Study Title	Example Final Report: Biacore SPR Evaluation
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Study Number	GBL_0000
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Report Reviewed by	Internal reviewer
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Contents

Signatures

The following project manager(s) were responsible for the overall conduct of this nonclinical laboratory study and for the data reported herein.

Project Manager:

Name:	Name of Principal Investigator, Ph.D.
Title:	Principal Investigator
Date:	

Background

The aim of the study is to determine the binding parameters (K_{on}, K_{off} and binding affinity, K_D) for angiotensin II (AT2) binding to Angiotensin Converting Enzyme 2 (ACE2) using Surface Plasmon Resonance (SPR). Additionally, using solution phase competition binding, to assess SARS-CoV2-Spike binding to immobilised ACE2 in the presence of soluble ACE2 or AT2.

Methods

Materials

Materials used in this study are summarised in Tables 1 and 2.

SPR Sensor Preparation

All experiments were performed using a Biacore T200 and carried out at 25 °C. The instrument was desorbed before use with the Biacore automated 'Desorb' routine and desorb solutions 1 and 2. A CM5 sensor was docked, running buffer changed to HBS-T and the sensor cleaned with a 30 second injection of 50 mM NaOH (10 µl/min flow rate).

Immobilisation pH Scouting

ACE2 protein was prepared at 20 µg/ml in 10 mM sodium acetate buffer at pH 5.5, 5.0, 4.5 or 4.0. ACE2 protein was injected onto the sensor surface at 10 µl/min for 120 seconds to determine the optimum pH for protein accumulation onto the surface. Sodium acetate buffer at pH 4.5 was selected as optimal for immobilisation. The sensor surface was then cleaned with a 30 second injection of 50 mM NaOH.

Ligand Immobilisation

The reference or active channel surface was activated for 7 minutes with a 1:1 mixture of 200 mM EDC and 50 mM NHS. ACE2 (in 10 mM sodium acetate immobilisation buffer at pH 4.5) was coupled to the surface, and then all unreacted EDC-NHS groups were inactivated with a 7 minute injection of 1 M ethanolamine.

The reference channel surface was prepared with EDC-NHS as above, followed by ethanolamine capping.

Protein immobilisation conditions are summarised in Table 3.

Binding Reproducibility Test

Running buffer was changed to HBS-ET. SARS-CoV2-Spike protein (spike) was prepared at 10 nM in HBS-ET and injected onto both sensor surfaces at 30 µl/minute for 2 minutes to observe protein binding. It was then allowed to dissociate for 5 minutes. The surface was regenerated by injecting Pierce Gentle Ag/Ab Elution buffer at 30 µl/minute for 10 seconds. Two further spike injection and regeneration cycles were carried out to confirm the

reproducibility of the binding response on successive binding cycles.

Binding cycle times are summarised in Table 4.

Kinetic Affinity Assays

AT2 was prepared as 625 nM solutions in HBS-ET and then diluted two-fold in series to give 625, 312.5, 156.3, 78.1, 39.1, 19.5, and 9.8 nM solutions. The lowest concentration of AT2 solution was injected onto both sensor surfaces at 30 µl/minute for 2 minutes to observe protein binding. It was then allowed to dissociate for 5 minutes. A regeneration step comprising a 10 second injection of Pierce Gentle Ag/Ab Elution Buffer, pH 6.6 was used after the protein dissociation period, followed by a fluidics cleaning step using 50% DMSO in running buffer, followed by a stabilization period of 60 seconds in running buffer, before beginning the next protein injection cycle. Buffer blanks were included before the lowest and after the highest protein concentration.

Analytes tested and assay binding cycles are summarised in Table 4.

Solution Phase Competition

Spike (10 nM) was prepared in HBS-ET alone or mixed with either AT2 (3 µM) or ACE2 (140 nM). Spike (10 nM) alone was injected at 30 µl/minute for 2 minutes to observe protein binding. It was then allowed to dissociate for 5 minutes, followed by regeneration as described above. A mixture of either spike and ACE2 or spike and AT2 was then injected using the same method to determine the competition binding response. This was followed by ACE2 or AT2 alone to determine their binding responses (if any) in the absence of spike. Finally, a repeat of spike alone was used to show consistency / reproducibility during the assay. Buffer blanks were included as the first and last cycles.

To determine if spike and AT2 bind to ACE2 at the same or different sites, samples of spike (10 nM) and AT2 (3 µM) were prepared as above. Spike alone was injected at 30 µl/minute for 2 minutes onto both surfaces to observe binding to ACE2. As soon after the spike injection as permitted by the instrument's sample handling process (about 120 seconds later) AT2 (3 µM) was injected (while a significant proportion of the spike protein remained bound to ACE2), to determine the effect of already-bound spike on AT2 binding. After a 5 minute dissociation period, the sensor was regenerated as above. The process was repeated injecting first spike then buffer (instead of AT2) to determine the response to spike alone. The process was then repeated, injecting buffer first (instead of spike) then AT2 to determine the binding of AT2 alone.

Finally, two sequential buffer injections were used to act as a cycle subtraction reference.

Data Analysis

Binding response data were analysed using Biacore T200 Evaluation software. Binding responses were reference channel subtracted (i.e., Fc2-Fc1) and buffer blank subtracted (double referenced).

Kinetic affinity: Data were globally fitted to a single-site binding model to determine binding association rate (k_a), dissociation rate (k_d) and kinetic affinity (K_D). Kinetic affinity values are reported in Table 5 along with Chi^2 values to indicate goodness of fit.

Equilibrium binding affinity: Data were also fitted to an equilibrium binding model. Binding response amplitudes averaged over the final 5 seconds of the injection period were fitted to a saturation binding curve, from which the R_{max} and K_D (concentration at half R_{max}) were determined. Values are reported in Table 5 along with Chi^2 values to indicate goodness of fit.

Solution phase competition: Data for each ligand injected alone or in combination with the second ligand were exported as *.csv files, imported in MS Excel and plotted. Where there was observable binding inhibition (Figure 6), response amplitudes for the ligands alone and when mixed were averaged over the final 5 seconds of the binding response. %Inhibition was calculated as:

[1 – (Measured Response / Theoretical Response)] x 100

Where observed, %Inhibition values are given in Table 6.

Where no binding inhibition was apparent, responses for each ligand injected alone were mathematically added to give the theoretical combined response. This was then compared against the actual response achieved when both ligands were injected mixed together or injected in sequence, to confirm the lack of inhibition.

Abbreviations

Angiotensin II (AT2); Angiotensin Converting Enzyme 2 (ACE2); SARS-CoV2-Spike (spike); HEPES Buffered Saline (HBS) with EDTA and 0.05% Tween 20 (HBS-ET); Flow-channel (Fc); Response Units (RUs); Association rate or on-rate (k_a or k_{on}); Dissociation rate or off-rate (k_d or k_{off}); Kinetic or equilibrium binding affinity constant (K_D).

Table 1. SPR Reagents

Name	Supplier	Cat no.	Lot no.	Composition
Biacore T200 instrument	Cytiva			
Series S CM5 sensor	Cytiva	100530	10344754	
Running Buffers (see each assay):	(in house)			All 0.22 µm filtered
HEPES buffered saline + 0.05% Tween 20 (HBS-T)				150 mM NaCl, 10 mM HEPES, pH 7.4 + 0.05% Tween 20
HEPES buffered saline + EDTA + 0.05% Tween 20 (HBS-ET)				150 mM NaCl, 10 mM HEPES, 3 mM EDTA, pH 7.4 + 0.05% Tween 20
Amine coupling kit:	Cytiva	BR100050	30649	
1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)				400 mM, aliquots stored at -20 °C, thawed on ice
N-hydroxy-succinimide (NHS)				100 mM, aliquots stored at -20 °C, thawed on ice
Ethanolamine				1 M, pH 8.5, aliquots stored at -20 °C, thawed on ice
Desorb solution 1	(in house)			Sodium dodecylsulphate (SDS), 0.5% aq. soln., 0.22 µm filtered
Desorb solution 2	(in house)			Glycine, 50 mM, pH 9.5, 0.22 µm filtered
NaOH for cleaning	(in house)			50 mM, 0.22 µm filtered
pH scouting and immobilisation solutions	(in house)			10 mM sodium acetate at pH 5.5, 5.0, 4.5 or 4.0, 0.22 µm filtered
Regeneration solutions	ThermoFisher	21027	IF114632	Pierce Gentle Ag/Ab Elution Buffer pH 6.6, 0.22 µm filtered

Table 2. Experimental Ligands and Analytes

Name	MW (Da)	Supplier	Cat no.	Lot no.	Quantity (µg)	Volume (µl)	Conc. (µg/ml)	Conc. (µM)	Reconstitution Date
Human ACE2, Fc-tagged	111,700	Acro Biosystems	AC2-H5257	C487P1-211KF1-XW	50	159	315	2.82	16/06/2020
Angiotensin 2	1046.2	Tocris	1158	24B	5,000	125	3,891	10,000	13/05/2020
SARS-CoV2-Spike	134,600	Acro Biosystems	SPN-C52H4		100	53	1,884	1.4	16/06/2020

Table 3. Summary of Ligand Immobilisation by Amine Coupling

Study Stage	Flow Channel	Sensor	Ligand	Immobilisation Method	Ligand Conc. (µg/ml)	Immobilisation Buffer pH	Injection Time (s)	RUs Immobilised
1	Fc1	CM5 2020-09-06	Activated-Capped	Amine coupling	NA	NA	EDC: 420 NHS: 420	NA
1	Fc2	CM5 2020-09-06	Fc-tagged ACE2	Amine coupling	20	4.5	360 (120+240)	9500

Table 4. Analyte Binding Assays Summary

Study Stage	Figure	Ligands	Analytes	Analyte Concentration (nM)	Flow channels	Association Time (s)	Dissociation Time (s)	Regeneration Solution	Regeneration Time (s)
Binding Reproducibility	Not shown	ACE2	Spike	10	Fc1+2	120	300	Pierce Gentle Ag/Ab Elution	10
Binding Affinity	1, 2	ACE2	AT2	9.8, 19.5, 39.1, 78.1, 156.3, 312.5, 625	Fc1+2	120	300	Pierce Gentle Ag/Ab Elution	10
Binding Competition	3	ACE2	Spike ACE2 Spike + ACE2	10 140 10 + 140	Fc1+2	120	300	Pierce Gentle Ag/Ab Elution	30
Binding Competition	4	ACE2	Spike AT2 Spike + AT2	10 3,000 10 + 3,000	Fc1+2	120	300	Pierce Gentle Ag/Ab Elution	30
Binding Competition	5	ACE2	Spike then AT2 Spike then buffer Buffer then AT2	10 + 3,000 10 + 0 0 + 3,000	Fc1+2	120	300	Pierce Gentle Ag/Ab Elution	30

Results

Summary

Binding Affinities

AT2 binding responses to immobilised ACE2 protein were globally fitted to a single site binding model to give a calculated kinetic affinity constant (K_D) of 12.8 nM (Figure 1, Table 5).

Binding response amplitudes at the end of the AT2 binding response interval were fitted to a single site saturation binding model to determine the concentration at half-maximal response (half-Rmax) to give an equilibrium affinity constant (K_D) of 26.7 nM (Figure 2, Table 5).

Table 5. Summary of Binding Affinity Data

Analyte	Ligand	Model	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)	Rmax (RU)	Chi^2 (RU^2)	Offset
AT2 (kinetic affinity)	ACE2	Kinetics - Single Site	2.57E+06	3.30E-02	1.28E-08	22.9	1.12	
AT2 (equilibrium)	ACE2	Equilibrium			2.67E-08	25.8	0.97	4.6

Competition Studies

The binding response for spike (10 nM) binding to immobilised ACE2 in the presence of soluble ACE2 (140nM) was reduced in comparison to binding of spike (10 nM) alone, indicating inhibition of spike binding (Figure 3). The percentage inhibition of spike binding by soluble ACE2 was 82.7%.

Table 6. Summary of Binding Inhibition Data

Competitors	Ligand	Model	Rmax (Spike)	Rmax (ACE2)	Rmax (Spike + ACE2)	% Inhibition
Spike + ACE2 (soluble)	ACE2	Solution-phase competition	57.1	4.9	13.9	82.7

When soluble AT2 (3 µM) alone, or spike (10 nM) alone, or spike preincubated with AT2, were bound to immobilised ACE2, there was no inhibition of spike binding (Figure 4). The response to both analytes binding in combination was equivalent to the mathematical sum of each analyte binding alone. This indicates that spike and AT2 bind to independent sites on ACE2.

Similarly, when spike (10 nM) was bound to ACE2, followed by injection of AT2 (3 µM) while spike was still bound, the binding response in combination was equivalent to the mathematical sum of each analyte alone, indicating no inhibition (Figure 5). This further indicates that spike and AT2 bind to independent sites on ACE2.

Figures

Figure 1. Kinetic affinity analysis for AT2 binding to ACE2.

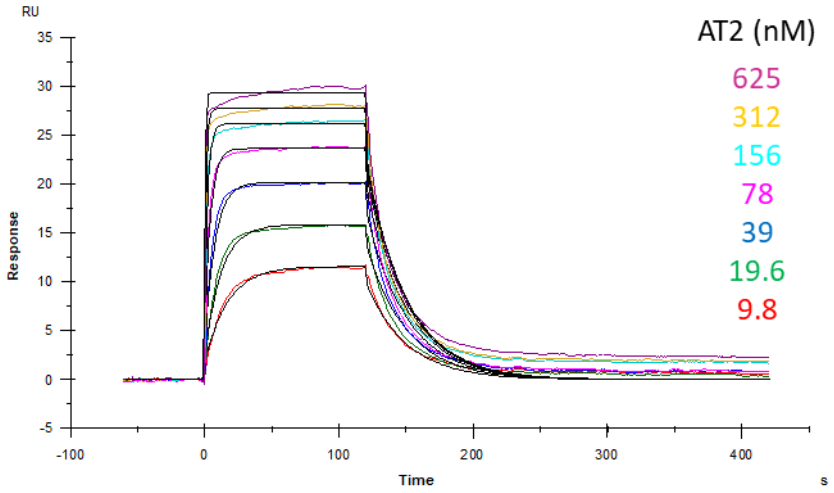
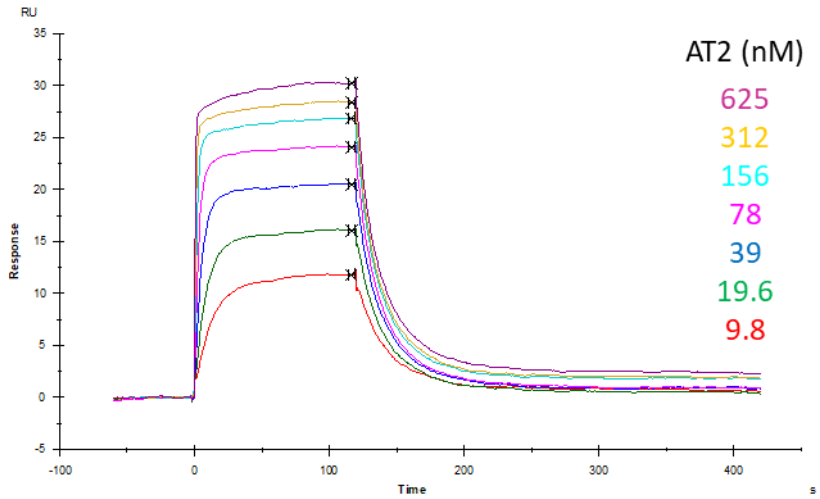


Figure 2. Equilibrium binding analysis for AT2 binding to ACE2.



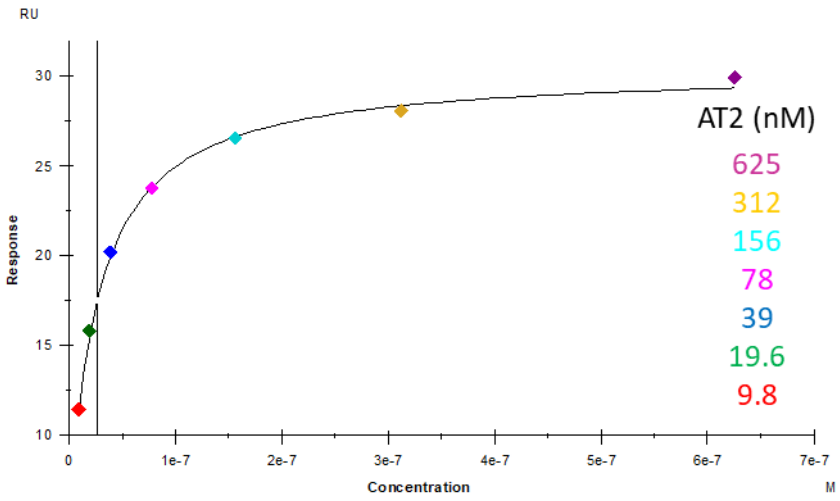


Figure 3. Solution phase competitive blockade of spike binding by preincubation with ACE2.

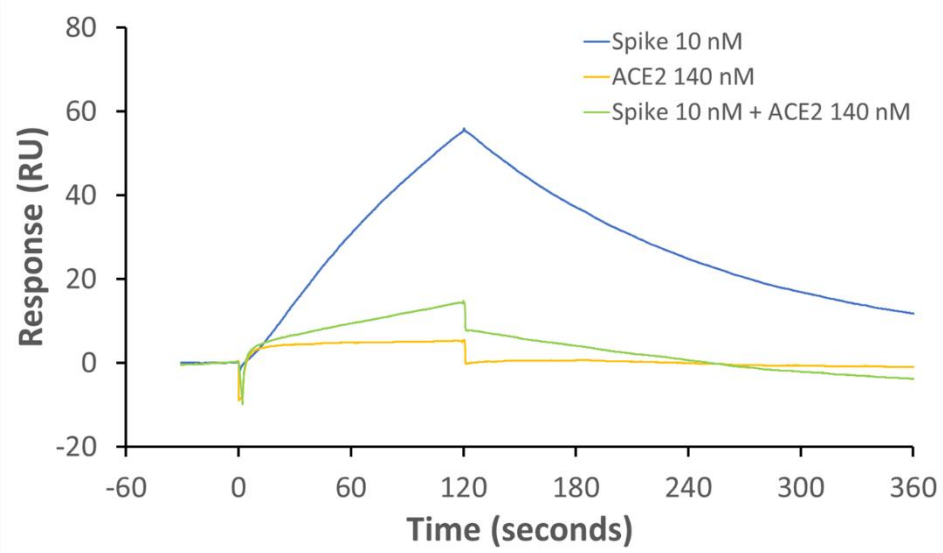


Figure 4. Lack of solution phase competition between spike and AT2.

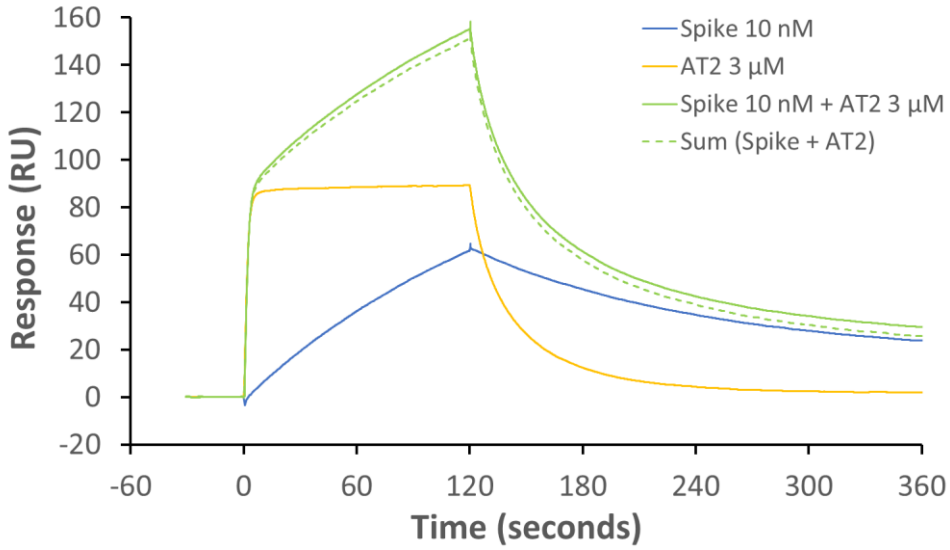
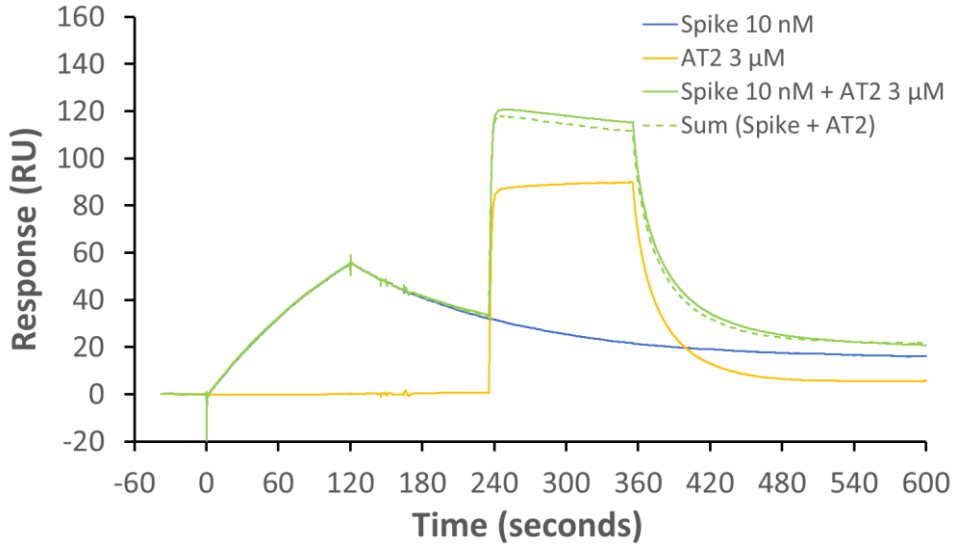


Figure 5. Sequential spike and AT2 binding at independent sites on ACE2.



Appendix

Method, Results (.blr), Analysis (.bme) and Summary Files

(09-06-2020)
1-pH scouting.blr
2-immobilization FC2.blr
3- immobilization FC1.blr
4- Spike reproducibility.blr
5-AngII affinity.blr
5-AngII affinity.bme
6-Spike + ACE2 and AngII.blr
7-AngII start at 10uM.blr
7-AngII start at 10uM.bme
8-Ang II and spike AngII Ace II rerun.blr
8-Ang II and spike AngII Ace II rerun.bme
9-ACE2 and Spike.blr
10-Spike binding test.blr
11-Spike AngII ACE2 test.blr
Spike + ACE2 Analysis.xlsx
Competition Binding SPR 09-Jun-2020.xlsx

(23-06-2020)
1-Immobilization FC 4.blr
2-Immobilization FC 3.blr
3-Spike-ACE2-AT2 binding 23jun2020.blr
3-Spike-ACE2-AT2 binding 23jun2020.bme
4-Spike-then-AT2 binding 24jun2020.blr
4-Spike-then-AT2 binding 24jun2020.bme
Spike + ACE2 Analysis.xlsx