

PROTOCOL FOR MEASURING  
SMALL MOLECULE INTERACTIONS  
USING BIACORE

*A Practical Guide  
to Experimental Design  
and Data Evaluation*

## **1. PREPARATION**

A successful small molecule assay on the Biacore requires careful preparation of running buffer, instrument, sensor surface, and samples. The process of setting up a small molecule assay follows the same general procedure (immobilization, binding, and regeneration) for all Biacore applications. Some of the steps may be more or less time consuming depending on how much is known about the ligand and the small molecules.

### *General Considerations*

All buffers used in the assay both as running buffer and for sample and reagent preparation, should be filtered through a 0.22 $\mu$  filter and degassed. Use Teflon or nylon filters for buffers containing DMSO: do not use cellulose acetate filters. Also avoid polystyrene containers for holding the buffer; glass bottles are recommended. Phosphate buffered saline (PBS) is recommended as a buffer for most small molecule assays. Many different “standard,, recipes are available for PBS and any of these are acceptable. Keep the phosphate concentration at 50 mM or higher to maintain sufficient buffering capacity to handle addition of DMSO to the buffer without seriously affecting the pH. Use ionic strength at or close to physiological levels (150 mM monovalent ions) to reduce non-specific binding of compounds to the sensor surface.

Other buffers may be used if required by the application however, organic buffers such as Hepes are not generally recommended, since they have been observed to interfere with ligands binding human serum albumin (HSA).

Make sure the buffer is compatible with other solutions used in the assay, in particular with regeneration solutions. Certain ligands (for example thrombin) are efficiently regenerated with 4 M MgCl<sub>2</sub>, which can cause precipitation at the injection interface between regeneration solution and phosphate-based running buffer. Alternative buffers must be used in such cases.

### *Solvents*

Many small molecules require organic solvents for solubility. Dimethyl sulfoxide (DMSO) is most commonly used: a recommended concentration for Biacore assays is 5%, however this will depend on the activity of the target or ligand in the presence of DMSO. The instrument fluidics can withstand concentrations up to 50% for short injections, for example for washing the flow system.

The running buffer should contain the same DMSO concentration as the samples, since DMSO has a high refractive index contribution and small differences in DMSO concentration will significantly effect the bulk response. Match the DMSO concentration between samples and running buffer as closely as possible. A 1% difference in DMSO concentration can result in a 1000 RU bulk shift.

When handling DMSO, particularly in concentrated form, make sure that all vessels and equipment withstand contact with the solvent. Certain plastics (polycarbonate and polysulfone) release contaminants into DMSO that have been observed to bind HSA and interfere with binding assays on the Biacore. Deterioration of the plastic on contact with DMSO is not always apparent. Running test analyses with negative controls (buffer containing DMSO) is recommended for all assays.

DMSO is incompatible with polycarbonate and polysulfone, moderately compatible with polystyrene and compatible with polypropylene.

DMSO from different suppliers may vary in quality, and may sometimes contain contaminants that interfere with the assay. Good results have been obtained using DMSO from Sigma (D 5879).

## **1.1 Buffer Preparation**

Prepare 1X PBS running buffer as follows:

1. Prepare a 10X concentrate of 67 mM Na<sub>2</sub>HPO<sub>4</sub>•2H<sub>2</sub>O, 12.5 mM KH<sub>2</sub>PO<sub>4</sub>, 70 mM NaCl and pH the solution with concentrated HCl according to the chart below so that the final pH is 7.4 when the 10X stock is diluted to 1X.

### **pH table for 10X PBS**

<u>%DMSO in final 1X buffer</u>	<u>pH of 10X PBS</u>	<u>pH of 1X PBS</u>
1	6.65	7.4
3	6.80	7.4
5	6.85	7.4
7	6.90	7.4
10	7.00	7.4

1X PBS (no DMSO); running buffer during immobilization

2. Add 50mL of 10X PBS to 450mL of H<sub>2</sub>O. Filter and degas. Set aside for immobilization procedure.

1X PBS (5% DMSO); running buffer during binding assay

3. Add 50mL of 10X PBS to 425mL of H<sub>2</sub>O. Add 25mL of DMSO (HPLC grade) to give ~5% DMSO 1X PBS, pH 7.4. Filter and degas. This will be the running buffer for the binding experiment.

1.05X PBS (no DMSO) for sample preparation

4. Add 52.5mL of 10X PBS to 447.5mL of H<sub>2</sub>O. Filter and degas. Set aside for sample preparation and dilutions.

Tips:

- \_ Make up fresh 1X PBS every day.
- \_ Avoid polystyrene containers; use clean glass bottles for buffer.
- \_ Use Teflon or nylon filters for buffers containing DMSO: do not use cellulose acetate filters.
- \_ Always keep buffers in clean bottles tightly capped before placing in the Biacore.
- \_ When the buffer is placed in the buffer compartment of the Biacore, keep the cap screwed down to prevent any evaporation or contamination of the buffer.

## 1.2 Instrument Preparation

In view of the low responses obtained from low molecular weight small molecules, it is essential for reliable results that the Biacore system is properly maintained at all times. To improve data quality, run the following cleaning procedure before every small molecule assay:

1. Set instrument temperature to 25°C.
2. Dock a clean maintenance chip.
3. Place distilled water in the buffer chamber and prime.
4. Run DESORB.
5. Prime 2X with water.

Automated Method for cleaning:

```
MAIN  
PRIME  
DESORB R2F3 R2F4  
PRIME  
PRIME  
APPEND STANDBY  
END
```

6. Dock a new CM5 research grade sensor chip.
7. Prime 3X with the 1X PBS running buffer (no DMSO).
8. Pre-condition the sensor chip as follows:
  - Set flow rate to 100  $\mu\text{L}/\text{min}$ .
  - Using the QUICKINJECT command, apply 2 consecutive 50  $\mu\text{L}$  pulses of each of the following reagents: 50 mM NaOH, then 10 mM HCl, 0.1% SDS, and 0.085%  $\text{H}_3\text{PO}_4$ .
  - Wash the IFC at the end of the injection series. (Go to *command* then *wash*)
  - Inject blank buffer to wash out any remaining cleaning solutions in the autosampler tubing.

Tips for improving data quality:

- \_ Check syringe plungers for debris and leaking.
- \_ If needed, clean syringes and replace o-rings on the plungers as directed under working tools.
- \_ Remove and clean connector block with distilled water then replace and check injection port.

### **1.3 Surface Preparation**

#### *General Considerations*

The target protein (ligand) should be immobilized to a high level under as mild pH conditions as possible. Performing the immobilization at pH 6 vs. pH 4 may be better even if it means using 100µg/ml of the target protein instead of 10µg/ml. Immobilizing at a pH closer to the pI of a protein helps to ensure a more active form of the protein in being immobilized. To estimate how much protein needs to be immobilized, calculate the theoretical maximum response,  $R_{max}$ , achievable when the lowest molecular weight compound under investigation binds to the protein. Theoretical  $R_{max}$  values of at least 30 are preferable. As an example, to achieve a theoretical  $R_{max}$  of 50 RU when a 358 Da compound binds to a 40 kDa target protein, the immobilization level required is calculated as follows:

$$R_{max} = MW_A/MW_L \times R_L \times S_M$$

Where  $MW_A$  is the molecular weight of the analyte

$MW_L$  is the molecular weight of the ligand

$R_L$  is the immobilization level in RU

$S_M$  is the molar stoichiometry

$$50 = 358/40,000 \times R_L \times 1$$

$$R_L = 40,000/358 \times 50$$

$$R_L = 5586 \text{ RU}$$

However, if the surface were only 50% active then 11,172 RU would need to be immobilized:

$$50\% \text{ activity: } 5586/0.5 = 11,172 \text{ RU}$$

#### *Reference Surface Considerations*

The use of a reference surface enables subtraction of bulk responses. The dextran matrix of a reference, without any immobilized protein, will have room for slightly more DMSO than when target is immobilized. A higher DMSO bulk response will then be obtained from the empty flowcell compared to the target flowcell during injection. Thus the response may be higher on the reference surface than the target surface. To correct for this difference a solvent correction assay can be performed. (Section 2).

Either unmodified or an activated and blocked dextran surface can be used as a reference surface. Which to choose depends on the properties of the compounds and their susceptibility to bind non-specifically to the modified/unmodified dextran.

### **1.4 Testing the Surface**

Once the surface has been prepared, the analyte binding activity should be tested before proceeding to further stages in assay development. The same protocol can also be used to test the activity of sensor chips that have been stored, to ensure that activity is retained.

To test the binding activity of the surface, a known binder should be used as analyte. If the analyte is a small molecule that requires DMSO for solubility, include the same concentration of DMSO in the running buffer to minimize the bulk refractive index shift during sample injection.

### **1.5 Preparation of Samples**

50  $\mu\text{M}$  is a suitable concentration to start with when sample binding levels are unknown.

To prepare a 50  $\mu\text{M}$  stock sample in 5% DMSO: Add 5  $\mu\text{l}$  of a 10 mM stock concentration in 100% DMSO to 995  $\mu\text{l}$  of 1.05X PBS no DMSO.

Serial dilutions can be made from the 50  $\mu\text{M}$  stock using the 1X PBS with 5% DMSO (running buffer) as the diluent.

Prepare 25  $\mu\text{M}$  sample by adding 500  $\mu\text{l}$  the 50  $\mu\text{M}$  stock to 500  $\mu\text{l}$  of the 1X PBS with 5% DMSO (running buffer).

The next dilution (12.5  $\mu\text{M}$ ) is prepared the same way using 25  $\mu\text{M}$  as the stock.

Six to eight concentrations should be prepared for an affinity / kinetic assay.

The total volume for each dilution is 500  $\mu\text{l}$ , which can be divided into 3 tubes with 150  $\mu\text{l}$  each.

Tips:

\_ Samples in 100% DMSO should be kept in glass vials and tightly capped.

\_ Certain plastics release contaminants into DMSO that have been observed to bind to human serum albumin and interfere with binding assays. DMSO is incompatible with polycarbonate, but is compatible with polypropylene.

## 2. SOLVENT CORRECTION

(Reference: “A calibration routine to improve the interpretation of low signal levels and low affinity interactions” Hakan Roos, Robert Karlsson, and Karl Andersson, Biacore AB)

The SPR signal reflects changes in refractive index (RI) as a result of increases in mass at the sensor surface. If the injected sample has a RI different from that of the running buffer an additional signal is obtained known as the bulk refractive index, also referred to as a solvent effect. The solvent effect can usually be removed by subtracting the reference flow cell from the active flow cell when the solvent effect is small (~100 RU). However, in most small molecule assays where DMSO is used as the solvent, the subtraction of the reference response does not eliminate the contribution of the solvent to the measured response because a higher DMSO bulk response will be obtained from the reference surface. Bulk solution is excluded from the volume occupied by ligand molecules on the ligand spot, so the bulk contribution to the relative response is smaller than on the reference spot (Figure 2.1).

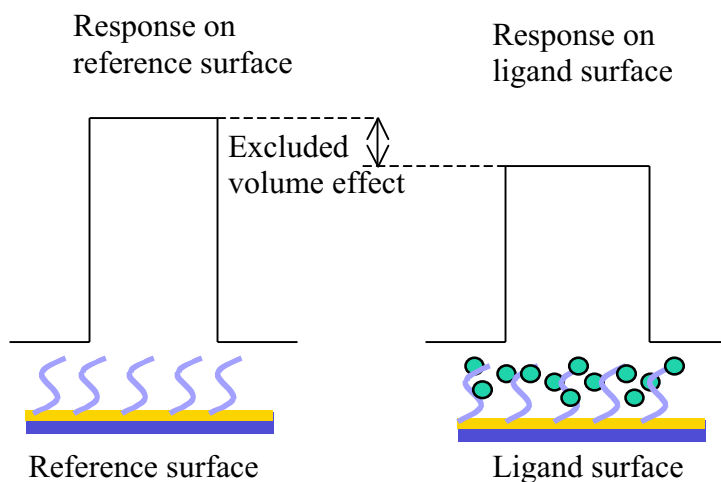


Figure 2.1

The difference is small (usually less than 10 RU) but it can be of the same order of magnitude as the responses expected from binding of low molecular weight lead compounds.



## 2.1 Is Solvent Correction Necessary?

Solvent correction is necessary under a combination of three circumstances that commonly arise when working with low molecular weight analytes such as drug candidates:

- The expected analyte responses are low.
- The ligand is a macromolecule immobilized at a high density on the surface (typically 10,000 RU or more).
- The absolute bulk response is high (typically 30,000 RU or more) and is subject to variations between sample solutions.

The combination of these three factors results in variations in the magnitude of the excluded volume effect that are significant in relation to the measured analyte response.

In drug discovery and development work, the analytes are often small molecules that give correspondingly low response values (typically of the order of 50-100 RU or less). High levels of immobilized ligands (several thousand RU) are used to maximize the analyte response. Addition of DMSO to samples and buffers gives a high bulk response. A difference of 1% in DMSO concentration corresponds to a difference of ~1200 RU in bulk response, so that small variations in DMSO concentration, unavoidable in the preparation of a large number of diverse samples, lead to significant differences in bulk response between samples. Solvent correction samples should generally be included in assay protocols for this kind of work: the decision as to whether to apply the solvent correction or not can be made when the results are evaluated. If data is collected during the dissociation phase, DMSO bulk responses are not an issue and the data may be handled by conventional reference subtraction.

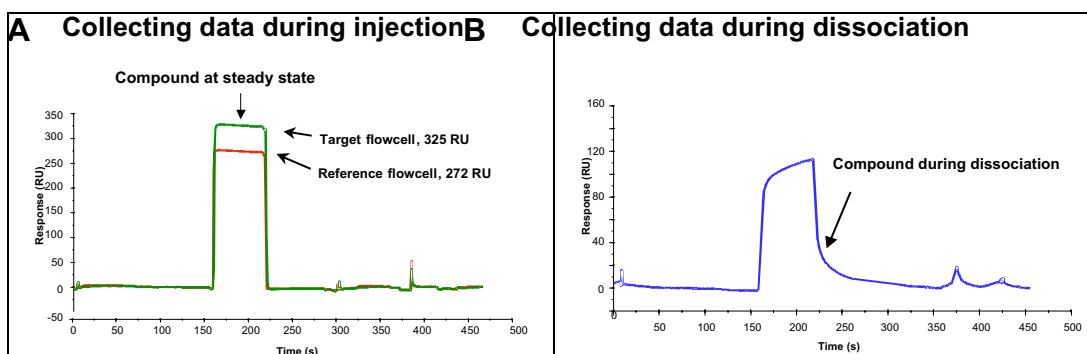


Figure 2.2

## **2.2 Solvent Correction Procedure**

The following procedure is a suggested protocol that uses 5% DMSO in samples and running buffer. Adjust the amounts and concentrations accordingly if a different DMSO concentration is being used. Prepare all solutions containing DMSO fresh each day.

1. Prepare a PBS buffer stock solution at 1.05X final concentration. (see section 1.1)
2. Prepare solvent correction solutions over a range of DMSO concentrations from 4.5% to 5.8% by adding 100% DMSO to 1900  $\mu$ l aliquots of 1.05X buffer. The table below gives suggested volumes for 8 solvent correction solutions.

	1	2	3	4	5	6	7	8
Nominal DMSO concentration	5.8%	5.6%	5.4%	5.2%	5.0%	4.85%	4.7%	4.5%
100% DMSO ( $\mu$ l)	116	112	108	104	100	97	94	90
1.05x buffer stock ( $\mu$ l)	1900	1900	1900	1900	1900	1900	1900	1900

3. Use these solutions for the solvent correction cycles in the assay procedure. The solutions should be freshly prepared for each assay.

### Tips:

\_ Do not store DMSO in plastic vessels. Make sure that all plastic equipment used in handling the solutions is resistant to DMSO. Use teflon or nylon membranes to filter DMSO solutions: do not use cellulose acetate membranes.

\_ Prepare all solutions carefully. Any mismatch in composition except for the DMSO concentration will affect the accuracy of the solvent correction.

\_ The exact DMSO concentrations in the solutions are not crucial, but it is important that the solutions cover an adequate range of bulk responses. If the range is too narrow, it may not be possible to correct the assay results for solvent effects.

### **2.3 Applying Solvent Correction**

Solvent correction is applied to assay results in the evaluation software, after the assay is completed. The following instructions are for applying solvent correction data to an assay performed on a Biacore 2000 or 3000 using BIAevaluation 3.0 or higher.

#### *Generate a DMSO Calibration Curve*

1. Open the file in BIAevaluation and choose Rpoint table only.
2. Extract the calibration data by selecting Data>filter. Under the Fc column choose the reference flow cell and under the Id column choose Bulk. The control flow cell and the Id for the calibration samples will depend on the user-defined method.
3. Once the data has been filtered for the control flow cell and the calibration solutions, copy and paste columns Cycle and RelResp into a new sheet by selecting File>New sheet.
4. Return to the window containing the original Rpoint table and filter the data to select the active flow cell and calibrators should still be under the Id column.
5. Once the data has been filtered for the active flow cell and the calibration solutions, copy and past RelResp to same sheet created in Step 3.
6. Change RelResp column headings to Fc 1 and Fc 2 and name the next empty column Fc 2-1.
7. In the Fc 2-1 column subtract Fc 1 from Fc 2 using the same method used in Excel.

#### EXAMPLE:

Cycle	Fc1	Fc2	Fc2-1
1	384.2	376.1	-8.1
2	181.9	177	-4.9
3	80	76.7	-3.3
4	-22.3	-24	-1.7
5	-127.3	-127.5	-0.2
6	487.3	476.6	-10.7
7	583.2	569.8	-13.4
8	279.4	272.9	-6.5

8. Select Data>Generate Calibration, set X Keyword Fc 2-1, Y Keyword Fc1. Choose Other and Line under Fitting Function.
9. View the calibration curve (example shown below) and determine if the fit is good. The fit may be improved by removing points that lie outside the range of sample bulk responses.

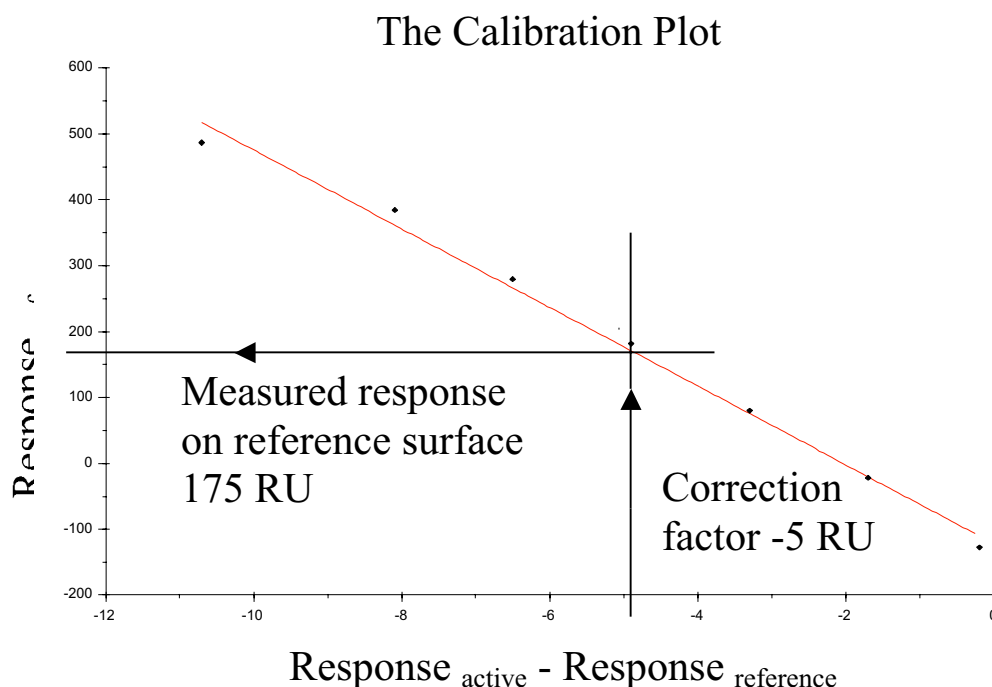


Figure 2.3

#### *Correct Sample Bulk Responses*

1. Return to the Rpoint table.
2. Filter for the reference flow cell and under Id column choose Plateau.
3. Copy and Paste columns Cycle and RelResp to sheet used to generate calibration curve.
4. Return to the Rpoint table.
5. Filter for the reference flow cell and under Id column choose Plateau.
6. Copy and Paste RelResp to sheet used to generate calibration curve.
7. Change RelResp column headings to Drug Fc1 and Drug Fc2.
8. To find the correction factor choose Data>Calculate values>Next.
9. Set Y keyword=Drug Fc1 (the samples bulk response in the reference flow cell). Click Finish.
10. Give the new column the heading Corr, and next column heading True.
11. In the True column subtract: Drug Fc2-Drug Fc1-Corr .

Repeat process for flow cells 3 and 4 substituting the data from Fc 3 and Fc 4 for both the calibration curve and correction of drug binding response. Using a new sheet for each flow cell is recommended.

### 3. EXPERIMENTAL PROCEDURE

#### 3.1 Method for Screening

The following MDL instructions are for performing the assay on Biacore 2000 or 3000.

```
MAIN
RACK 1 Thermo_A
RACK 2 Thermo_C
    NORMALIZE r1a1
    PRIME
    PRIME
    PRIME
    PRIME
    PRIME
FLOWCELL 1,2,3,4
LOOP SAMPLES ORDER
    APROG  SCREEN %POS %ID %CONC
ENDLOOP
    APPEND  STANDBY
END

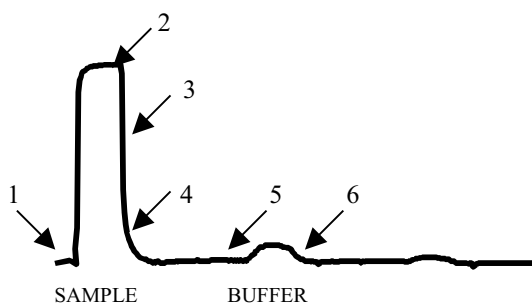
DEFINE APROG SCREEN
    PARAM %POS %ID %CONC
    FLOW 50
FLOWPATH 1,2,3,4
    * KINJECT r1a2 50 1          !BLANK BUFFER INJECTION

-0:20 RPOINT -B BASELINE  !TO CHECK FOR CARRY-OVER.
1:20 RPOINT CARRYOVER
    * KINJECT %POS 50 60
    EXTRACLEAN
-0:20 RPOINT -B BASELINE
0:50 RPOINT BULK
1:10 RPOINT DISS1
1:40 RPOINT DISS2
END
```

DEFINE LOOP SAMPLES

```
LPARAM %POS %ID %CONC
TIMES 2
  r2a1 BUFFER 0 !RUN 10 BUFFER INJECTIONS AT THE BEGINNING
  r2a2 BUFFER 0 !OF THE SCREEN TO STABILIZE THE SYSTEM.
  r2a3 BUFFER 0
  r2a4 BUFFER 0 !THROUGHOUT THE SCREEN, INCLUDE BLANK
  r2a5 BUFFER 0 !BUFFER INJECTIONS TO BE USED IN THE
  r2a6 BUFFER 0 !DOUBLE REFERENCING.
  r2a7 BUFFER 0
  r2a8 BUFFER 0
  r2a9 BUFFER 0
  r2a10 BUFFER 0
  r2b1 CALIBRATION 1 !RUN CALIBRATORS EVERY 20 SAMPLES.
  r2b2 CALIBRATION 2
  r2b3 CALIBRATION 3
  r2b4 CALIBRATION 4
  r2b5 CALIBRATION 5
  r2b6 CALIBRATION 6
  r2b7 CALIBRATION 7
  r2b8 CALIBRATION 8
  r2c1 compound_1 10uM
  r2c2 compound_2 10uM
  r2c3 compound_1 100uM
  r2c4 compound_2 100uM
END
```

**3.2 Report Points**



- 1- sample baseline
- 2- bulk response, (binding level)
- 3- dissociation 1, (stability early)
- 4 -dissociation 2, (stability late)
- 5- carry-over baseline
- 6- carry-over control

## 4. DATA EVALUATION

### References:

**A. Frostel-Karlsson, A. Remaeus, H. Roos, K. Anderson, P. Borg, M. Hamalainen, R. Karlsson** (2000) Biosensor Analysis of the Interaction between Immobilized Human Serum Albumin and Drug Compounds for Prediction of Human Serum Albumin Binding Levels. *Journal of Medicinal Chemistry*, 43, 1986-1992.

Data evaluation and analysis are report point - based. All plots can be constructed in Excel.

### 4.1 Quality Control Plots

#### 1. Baseline stability

Plot the baseline report point (report point 1) versus cycle number. This should be done using raw data, i.e. the data should *not* be Y-transformed, reference subtracted, molecular weight corrected or solvent corrected.

#### 2. Carry-over

Plot the carry-over report point (report point 6) versus cycle number. The data should be Y-transformed to baseline-zero, reference subtracted and solvent corrected.

#### 3. Binding to control surface

Plot the bulk response (report point 2) versus cycle number for Fc 1, reference flow cell. The data should be raw data, i.e. *not* reference subtracted, Y-transformed or solvent corrected.

#### 4. Control binding levels

Plot the bulk response (report point 2) for positive and negative control compounds versus cycle number for Fc 2, experimental flow cell. The data should be Y-transformed, reference subtracted, solvent corrected and *not* molecular weight corrected.

Generate these Quality Control Plots for the Fc 2, experimental flow cell, using the Fc 1 surface as the reference.

### 4.2 Data presentation and analysis

Plot the following versus cycle number for all compounds. All data should be Y-transformed to baseline-zero, reference subtracted, solvent corrected and molecular weight corrected.

- Bulk response (binding level), report point 2
- Dissociation 1 (stability early), report point 3
- Dissociation 2 (stability late), report point 4

Plot bulk response (report point 2) versus dissociation 1 (report point 3) or dissociation 2 (report point 4) depending on the magnitude of the off rates in the data set.

For “hits,, found during the experiment, a compound characterization (kinetics) run can be performed. The kinetic data is summarized in a  $k_{on}$  versus  $k_{off}$  plot, where lines are drawn representing constant affinity,  $K_D$ , values.

Set “Hit,, Criterion. Often control compounds can be used to define hits as “high,, or “low,, binders. Also groups of compounds may be evident on bulk response (report point 2) versus dissociation 1 (report point 3) plots.

For fast kinetic interactions, where dissociation 1 (report point 3) may not contain useful values, rank compounds by  $R_{eq}$  values, bulk response (report point 2) at same analyte concentration.