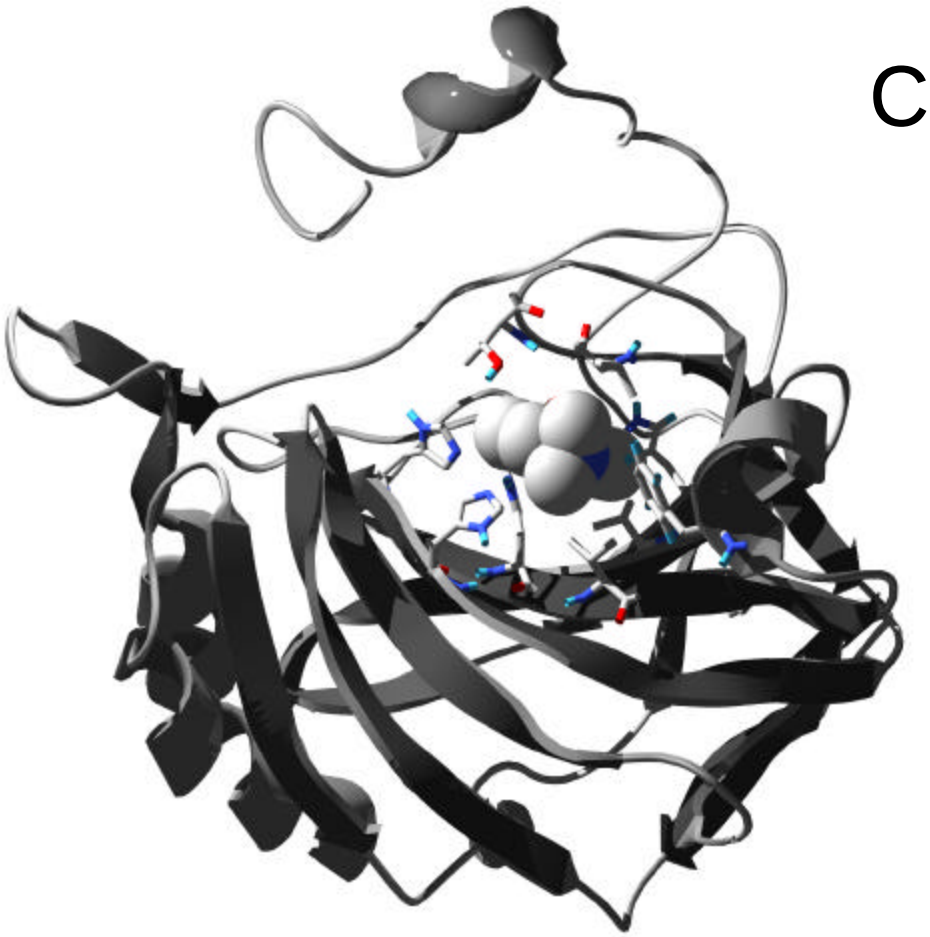
The MIRG 2002 Study:

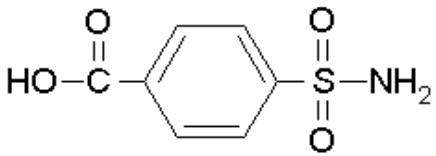
Assembly State, Thermodynamic and Kinetic Analysis of an Enzyme/Inhibitor Interaction

Model System



Carbonic Anhydrase II

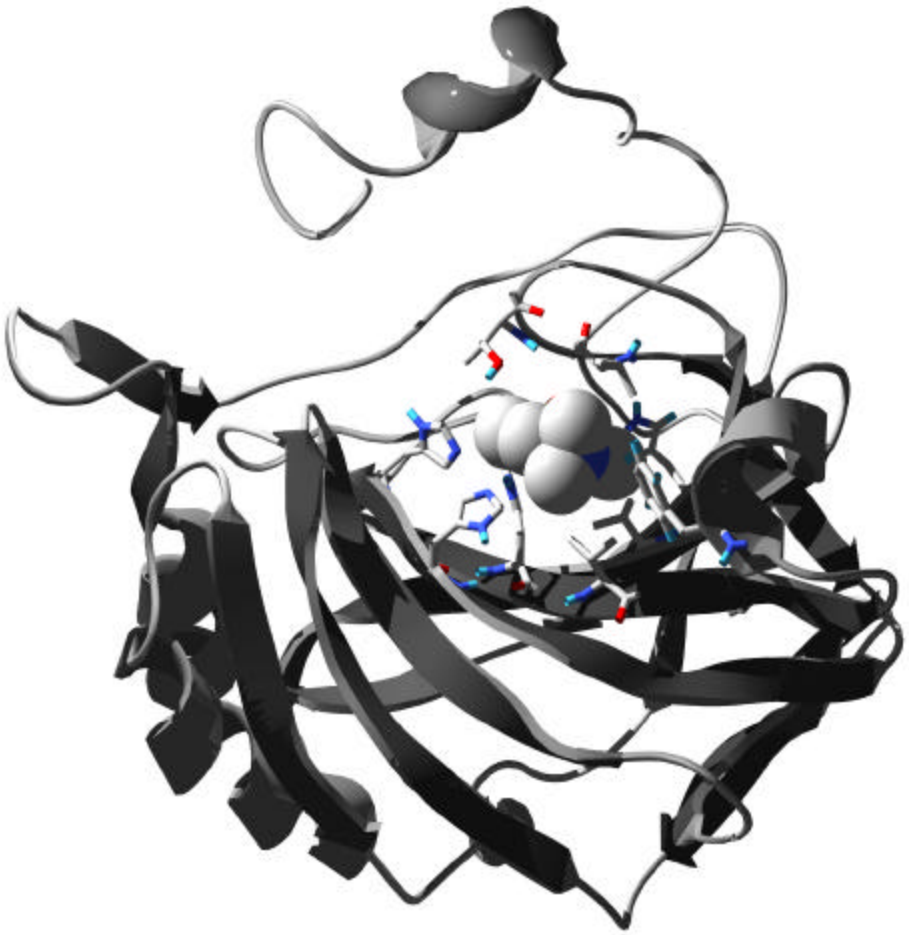
(CA-II = ~30 kDa)



4-Carboxybenzenesulfonamide

(CBS = 201.2 Da)

Goals



AUC – Mass & assembly state

ITC – Affinity & Thermodynamics

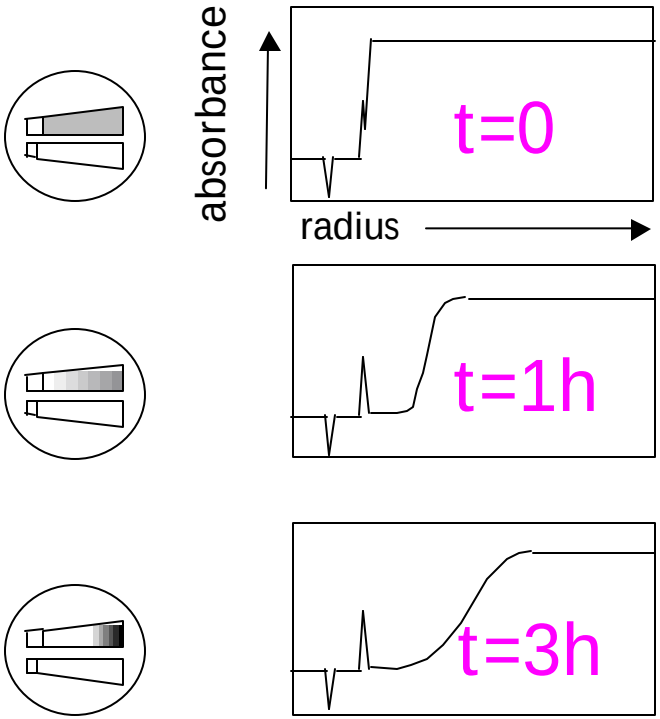
SPR –Affinity & Kinetics

Analytical Ultracentrifuge

Two Types of Experiments

Velocity

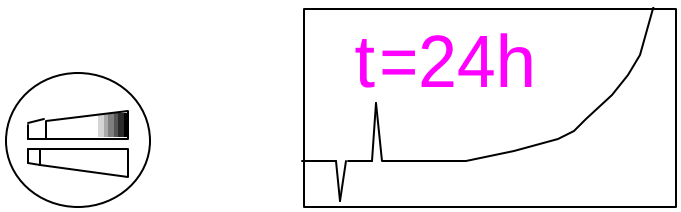
55k rpm



M, D,
sed coefft

Equilibrium

8-40k rpm



M, Kd,
stoichiometry

AUC PARTICIPANTS

- Samples sent to 13 groups
- Results returned so far by 5 groups

AUC SAMPLES

- CA-II supplied as lyophilised powder (" 93% CA-II)
- Pre-run exhaustive dialysis against 20 mM sodium phosphate, 150 mM NaCl, pH 7.4
- Experimental conditions: 20°C (velocity) and 4°C (equilibrium)
- vbar calculated from a.a. sequence based on consensus volumes (Perkins 1986)

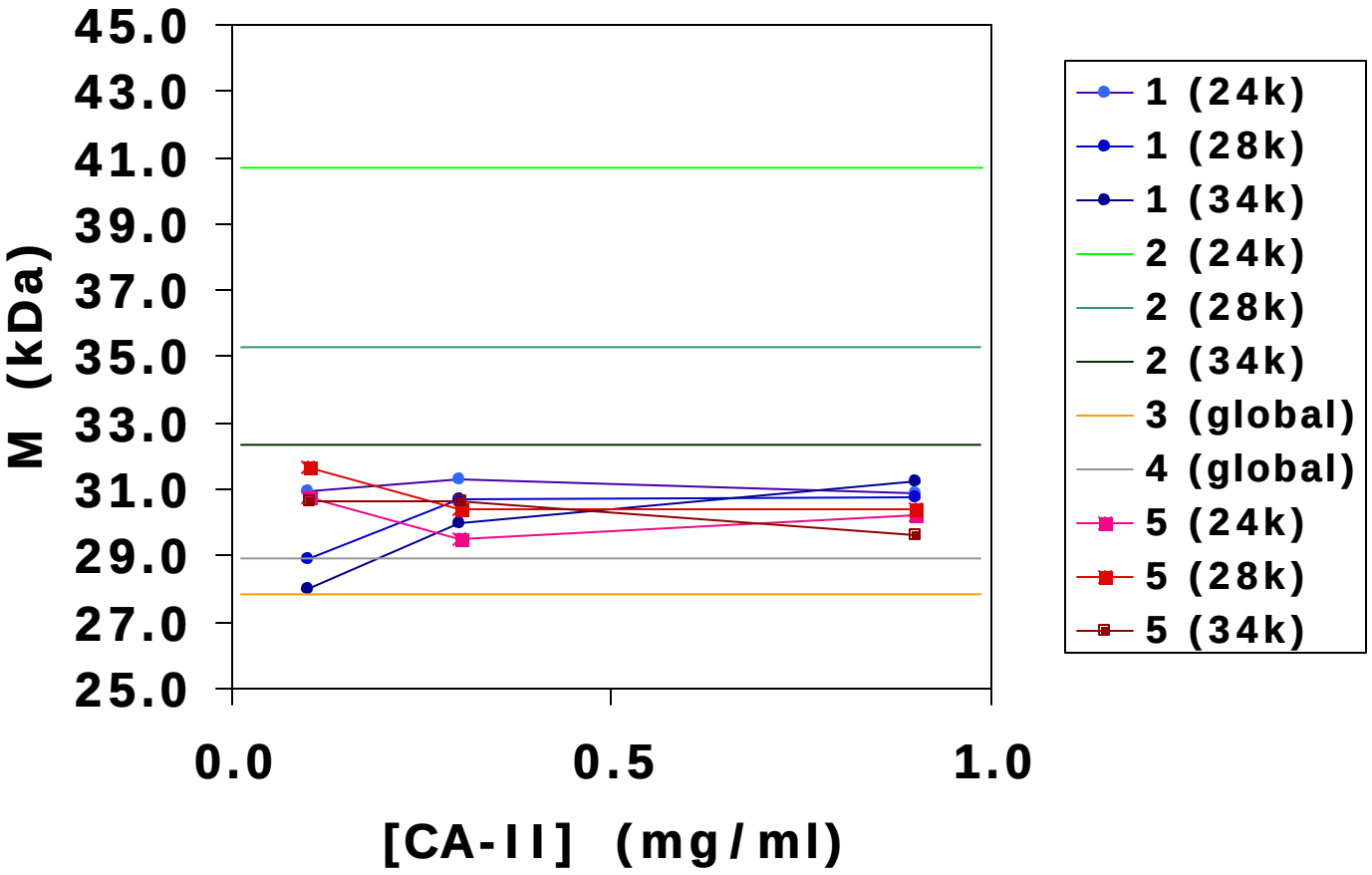
EQUILIBRIUM

- 3 concentrations
  - (0.9, 0.3, 0.1 a.u. absorbance optics)
  - (1.0, 0.33, 0.01 mg/ml interference optics)
- 3 rotor speeds (24k, 28k, 34k rpm)
- Analyse with favourite software
- Determine molecular weight

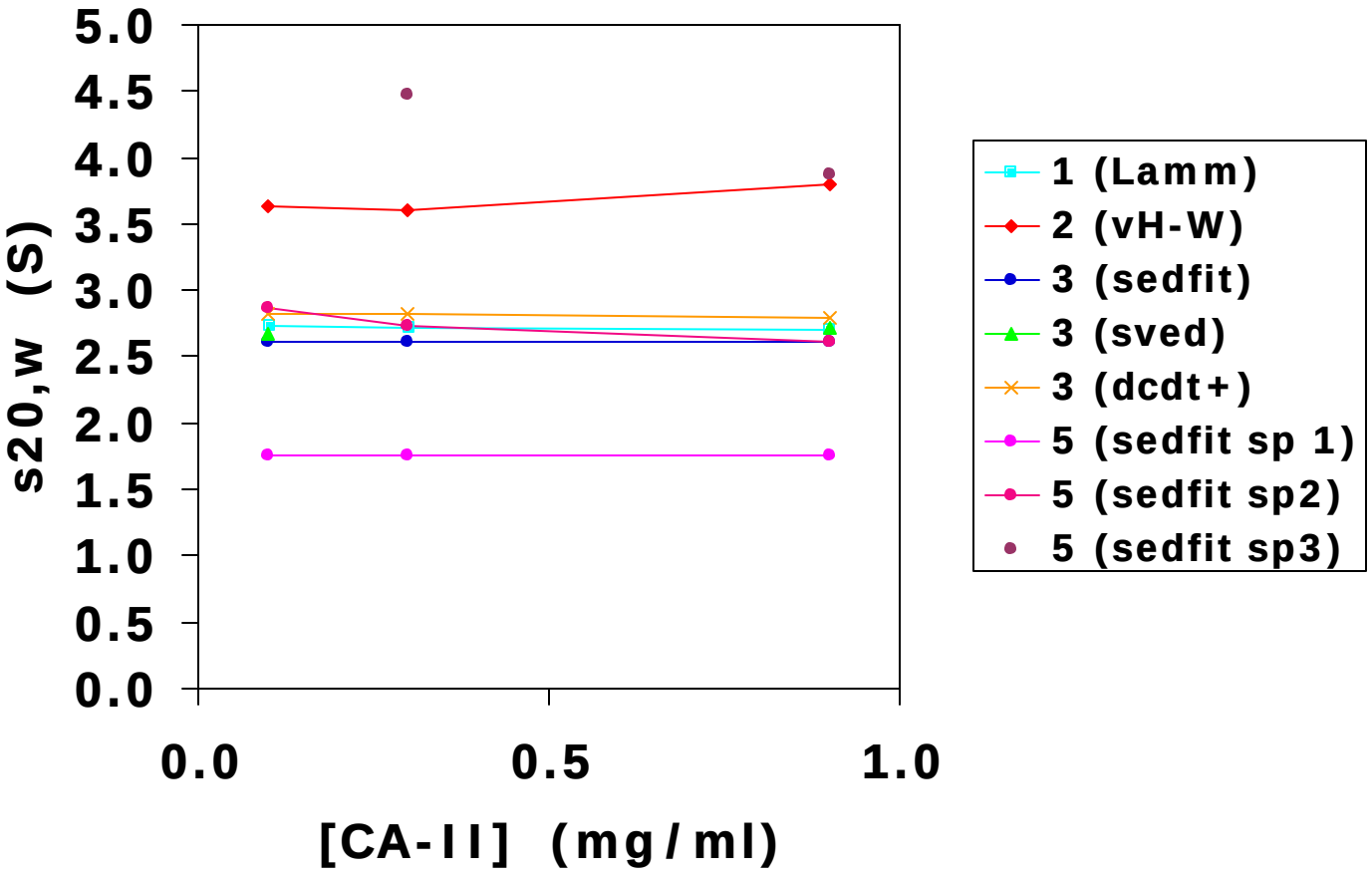
VELOCITY

- 3 concentrations
  - (0.9, 0.3, 0.1 a.u. absorbance optics)
  - (1.0, 0.3, 0.2, 0.01 mg/ml interference optics)
- Rotor speed 50k rpm
- Analyse with favourite software
- Determine molecular weight (and sedimentation coefficient)

AUC (Equilibrium) Results



AUC (Velocity) Results



Summary

- AUC able to verify integrity of sample
- Results sensitive to both method *and* analysis
- AUC community is *very* heterogeneous
- AUC activists are very poor at responding to deadlines
- AUC part of MIRG project is very much ongoing

Titration Calorimetry

Summary: ITC Set-up

Number of participants: 12

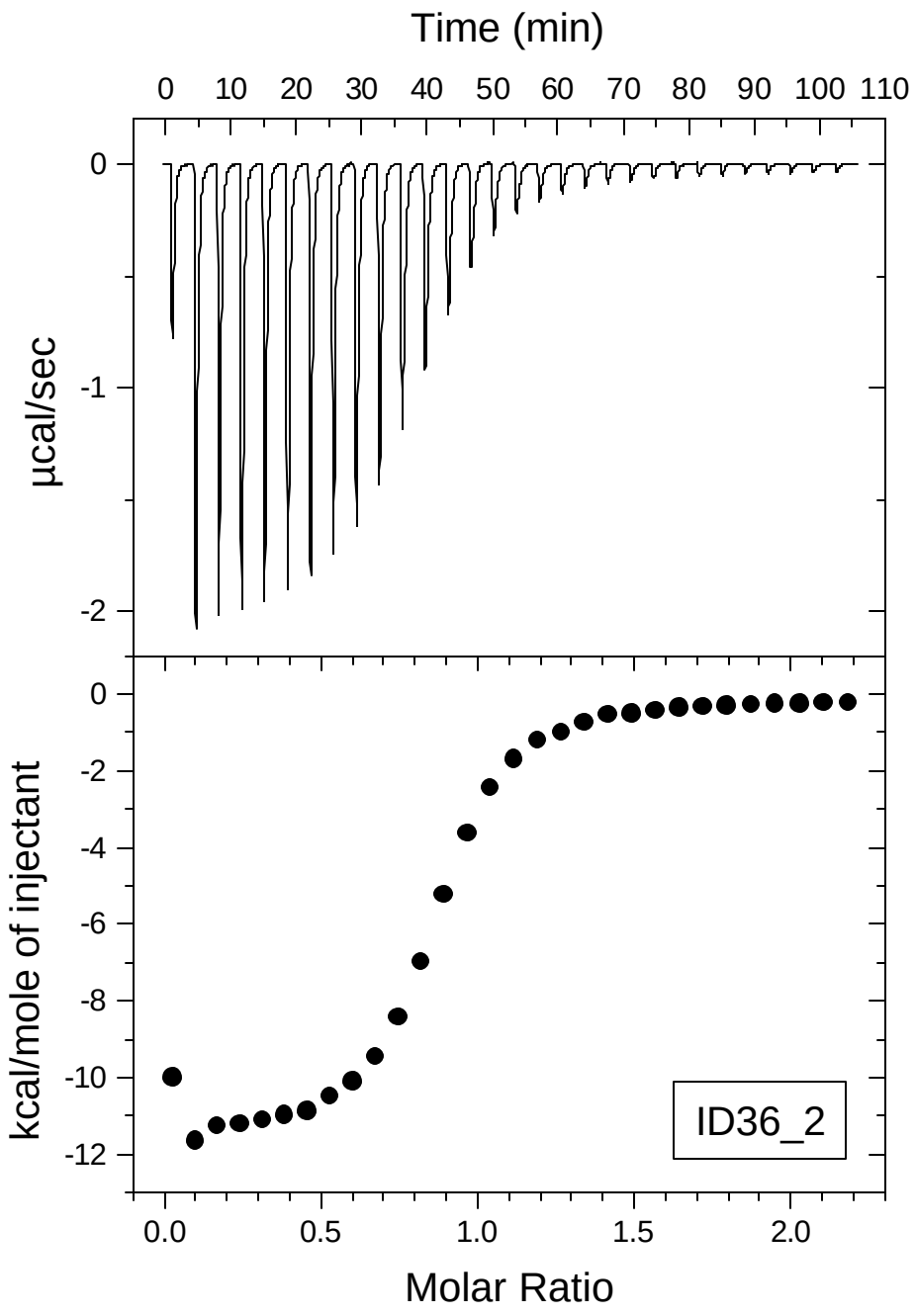
Instruments used: MicroCal VP-ITC (9 submissions)
MicroCal MCS-ITC (2 submissions)
CSC 4200 (1 submission)

Total number of titrations: 27

C-value range: 6 to 134

Chi^2 range: 1114 to 117140

Variations in Instrumental Set-up				
ID#	Make	[CAII] (μM)	[CBS] (μM)	Inj. vol. (μL)
9	MC MCS-ITC	16	390	15 x 10
22	CSC 4200	70.8	556	21 x 10
24	MC VP-ITC	42	400	2 + 31 x 8
26	MC VP-ITC	22.65 or 22.9	870 or 642	0.1 + 60 x 4.5
28	MC VP-ITC	23.4 or 39	665	1 + 25 x 10
30	MC VP-ITC	35 or 37	780 or 380	1 + 25 x 10
36	MC VP-ITC	30.5 or 56.1	1144	2 + (24 or 29) x 5
37	MC VP-ITC	31.5	384	14 x 17 or 30 x 8
42	MC VP-ITC	8.61	100	2 + 28 x 10
43	MC VP-ITC	28	896	3 +39 x 3
44	MC VP-ITC	11.4	114	1.5 +15 x 18.7
48	MC MCS-ITC	21.4 to 23.4	300	2 + 19 x 10



ID 36_2
MicroCal VP-ITC
1144 µM CBS
56.1 µM CAII
C-value = 51
2 + 29 x 5 µL inj.
210 second intervals
310 rpm stir speed

Reported average values:

N = 0.87 (±0.03)
K = 1.11 (±0.05) x 10^6 M^{-1}
ΔH = -11.54 (±0.007) kcal/mol

Binding Parameters from ITC Data Submissions			
ID#	Stoichiometry	Ka ($M^{-1}x10^{-6}$)	ΔH (kcal/mol)
9	1.01	1.23	-8.93
22	0.95	1.44	-7.19
24	0.81	1.24	-11.8
26	0.93	0.84	-10.8
28	0.987	0.84	-10.2
30	0.921	1.08	-10.95
36	0.87	1.12	-11.54
37	0.961	0.92	-12.2
42	1.252	1.29	-9.33
43	1.000	1.01	-10.51
44	0.9	1.1	-13
48	0.52	1.3	-12
Average	0.93	1.1	-10.7
Stand. Dev.	0.17	0.2	1.6

ITC Summary

ITC Set-up

Number of participants: 12

Instruments used: MicroCal VP-ITC (9 submissions)
MicroCal MCS-ITC (2 submissions)
CSC 4200 (1 submission)

Total number of titrations: 27
C-value range: 6 to 134
Chi^2 range: 1114 to 117140
CBS molar extinction coefficient range: 1110 to 1430 M^{-1} cm^{-1}

ITC Summary

ITC Thermodynamic Parameters

Average of reported thermodynamic parameters:
n = 0.92 ($\pm$0.17)
Ka = 1.1 ($\pm$0.2) x 10^6 M^{-1}
ΔH = -10.7 ($\pm$1.6) kcal/mol

Average of reported thermodynamic parameters after removing anomalous data set:
n = 0.96 ($\pm$0.11)
Ka = 1.1 ($\pm$0.2) x 10^6 M^{-1}
ΔH = -10.6 ($\pm$1.6) kcal/mol

Average of partially optimized* thermodynamic parameters:
n = 0.94 ($\pm$0.10)
Ka = 1.1 ($\pm$0.3) x 10^6 M^{-1}
ΔH = -10.5 ($\pm$1.0) kcal/mol

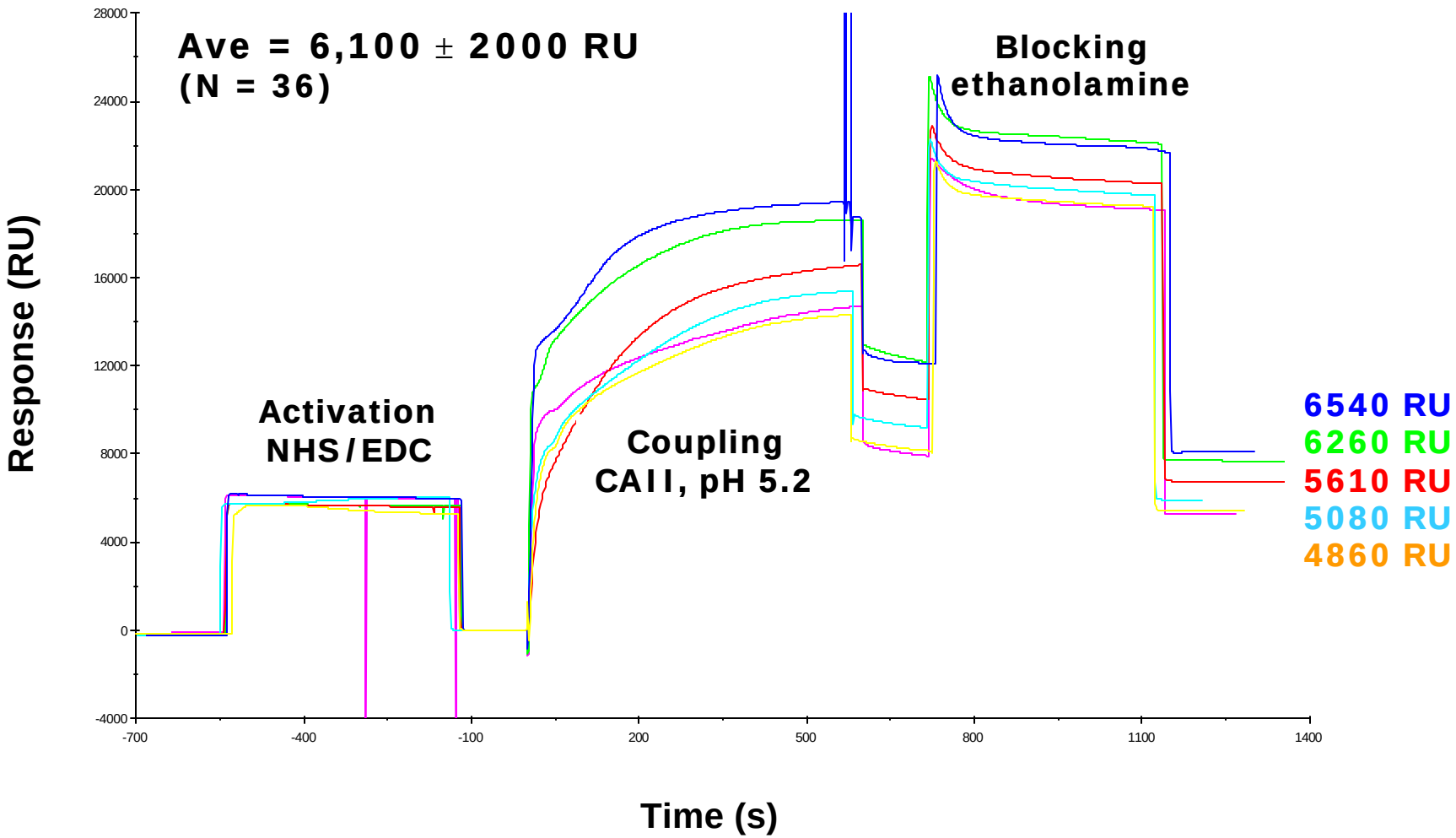
*Removed anomalous data set, normalized CBS concentration and varied some of the dilution control correction constants

Biosensor Experiments

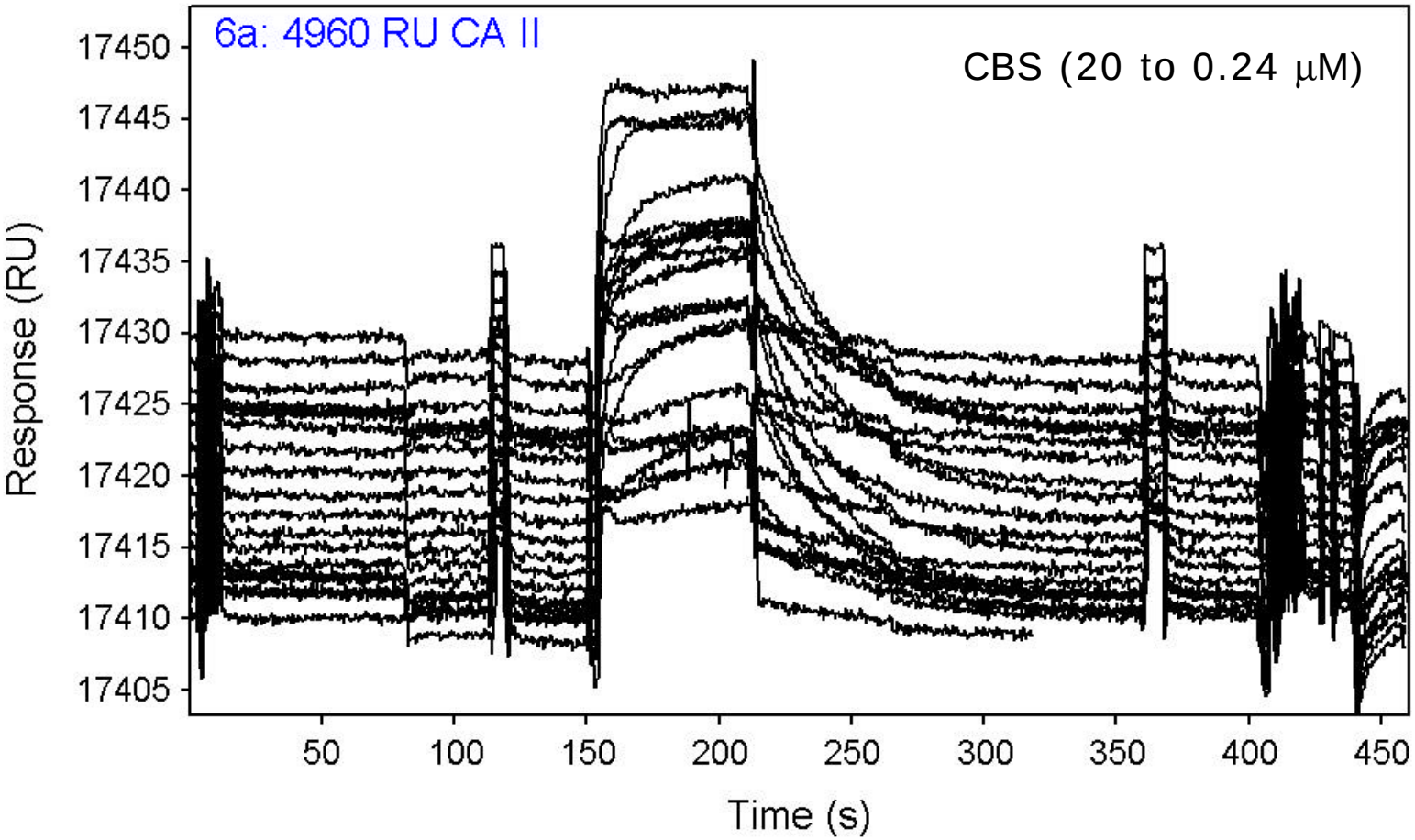
- 200 μg CAII powder
- 100 μL of a 20 mM CBS in PBS
- Detailed protocol

⇒ Cleaning
⇒ Blank injections
⇒ Immobilize CAII
⇒ Inject CBS (20 to 0.24 μM)
⇒ Data processing and fitting

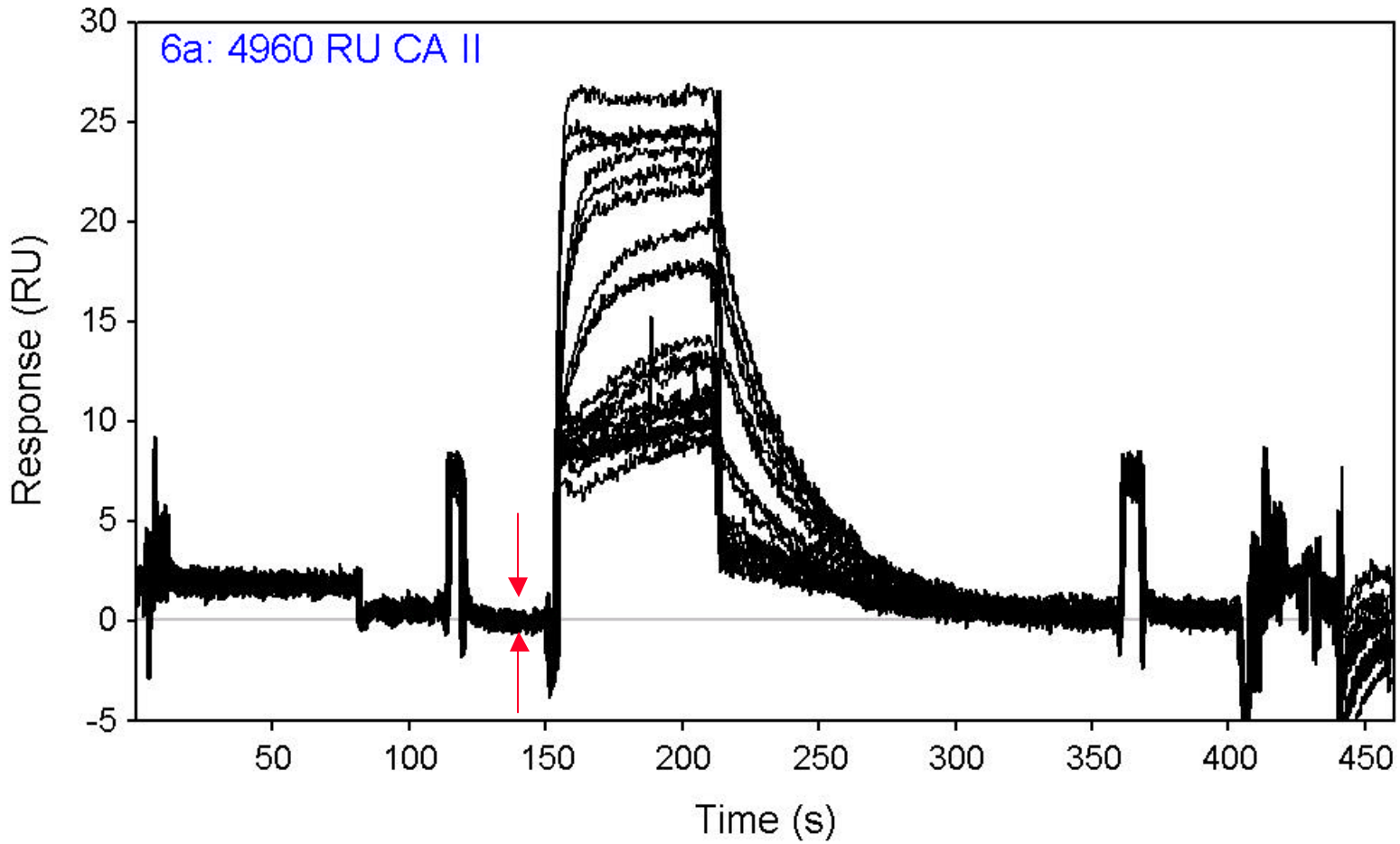
Immobilization



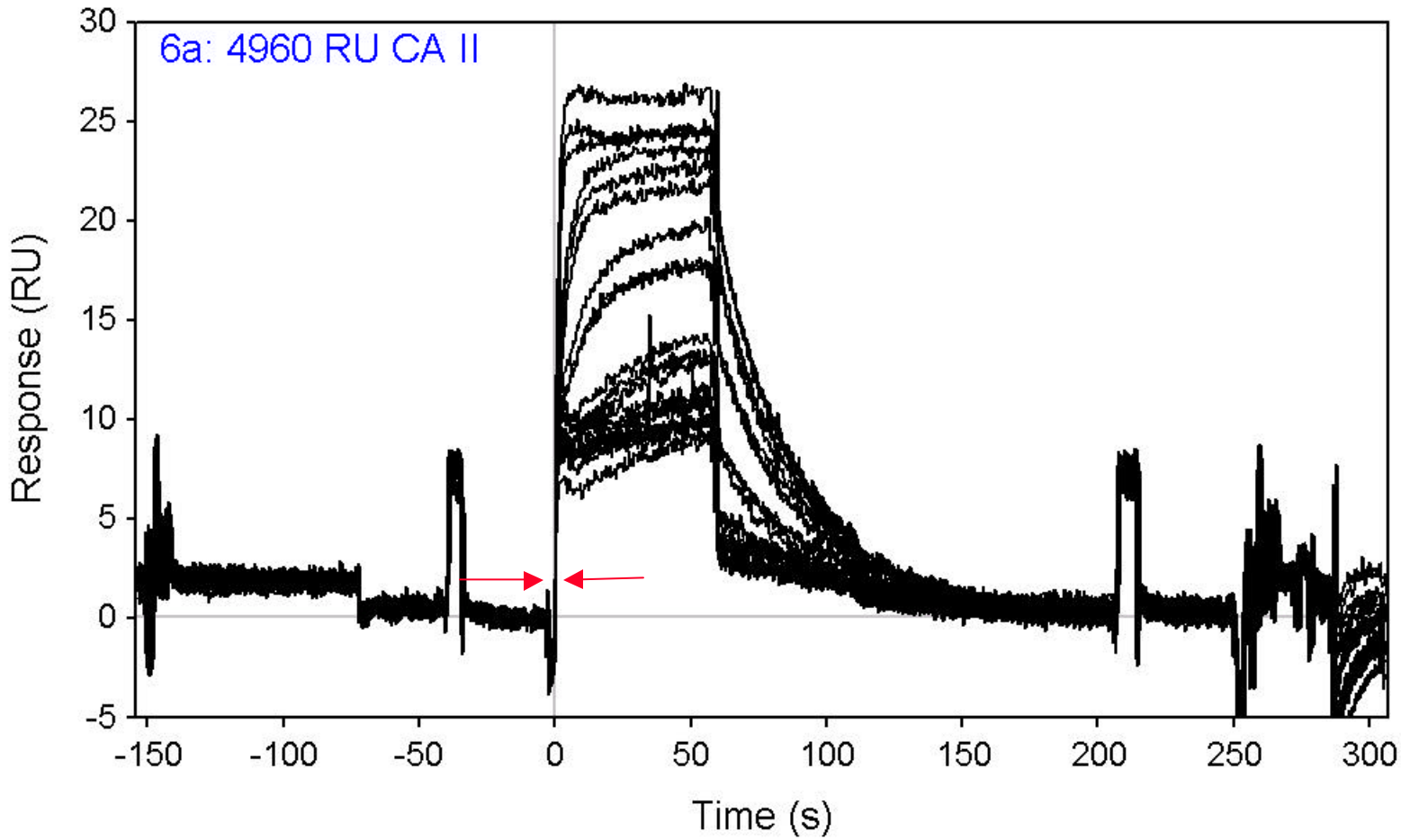
Data Processing



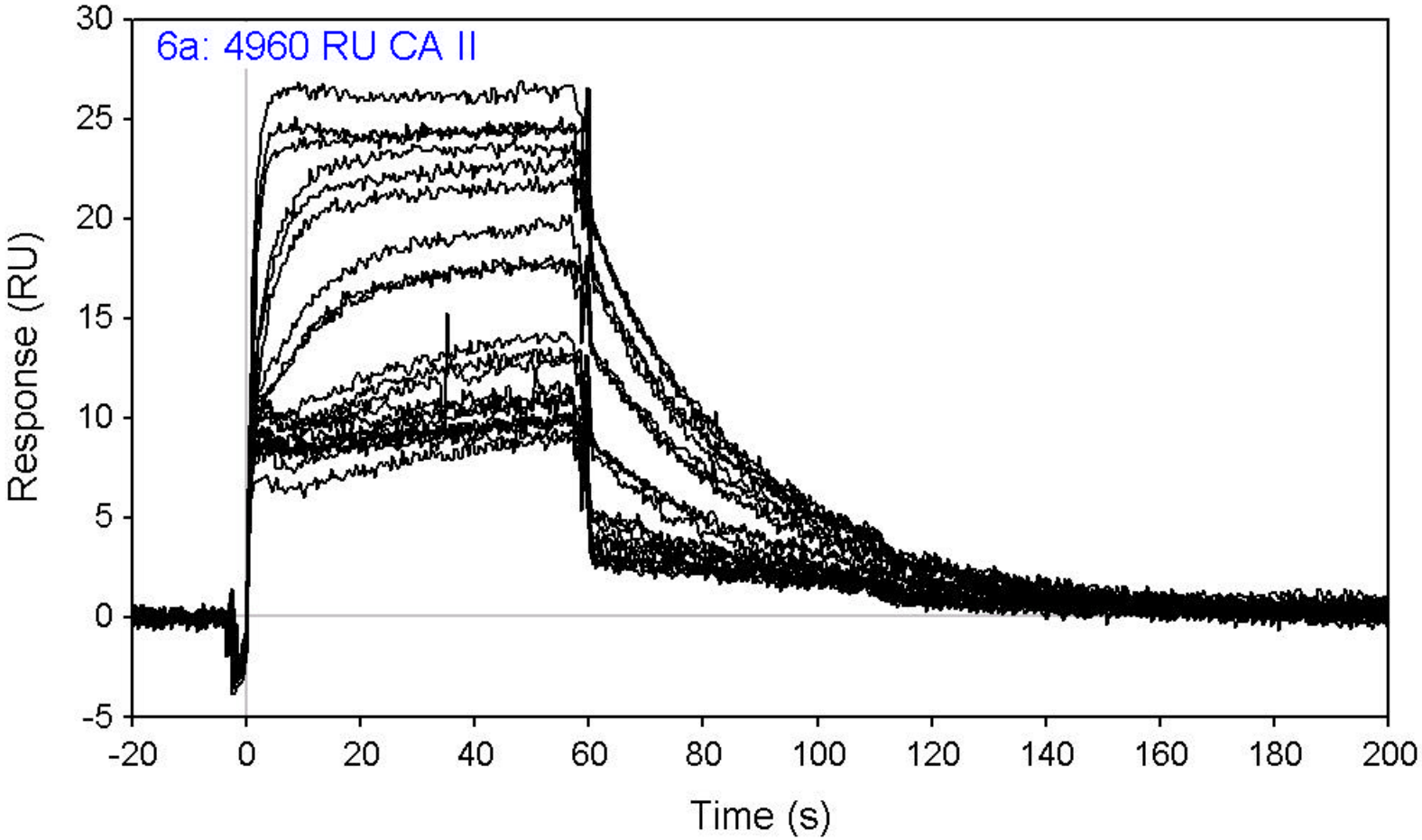
Zero on the Y-Axis



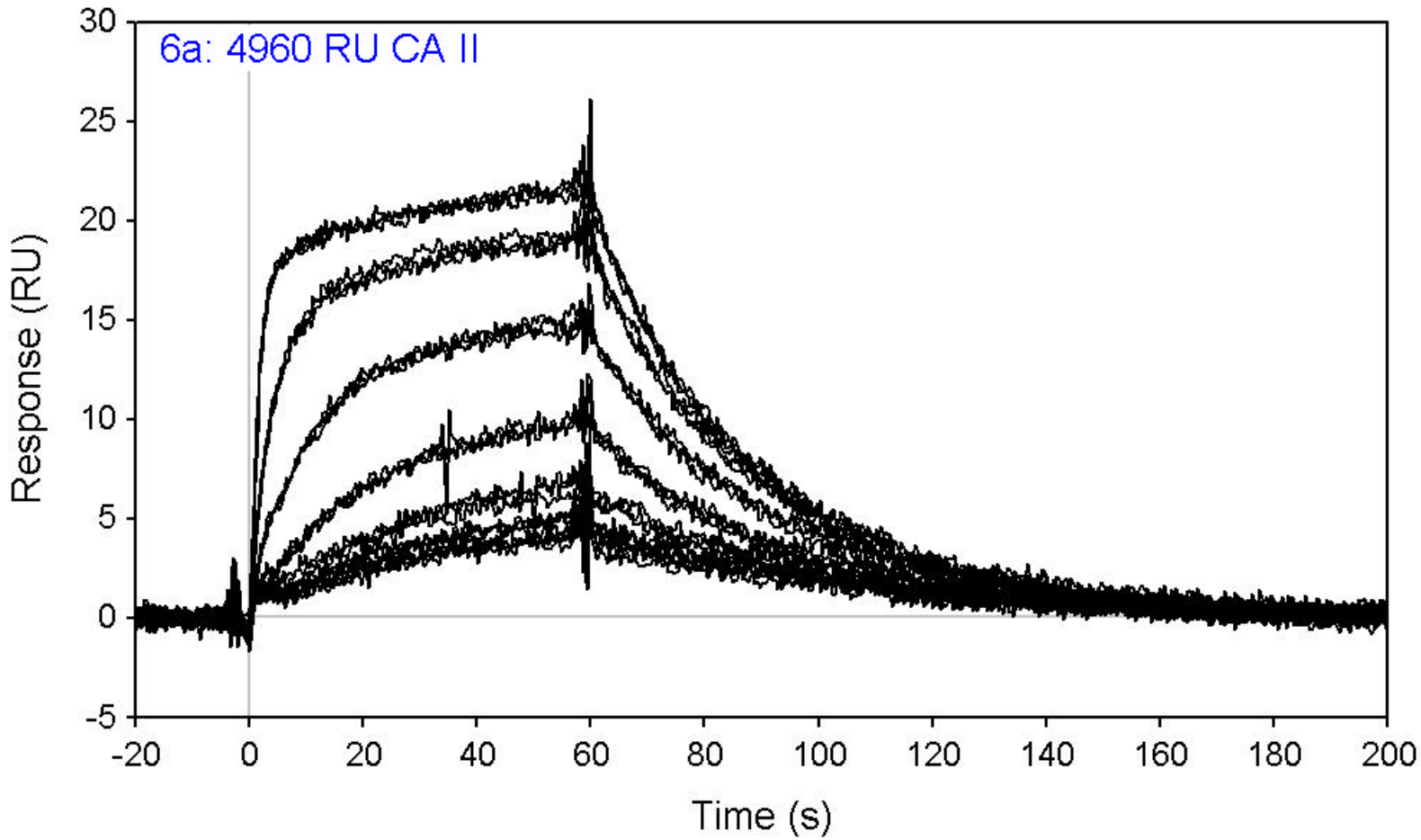
Zero on the x-Axis



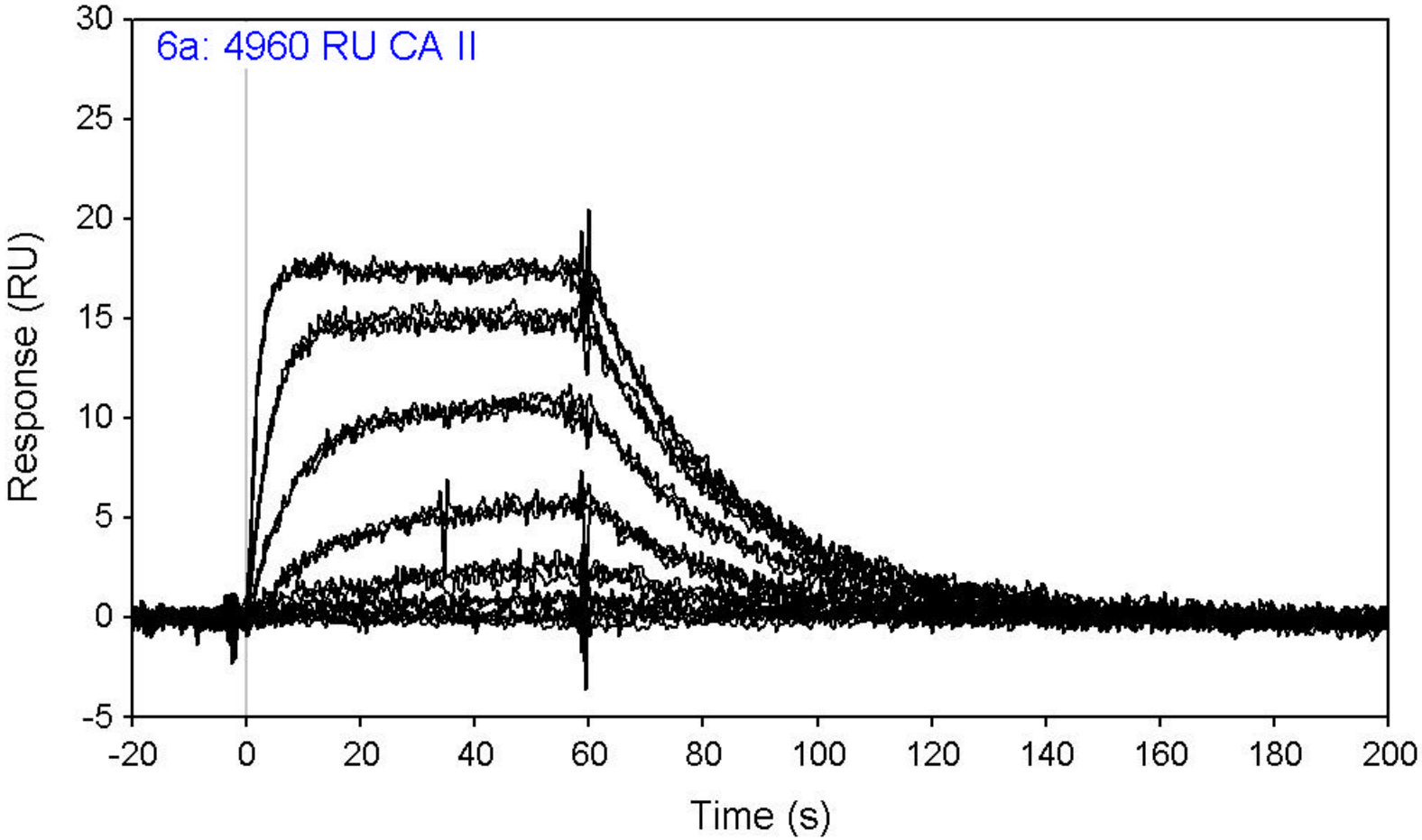
Crop Data


6a: 4960 RU CA II
Response (RU)
30
25
20
15
10
5
0
-5
-20
0
20
40
60
80
100
120
140
160
180
200
Time (s)

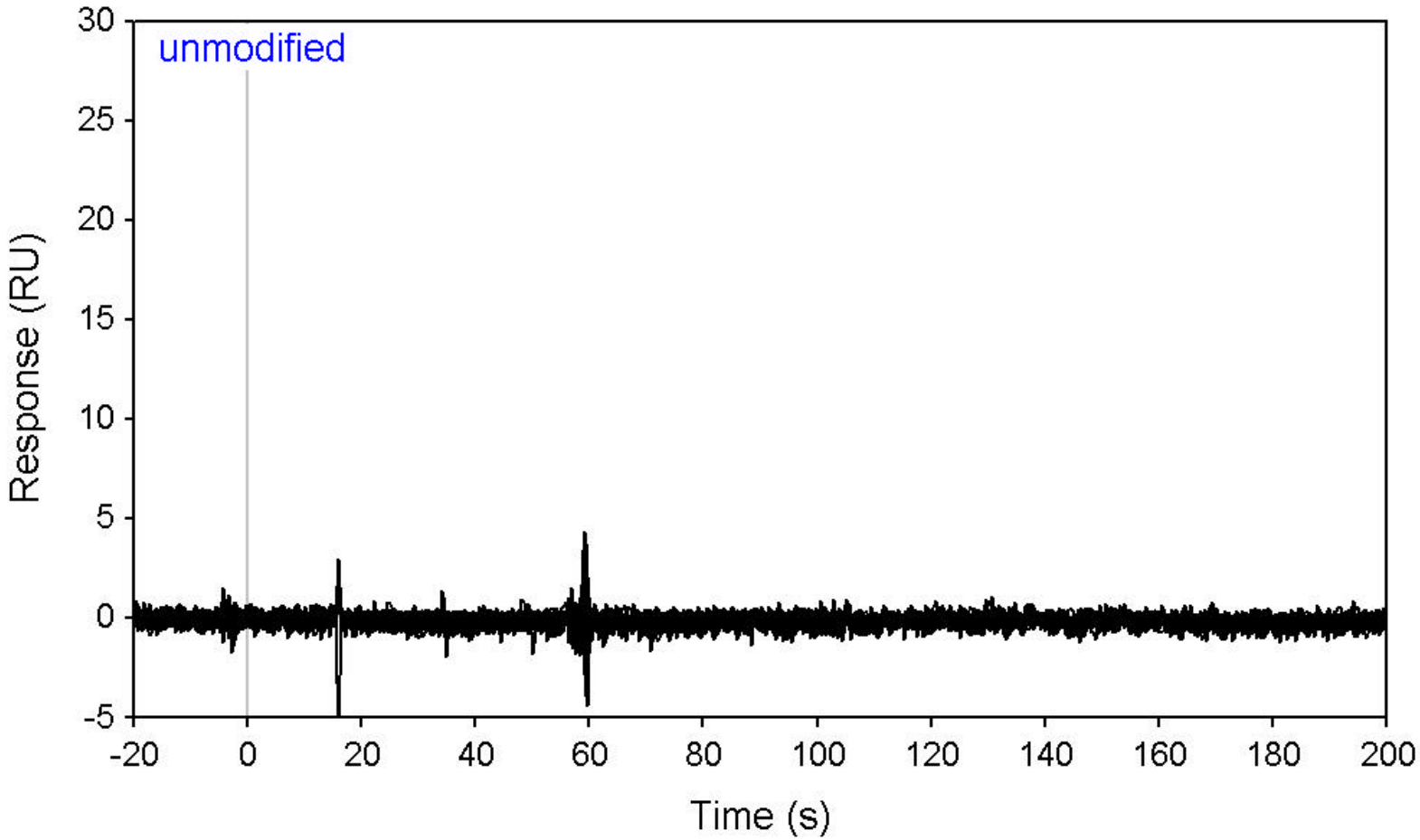
Subtract Reference Surface



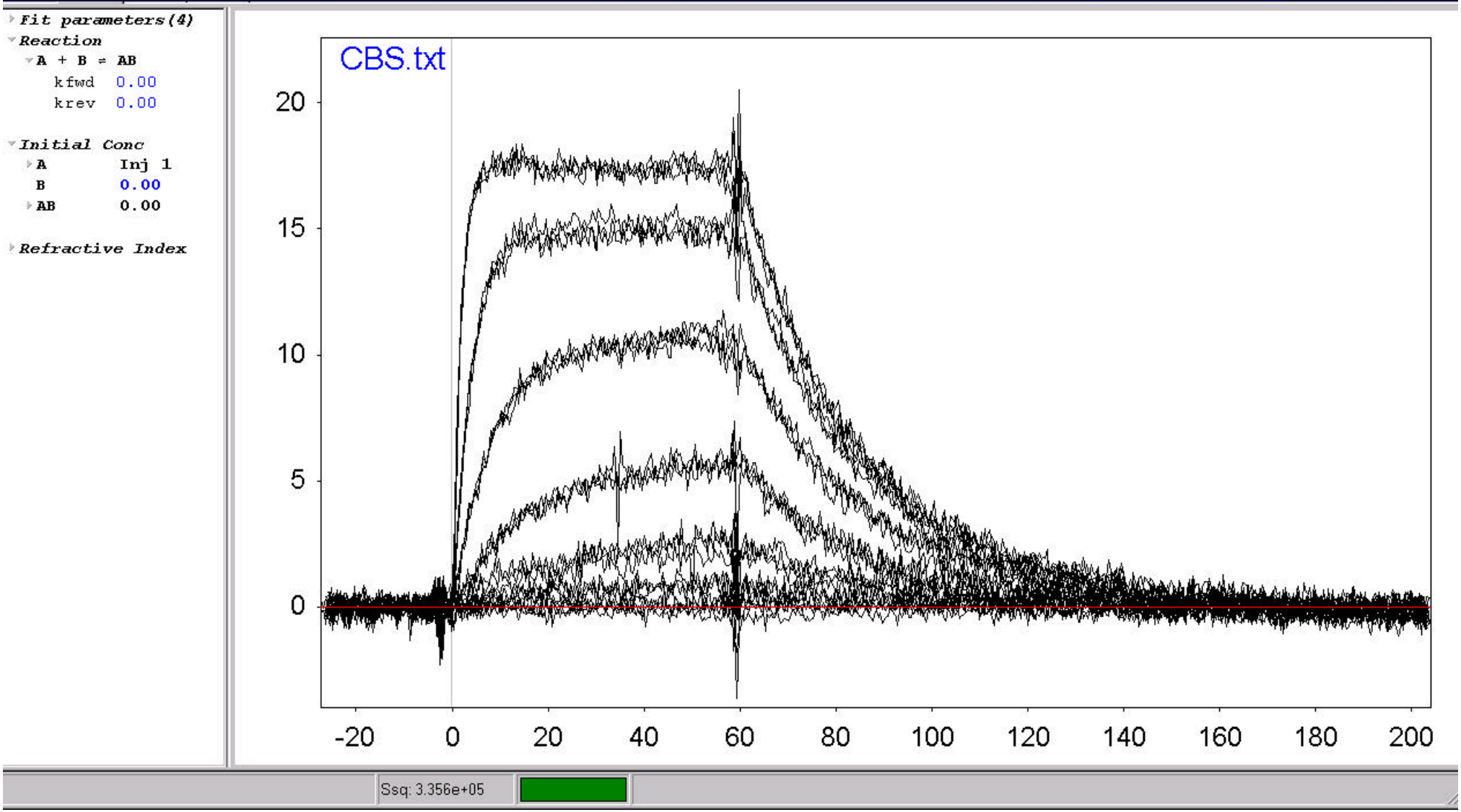
Subtract Blank Injections



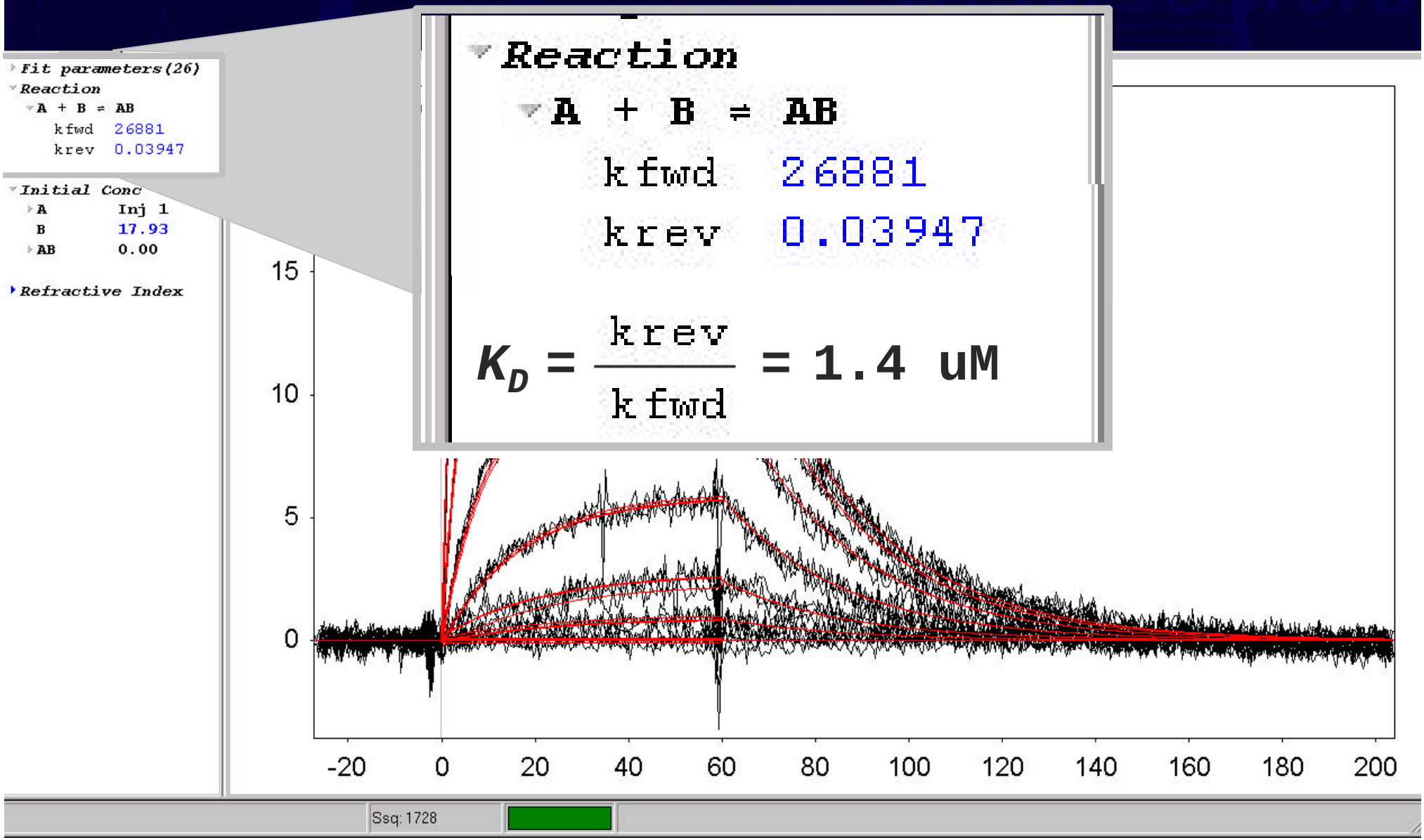
Check Reference Surface



Global Data Fitting



Final Fit


Fit parameters (26)
Reaction
A + B ⇌ AB
kfwd 26881
krev 0.03947
Initial Conc
A Inj 1
B 17.93
AB 0.00
Refractive Index
Reaction
A + B ⇌ AB
kfwd 26881
krev 0.03947
$K_D = \frac{krev}{kfwd} = 1.4$ uM
15
10
5
0
-20
0
20
40
60
80
100
120
140
160
180
200
Ssq: 1728

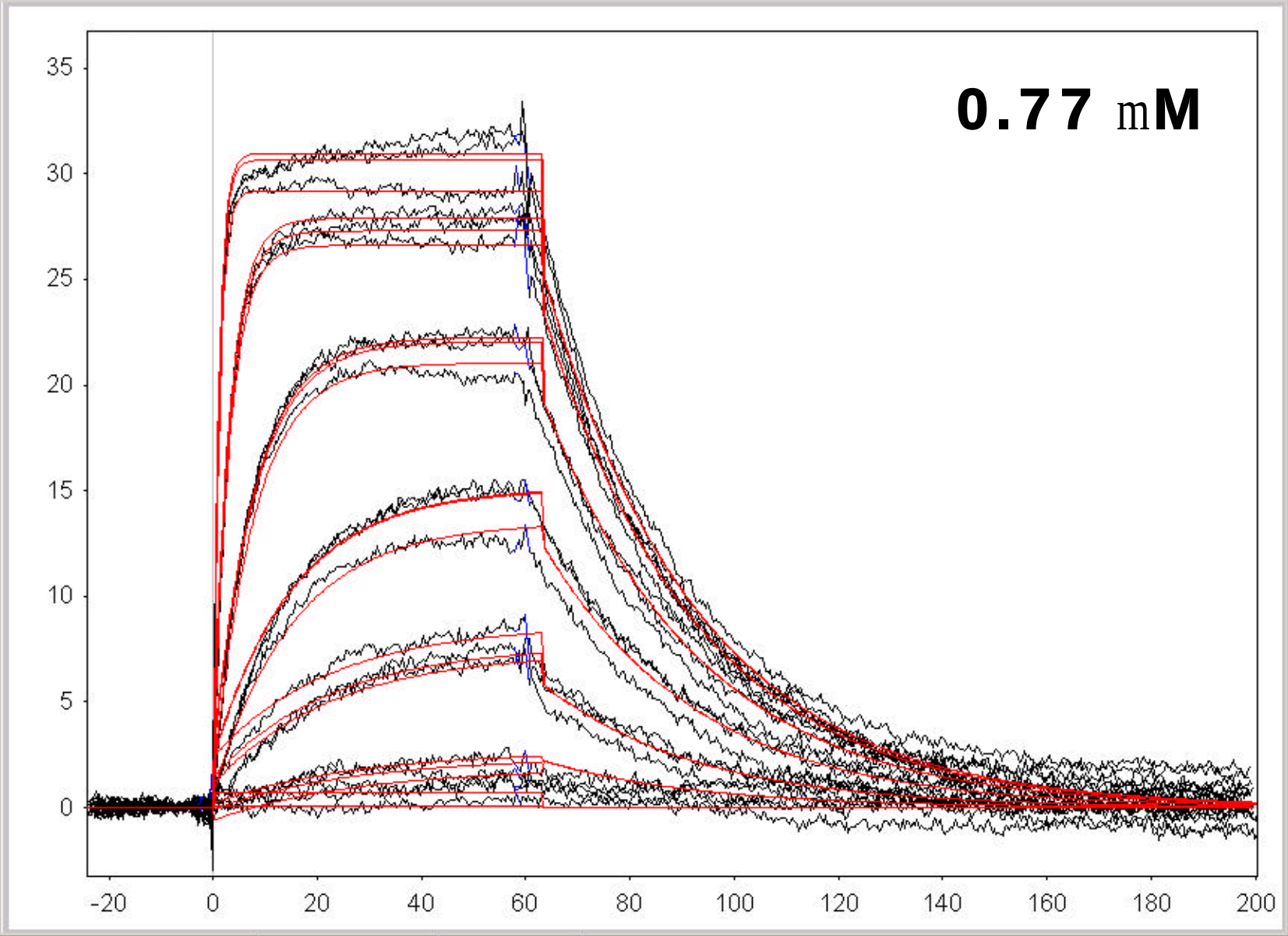
1

Fit parameters(25)
Reaction
A + B = AB
kfwd 43900
krev 0.03389

Initial Conc
A Inj 1
B 26.66
AB 0.00

Refractive Index

3000



Ssq: 3154

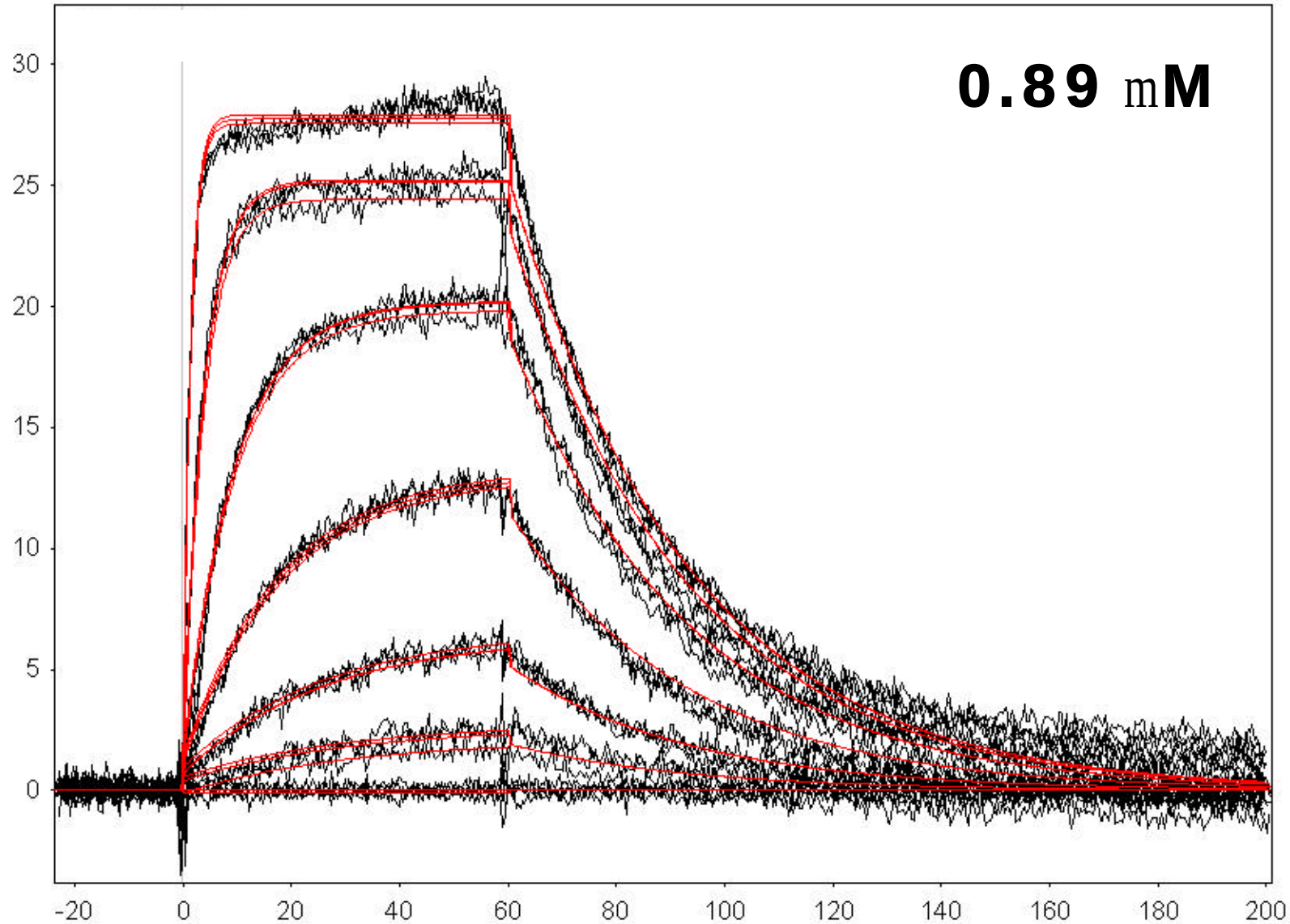
14

Fit parameters(27)
Reaction
A + B = AB
kfwd 33920
krev 0.03038

Initial Conc
A Inj 1
B 26.29
AB 0.00

Refractive Index

2000



Done
Ssq: 1913

35

Fit parameters (23)

Reaction

A + B ⇌ AB

kfwd 44613

krev 0.01808

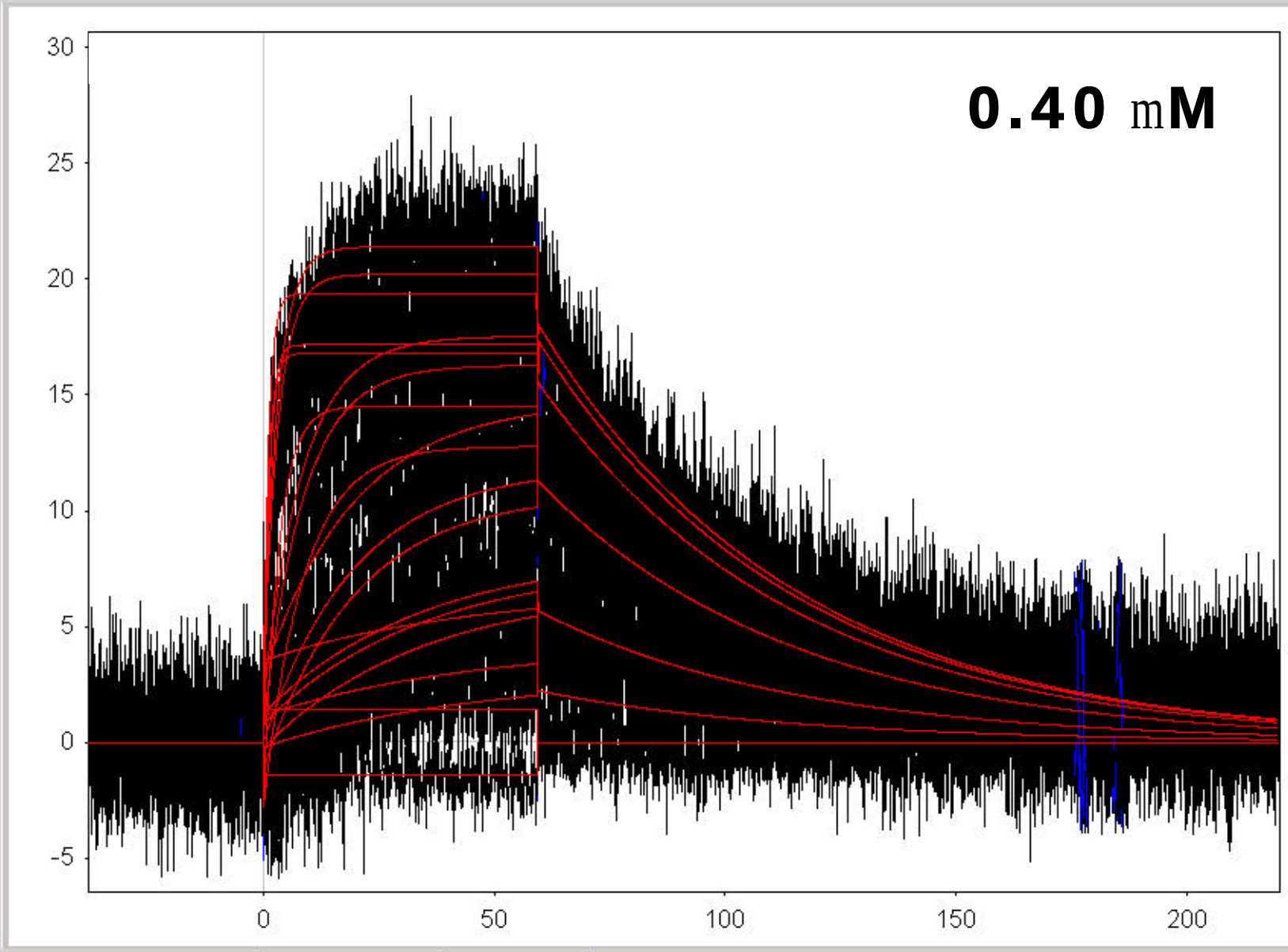
Initial Conc

A Inj 1

B 18.36

AB 0.00

Refractive Index



1000

Done

Ssq:

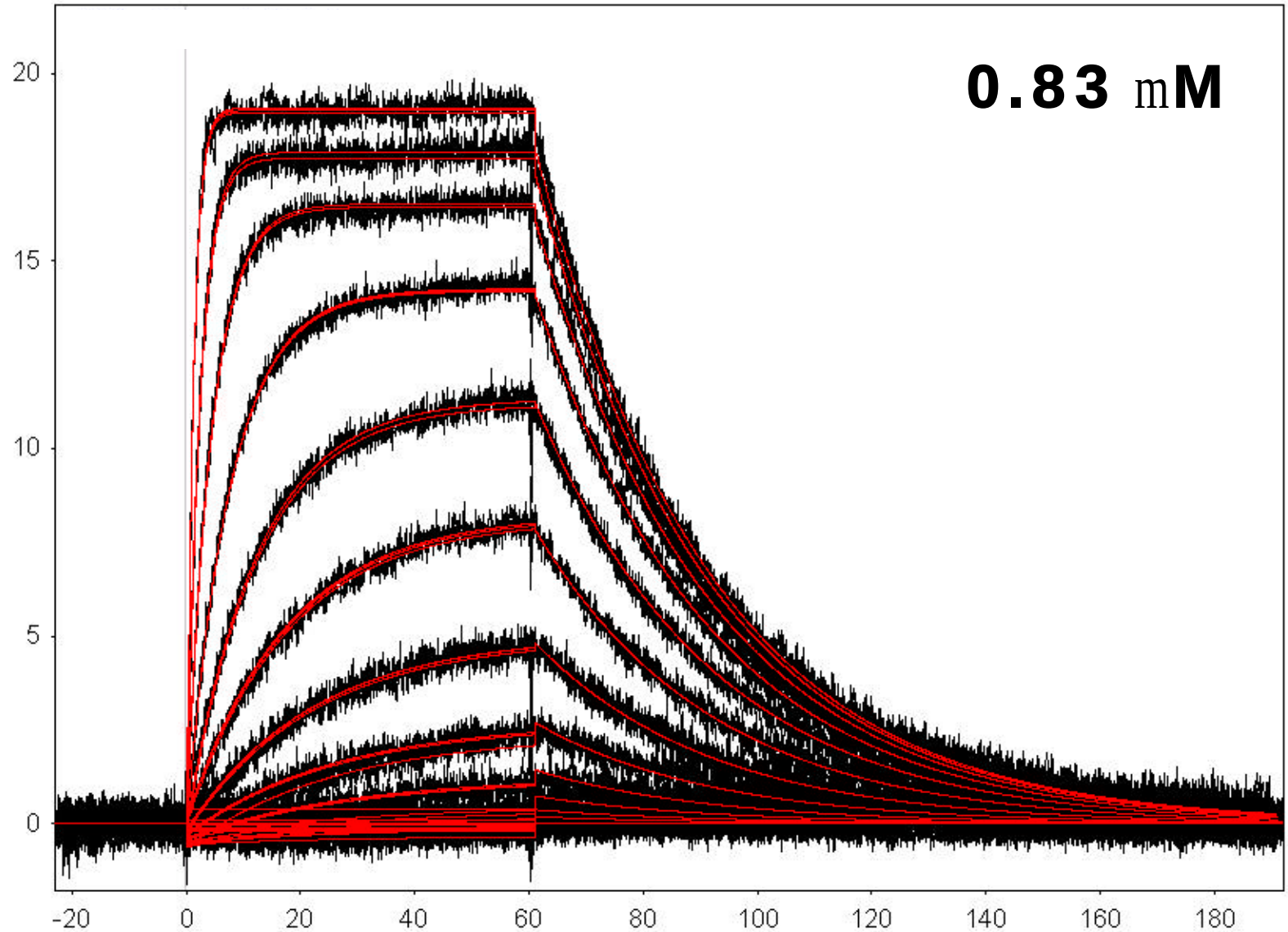
36

Fit parameters(48)
Reaction
A + B = AB
kfwd 39292
krev 0.0325

Initial Conc
A Inj 1
B 18.67
AB 0.00

Refractive Index

S51

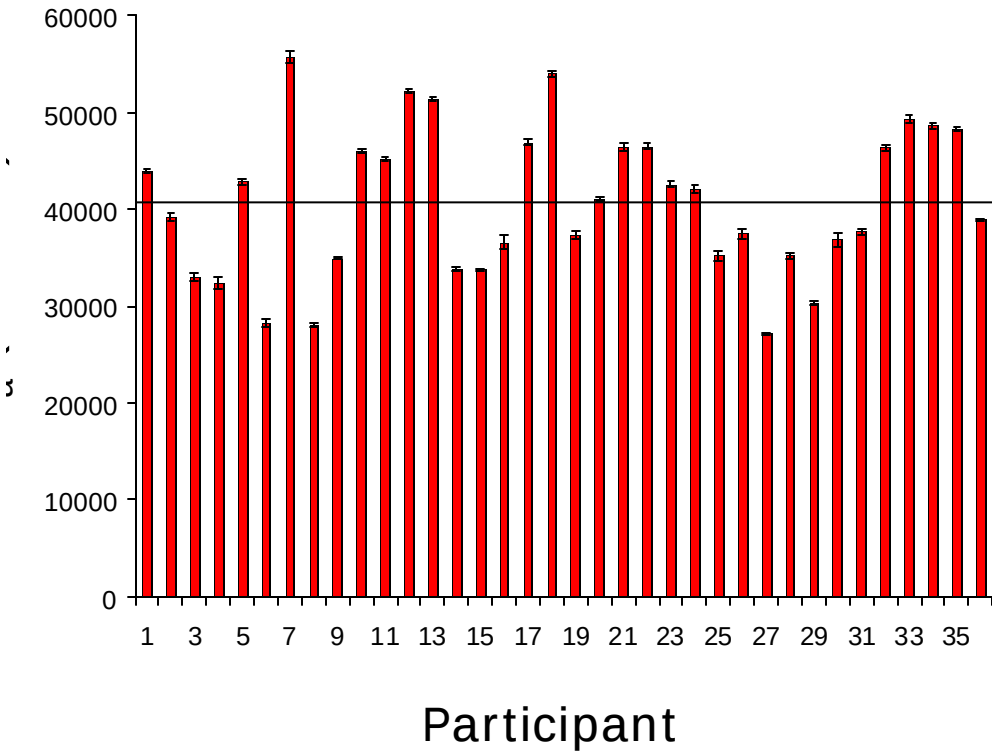


Done | Ssq: 1913

Kinetics

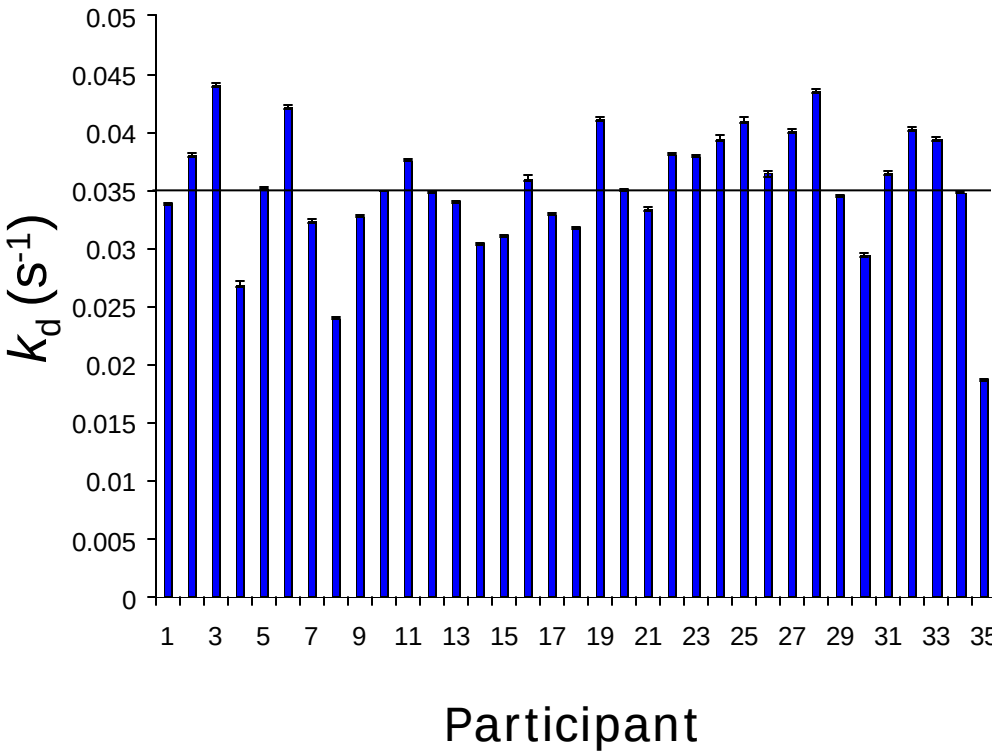
Association Rate

k_a = 40,700 ± 7,600 (M^{-1} s^{-1})

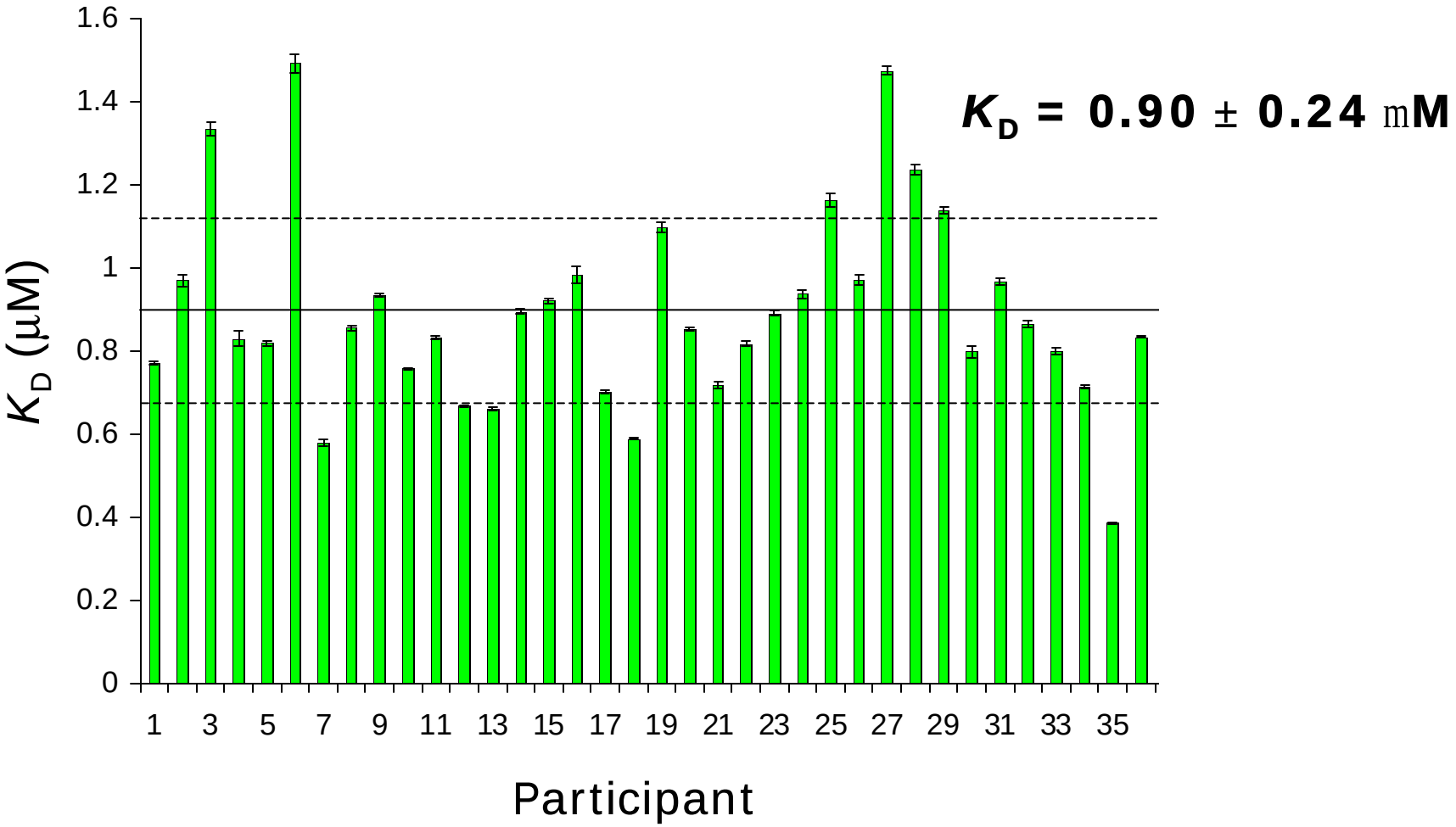


Dissociation Rate

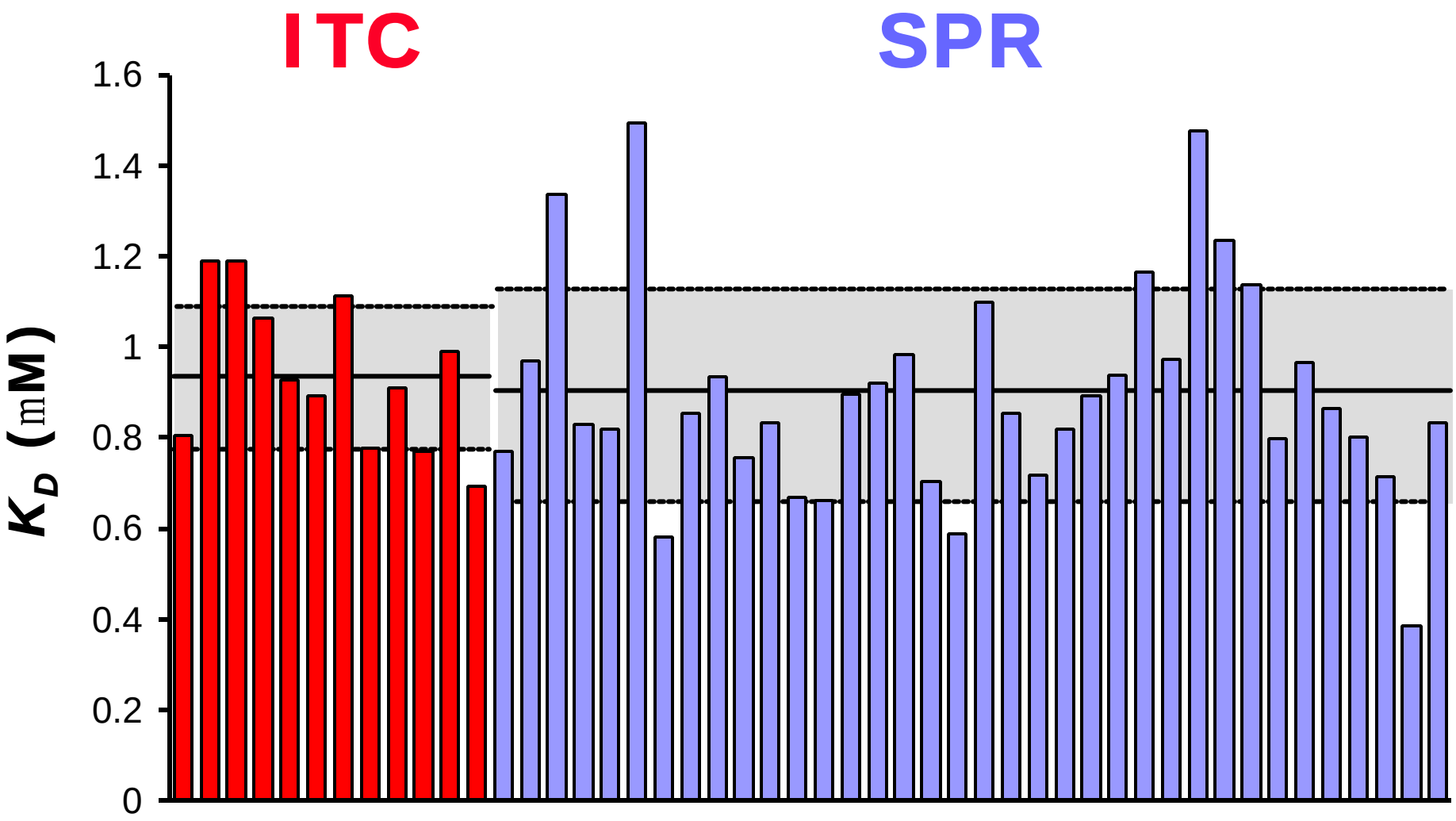
k_d = 0.035 ± 0.005 (s^{-1})



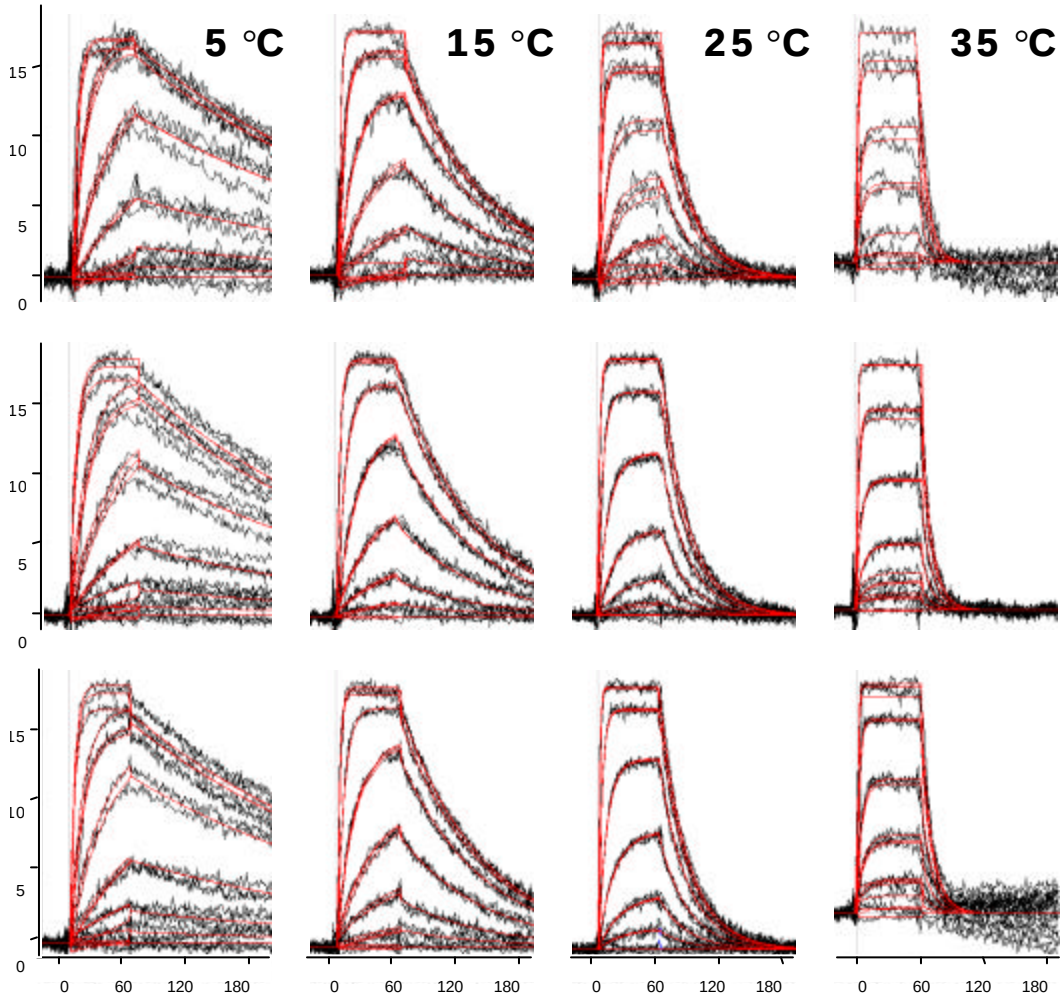
Affinity

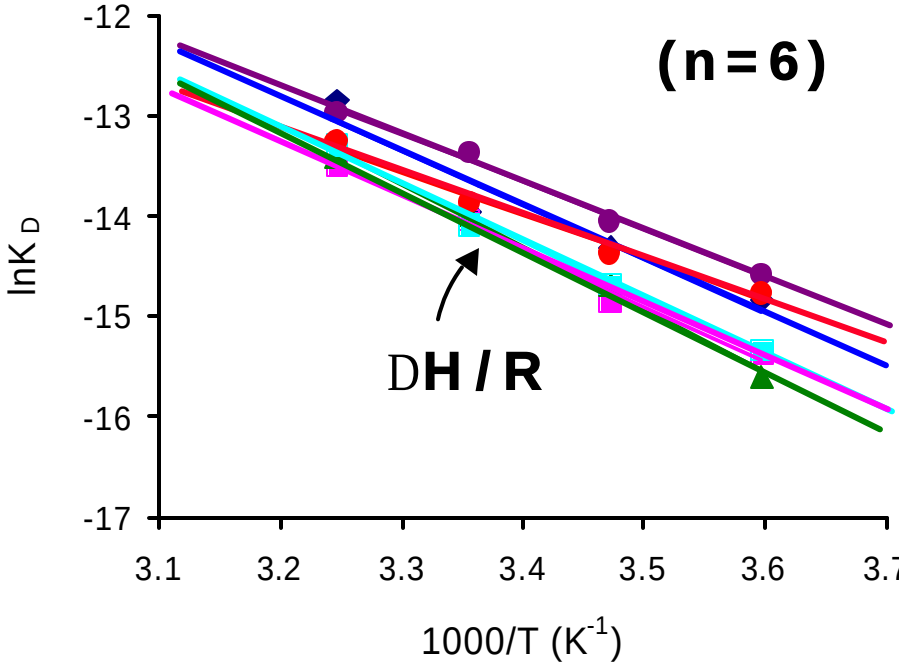

K_D = 0.90 ± 0.24 mM
K_D (μM)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35
Participant

Affinity Comparison



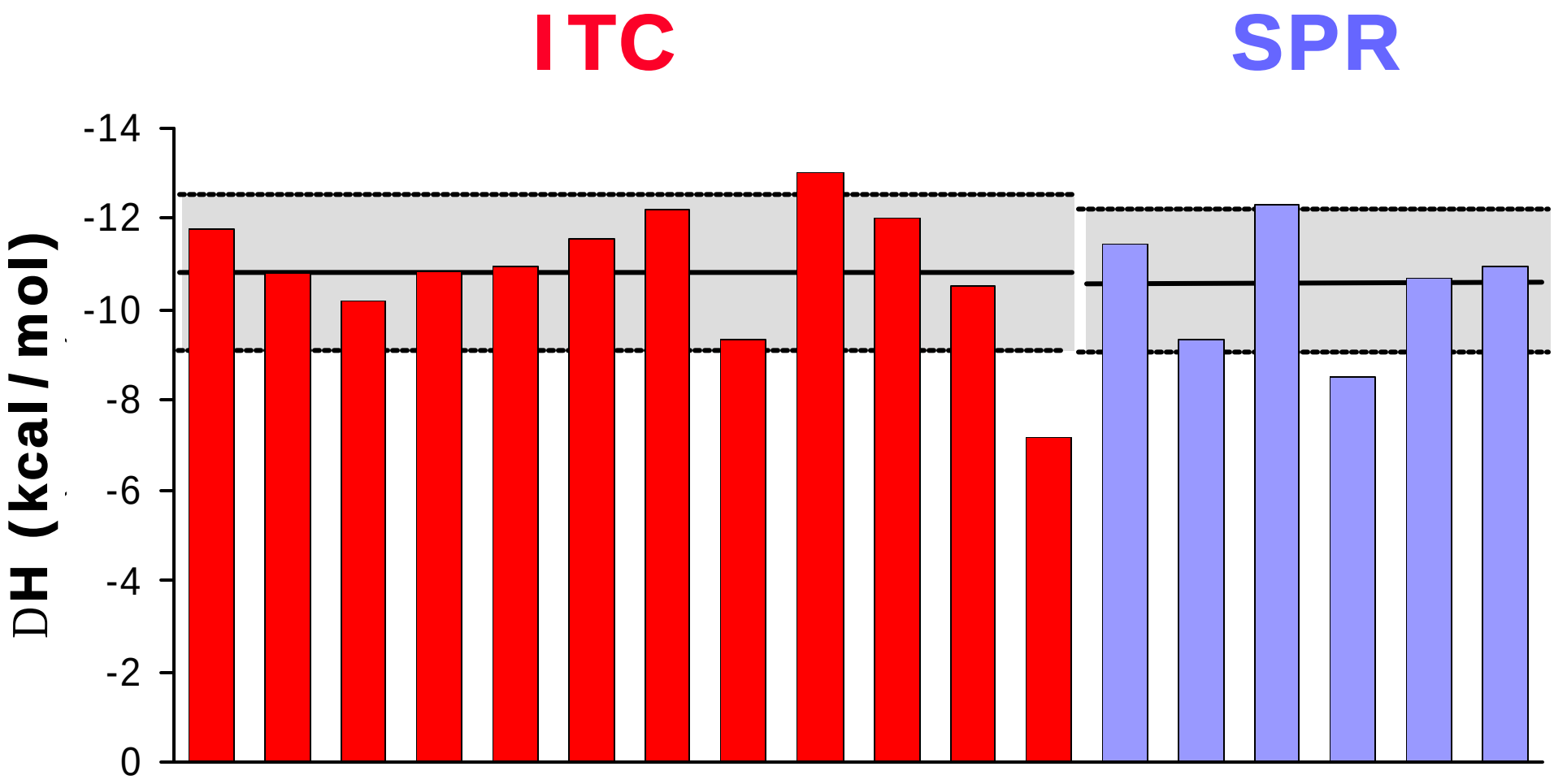
Van't Hoff Analysis





$$\Delta H^{vant} = -10.6 \pm 1.4 \text{ kcal/mol}$$

Enthalpy Comparison



Data Summary

	AUC	ITC	SPR
MW (28.98 kDa)	27.83 ± 0.75		
Stoichiometry		0.94 ± 0.10	
K_D (mM)		0.94 ± 0.17	0.90 ± 0.24
DH (kcal/mol)		–10.8 ± 1.6	–10.6 ± 1.4
k_a (M^{-1} s^{-1})			40,700 ± 7,600
k_d (s^{-1})			0.035 ± 0.005

20 mM Phosphate, 150 mM NaCl, pH 7.4, 25ºC

Acknowledgements

Poster R1
www.abrf.org