

A Surface Plasmon Resonance Method to Study HCV NS5B Inhibitors

Melanie Wong and Giuseppe A. Papalia*

Biology Department, Gilead Sciences, Inc, Foster City, USA

*For correspondence: jpapalia@gilead.com

[Abstract] Surface Plasmon Resonance (SPR) technology is a well-established platform used to evaluate the kinetic parameters of protein-small molecule interactions. Below, we describe the use of the ProteOn XPR36 biosensor from Bio-Rad (Hercules, CA) to evaluate the binding of small molecule inhibitors to recombinant NS5B protein. The high pI (> 9) of this construct allows for chemical immobilization using HEPES-buffered saline at pH 7.5. This is in contrast to traditional biosensor protocols that use both low pH and ionic strength. The use of a more physiological buffer to immobilize this enzyme leads to improved surface activity.

Materials and Reagents

1. HCV NS5B inhibitors
2. Purified recombinant HCV NS5B Δ C21 soluble protein [cloned and purified according to Boyce *et al.* (2014) and Hung *et al.* (2011)]
3. ProteOn GLH Sensor Chip (Bio-Rad Laboratories, catalog number: 176-5013)
4. HEPES solution (1 M) (Sigma-Aldrich, catalog number: H3537-1L)
5. $MgCl_2$ (1 M) (Sigma-Aldrich, catalog number: M1028-100ML)
6. EDTA (0.5 M) (Sigma-Aldrich, catalog number: E7889-100ML)
7. NaCl (5 M) (Sigma-Aldrich, catalog number: S6546-1L)
8. KCl (2 M) (Life Technologies, Ambion[®], catalog number: AM9640G)
9. Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) (Sigma-Aldrich, catalog number: C4706-10G)
10. Surfactant P20 (10% v/v) (General Electric Company, catalog number: BR-1000-54)
11. DMSO (Sigma-Aldrich, catalog number: 472301-100ML)
12. ProteOn Amine Coupling Kit (Bio-Rad Laboratories, catalog number: 176-2410) containing 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC or EDC), sulfo-N-hydroxysuccinimide (Sulfo-NHS) and ethanolamine-HCl
13. Preconditioning Reagents (see Recipes)
14. Immobilization Buffer (see Recipes)
15. Running Buffer (see Recipes)
16. Running Buffer with 5% DMSO (see Recipes)

Equipment

1. ProteOn Standard & Deep-Well Microplates (Bio-Rad Laboratories, catalog numbers: 176-6020 and 176-6023)
2. ProteOn Microplate Sealing Film (Bio-Rad Laboratories, catalog number: 176-6040)
3. Nalgene Rapid-Flow Filter Units and Bottle Top Filters, PES Membrane, Sterile (VWR International, catalog number: 16211-056) (for the 1 L size)
4. ProteOn XPR36 instrument (Bio-Rad Laboratories)
5. BenchTop Centrifuge for microfuge tubes
6. BenchTop Centrifuge to spin ProteOn 96-well assay plate

Software

1. ProteOn Manager Software Version 3.1.0.6
2. Scrubber software designed for ProteOn data analysis (BioLogic Software)

Procedure

1. Prime the system with distilled water. Precondition a GLH chip using 1-min pulses each of 100 mM HCl, 50 mM NaOH, 0.5% SDS and 10% DMSO using a flow rate of 30 μ l/min in both the horizontal and vertical directions. Seal all microplates used with ProteOn sealing film.
2. Prime the system with Running Buffer. Activate all 6 ligand channels (L1-L6) in the vertical direction using EDC and sulfo-NHS as prescribed by the manufacturer using a flow rate of 30 μ l/min and a contact time of 5 min.
3. Dilute NS5B protein in Immobilization Buffer to a concentration of 30 μ g/ml and inject using a flow rate of 30 μ l/min for 5 min in the vertical direction into the ligand channel (s) to obtain 5,000-10,000 RU of immobilized protein (see Notes). Leave the L1 channel open as a reference channel.
4. When calculating the theoretical maximum response, R_{\max} , the activity of the protein should be taken into account. R_{\max} is calculated as $R_L * (MW_A/MW_L) * n$, where R_L is equal to the amount of ligand immobilized, MW_A is the molecular weight of the analyte, MW_L is the molecular weight of the ligand, and n is the number of binding sites per ligand molecule. Assuming single-site binding (*i.e.* $n = 1$), a small molecule analyte having an average molecular weight of 500 Da interacting with a surface comprised of 10,000 RU of NS5B Δ C21 (M.W.~ 64,300 Da) should yield a theoretical R_{\max} of ~ 78 RU (*i.e.* 10,000 RU x 500 Da/64,300 Da) or 39 RU if the surface is only 50% active.

5. Deactivate all vertical channels (L1-L6) by injecting ethanolamine-HCl for 5 min at flow rate of 30 μ l/min.
6. Rotate the multi-channel module (MCM) as all subsequent injections will occur in the horizontal direction. Prime the system with Running Buffer containing 5% DMSO and also use this buffer to inject buffer three times to help stabilize the system after rotation.
7. To account for excluded volume effects, a set of five solutions between 4.5% and 5.5% DMSO (DMSO concentration series) are injected for the software to generate a DMSO excluded volume

calibration (EVC) curve. Prepare a 5.5% DMSO solution by adding 15 μ l of 100% DMSO to 3,000 μ l of Running Buffer with 5% DMSO, and a 4.5% DMSO solution by adding 333 μ l of Running Buffer to 3,000 μ l of Running Buffer with 5% DMSO. Mix the 5.5% and 4.5% DMSO solutions according to the volumes prescribed below to obtain the five different solutions. Five separate injections are performed to generate a DMSO concentration series each corresponding to the injection of one of the DMSO solutions. The software will use this series of injections to generate a DMSO calibration curve to correct for excluded volume effects.

5.5% DMSO buffer	4.5% DMSO buffer
1200 μ l	0 μ l
900 μ l	300 μ l
600 μ l	600 μ l
300 μ l	900 μ l
0 μ l	1200 μ l

8. When designing the analyte injection sequence, include buffer injections between every injection of compound (or as space permits in the 96-well plate).
9. Thaw compounds, typically stored in 100% DMSO in microfuge tubes at -20 $^{\circ}$ C, and quickly spin them in a microfuge centrifuge.
10. Serially dilute the compounds in DMSO to achieve a set of five concentrations (four-fold dilutions in this example) at 20 times the desired final assay concentration. Transfer the diluents to wells in the microplate containing an appropriate volume of Running Buffer to yield a final DMSO concentration of 5% (*i.e.* matching the % of DMSO in Running Buffer with DMSO). These will be injected as Analytes A2-A6. Add 100% DMSO to the same volume of Running Buffer to the well corresponding to injection of Analyte A1 to yield a final DMSO concentration of 5%. Injection from well A1 will serve as a blank and be used to double-reference the data.
11. Centrifuge the plate using a quick 15-30 sec pulse to ensure collection of any compound solution stuck to the walls of the microplate well.

12. Inject compounds at 100 $\mu\text{l}/\text{min}$ for a contact time of 90 sec and for variable dissociation times ranging between 60 sec and one hour (as required by individual compounds). Absent any knowledge about a compound's affinity, preliminary experiments involving injection at a single concentration may have to be performed in order to gauge optimal dissociation times for each compound.
13. Data can be analyzed using ProteOn Software but can also be analyzed using a version of Scrubber software designed for ProteOn data. Double reference all signals (using L1 & A1) and correct for excluded volume effects by applying the DMSO calibration curve.
14. Sensorgrams for all the concentrations tested should be fit simultaneously, *i.e.* globally. In general, data should be always first fit to a simple 1:1 kinetic model which yields the equilibrium dissociation constant (K_D), the maximum binding capacitance of the surface R_{max} , (which may differ from the theoretical maximum binding capacitance) and the rate constants of association (k_a) and dissociation (k_d). In a simple 1:1 kinetic model, single values of k_a , k_d and R_{max} are obtained. Both Scrubber and ProteOn fitting routines solve for the parameters k_a and k_d . K_D is then calculated as the ratio of k_d/k_a . Use of other more complex models that require more than one value for any of these parameters should be explicitly stated. Filibuvir binding to NS5B ΔC21 (see Figure 1) is an example of a small molecule binding showing simple 1:1 kinetics.

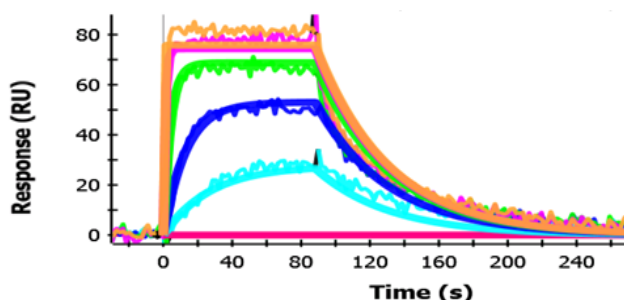


Figure 1. Filibuvir Binding to NS5B ΔC21 . Sensorgrams of Filibuvir binding are shown. Filibuvir was injected for a contact time of 90 sec at concentrations of (from top to bottom) 4 μM (orange), 1 μM (pink), 0.25 μM (green), 0.0625 μM (blue) and 0.0156 μM (cyan). Dissociation was monitored for 180 sec. The fit to the data are shown as solid lines.

Notes

The physiological immobilization buffer used can readily pre-concentrate sufficient NS5B enzyme onto the carboxyl surface. The ligand activity increased from 45% to 99% for

NS5B Δ C21 when opting to dilute protein in HEPES-buffered saline rather than standard low pH (5.5) 10 mM sodium acetate immobilization buffer.

Recipes

1. Preconditioning Reagents

100 mM HCl

50 mM NaOH

0.5% SDS

10% DMSO

2. Immobilization Buffer

10 mM HEPES (pH 7.5)

150 mM NaCl

Filter sterilize (0.45 μ m)

3. Running Buffer

50 mM HEPES

5 mM MgCl₂

10 mM KCl

1 mM EDTA

1 mM TCEP

0.01% P20

Adjust pH to 7.5 with NaOH

Add dH₂O to the desired volume

Filter sterilize (0.45 μ m)

4. Running Buffer with 5% DMSO

Add 100% DMSO to Running Buffer to a final concentration of 5% DMSO

For example, add 50 ml of DMSO to Running Buffer to a total volume of 1 L.

Acknowledgments

A brief description of this protocol has previously appeared in Boyce *et al.* (2014).

References

1. Boyce, S. E., Tirunagari, N., Niedziela-Majka, A., Perry, J., Wong, M., Kan, E., Lagpacan, L., Barauskas, O., Hung, M., Fenaux, M., Appleby, T., Watkins, W. J., Schmitz, U. and Sakowicz, R. (2014). [Structural and regulatory elements of HCV NS5B polymerase - beta-](#)

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2. Hung, M., Wang, R. and Liu, X. (2011). [Preparation of HCV NS3 and NS5B proteins to support small-molecule drug discovery.](#) *Curr Protoc Pharmacol* Chapter 13: Unit13B 16.