Small molecule interaction

Goal:

This protocol describes how to collect binding data for a small molecule/protein interaction. Specifically, it outlines how to measure the binding of 4-carboxybenzenesulfonamide (CBS) to surface-immobilized carbonic anhydrase II (CAII). Binding data will be collected on CAII surfaces immobilized at three different densities. The original methods described here are based on the control software of the BIAcore 2000 and 3000 platforms. We have modified the protocol in some areas to accommodate the differences between the BIAcore 3000 and the T200. Part of the Scrubber tutorial takes you through the analysis of the same experiment.

Strategy:

The protocol has been broken down into the following six steps:

- Step 1: Clean instrument
- Step 2: Prepare, filter and degas buffer
- Step 3: Precondition chip
- Step 4: Immobolize CAII
- Step 5: Normalize instrument
- Step 6: Prepare CBS and collect kinetic data

Reagents: We use 10X PBS provided by GEHealthcare. GE Healthcare also offers 10X HBS-EP$^+$, another commonly used running buffer.

- Sigma Aldrich Corp., St. Louis, MO
    - Carbonic anhydrase II (C-2522)
- Argos Organics, USA
    - 4-carboxybenzenesulfonamide (CBS; 393171000)
- GE Healthcare
    - Amine coupling kit (BR-1000-50)
    - 10 mM sodium acetate buffer, pH 5.0 (BR-1003-51)
    - 70% glycerol Normalizing solution (found in BIAmaintenance Kit, type 2, BR-1006-51 for T200 or BIAmaintenance kit, BR-1006-66 for the 3000).
    - 0.5% SDS, filtered and degassed (Desorb 1 found in maintenance kits above)
    - 50 mM glycine, pH 9.5, filtered and degassed (Desorb 2 found in maintenance kits above)
    - 10X PBS (BR-1006-72) for use as running buffer
- General
    - 100 mM HCl
    - 50 mM NaOH (BR-1003-58)

- 0.5% SDS (w/v)
- milliQ water

Equipment

- GE/Biacore optical biosensor (3000, T200)
- CM5 sensor chip (BR-1000-12)
- Series S CM5 sensor chip (BR-1005-30)
- Bench top ultracentrifuge w/ appropriate tubes
- Pipetman or equivalent w/ appropriate tips

Accessories

- 4.0 mL borosilicate screw-top vials, outer diameter 16 mm (GE Healthcare; BR-1002-09), equipped with polypropylene screw caps and high quality silicon/PTFE septa (GE Healthcare; BR-1002-11) or 4 mL plastic vials with an 11 mm OD (BR 1006-54) with kraton G ventilated caps, type 5 (BR-1006-55) for the T200.
- 0.8 mL rounded polypropylene micro-vials, outer diameter 7 mm (GE Healthcare; BR-1002-12), equipped with penetrable polyethylene snap caps for the 3000 (BR-1002-13) or kraton G ventilated caps, type 3 (BR-1005-02) for the T2200.
- 1.5 mL Eppendorf tubes for making dilutions

Step 1: Clean Instrument

Untitled 1Before beginning an experiment, the instrument should be cleaned to improve performance. A standard *Desorb* cleaning is performed here before the experiment and must be performed after a series of experiments. Additional cleaning or maintenance procedures may be done if necessary.

1. Set temperature to 25°C.
2. Prime with filtered, degassed milliQ water
3. Undock the maintenance chip. Note the differences between the CM5 sensor/maintenance chip for the 3000 and the Series S CM5 sensor/mainenance chip for the T200.
4. Re-Dock the maintenance chip. Desorb reagents will destroy a research grade sensor chip.
5. Prime with distilled water
6. Install Rack base: THERMO_A in position R2 on right side (BIAcore 3000) or Sample & Reagent Rack 1 (T100).
7. Desorb instrument using standard 0.5% (w/v) SDS and 50 mM glycine, pH 9.5 solutions following the on-screen instructions on each instrument.
8. Prime with water.

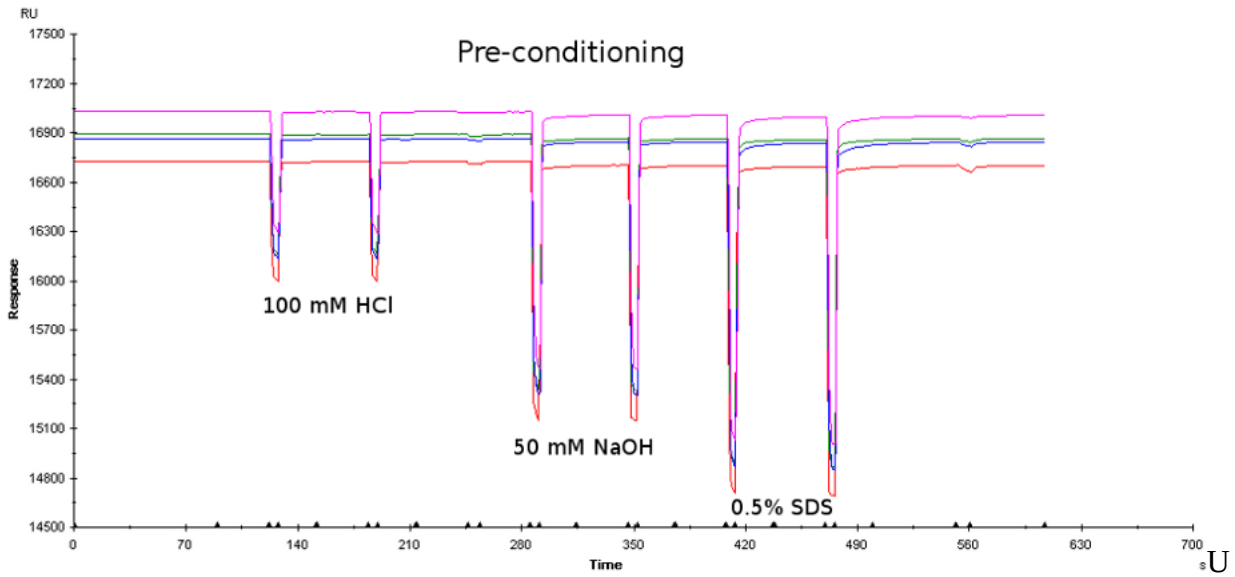
Step 2: Filter and degas buffer

1. Dilute 100 mL of 10X PBS from GE Healthcare to 1000 mL of millQ water, filter, and degas.
2. Filter and degas buffer

Step 3: Precondition chip

When using a new biosensor chip, the chip's surface should be preconditioned prior to immobilizing the ligand. The preconditioning process uses sequential injections of acid, base, and a denaturing agent. This cleans and hydrates the dextran layer.

1. Undock the maintenance chip and remove. Place in container.
2. Dock the new research-grade CM5 or Series S CM5 sensor chip provided to you with this protocol.
3. Replace the water with the 1X PBS running buffer
4. Prime the instrument three times with the running buffer using Tools/Working Tools/Prime or Tools -> Prime. A method is available on the B3000 to do this for you. Priming 3X on the T200 is manual
5. Pipet 250 µl 100 mM HCl, 50 mM NaOH, and 0.5% SDS respectively into 7 mm vials, cap and place in positions A1, A2, and A3 of a Thermo_A rack and place the rack in position R2. Do the same for the T200 using the kraton G caps and placing the reagents in A1, A2, and A3 of the Sample and Reagent Rack 1.
6. Ensure that Rack base:Thermo_A is recognized in position 2 on the BIAcore 3000 and the Sample & Reagent Rack 1 is selected on the T200.
7. On the B3000, select Run: Run Sensogram. On the T200, select Run:Manual
8. Select Flow and Detection over flow cells 1-2-3-4.
9. Define a flow rate of 100 µL/min.
10. Once the sensorgram begins, a queue window will open on the Biacore 3000 - choose Command:Inject:Quickinject. For the T200, use the Regeneration injection and a 6 second contact time.
11. Define the sample positions R2A1 and select a volume of 10 µL. Click on More and select Extraclean. Repeat so that there are two Quickinjects from R2A1 with extraclean. You will need to do this for each injection.
12. Repeat steps 9 through 11 to inject two aliquots of each reagent. Note that sample position should be changed to reflect the position of the reagent in the rack.
13. At the end of the preconditioning step, you should obtain a profile that resembles the profile shown in the figure.



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Step 4: Immobilize CAII

This step outlines how to immobilize the CAII on three flow cells at different densities.

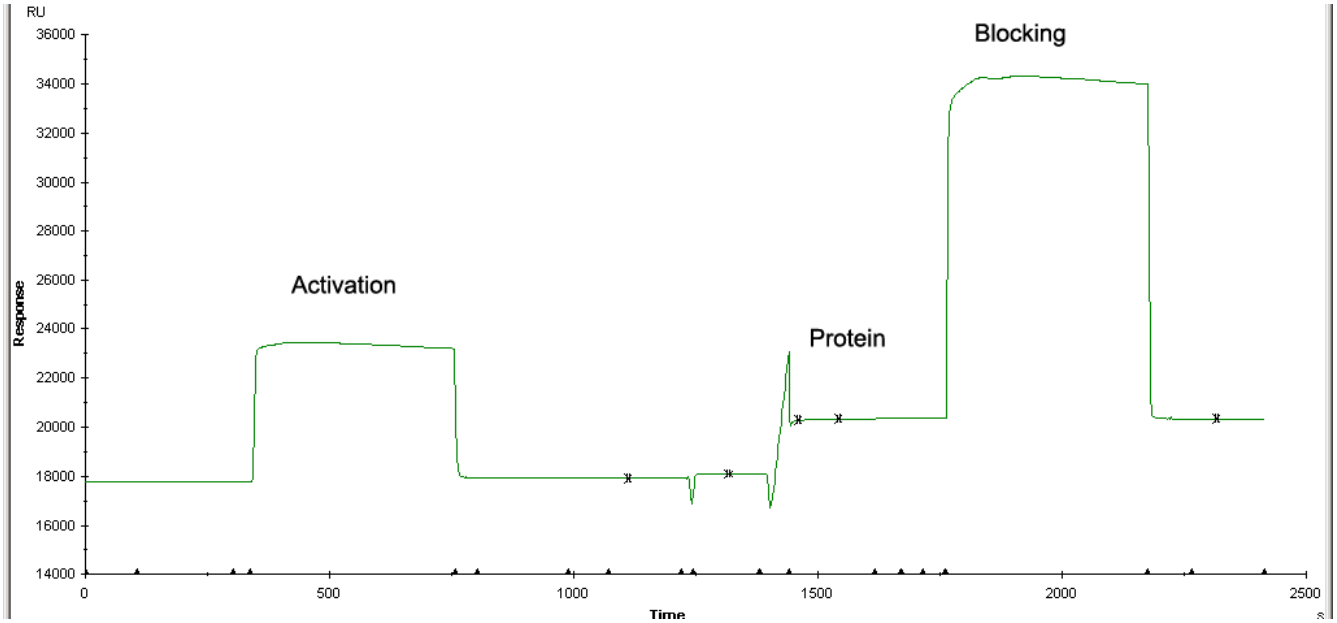
- Centrifuge the lyophilized CAII pellet contained in the Eppendorf tube in a bench top centrifuge.
- Dissolve the CAII pellet in 1 mL of 10 mM sodium acetate, pH 5.0. The concentration is 1 mg/ml. Centrifuge the solution for 5 min in an ultracentrifuge at 100,000 rpm, 4°C.
- Dilute the supernatant 1:10 with 10 mM sodium acetate, pH 5.0. The working concentration is 0.1 mg/ml.
- Start new sensorgram (Run:Sensorgram on 3000; Manual Run on T200).
- To create a high-capacity surface on FC4, select flow path FC4 only.
- Set flow rate to 5 µL/min.
    1. Place 500 µL ethanolamine in R2A4 or in A2 on the T200. Thaw and swirl 1 tube each of NHS and EDC. In the 3000, place one tube in R2A1, the other in R2A2 (it doesn't matter which) and an empty, capped tube in R2A3. Go to Command:Mix to automatically prepare a 50% mixture to be placed in R2A3. Use Inject:Quickinject with extraclean to inject 35 µL of the mixture from R2A3 over the surface of FC4. For the T200, manually prepare a 50% solution (mix 100 µL of each in an Eppendorf tube and transfer to a 7 mm tube and cap) of NHS and EDC and place in A1. Use the Regeneration injection to inject 35 µL over the surface of FC4. Activation is time sensitive, so you will need to work quickly.
    2. Once completed, Quickinject 10 µL of clarified CAII over the surface of FC4 at the same 5 µL/minute or use the Inject command on the T200. Calculate the "preliminary" immobilization level. If 5,000 RU or greater are immobilized on the surface, proceed to the next step. If not, inject an amount which you estimate will give you 5,000 RU by changing the volume. Continue to inject until the required RU are immobilized on the

surface, but remember to work quickly.

3. To deactivate remaining activated groups, Quickinject with extraclean 35 µL of ethanolamine from R2A4 or Regeneration Injection 35 µL from A2.
4. Calculate the immobilization level as the difference from immediately before the coupling step and after the deactivation step. The density should be approximately 5,000 RU.

- Repeat the steps for FC3 to immobilize 4,000 RU and block.
- Repeat the steps for FC2 to immobilize 3,000 RU and block.
- Activate FC1 and block immediately without immobilizing any protein. This is your reference Flow Cell.

The figure shows activation of a flow cell, capture of protein and blocking. Approximately 2,000 RU of protein were captured on this surface.



Steps 5 and 6: Normalization and preparation of CBS

- Place 250 µL of 70% glycerol in R2F2 in the B3000 (9 mm glass vial with cap). Place 250 µL of 70% glycerol in a 7 mm plastic vial with the appropriate kraton G cap in the T200 and place in C15.
- In the B3000, run the Normalize procedure from the Tools:Working tools. On the T200, the normalize procedure is programmed into the beginning of the assay.
- Dilute the 1 mM CBS stock solution 100-fold to a final concentration of 10 µM. To do this, prepare a total volume of 1.0 mL by adding 10 µL of the 1 mM CBS to 990 µL of freshly degassed 1X PBS running buffer.. This 10 µM sample is called sample 1.
- Prepare four additional samples of the small molecule using three-fold serial dilutions as follows.
    - Mix 0.5 mL of sample 1 and 1.0 mL of running buffer. This is sample 2. It has a

concentration of 3.3 µM.
    - Mix 0.5 mL of sample 2 and 1.0 mL of running buffer. This is sample 3. It has a concentration of 1.11 µM.
    - Mix 0.5 mL of sample 3 and 1.0 mL of running buffer. This is sample 4. It has a concentration of 0.37 µM.
    - Mix 0.5 mL of sample 4 and 1.0 mL of running buffer. This is sample 5. It has a concentration of 0.12 µM.
- Label nine 7-mm plastic vials with a "0" and aliquot 200 µL of running buffer into each and cap. Tap and inspect to make sure no air is trapped in the bottom.
- Label three 7-mm plastic vials with a "1", 3 vials with a "2" and continue through "5".
- Pipet 150 µL of sample 1 into each of the 3 vials marked "1" and continue through "5".
- Label one 4-mL vial with a "0" and fill it with 4 mL of running buffer. This will serve for injection of startup "blank" samples. Label one 4-mL vial with "wash" and fill it with 4 mL of running buffer. This will serve as wash after each injection. Cap appropriately.

BIAcore 3000

For the BIAcore 3000, make sure that the rack THERMO_A is placed in the right hand slot. Place the large vial marked "0" in R2F3 and the large vial marked "W" in R2F4.

Visually examine each vial for trapped air bubbles on the bottom before placing them in the rack. Place one vial containing 0.12µM in R2A1, 0.37 µM in R2A2, 1.11 µM in R2A3, 3.33 µM in R2A4, and 10.0 µM in R2A5. Place tubes containing buffer only in R2A6 - R2A8. Repeat for the second replicate from lowest to highest concentration followed by three buffer blanks and repeat again for the third replicate followed by the last three buffer blanks.

The following is the BIAcore 3000 program that will be used to collect the kinetic data of the CBS/CAII interaction. The program is a simple text file that can be written in any simple text editor such as Notepad. The first column is a sequential list of steps starting with s1, the second column is the position that the sample will be drawn from, and the third column following the exclamation (!) is a comment section.

```
DEFINE LOOP cycle1
LPARM %s1 %p1

times1
s1 r2f3 !initial buffer injections to prepare the instrument
s2 r2f3 !
s3 r2f3 !
s4 r2f3 !
s5 r2f3 !
s6 r2f3 !
```

s7 r2f3 !
s8 r2f3 !
s9 r2f3 !
s10 r2f3 !
s11 r2a1 !0.12µM
s12 r2a2 !0.37µM
s13 r2a3 !1.11µM
s14 r2a4 !3.33µM
s15 r2a5 !10.0µM
s16 r2a6 !buffer blanks
s17 r2a7 !
s18 r2a8 !
s19 r2a9 !0.12µM
s20 r2a10 !0.37µM
s21 r2b1 !1.11µM
s22 r2b2 !3.33µM
s23 r2b3 !10.0µM
s24 r2b4 !buffer blanks
s25 r2b5 !
s26 r2b6 !
s27 r2b7 !0.12µM
s28 r2b8 !0.37µM
s29 r2b9 !1.11µM
s30 r2b10 !3.33µM
s31 r2c1 !10.0µM
s32 r2c2 !buffer blanks
s33 r2c3
s34 r2c4
END

DEFINE APROG analyse1
PARAM %s1 %p1
flow 50 !flow rate
flowpath 1,2,3,4 !flow path is all four flow cells
kinject %p1 50 180 !perform a kinject using a volume of 50µL for a 60s contact time, 180s dissociation

quickinject r2f4 15 !regeneration with wash solution and using extraclean
extraclean
END

MAIN

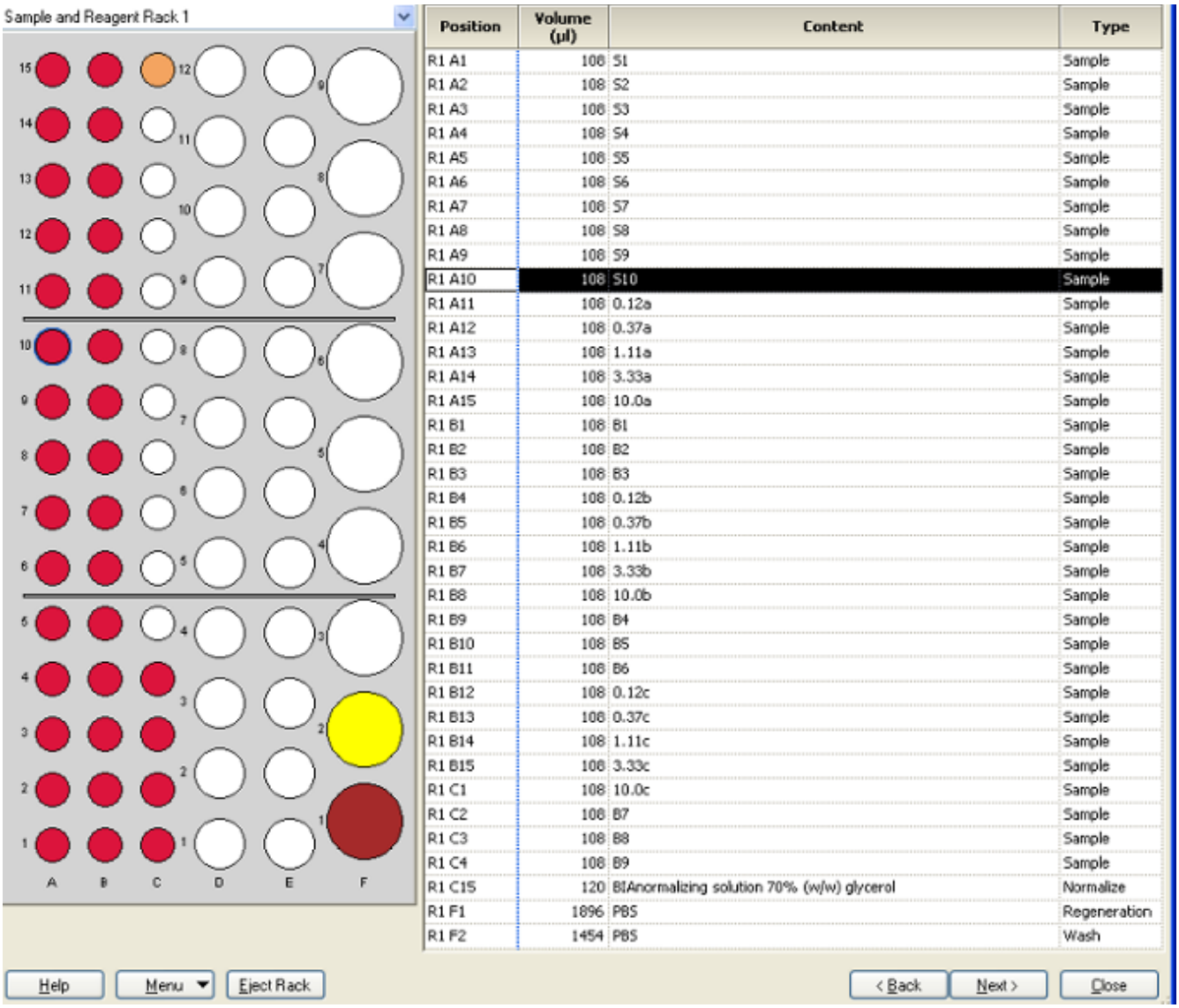
```
DETECTION 1,2,3,4 !detection is on all four flow cells

LOOP cycle1 STEP
APROG analyse1 %s1 %p1
ENDLOOP

append continue !instrument goes into standby after all cycles in the loop are completed
END
```

T200

For the T200, use the Sample & Reagent Rack. Place the tube containing the normalize solution in C15. Place ten 7 mm buffer tubes in A1 - A10, the first replicate of sample tubes from lowest to highest concentration (A11 - A15), and three buffer blanks in B1, B2, and B3. Repeat for the second replicate from lowest to highest concentration followed by three buffer blanks and repeat again for the third replicate followed by the last three buffer blanks.



Position	Volume (µl)	Content	Type
R1 A1	108	S1	Sample
R1 A2	108	S2	Sample
R1 A3	108	S3	Sample
R1 A4	108	S4	Sample
R1 A5	108	S5	Sample
R1 A6	108	S6	Sample
R1 A7	108	S7	Sample
R1 A8	108	S8	Sample
R1 A9	108	S9	Sample
R1 A10	108	S10	Sample
R1 A11	108	0.12a	Sample
R1 A12	108	0.37a	Sample
R1 A13	108	1.11a	Sample
R1 A14	108	3.33a	Sample
R1 A15	108	10.0a	Sample
R1 B1	108	B1	Sample
R1 B2	108	B2	Sample
R1 B3	108	B3	Sample
R1 B4	108	0.12b	Sample
R1 B5	108	0.37b	Sample
R1 B6	108	1.11b	Sample
R1 B7	108	3.33b	Sample
R1 B8	108	10.0b	Sample
R1 B9	108	B4	Sample
R1 B10	108	B5	Sample
R1 B11	108	B6	Sample
R1 B12	108	0.12c	Sample
R1 B13	108	0.37c	Sample
R1 B14	108	1.11c	Sample
R1 B15	108	3.33c	Sample
R1 C1	108	10.0c	Sample
R1 C2	108	B7	Sample
R1 C3	108	B8	Sample
R1 C4	108	B9	Sample
R1 C15	120	B1Anormalizing solution 70% (w/w) glycerol	Normalize
R1 F1	1896	PBS	Regeneration
R1 F2	1454	PBS	Wash