIAevaluation 4.1 software. The experimental sensogram (Fc1) is subtracted from reference sensogram (Fc2) and the air spikes and regeneration phases are removed

13. Export the resultant data file after the above modifications as text document and use Microsoft Excel program to make the necessary changes to prepare publication quality graphs.

3.5.2 Kinetic Analysis

1. Dock the CM-5 chip immobilized with 250 RU MBP-p33C in Flow cell 1 and 250 RU MBP in Flow cell 2 (12).
2. Prime the system with running buffer as guided by the "Prime" wizard.
3. Start a sensogram from BIAcore X control software and in the "Flowcell" dialogue box choose multichannel detection mode, flow path Fc1–2 and Fc2 (MBP) as reference cell.
4. Set the flow rate at 40 µl min^{-1}.

5. Dilute MBP-p33C to 25, 50, 75, 100, 250, and 500 nM in the running buffer.
6. Inject 100 µl (2.5 min) of 25 nM MBP-p33C in the running buffer over the p33C chip surface. The protein flows through both Fc1 (coupled with p33C) and Fc2 (coupled with MBP) and a sensogram for each flow cell is generated in real time.
7. After protein injection, allow the running buffer to pass for additional 3 min to record the dissociation phase of protein interactions.
8. Inject 10 µl of regeneration buffer to remove any bound proteins and bring the chip surface back to base level for the next binding assay.
9. Stop the sensogram and save the data.
10. Repeat steps 6–9 for 50, 75, 100, 250 and 500 nM p33C proteins.
11. Open the data file in BIAevaluation software, select both sensogram Fc1 and Fc2 to display graph, remove any air spikes and the regeneration phase data set to highlight the association and dissociation phases of the interactions in the graph.
12. Zero baseline on *Y*-axis using "Y-transform" menu.
13. Subtract the experimental data (MBP-p33C) from data obtained with the reference surface (MBP) using Y-transform menu and save.
14. Repeat steps 12 and 13 for sensograms generated for each protein concentration.
15. Add all the reference-subtracted sensograms from step 13 in one data file and overlay using "Overlay Plot" menu.
16. Align the curves on X-axis to the injection-start time point using X-transform menu.
17. Fit the data using "Simultaneous ka/kd" menu and follow the instructions as guided by this application wizard.
18. Evaluate the curve-fitting first by visual assessment of deviations of experimental data from mathematically fitted data. The data shown in Fig. 4 represent a well-fitted experimental data.



	$K_{on}(10^4 M^{-1}s^{-1})$	K_{off} $(10^{-3}s^{-1})$	K_D $(10^{-7}$ M)
p33C (151-296)	2.11	4.74	2.25

Fig. 4 Fitted Kinetic data showing interaction between p33C–p33C proteins. Varying concentrations of highly purified p33C were used (25, 50, 75, 100, 250, and 500 nM) to obtain the above data. The experimental data is fitted to 1:1 binding model using BIAevaluation software. The dark line represents experimental data and the grey line represents mathematical fitting. The table below shows the kinetic parameters derived from experimental data

19. Export the resultant kinetic data file after fitting as text document and use Microsoft Excel program to make the necessary changes to prepare publication quality graphs.

4 Notes

1. Before starting the BIAcore X instrument, go through the maintenance logbook to be sure that the instrument is in good condition and the signal quality in the flow cells is good. Desorbing and sanitization need to be done to clear the flow path off protein or other residues.
2. The purity of the protein is a very important factor for kinetic analysis. The active concentration of impure sample is very difficult to quantitate. Impure samples also contribute to nonspecific binding and bulk effect. Therefore, it is essential to use highly pure protein samples for kinetic analysis. Purity is not a major problem in qualitative (yes/no) type of binding assay provided a reference surface is prepared with a known nonbinding mutant protein.
3. The response signal of the protein being passed on the chip surface is dependent on protein molecular weight. Therefore, BIAcore recommends immobilizing the lower molecular weight proteins onto the chip.
4. The nonspecific binding of active proteins or impurities to chip surface or to immobilized protein on the chip and bulk refractive change due to differences in buffer composition during association phase can be normalized by subtracting the reference cell response from experimental data. Therefore, reference cell preparation assumes critical importance in SPR experiments. The good reference surfaces are the ones immobilized with mutant test protein that lost binding ability. In our case, we found that MBP in reference cell works well for this purpose because all our assay proteins are MBP fusions.
5. All the buffers used for SPR experiments should be filtered and degassed daily to avoid clogging the micro flow circuit. If the buffers are not degassed, there will be frequent air spikes in the sensogram making it difficult to get reproducible information from the experimental data.
6. Biochemical characteristics such as aggregation state of proteins and stoichiometry of the protein interaction influence the outcome of the kinetic analysis. We checked the heterogeneity of our protein samples in gel filtration column and found majority of the proteins eluted in a single peak.
7. Regeneration conditions in our experiments are different for different binding partners. In general, 50 mM NaOH worked well. But for some truncated p33 mutant proteins, we used 10 mM glycine–HCl pH 2.5, which provides milder regeneration condition than 50 mM NaOH. BIAcore sells a set of regeneration buffers and the BIAcore handbooks also list many other regeneration solutions to try.

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