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Biacore® X
Getting Started

Edition October 2002

Biacore® X Getting Started
Edition October 2002 (Version AA).

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Biacore AB,
Rapsgatan 7
SE-754 50 Uppsala,
Sweden.

www.biacore.com

Biacore X Getting Started 3
Introduction 3
Requirements 3
References 4
Contents of the Getting Started Kit 4
Ordering information 5

Background information 6
Biacore terminology 6
Sensor surface properties 7
Immobilization 8
Amine coupling 8
Conditions for immobilization 8
Example of immobilization pH-scouting 9
Immobilization levels 11
Ligand requirements 12
Interaction analysis 12
Sample requirements 12
Regeneration 14
Testing regeneration conditions 14
Choice of regeneration conditions 16

Exercises 19
Description of the experimental set-up 19
Loading the sample loop 19
Loading instructions 19
Sample loading 20
Exercise 1. Start up 23
Startingt the system 23
Inserting a sensor chip and priming with buffer 23
Removing a sensor chip 25
Normalizing the signal response 26
Exercise 2. Effect of buffer on pre-concentration 27
Exercise 3. Immobilization 31
Immobilization results 36
Exercise 4. Interaction analysis 38
Interaction analysis results 41
Evaluation of the results 42
Exercise 5. Interaction analysis – multiple binding 47

Biacore routine maintenance 51

Biacore X Getting Started

Introduction

This Getting Started handbook is designed as a self-study guide to introduce you to the basic operations of Biacore X, Biacore X Control Software and BIAevaluation Software.

The handbook, together with the associated reagent kit, will take you through the main steps in a Biacore experiment. The sections of this handbook marked with a shaded border provide important background information that will help you carry out the exercises described in the later sections.

The reagents in the Getting Started Kit are for training purposes only and are supplied in quantities allowing for two repeated exercise sessions, following the instructions in this handbook. Biacore AB can accept no responsibility for results obtained with these reagents in any other context.

The reagents should be stored at between +4 and +8°C and should be used within one week of opening.

Requirements

The following are required for completing the Getting Started exercises:

- Time: approximately one day
- Biacore X instrument
- Familiarity with PC and Windows
- Getting Started Kit (take out from the refrigerator 1-2 hours before starting the analyses)
- Sensor Chip CM5 (not included in the Getting Started Kit)
- Amine Coupling Kit (not included in the Getting Started Kit)
- Micropipettes; 2-10 µl, 10-100 µl and 100-1000 µl and tips

References

For further details on the topics discussed in this booklet refer to:

- Biacore X Instrument Handbook
- Biacore Advisor Tutorial (CD-ROM)
- BIAevaluation Software Handbook

Contents of the Getting Started Kit

The contents of the Getting Started Kit are listed in Table 1. All solutions, except for the ligand, analyte and enhancement molecule preparation are ready for use. The ligand, analyte and enhancement molecule preparation should be diluted as described in the exercises and then used immediately.

Table 1. *Contents of the Getting Started Kit. For in vitro use only. Storage: +4 to +8°C for all solutions.*

Reagent/item, quantity	**Specification**
Running buffer (200 ml), 2pcs	HBS-EP buffer, 10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) Surfactant P20
Ligand (50 µl)	Monoclonal mouse-anti-human β2-microglobulin
Analyte (50 µl)	Human β2-microglobulin
Enhancement molecule (50 µl)	Polyclonal rabbit-anti-human β2-microglobulin
Immobilization buffer (1 ml), 2 pcs	10 mM sodium acetate, pH 5.0
Regeneration solution (1 ml), 2 pcs	10 mM glycine-HCl, pH 2.5
BIAnormalizing solution 70% (1.5 ml)	70% (w/w) glycerol (for Biacore 3000 and Biacore X)
BIAnormalizing solution 40% (1.5 ml)	40% (w/w) glycerol (for Biacore 2000)
Glass vials, 2 pcs	1.8 ml borosilicate vials, diam. 9 mm.
Plastic vials, 28 pcs	0.8 ml polypropylene microvials, diam. 7 mm.
Caps for 7 mm plastic vials and 9 mm glass vials, 30 pcs	Thin polyethelene snap caps, diam. 7 mm.
Plastic screw top vials and caps, 6 pcs of each	2.0 ml polypropylene screw top vials, diam. 10.8 mm and **non-penetrable** screw caps with o-ring seal

Ordering information

Ordering information is given in Table 2. For further information, please contact your local Biacore representative.

***Table 2**. Ordering information.*

Item	Code No.
HBS-EP buffer (6 x 200 ml)	BR-1001-88
Sensor Chip CM5 Research Grade (package of 3 chips)	BR-1000-14
Amine Coupling Kit	BR-1000-50
BIAmaintenance Kit	BR-1002-83
Biacore X Instrument Handbook	BR-1002-81
BIAevaluation Software Handbook	BR-1002-29
Biacore Advisor Tutorial, CD-ROM	BR-1005-44
Plastic vials, 7 mm (package of 1000 vials)	BR-1002-12
Plastic screw top vials and caps 10.8 mm (package of 1000 vials and 1000 caps)	BR-1002-14
Caps for 7 mm vials (package of 1000 caps)	BR-1002-13
Glass vials 9 mm, (package of 600 vials)	BR-1002-07

Background information

This chapter is intended to provide the basis for a more detailed understanding of the main steps in a Biacore assay. The information goes beyond what is explicitly covered in the exercises and is, therefore, a complement to the exercises.

Biacore terminology

- When molecular interactions are studied using Biacore, one of the interactants is immobilized on the sensor surface while the other is passed over that surface in solution. In Biacore terminology, ***ligand*** refers to the immobilized component and the interactant present in the sample injected over the surface is referred to as the ***analyte*** (Figure 1).

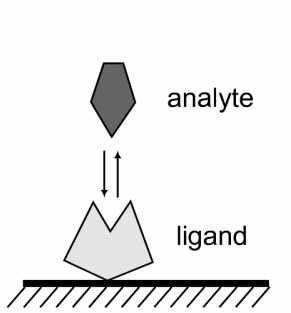


Figure 1. *"Ligand" refers to the immobilized interactant and "analyte" refers to the other interactant present in the sample injected over the sensor surface.*

- The response is measured in ***resonance units*** (RU) and is proportional to the molecular mass on the surface. For an interactant of a given mass, therefore, the response is proportional to the number of molecules at the surface.

- A ***sensorgram*** is a plot of response against time, showing the progress of the interaction. The sensorgram is displayed on the computer screen during the course of an analysis.

There are three major steps in a Biacore assay. These are:

1. ***Immobilization:*** The process by which the ligand is attached to the sensor chip surface.

2. ***Interaction analysis:*** The analyte is injected over the sensor chip surface and the interaction between the analyte and the immobilized ligand is monitored.

3. ***Regeneration:*** The process of removing bound analyte from the ligand on the surface.

Sensor surface properties

Sensor Chip CM5, which is used in the Getting Started Kit, is a glass slide coated with a thin layer of gold, to which a matrix of carboxymethylated dextran is covalently attached (Figure 2). The gold is required for generation of the surface plasmon resonance (SPR) response. The dextran matrix allows covalent immobilization of biomolecules using well-characterized chemistry and provides a hydrophilic environment suitable for a wide variety of protein interactions.

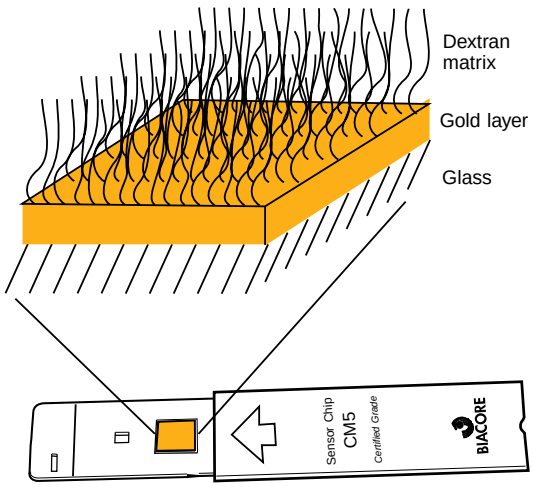


Figure 2. *Schematic illustration of the surface structure of Sensor Chip CM5.*

In addition to Sensor Chip CM5, which is the most versatile chip, Biacore offers a range of sensor chips with different properties that allow immobilization of biomolecules with different characteristics. Refer to the Biacore Catalogue (BR-9002-70) for further information.

Immobilization

There are different ways of immobilizing substances to the sensor surface. The choice of immobilization method depends on the properties of the substance. The immobilization approaches may be directed towards amine, carboxyl, thiol or hydroxyl groups on the ligand, or may use specific tags attached to the ligand.

Amine coupling

Amine coupling chemistry is the most widely applicable approach for covalently attaching biomolecules to the sensor chip surface and is suitable for the ligand included in the Getting Started Kit. With this method, the dextran matrix on the sensor chip surface is first activated with a mixture of 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) to give reactive succinimide esters. Ligand is then passed over the surface and the esters react spontaneously with amino groups or other nucleophilic groups to link the ligand covalently to the dextran (Figure 3). After the injection of ligand, ethanolamine is passed over the sensor surface to deactivate remaining active esters.

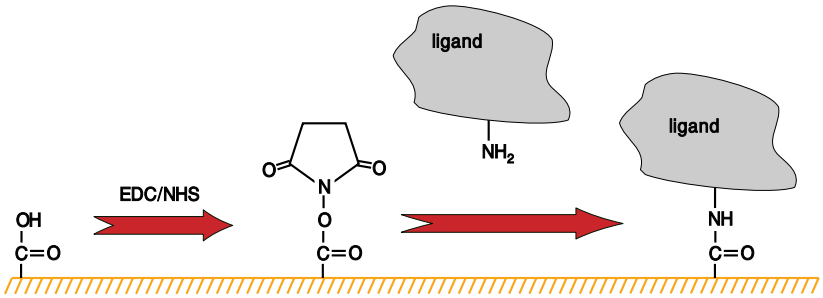


Figure 3. *Amine coupling of ligands to the sensor chip surface.*

Conditions for immobilization

At the concentrations of ligand commonly used for immobilization (20-200 µg/ml), the expected levels of immobilized ligand would be low in the absence of a mechanism that attracts the ligand molecules to the sensor surface. The main mechanism for this concentration process is electrostatic attraction of the ligand to the surface. This attraction is referred to as ***pre-concentration*** and can increase the concentration of ligand on the surface several thousand-fold.

The carboxymethylated dextran matrix of the sensor chip carries a net negative charge at pH values above approximately 3.5. The pH of the

immobilization buffer should therefore be higher than this and lower than the isoelectric point of the ligand in order to achieve efficient pre-concentration. Although coupling in 10 mM sodium acetate buffer (pH 4.5) works well for many ptoteins, the choice of pH in determining the success of immobilization can be critical. If other conditions are used, the following should be considered:

- The ionic strength should be low (10 mM monovalent cations recommended) for the electrostatic attraction to occur.

- Buffer components containing primary amine groups and other strong nucleophilic groups (e.g. Tris or sodium azide) must be avoided for amine coupling, as these will compete with the ligand for active esters on the sensor chip surface.

Many proteins show limited stability in low ionic strength solutions and at low pH. The ligand solutions should therefore be prepared directly before use.

Example of immobilization pH-scouting

The experimental procedure of finding the appropriate immobilization pH is referred to as pH-scouting. The example below shows how to set up an immobilization pH-scouting experiment and how to assess the results. The analyses in the example were performed with a Biacore 3000 instrument. The response levels may therefore be slightly higher than those expected using a Biacore X instrument. The principles, however, are the same.

Example 1

In this example, the ligand immobilized was the monoclonal anti-β2-microglobulin antibody included in the Getting Started Kit. The ligand was diluted in sodium acetate buffers with different pH (pH 5.5, pH 5.0, pH 4.5 and pH 4.0) to a final concentration of 30 µg/ml in each sample. The flow rate was 10 µl/min and the contact time was two minutes. *If the sample is limited, ligand consumption may be minimized by setting the flow rate to 5 µl/min and/or a lower ligand concentration may be used.*

After the last ligand injection, a wash solution was injected to remove any remaining ligand molecules. *A short pulse of 1 M ethanolamine-HCl pH 8.5 (included in the Amine Coupling Kit) or 50 mM NaOH is commonly used for washing surfaces that have been used for pre-concentration/pH-scouting experiments.*

As shown in Figure 4, the attraction of the protein into the dextran matrix on the sensor chip surface increased with decreasing pH of the buffers. This is explained by the fact that the protein is less positively charged at pH 5.5 compared to pH 4.0, which affects the attraction to

the negatively charged dextran matrix. pH 5.5, 5.0 and 4.5 all gave a satisfactory pre-concentration effect. At pH 4.0, a plateau was reached at the end of the injection, which indicates that the maximum level of attraction was reached at that pH.

It should be pointed out, however, that pH-scouting does not provide a conclusive answer regarding suitable immobilization conditions. It is well known that amine coupling is less efficient at low pH and in order to optimize the conditions and verify the choice of pH, the entire immobilization procedure has to be performed.

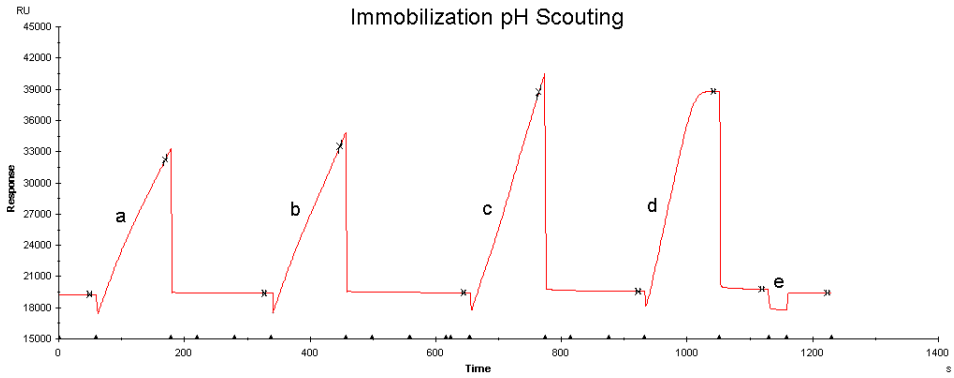


Figure 4. *Sensorgram showing the SPR responses generated by the ligand anti-β2-microglobulin at different immobilization buffer pH. a) pH 5.5, b) pH 5.0, c) pH 4.5, d) pH 4.0. e) Injection of a wash solution to remove any traces of anti-β2-microglobulin from the sensor chip surface. The experiment was performed with a Biacore 3000 instrument.*

In Figure 5, the sensorgrams from amine couplings of monoclonal anti-β2-microglobulin at pH 5.5, 5.0, 4.5 and 4.0 are shown. Although the pre-concentration response was very high at pH 4.0 (Figure 4), the final immobilization level was lower than at the other pH values tested. This clearly demonstrates the pH-dependence of the amine coupling chemistry and pH 4.0 was therefore excluded in this case.

As stated previously, the pre-concentration effects at pH 5.5, 5.0 and 4.5 were all acceptable (Figure 4). Since the immobilization level at pH 5.5 was higher than the immobilization levels at pH 5.0 and 4.5, pH 5.5 could appear to be the best choice (Figure 5). By looking at the sensorgrams (Figure 5), however, it becomes evident that at pH 5.5 the response after attraction and covalent coupling has not reached the maximum level. At pH 5.0, the sensorgram begins to flatten out after the covalent coupling (the maximum level is almost reached), which may contribute to an increase in the robustness of the assay set-up. At pH 4.5, the plateau is more evident, but at the same time the immobilization level has dropped. Consequently, pH 5.0 should be

chosen for immobilization of monoclonal anti-β2-microglobulin in this case.

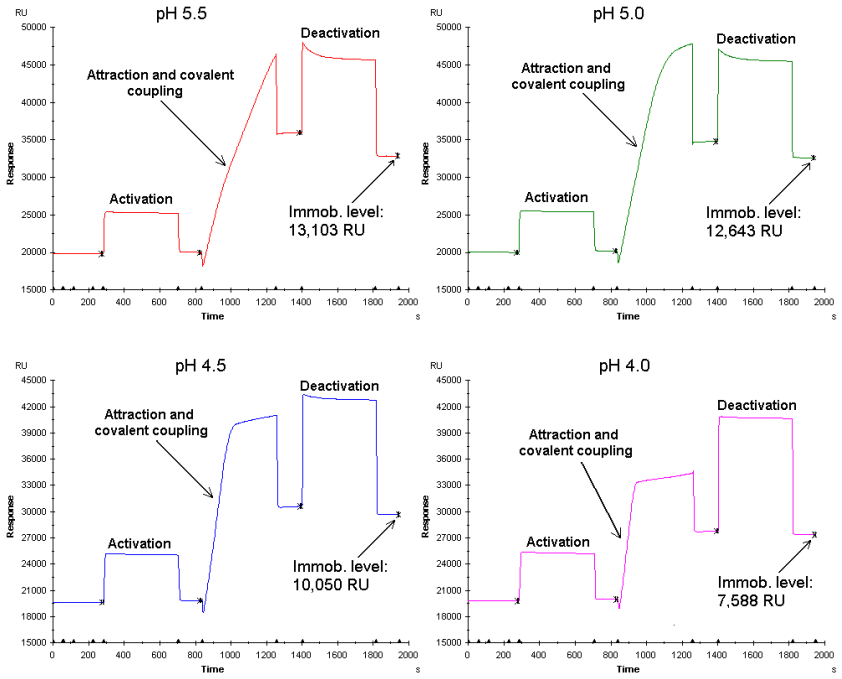


Figure 5. *Sensorgrams showing the immobilization of anti-β2-microglobulin via amine coupling at pH 5.5, pH 5.0, pH 4.5 and pH 4.0. For further information on sensorgrams from amine couplings, refer to page 36. The experiment was performed with a Biacore 3000 instrument.*

Immobilization levels

The binding capacity of the surface will depend on the levels of immobilized ligand. The term, maximum response (R_{max}) is often used in connection with Biacore experiments and describes the binding capacity of the surface in terms of the response at saturation. A theoretical R_{max} value can be calculated using the formula below:

$$R_{max} = \frac{\text{analyte MW}}{\text{ligand MW}} \times \text{immobilized amount} \times \text{stoichiometric ratio}$$

A theoretically calculated R_{max} is often higher than the experimentally derived R_{max} for the same interaction. There are many potential explanations for this, e.g. that the ligand is not fully active or that there is sterical hindrance of the interaction.

Different applications may require different binding capacities. A low R_{max}, for example, is often beneficial in kinetic analyses, while higher levels are advantageous in concentration measurements.

Ligand requirements

The quality and purity of the ligand have an important effect on determining the specificity and analyte binding capacity of the surface. The ligand should be as specific as possible for the analyte as the selectivity of the assay is determined by specificity. If necessary, an enhancement molecule can be used to increase the sensitivity and/or specificity of the assay.

The purity of the ligand is of vital importance for the experimental results. Impurities may be immobilized on the sensor chip and could affect the analyte binding capacity of the surface. Sensor surfaces immobilized with impure ligand solutions may give rise to results that are difficult to interpret. It is therefore recommended to use ligand solutions that are as pure as possible.

Cross-reactivity (i.e. binding of analyte-related molecules to the ligand) and non-specific binding (i.e. general binding to the ligand) can be controlled by the choice of ligand. Note that these factors are generally applicable to the vast majority of immunoassay formats and are not specific to Biacore assays.

Interaction analysis

During injection of the analyte, the binding of analyte to ligand takes place and is monitored in real time on the screen. After the analyte injection, when buffer flows over the surface, the dissociation of the analyte/ligand complex is monitored. Although analytes in crude sample matrices can be measured and the need for sample purification is low, a few guidelines on sample requirements are given below.

Sample requirements

The detection principle in Biacore technology measures changes in refractive index (RI) that are related to changes in mass close to the sensor surface. The design of the opto-interface allows for measurements of samples in crude environments, such as serum and cell culture supernatants.

Sample environments that differ greatly from the running buffer will cause a bulk refractive index (RI) effect that is common during an injection. Bulk refractive index effects do not affect the binding. Our recommendation, however is that the samples should be diluted in running buffer to minimize bulk shifts.

The bulk RI contribution of the sample requires that measurement points (report points) are set before and immediately after an injection where running buffer flows over the surface. The relative response then reflects the amount bound (Figure 6).

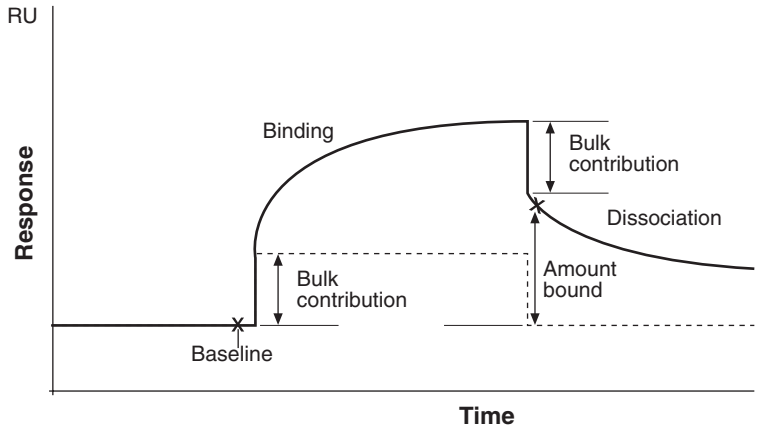


Figure 6. *Contribution of bulk refractive index to the response.*

One useful feature of Biacore X Control Software is that the bulk contribution can be automatically subtracted. Flow cell 1 can be used as a reference cell for in-line reference subtraction, providing direct recording and display of blank-corrected sensorgrams. The reference curve subtraction automatically corrects for the small time delay between the serial flow cells. The principle of reference subtraction is illustrated in Figure 7.

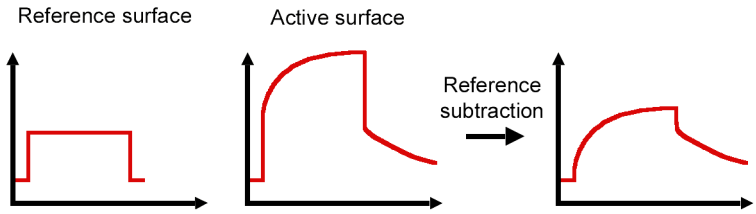


Figure 7. *The effect of reference subtraction. The sensorgram from the reference surface shows the contribution of the bulk, whereas the sensorgram from the active surface shows the actual binding response plus the bulk contribution. In the reference-subtracted sensorgram, only the binding response is shown.*

Although the reference-subtracted sensorgram shows the actual binding response, it is extremely important to inspect the sensorgrams from both the reference and active surfaces. The sensorgram from the reference surface, for example, will reveal non-specific binding. Remember that non-specific binding cannot be accurately corrected by subtraction of the response on a reference surface, since the binding is not necessarily

identical on the active and reference surface. In Exercises 4 and 5, automatic reference subtraction will be used.

Regeneration

Regeneration is the process of removing bound analyte from the ligand on the sensor chip surface after analysis of a sample.

Efficient regeneration (removing bound analyte without affecting ligand activity) is crucial to a successful assay. If the regeneration is incomplete or the binding activity of the surface is reduced, the performance of the assay will be impaired and the useful lifetime of the sensor chip will be shortened. Time spent on establishing suitable regeneration conditions, therefore, is a valuable investment.

The choice of conditions for regeneration is dictated by the stability and nature of the ligand and analyte. Our experience has shown that while different applications may need individually tailored regeneration conditions, many surfaces can be regenerated with brief exposure to acidic (glycine-HCl) or basic (NaOH) solutions.

If the sensor surface is not efficiently regenerated with high or low pH (either because the analyte is not fully removed or because the ligand loses activity), other conditions may be tested alone or in combination with high or low pH. These include :

- Up to 100% ethylene glycol
- High ionic strength (e.g. ≤5 M NaCl or ≤4 M $MgCl_2$)
- Low concentrations of SDS (≤0.5%)

Testing regeneration conditions

When exploring appropriate regeneration conditions, it is important to test an interval of different conditions, such as pH or ion concentrations. Our recommendation is to use mild conditions first and then progressively increase the harshness of the treatment until suitable conditions are established. In this way, the risk of damaging the ligand on the sensor surface is minimized.

Exploratory testing for regeneration conditions involves injection of analyte over the surface, followed by regeneration solution. The extent to which the analyte is removed is indicated by the response after the injection of regeneration solution. A second injection of analyte is necessary to test whether the ligand is still active and binds the same amount of analyte as before exposure to the regeneration solution (Figure 8).

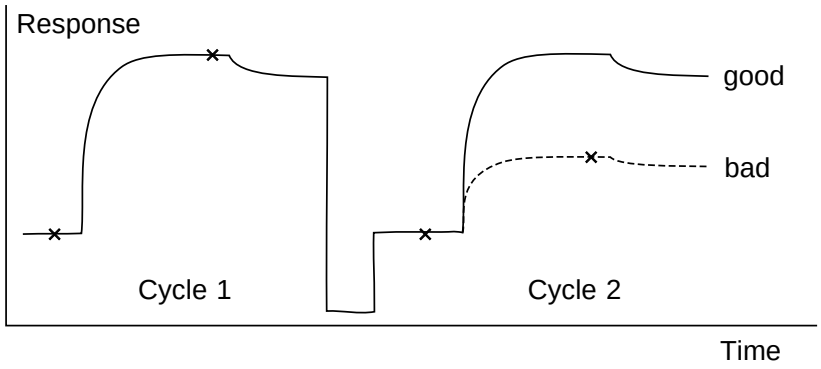


Figure 8. *Efficient regeneration removes all bound analyte. A second injection of analyte reveals whether the ligand is still fully active.*

Injection of regeneration solution often gives a considerable bulk response because the refractive index of the regeneration solution is not matched with the running buffer. The relative bulk response may be either positive or negative depending on the solution used.

The analyte injections described above may be used when it is to be established whether a particular regeneration strategy is worth pursuing. To establish regeneration conditions reliably and reveal trends in regeneration efficiency and ligand activity, it is necessary to perform a series of repeated cycles of analyte injection and regeneration. At least five cycles of analyte binding and regeneration are recommended in testing new regeneration conditions. Once the conditions have been found, more extensive tests may be performed to establish that the assay is stable through large numbers of repeated cycles.

General guidance on how to interpret the trends in analyte binding response and baseline response is given below:

- **Ideal regeneration:** The analyte response is constant after repeated injections and within ±10% of the level reached in the first injection.

- **Conditions are too mild:** The analyte response decreases and the baseline response increases. The decrease in analyte response is caused by incomplete removal of the analyte from the surface so that the baseline increases. Slightly harsher conditions or a change in the type of regeneration solution should be tested.

- **Conditions are too harsh:** The analyte response decreases and the baseline response is constant or decreases. The drop in analyte response without a corresponding increase in baseline response indicates that the surface is losing analyte binding capacity. A different type of regeneration solution should be tested. A new surface may have to be prepared if the ligand has been extensively damaged.

Choice of regeneration conditions

Example 2
In this example, regeneration solutions with different pH were tested. Each cycle consisted of an injection of analyte followed by a single injection of regeneration solution. Each regeneration solution was tested over five identical cycles.

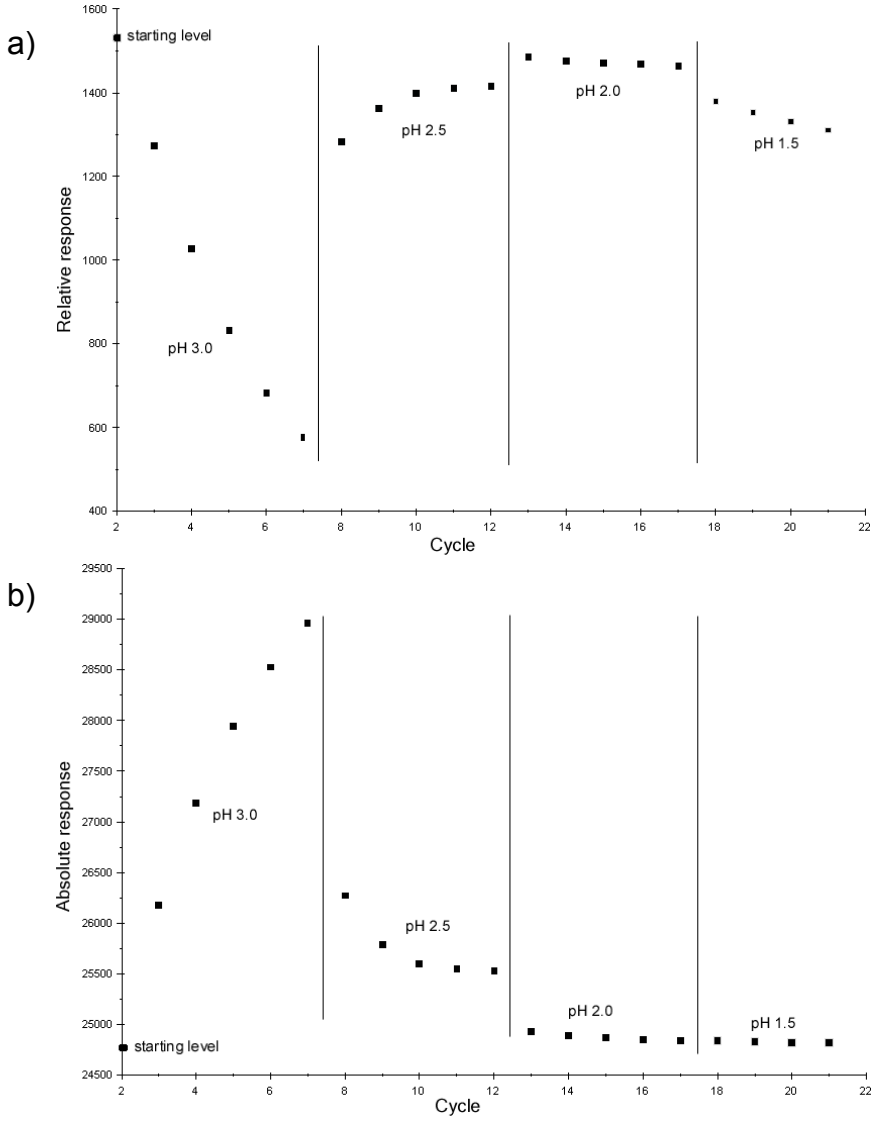


Figure 9. *Regeneration solutions of different pH were tested to find the best regeneration condition for the ligand and analyte. a) The sample response after each analyte injection (relative to the baseline). b) The baseline level after each regeneration cycle. The report points from the first cycle give the starting levels (far left in the diagrams). Thereafter, the points are grouped according to the regeneration conditions tested.*

At pH 3.0, analyte binding decreased and the baseline response increased (Figure 9). This suggests that the analyte was not removed from the surface and the binding site on the ligand remained occupied. Consequently, more material accumulated on the surface, allowing less analyte to bind in each cycle. The first condition (pH 3.0) therefore appeared to be too mild.

pH 2.5 showed some improvement, but the regeneration was still not good enough, since several cycles were needed to reach a stable sample response. In addition, the baseline was still at a higher level than the starting value.

The third condition (pH 2.0) is acceptable. Both the sample response and the baseline were close to the starting values after each cycle.

The fourth condition (pH 1.5) was too harsh, since the sample response decreased. The baseline, however, was still acceptable.

The conclusion from this experiment would be to use a regeneration solution at pH 2.0. The next step would be to test that the surface remains active and is not harmed by this solution during the number of cycles that the assay is intended to cover.

Example 3
This example is similar to the previous example, but shows the regeneration scouting for the analyte and ligand included in the Getting Started Kit. As in the pH-scouting example on pages 9-11, the experiment was performed with a Biacore 3000 instrument and the response levels given may differ from those expected using Biacore X.

To establish appropriate regeneration conditions for the monoclonal anti-β2-microglobulin (the ligand) and the analyte β2-microglobulin, repeated injections of analyte followed by regeneration solution were performed. Four different glycine-HCl regeneration solutions with pH 3.0, 2.5, 2.0 and 1.5, respectively, were examined by five repeated injections. The results are shown in Figure 10. At pH 3.0, both the sample response and the baseline level increased, indicating that this regeneration solution was too mild. At all the other pH values tested, the responses were more stable, returning approximately back to the level of the starting value.

This example illustrates that clear-cut differences in the response to different regeneration solutions cannot always be seen. In this case, pH 2.5 was chosen for further testing, although both pH 2.0 and 1.5 gave a similar effect.

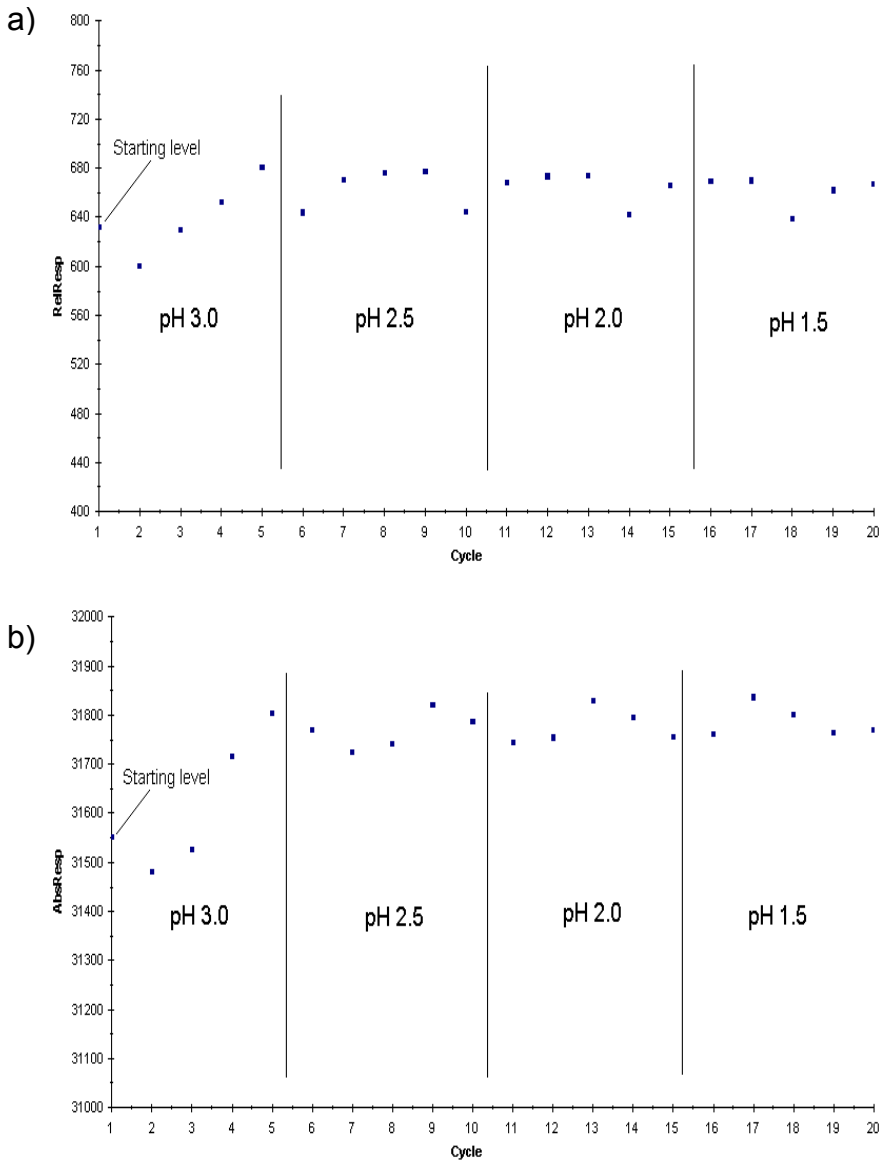


Figure 10. *Glycine-HCl solutions at different pH were tested to establish appropriate regeneration conditions for the ligand anti-β2-microglobulin and the analyte β2-microglobulin. a) The sample response after each analyte injection (relative to the baseline). b) The baseline level after each analyte injection. The experiment was performed with a Biacore 3000 instrument.*

Exercises

Description of the experimental set-up

The experiments described in this handbook exploit the binding of β2-microglobulin to immobilized monoclonal anti-β2-microglobulin antibodies. The following steps are described:

- Start up of the system.
- The pre-concentration effect: Illustration of the importance of the ion strength and pH of the buffer used for immobilization.
- Immobilization of monoclonal anti-β2-microglobulin via amine coupling.
- Interaction analysis: Injection of two different concentrations of β2-microglobulin and evaluation of the results.
- Multiple binding: Injection of an enhancement molecule to amplify the signal from a low concentration of analyte.
- Maintenance routines.

Upon completion of the Getting Started experiments you should have a basic understanding of the main steps in a Biacore assay and how to handle the instrument and the software. You should also be ready to incorporate your own reagents into similar protocols.

Loading the sample loop

Loading instructions

1. Place the pipette tip firmly in the connector block injection port (Figure 11).
2. Press the pipette plunger down to the first stop. If loading slows down significantly, press the plunger down to the second stop. Loading 100 µl of sample with normal viscosity for protein solutions should take about 15 s.
3. Watch the surface of the sample in the pipette tip. As soon as it disappears into the connector block, lift the pipette and release the plunger in a single movement. In this way you will avoid pushing air into the sample loop after the sample. Take care not to

withdraw sample back into the pipette tip from the injection port as you release the plunger.

WARNING!
Liquids are injected into the IFC against a back pressure and can splash when a pipette containing solution is removed. Wear protective glasses when loading hazardous solutions.

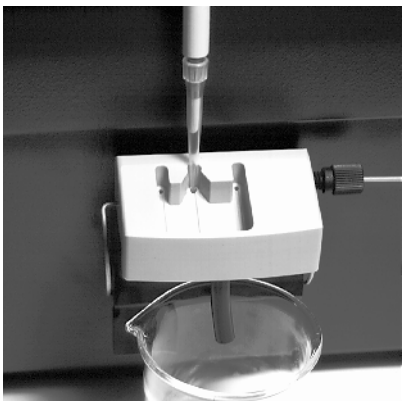


Figure 11. Loading the sample loop using a micropipette.

Sample loading

To minimize sample dispersion at the beginning and end of the injection, it is recommended to introduce small air and sample segments (about 5 µl each) into the pipette tip before loading. This will separate the sample from running buffer and prevent mixing (Figure 12).

1. Aspirate the recommended volume of sample to be loaded. Do not use more than 110 µl of sample as larger volumes will push the air bubble beyond the sample loop and defeat the purpose of the air bubble technique.

2. Lift the pipette tip and aspirate 3-5 µl of air.

3. For higher precision aspirate about 5 µl of sample followed by a second 3-5 µl air bubble.

4. Load the sample into the IFC so that the air bubble(s) separates the sample from the buffer already in the IFC (Figure 12).

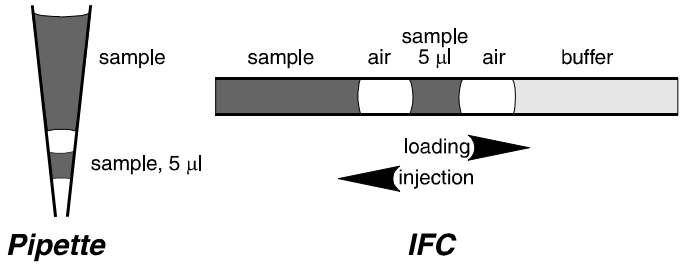


Figure 12. *Air bubble technique for loading sample into the IFC, showing sample with air bubble in the pipette tip (left) and in the IFC sample loop (right). Use two bubbles separated by about 5 µl sample for maximum precision.*

The use of air bubbles during loading is more important for viscous samples that tend to "drag" on the sample loop walls during injection. The effect of using air bubbles during loading for injection is shown in figure 13.

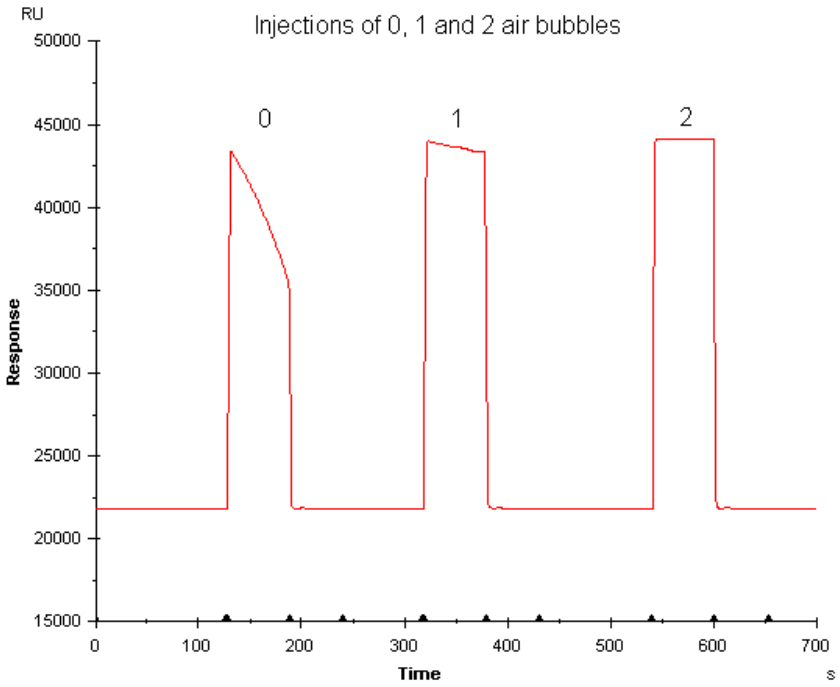


Figure 13. *Sensorgram obtained from injections of BIAtest solution (15% sucrose in HBS buffer), using 0, 1 or 2 air bubbles during loading. Note the significant sample dispersion when no air bubbles are used.*

If you feel uncertain about the sample loading technique, practise loading and injecting sample before performing the exercises in this Getting Started Handbook. A good way to practise, is to inject 15% glycerol or sucrose in buffer (e.g. BIAtest solution, which is included in

the BIAmaintenace kit) using a flow rate of 10 µl/min. The change in refractive index will give a response in the sensorgram, which ideally should be a square profile (Figure 13). If the leading edge of the pulse is distorted, you have probably injected air after the sample. If the trailing edge is distorted, you have failed to introduce a sufficient air bubble before the sample.

Exercise 1. Start up

This section deals with starting up the system, docking the sensor chip, priming the liquid system and normalizing the signal response. The priming procedure flushes the liquid system with running buffer. This should be done when a new sensor chip is inserted and/or the running buffer is changed.

Starting the system

1. Switch on the Biacore X processing unit, PC and printer. If the processing unit has been switched off, temperature stabilization of the unit will take approximately one hour. Wait until the yellow **Temperature** lamp on the front panel of the processing unit lights steadily.

2. Take out the Getting Started Kit from the refrigerator and equilibrate to room temperature. If you are using your own buffer, it must be filtered (0.22 µm) and degassed before use.

3. Open Windows Explorer: Click on the **Start** menu and select **Programs: Windows Explorer**. Find and open the "BIA Users" folder. Choose **File: New Folder** and name the new folder, for example "Tutorial". Close Windows Explorer by choosing **File: Close**.

4. Start Biacore X Control Software: click on the **Start** menu and select **Biacore X Control Software** from the **BIA Programs** menu.

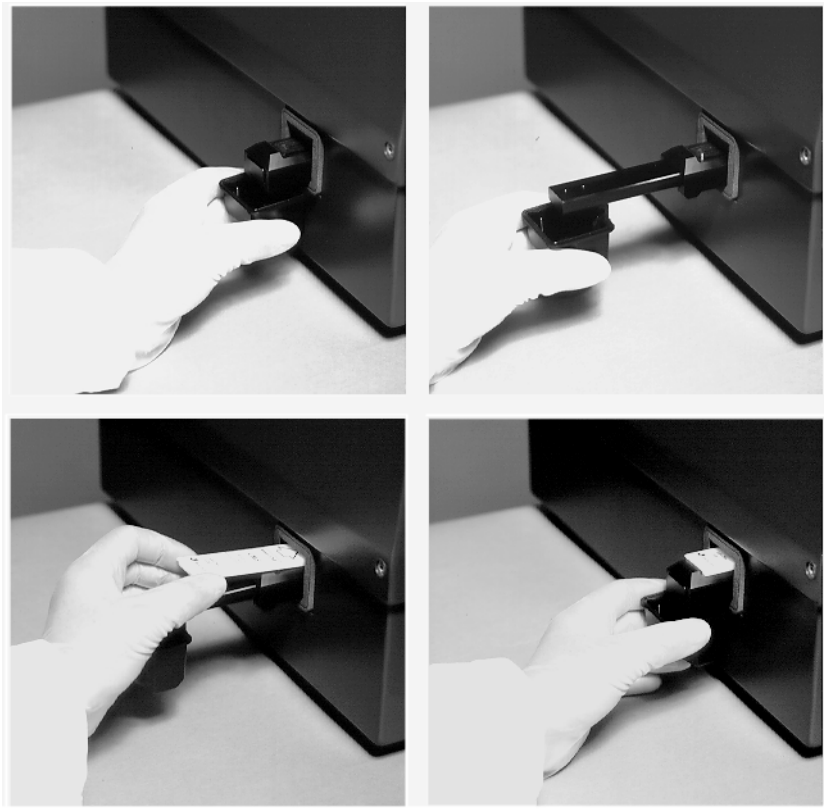
Inserting a sensor chip and priming with buffer

5. Place the buffer package next to the instrument and place a waste beaker beneath the waste outlet. Insert the buffer tubing into the buffer package.



6. If there is *not* a sensor chip in the instrument, a **Dock** dialogue box will be seen on the screen. Insert the new sensor chip (see steps 7-10).

7. Open the sensor chip port cover and pull out the slide.



8. Place the sensor chip on the slide, with the arrow pointing towards the instrument (make sure that the sensor chip is properly placed on the guiding pin).

9. Push the slide into the instrument and close the sensor chip port cover; the cover should snap into the closed position.

10. Click on **Dock**.

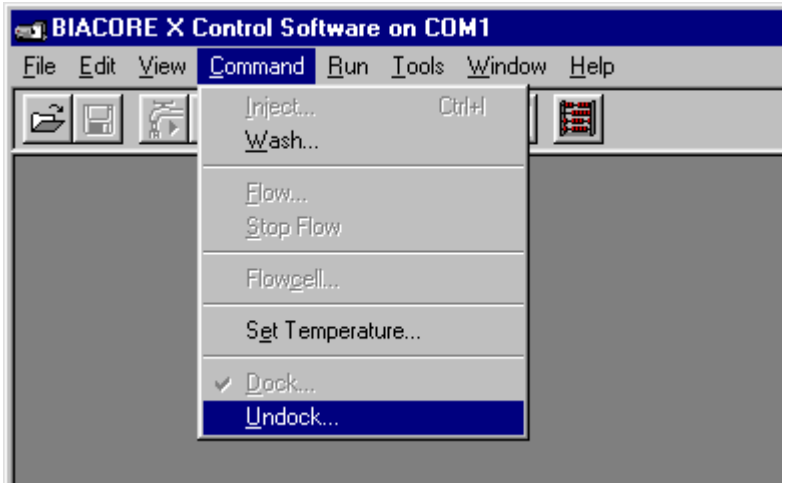
11. If the **Sensor Chip** lamp is flashing or lights steadily, a sensor chip is already inserted or docked: remove this sensor chip before inserting the new sensor chip (see steps 13-14).

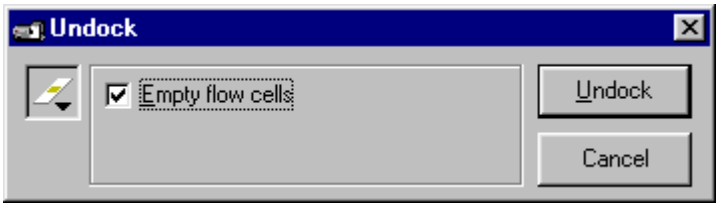
12. A dialogue box for the **Prime** procedure will be shown, click on **Start** in this box to start priming the system. When the method is finished (after about 3 minutes), click on **Exit.**

Removing a sensor chip

13. If a sensor chip is docked, a **Continue** or **Prime** dialogue box is shown. If the **Continue** box, which indicates a continuous buffer flow, is displayed, click on **Stop**. If the **Prime** dialogue box is displayed, click on **No.**

14. Choose **Command:Undock**. Check the **Empty flow cells** option and click on **Undock** in the dialogue box. Wait until the **Sensor Chip** lamp is flashing (this indicates that a sensor chip is inserted but not docked). Open the sensor chip port cover and remove the sensor chip.

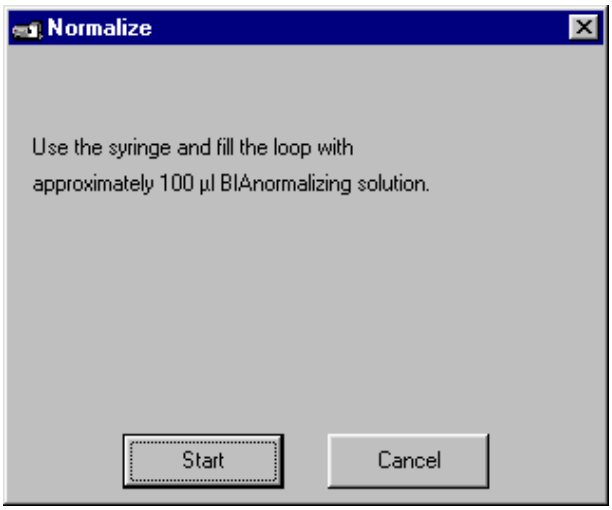




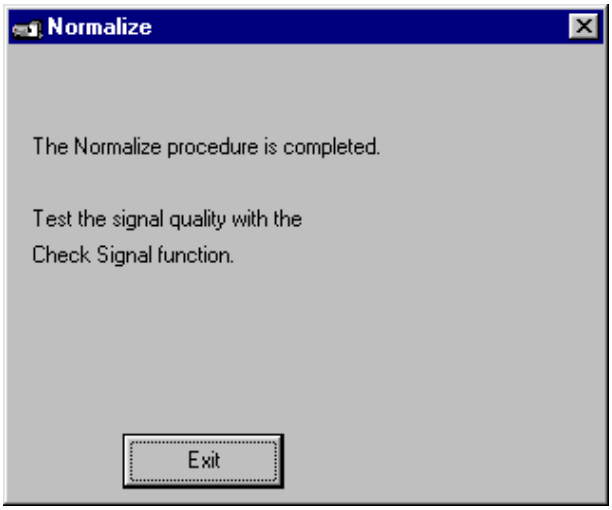
Normalizing the signal response

15. It is recommended to run the normalize procedure whenever the temperature has been changed or a when a new chip has been docked and maximum sensitivity is required. This procedure calibrates the detector unit. Choose **Tools: Working Tools: Normalize**.

16. Follow the instruction in the dialogue box: load the sample loop with 100 µl BIAnormalizing solution 70%. The loading of the sample loop is described on page 19. Start the normalization procedure by clicking on **Start**.



17. When the Normalize procedure is completed, click on **Exit**.



Exercise 2. Effect of buffer on pre-concentration

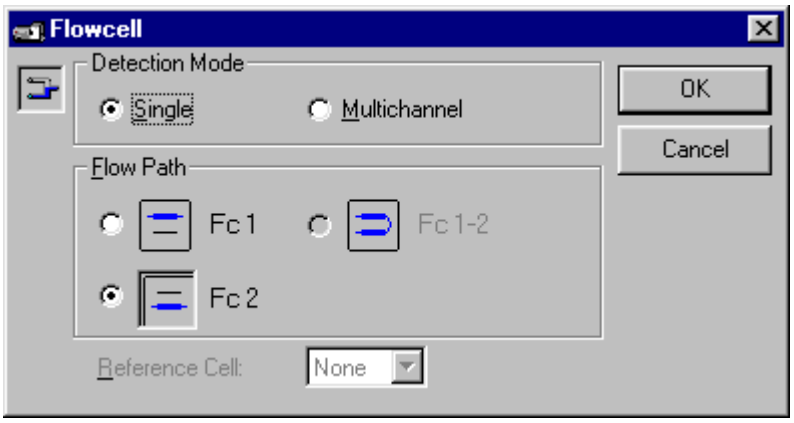
In this exercise, the importance of using a buffer with appropriate pH and ionic strength to obtain sufficient pre-concentration is demonstrated.

1. Make sure that no liquid is condensed in the ligand tube as this may affect the ligand concentration. If needed, spin the tube before diluting the ligand.

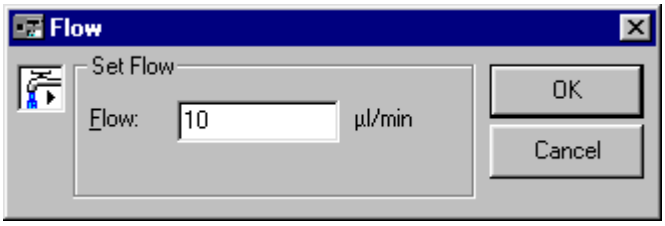
2. Make dilutions of the ligand in both HBS-EP (running buffer) and immobilization buffer (10 mM sodium acetate, pH 5.0). Mix 3 µl ligand stock solution with 72 µl HBS-EP and 3 µl ligand with 72 µl sodium acetate buffer. Use 7 mm plastic vials and cap all vials.

3. Start a sensorgram by choosing **Run: Run Sensorgram**.

4. Biacore X operates in one of two detection modes. The detection mode is set when starting a sensorgram and cannot be changed during the run. In Single mode, only one flow cell is used for the analysis and the sensorgram displays the response signal corresponding to the chosen flow cell. In Multi mode, one or two cells may be used. The response signals from the flow cells are displayed in separate sensorgrams, which are differentiated by different colors on the screen. Choose single detection in flow cell 2 by selecting Detection Mode: **Single** and Flow Path: **Fc2** in the **Flowcell** dialogue box. Click on **OK**.



5. In the **Flow** dialogue box, enter the **Flow:** 10 µl/min and click on **OK**.



6. Inject 10 µl of the ligand diluted in HBS-EP (running buffer) over the non-activated surface:

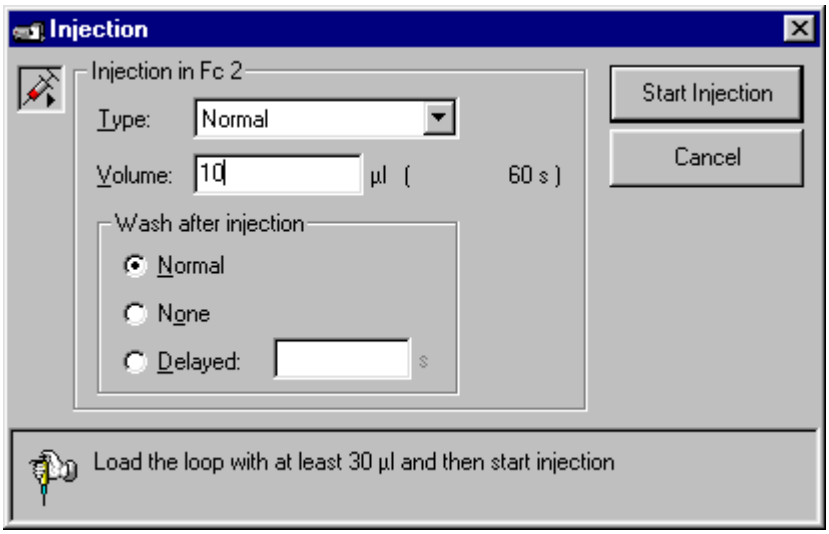
   a. Select **Command: Inject**.

   b. Enter **Volume:** 10 µl. The minimal volume that the loop has to be loaded with is given at the bottom of the dialogue box.

   c. Fill your pipette tip with sample and air bubbles according to the technique described on page 20: 30 µl sample + 5µl air + 5µl sample + 5 µl air.

   d. Load the injection loop carefully.

   e. Click on **Start Injection**.



7. Wait until the baseline is stable after the previous injection before you repeat step 6a-e to inject the ligand diluted in 10 mM sodium acetate buffer.

8. To inject the wash solution after the last sample injection select **Command: Inject** again and enter **Volume:** 5 µl. Load the loop with

at least 25 µl glycine-HCl, pH 2.5 (the minimum loading volume). Click on **Start Injection**.

9. Select **Run: Stop Sensorgram** to stop the data collection. Click on **Yes**.

10. To save the results, choose **File: Save As** and choose the directory "Tutorial". Enter a file name, "date-Exercise2"and click on **Save**.

11. Compare your sensorgram with Figure 14. The difference in electrostatic attraction between the two different buffers is striking. The response of the first injection is very low, but it is possible to zoom in on that area by using the left mouse button. Double-click anywhere in the sensorgram to zoom out.

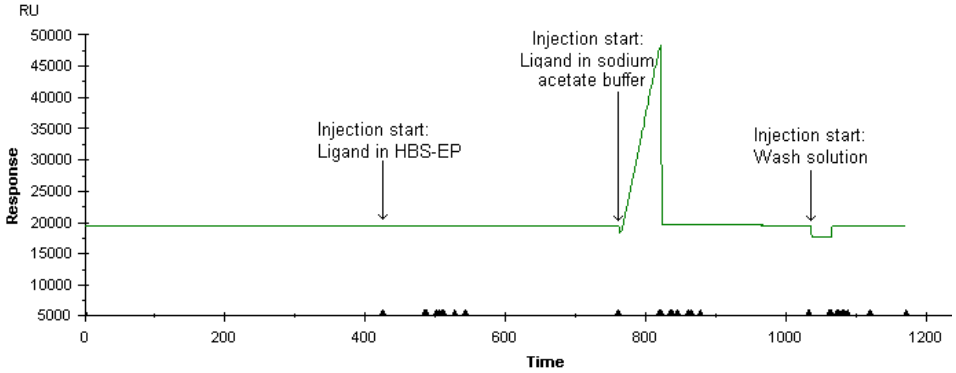
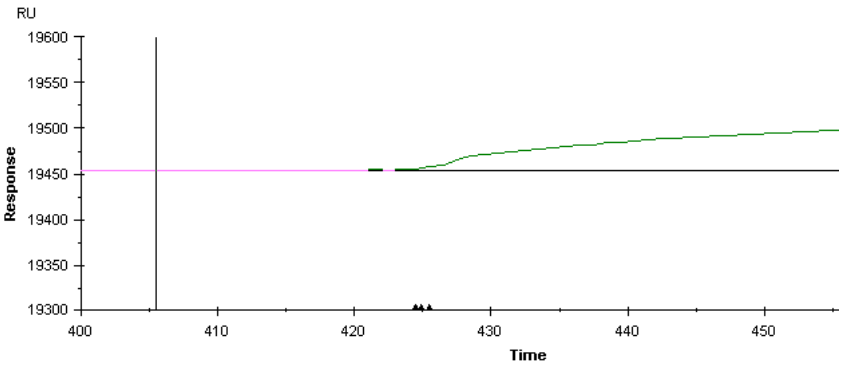


Figure 14. *The sensorgram shows that the electrostatic attraction of the ligand to the dextran matrix is heavily dependent on the pH and the ionic strength of the buffer.*

12. To quantify the response level after the first injection (ligand in running buffer), you first have to define a baseline. Zoom in on the area just before the first injection. Select **View: Reference Line** to activate the reference line. Drag the reference line to a point 10-20 s before the injection start. Select **View: Baseline** to display a baseline. This sets the baseline level to zero. The response level (0.0 RU) is now displayed at the bottom right on the screen.



13. Drag the reference line to a time point at the end of the ligand injection. As you move the reference line along the sensorgram the response level at the different time points can be read at the bottom right on the screen. Write down the response you observe after the first ligand injection on the line in step 14 below.

14. Drag the reference line to a time point at the end of the second ligand injection (ligand in sodium acetate buffer) and write down the response level below:

    Response level after the first injection:_____

    Response level after the second injection:_____

15. Select **View: Baseline** to remove the baseline and **View: Reference Line** to remove the reference line.

16. Close the sensorgram window by choosing **File: Close**.

Conclusion

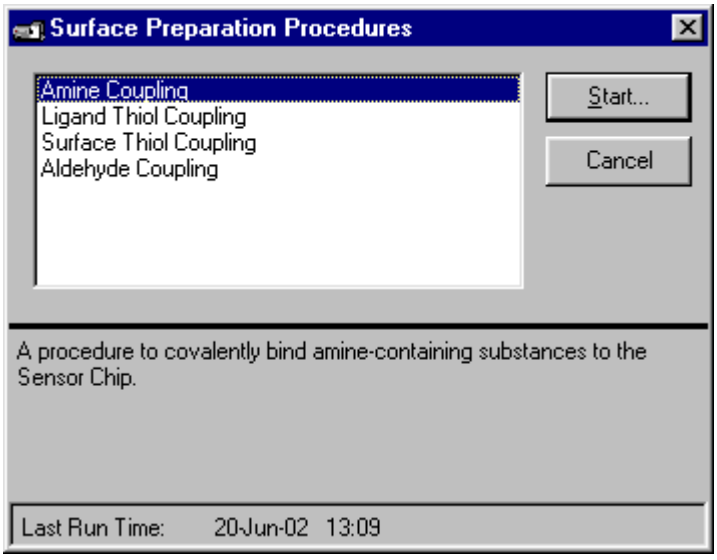
This exercise illustrates the importance of the ionic strength and the pH of the buffer on pre-concentration. Dilution of the ligand in HBS-EP causes a small pre-concentration effect, while dilution in 10 mM sodium acetate buffer, pH 5.0, causes a much higher pre-concentration. Consequently, the choice of immobilization buffer greatly influences the results in this case.

Exercise 3. Immobilization

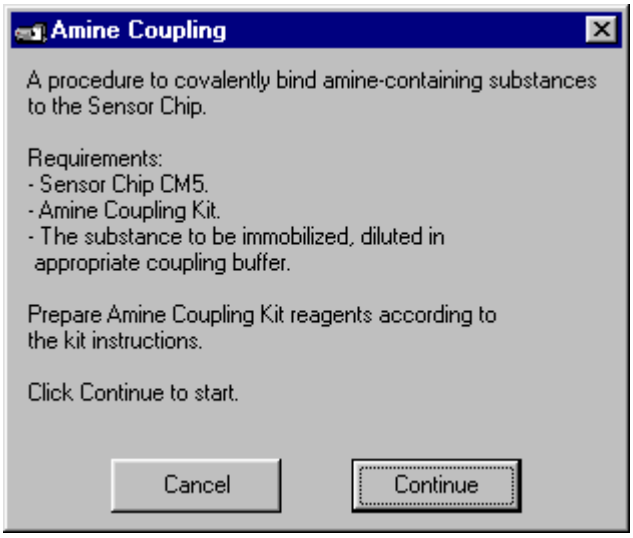
In this exercise the ligand (monoclonal anti-β2-microglobulin) is immobilized on the surface of Sensor Chip CM5. An application wizard guides you through the different steps in setting up an immobilization template. The template can be saved and used to simplify further immobilizations.

1. Prepare EDC and NHS solutions according to the instructions in the Amine Coupling Kit. A ready-to-use Ethanolamine solution is provided in the Amine Coupling Kit.

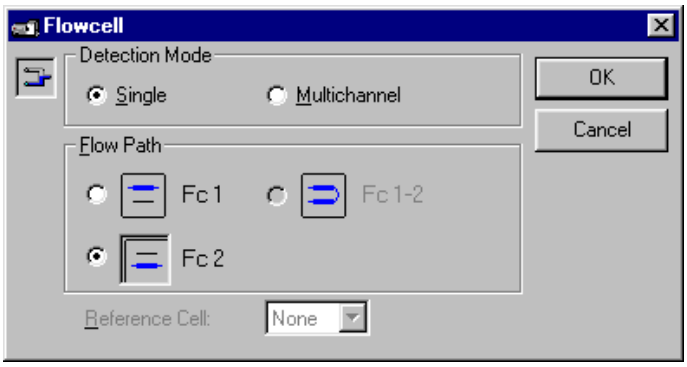
2. In the **Tools** menu, choose **Surface Preparation Procedures**. Select **Amine Coupling** and click on **Start**.



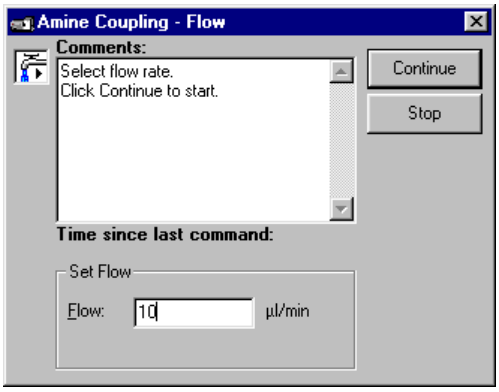
3. In the **Amine Coupling** dialogue box, read the instructions and click on **Continue**.



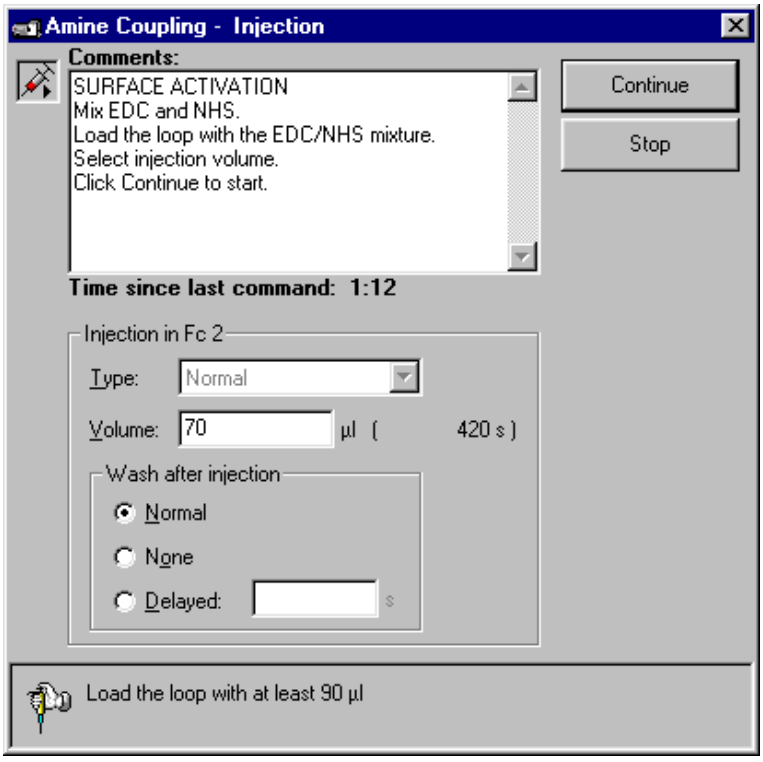
4. In the **Flow Cell** dialogue box, tick the **Single Detection Mode** option and choose detection in **Fc2**. Click on **OK**.



5. In the **Flow** dialogue box, set the flow rate to 10 µl/min. Click on **Continue**.

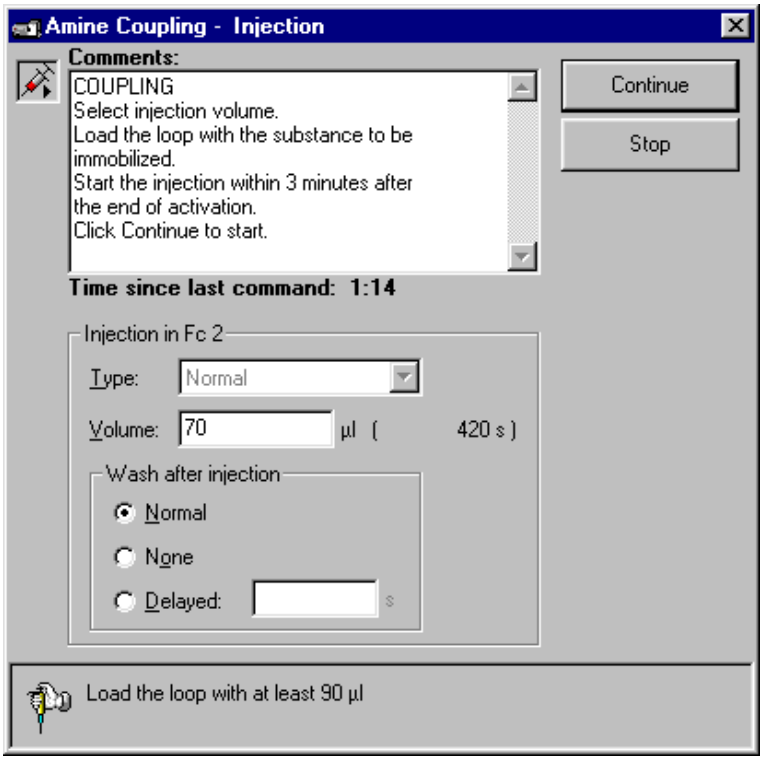


6. Read the information in the subsequent dialogue box: Enter **Volume:** 70 µl. Mix 60 µl EDC with 60µl NHS and load the injection loop with at least 90 µl of the mix. Use the air bubble technique to avoid dispersion (90 µl sample + 5µl air + 5µl sample + 5 µl air). Click on **Continue**. The injection will take 7 minutes.

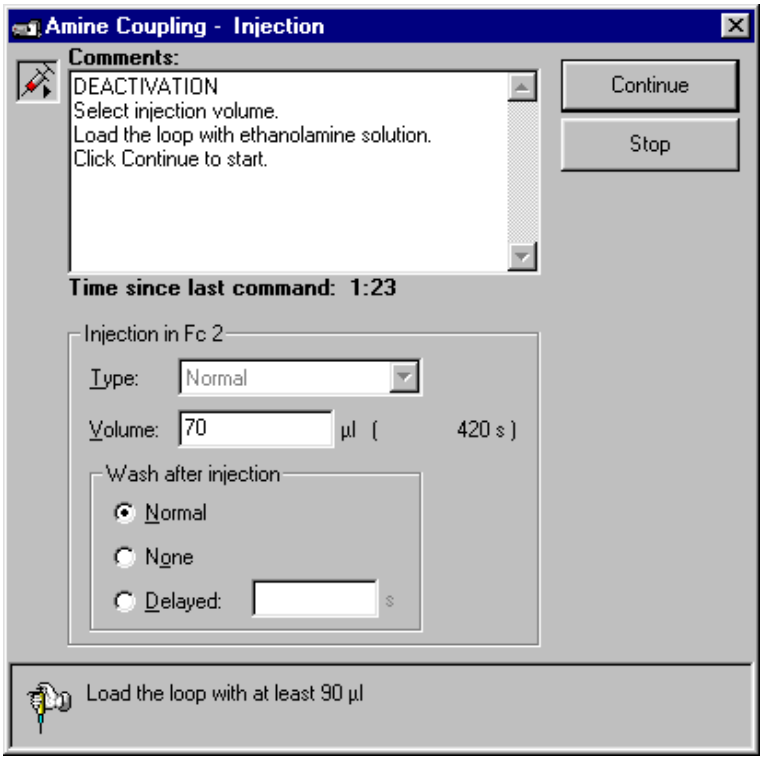


7. While waiting for the EDC/NHS injection to be finished, dilute the ligand in 10 mM sodium acetate buffer, pH 5.0: mix 6 µl ligand stock solution with 144 µl sodium acetate buffer and cap the vial. The choice of immobilization buffer is based on the pH-scouting in Example 1 on pages 9-11.

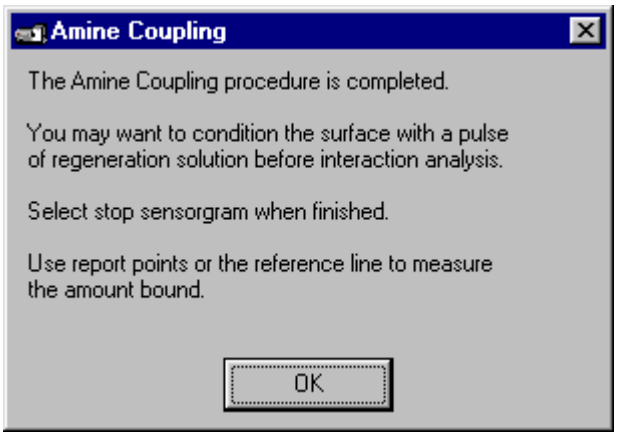
8. Follow the instructions in the subsequent dialogue box to immobilize the ligand on the sensor chip surface: enter the **Volume**: 70 µl (i.e. injection for7 minutes), load the loop with at least 90 µl of the ligand diluted in sodium acetate buffer (use the air bubble technique). Click on **Continue**. The injection of ligand has to be started within 3 minutes after the end of the injection of EDC/NHS in the previous step.



9. To deactivate any remaining active esters, ethanolamine (from the Amine Coupling Kit) should now be injected. Enter **Volume:** 70 µl and load the loop with at least 90µl as in the previous steps. Click on **Continue** to start the injection.



10. Once the Amine Coupling procedure is completed a dialogue box stating this will be displayed. Click on **OK** in this dialogue box.



11. Select **Run:Stop Sensorgram** and click on **Yes**. To save the results, choose **File: Save As** and choose the directory "Tutorial". Enter a file name, "date-Exercise3"and click on **Save**.

Immobilization results

12. Compare your sensorgram with the sensorgram in Figure 15.

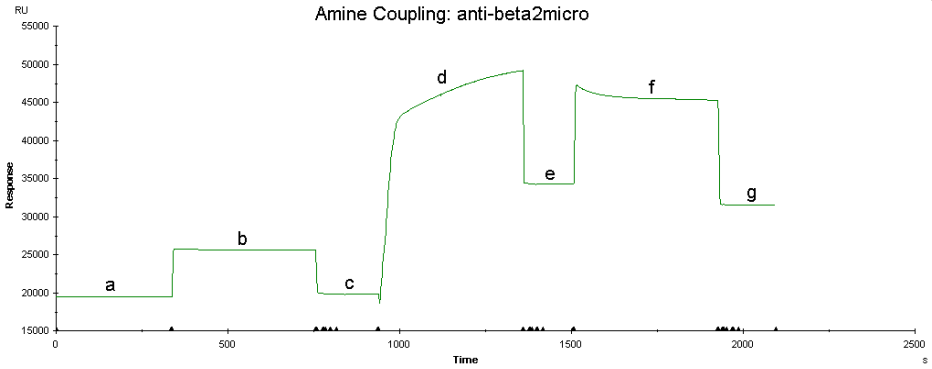


Figure 15. *Immobilization sensorgram.*

a) Baseline.
b) EDC/NHS activation of the dextran matrix.
c) Baseline after activation.
d) Attraction and covalent coupling of the ligand to the dextran matrix
e) Buffer washes away loosely associated ligand. The response level at this point gives a first indication of the amount of immobilized ligand.
f) Deactivation and further washing away of loosely associated ligand.
*g) The difference in response between points **a** and **g** reflects the amount immobilized.*

13. To find out the level of ligand bound to the surface, report points have to be added at appropriate positions in the sensorgram: Select **View: Reference Line** to activate the reference line. Drag the reference line to the starting baseline of the sensorgram (position a in Figure 15).

14. Add a report point by selecting **Edit: Add Report Point**. Enter the name "Baseline" in the **Id** field. The response recorded for the report point will be averaged over the time window specified by the **Window** parameter (use the default value of 5 s). Check the baseline option in order to use this report point as a baseline for relative responses at other report points. Click on **OK**.

15. Drag the reference line to a position after the ethanolamine injection (position g in Figure 15). Choose **Edit: Add Report Point**, enter **Id:** Ethanolamine. Use the default value of 5 s for the Window parameter and click on **OK**. This response level is given relative to the baseline response (position a) and represents the amount of

ligand covalently bound to the surface. An immobilized level of more than 8000 RU is satisfactory for this exercise.

16. Calculate the theoretical maximum binding capacity (R_{max}) of your surface using the equation below (for more background information, see page 11). The molecular weight of β2-microglobulin is 11800 Da and the molecular weight of the monoclonal anti-β2-microglobulin is 150000 Da. Remember that the ligand is an antibody when entering the stoichiometric ratio in the equation. This means that each antibody can theoretically bind two analyte molecules. Consequently, the stoichiometric ratio is 2.

$$R_{max} = \frac{\text{analyte MW}}{\text{ligand MW}} \times \text{immobilized amount} \times \text{stoichiometric ratio}$$

Your theoretical R_{max} value: _____

17. Close the sensorgram window.

Exercise 4. Interaction analysis

In this exercise, two concentrations of β2-microglobulin are injected over the immobilized anti-β2-microglobulin antibody.

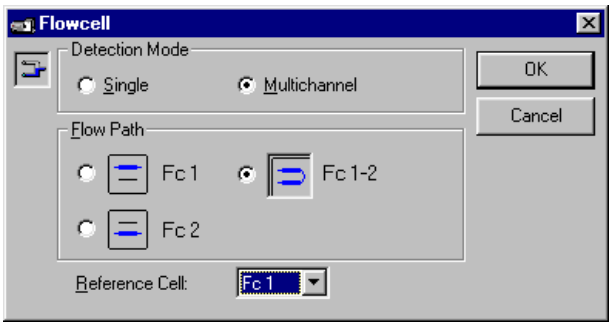
1. Prepare your samples:

   a. Dilute the analyte by mixing 10 µl analyte stock solution with 990 µl HBS-EP (running buffer) in a 2 ml plastic vial. This will be referred to as the high concentration of β2-microglobulin.

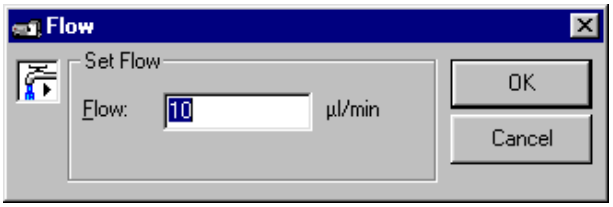
   b. Pipette 100 µl of the diluted analyte solution to another 2 ml plastic vial and mix with 900 µl HBS-EP. This will be referred to as the low concentration of β2-microglobulin. This analyte solution will also be used in Exercise 5.

2. Start a sensorgram by choosing **Run: Run Sensorgram**.

3. In the **Flowcell** dialogue box, check the **Multichannel** option and then select **Fc1-2**. Select **Fc1** from the reference cell drop down menu. Click on **OK**.



4. In the **Flow** dialogue box, enter the **Flow:** 10 µl/min and click on OK.



5. Inject 30 µl of the “low-concentration” analyte solution:

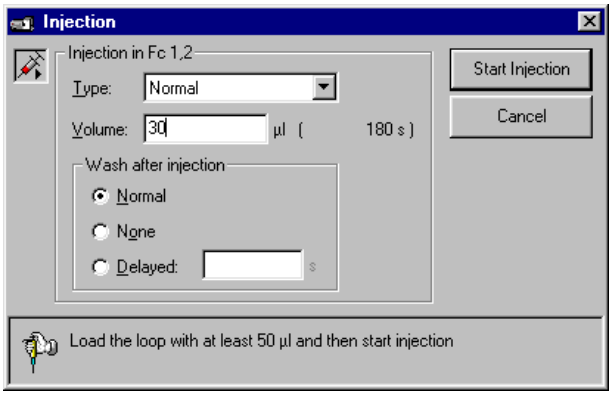
    a. Select **Command: Inject**.

    b. Enter **Volume:** 30 µl. The minimum volume with which the loop has to be loaded is given at the bottom of the dialogue box (in this case 50 µl).

    c. Fill your pipette tip with sample and air bubbles according to the technique described on page 20: 50 µl sample + 5µl air + 5µl sample + 5 µl air.

    d. Load the injection loop carefully.

    e. Click on **Start Injection**.

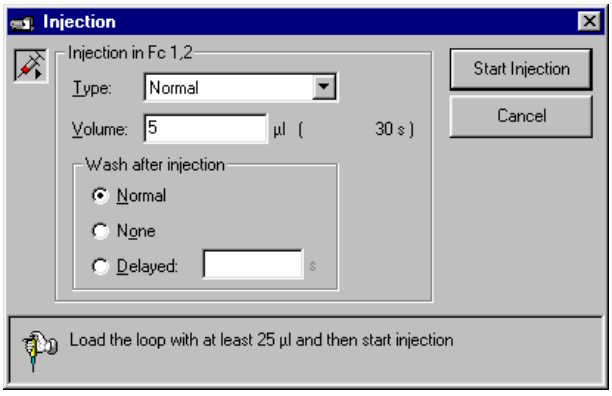


6. Wait until the injection is finished and then inject 5 µl of the regeneration solution (glycine HCl, pH 2.5):

    a. Select **Command: Inject**.

    b. Enter **Volume:** 5 µl.

    c. Fill your pipette tip with sample and air bubbles: 25 µl sample + 5µl air + 5µl sample + 5 µl air and load the loop carefully. Click on **Start Injection**.



7. Repeat steps 5a-e to inject the “high-concentration” analyte solution.

8. Wait until the injection is completed and repeat steps 6a-c to inject regeneration solution.

9. When the injection of regeneration solution is completed, choose **Run: Stop Sensorgram** to stop the data collection. Click on **Yes**. To save the results, choose **File: Save As** and choose the directory “Tutorial”. Enter a file name, “date-Exercise4”, and click on **Save**.

Interaction analysis results

10. Compare your sensorgrams with Figure 16 (zoom in on the sensorgrams). In this particular exercise, reference subtraction (Fc2-1) is not crucial, since the analyte was diluted in running buffer, i.e. there is no significant bulk contribution. By looking at the response from the reference surface any non-specific binding can be revealed. Reference subtraction is discussed in more detail in the theory section on page 13.

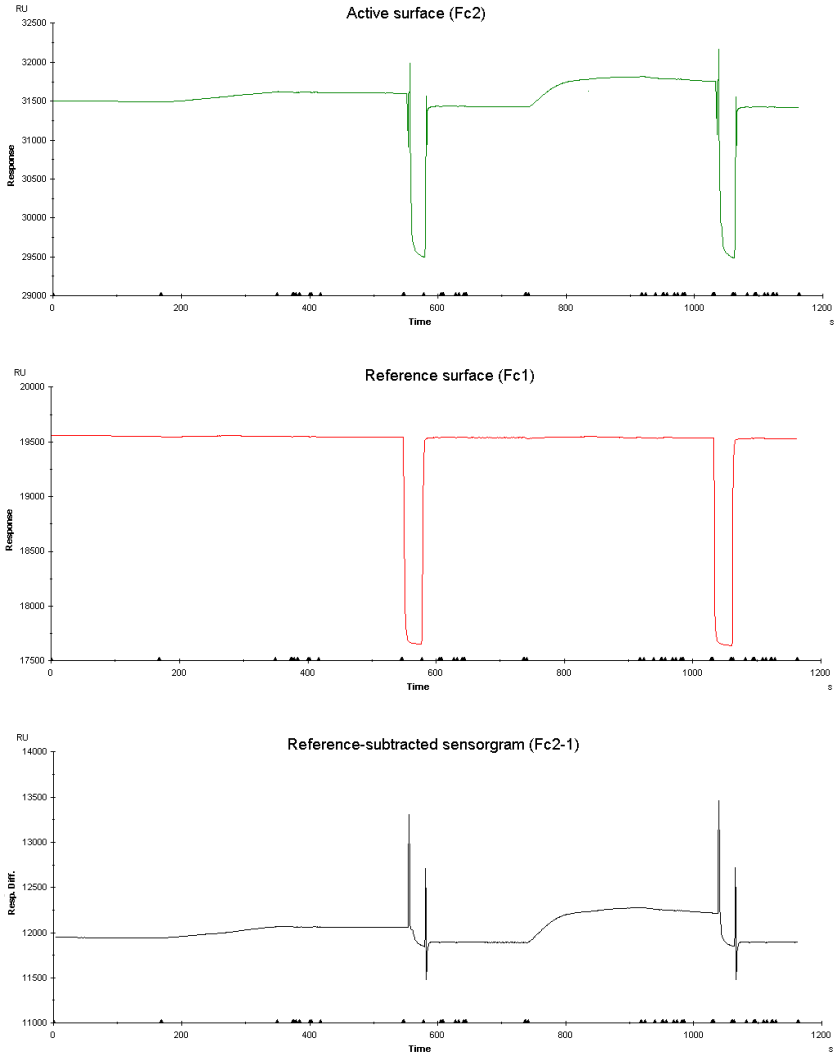
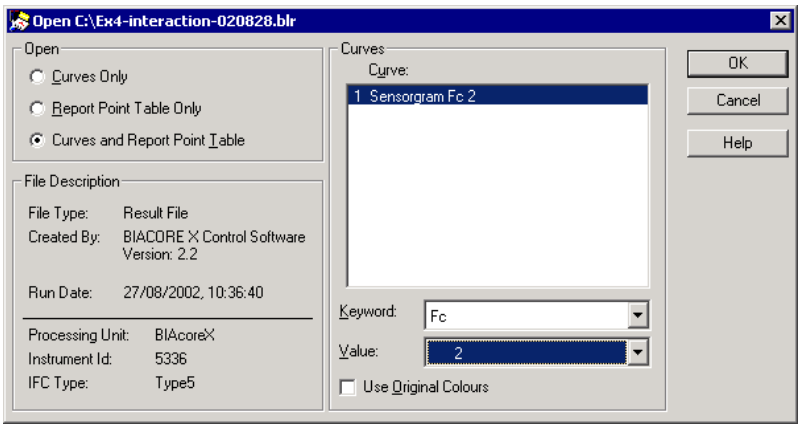


Figure 16. *Sensorgrams from the active and reference surfaces during injections of β2-microglobulin. The spikes in the reference-subtracted sensorgram derive from the small time difference between flow cells 1 and 2.*

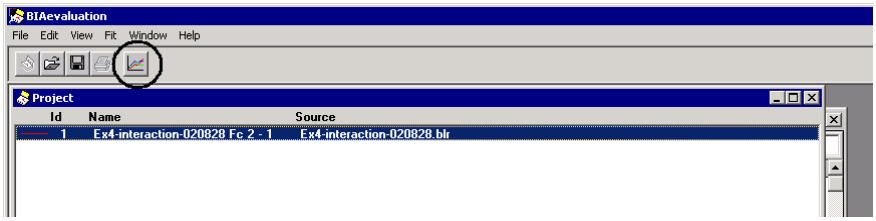
Evaluation of the results

11. Start BIAevaluation Software: click on the **Start** menu and select **BIAevaluation Software** from the **BIA Programs** menu.

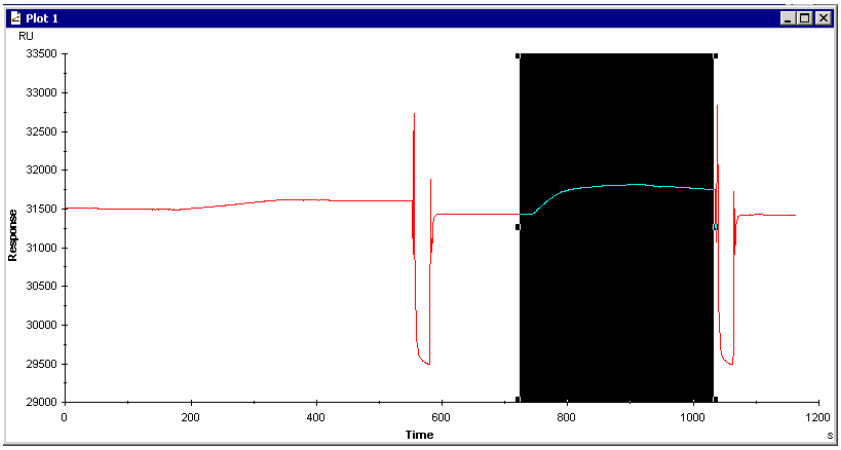
12. Choose **File: Open,** and open your results file from Exercise 4. Check the **Open Curves and Report Point Table** option and select **Value** 2 from the drop-down menu. Click on **OK**.



13. The available curve is now displayed in the Project window. The report point table is opened in a spreadsheet. Select the curve and click on the **Plot Overlay** button in the toolbar.

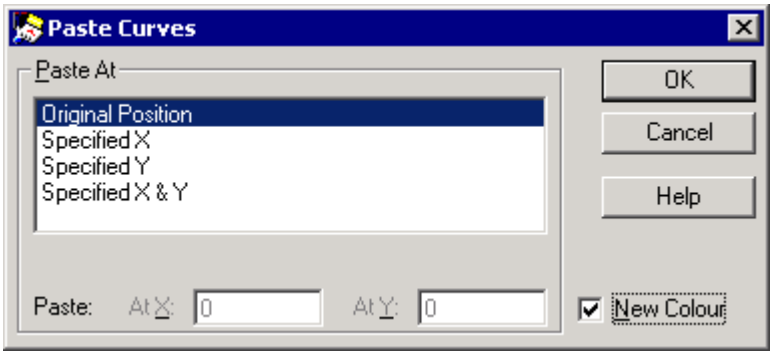


14. A plot of the sensorgram is now displayed. First, the two injections have to be aligned to the same starting point. Select the area covering the second analyte injection by using the right mouse button.

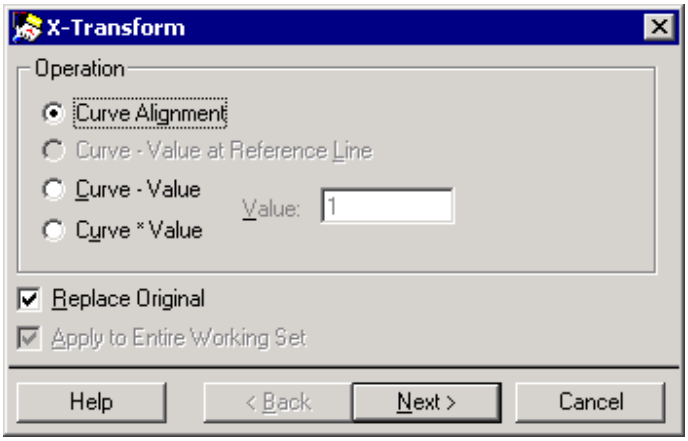


15. Choose **Edit: Cut** to cut out the selected part of the curve.

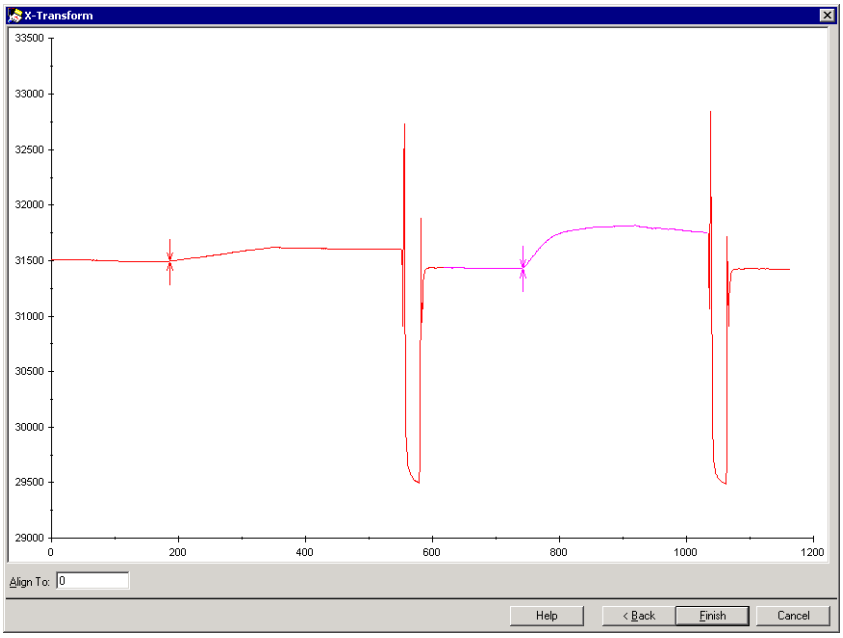
16. To view the second injection in a different colour than the first, tick the option **New Colour** and choose **Edit: Paste Curves**. Click on **OK**.



17. Choose **Calculate: X-transform** and select **Curve Alignment** and click on **Next**.

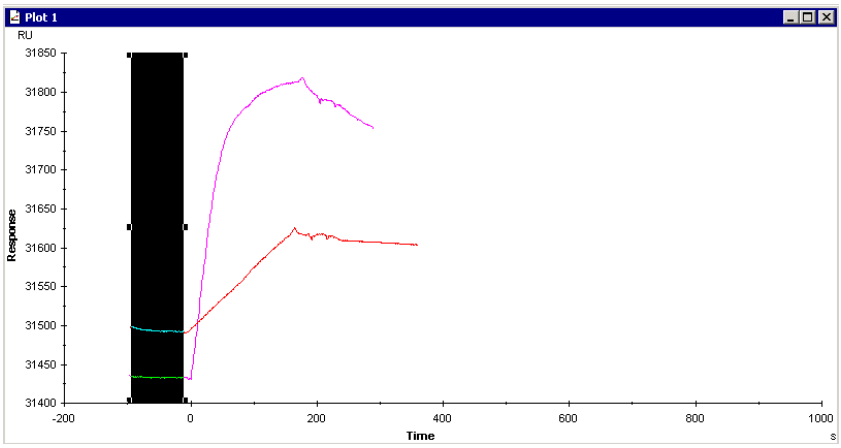


18. Each curve will now be assigned an arrow in the same colour as the curve. Move the arrows to the corresponding start of injection and click on **Finish**. This will align the curves to the same starting point.

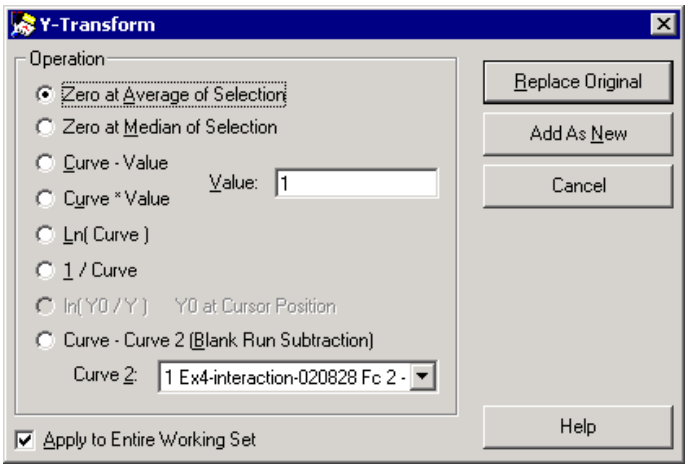


19. Cut away the regeneration-injections from the sensorgram by selecting the corresponding areas of the curve (use the right mouse button) and choosing **Edit: Cut**.

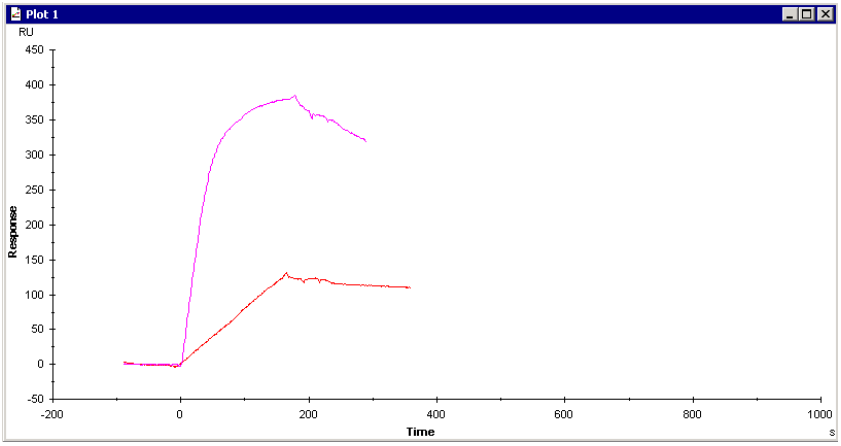
20. To adjust the curves to a common baseline, select an area before the injection start by using the right mouse button.



21. Choose **Calculate: Y-Transform** and check the **Zero at Average of Selection** option. Click on **Replace Original**.



22. The sensorgrams are now adjusted to a common baseline and the difference in the response between the high and low concentration of analyte is clearly seen.



23. Close the plot window and look at the report point table in which the data from your results file is displayed. Refer to the BIAevaluation Software Handbook for more detailed descriptions of how the data in the table can be sorted, filtered and plotted in different ways.

Conclusions

This exercise provides a simple illustration of the fact that under given experimental conditions, the SPR response is related to analyte concentration.

Exercise 5. Interaction analysis – multiple binding

In this exercise, a low concentration of analyte is injected over the reference surface and the surface with the immobilized ligand. To illustrate enhancement molecules can be used to either amplify a response or to confirm the identity of the bound analyte, a polyclonal anti-β2-microglobulin antibody is injected after the analyte.

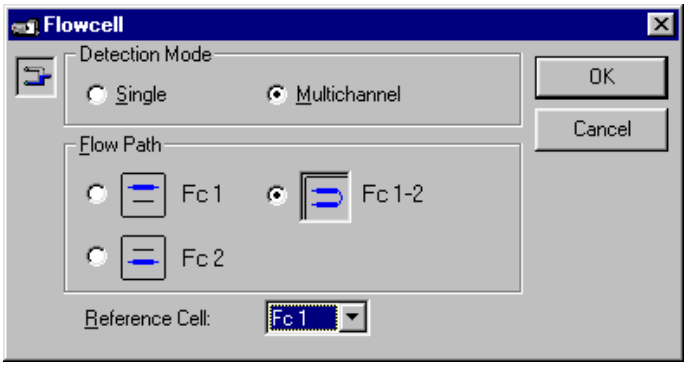
1. Prepare your samples:

   a. Dilute the polyclonal anti-β2-microglobulin by mixing 10 µl antibody stock solution with 990 µl HBS-EP (running buffer) in a 2ml plastic tube. Cap the vial.

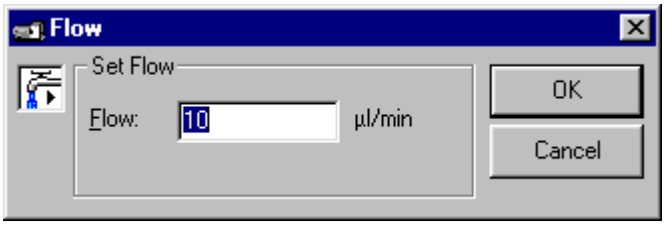
   b. The vial with "low-concentration" analyte solution from Exercise 4 will be used in this exercise.

2. Start a sensorgram by choosing **Run: Run Sensorgram**.

3. In the **Flowcell** dialogue box, check the **Multichannel** option and then select **Fc1-2**. Select **Fc1** from the reference cell drop down menu. Click on **OK**.



4. In the **Flow** dialogue box, enter the **Flow:** 10 µl/min and click on OK.



5. Inject 30 µl of the “low-concentration” analyte solution:

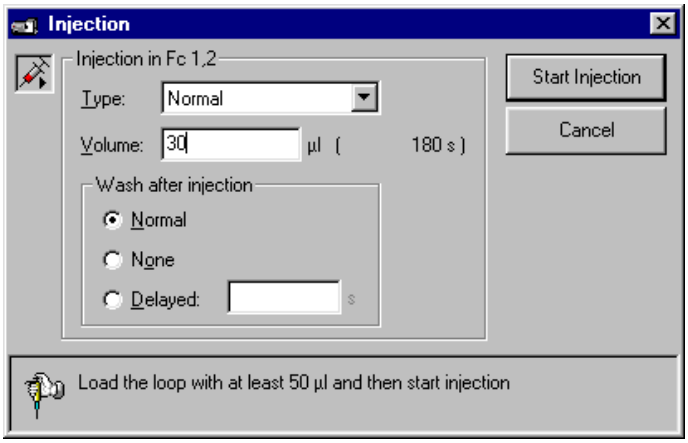
    a. Select **Command: Inject**.

    b. Enter **Volume:** 30 µl. The minimal volume with which the loop has to be loaded is given at the bottom of the dialogue box (in this case 50 µl).

    c. Fill your pipette tip with sample and air bubbles according to the technique described on page 20: 50 µl sample + 5µl air + 5µl sample + 5 µl air.

    d. Load the injection loop carefully.

    e. Click on **Start Injection**.



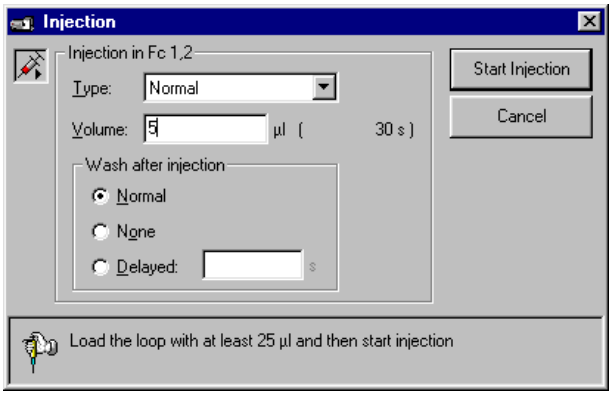
6. Wait until the injection is finished and then repeat steps 5a-e to inject the polyclonal anti-β2-microglobulin (enhancement molecule).

7. Inject 5 µl of the regeneration solution (glycine HCl, pH 2.5):

    d. Select **Command: Inject**.

    e. Enter **Volume:** 5 µl.

    f. Fill your pipette tip with sample and air bubbles: 25 µl sample + 5µl air + 5µl sample + 5 µl air and load the loop carefully. Click on **Start Injection**.



8. Select **Run: Stop Sensorgram** to stop the data collection. Click on **Yes**.

9. To save the results, choose **File: Save As** and choose the directory "Tutorial". Enter a file name, "date-Exercise5"and click on **Save**.

10. Compare the sensorgram from Fc2 with that shown in Figure 17.

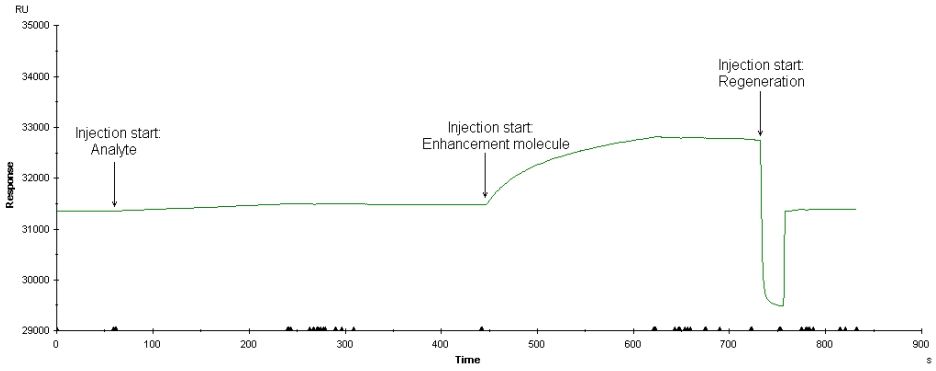


Figure 17. *The sensorgram clearly shows the enhancing effect of the injection of polyclonal anti-β2-microglobulin (the enhancement molecule) compared to the response generated by the injection of the low concentration of analyte.*

11. Select **View: Reference Line** to activate the reference line. Drag the reference line to the starting baseline of the sensorgram.

12. Add a report point by selecting **Edit: Add Report Point**. Enter the name "Baseline" in the **Id** field, use the default value of 5 s for the **Window** parameter. Click on **OK**.

13. Drag the reference line to a position just after the analyte injection. Choose **Edit: Add Report Point,** enter **Id:** beta2-micro. Use the default value of 5 s for the Window parameter and click on **OK**.

14. Drag the reference line to a position just after the injection of the polyclonal anti-β2-microglobulin. Choose **Edit: Add Report Point**, enter **Id:** Polyclonal anti-beta2-micro and click on **OK**.

15. The report points are now listed in the results table below the sensorgram.

16. Remove the reference line by choosing **View:Reference Line**.

17. Look at the sensorgram from Fc1 (the reference cell) to determine whether there is any non-specific binding to the dextran matrix.

18. Choose **Tools: Working Tools** and select **Continue**. Click on **Start** and follow the instructions provided on the screen. A continous flow of buffer is then started and the instrument can be left for up to four days (providing that there is enough buffer left).

Conclusions

In this exercise the effect of an enhancement molecule is clearly seen. Enhancement molecules can be used in situations other than the signal amplification illustrated in Exercise 5. It is also possible to enhance specificity by using a secondary binder. For example, if a ligand binds a family of analyte species, the secondary binder should be able to distinguish between these different analyte species.

Yet another application would be to analyze fusion proteins, where it may be desirable to confirm that the first response reflects proteins that contain both parts. In this case, the ligand would be directed against one part of the protein and the secondary binder directed against the other.

In addition, secondary binders are commonly used in analyses of different types of multi-component complexes.

Biacore routine maintenance

Regular maintenance of Biacore X is of the utmost importance for reliable results. It is important to avoid contamination, such as microbial growth and adsorbed proteins in the liquid handling system.

In Biacore X Control Software, maintenance procedures can be found under **Tools: Working Tools**. In the BIAmaintenance Kit (BR-1002-83) you will find the solutions required to run the different procedures. Use the Maintenance Chip when running methods **Desorb** and **Sanitize**. Inspect both sides of the chip before use. If necessary, clean the surface with water and wipe it dry.

The local field service representative provides preventive maintenance service every 6 or 12 months. Biacore personnel will be happy to discuss the service frequency most suitable to your situation.

Daily maintenance

- Filter and degas buffers.
- After running an experiment, maintain the buffer flow by selecting **Tools: Working Tools** and choosing **Continue**. If you intend to leave the instrument for more than four days, shut down the instrument by selecting **Tools: Working Tools** and choosing **Shutdown**.
- Flush the system by selecting **Tools: Working Tools** and choosing **Prime**.

Weekly maintenance

Cleaning the liquid system

- Run **Desorb** from the menu **Tools: Working Tools**.

Removal of salt residues

- Rinse the connector block and injection port on site with deionized water.
- Wipe the injection needle and vial dislodger with wet tissue.

Inspection of pumps

- Inspect pump tip for leakage (look for liquid beneath the tip).

- Inspect pump tip for contamination (look for residues on the top of the tip).

- If cleaning or adjustment of the tip becomes necessary, follow instructions according to **Syringe/Tip** under the **Tools: Service Tools** menu.

Monthly maintenance

Cleaning the liquid system

- Run **Desorb** from the menu **Tools: Working Tools**.

- Run **Sanitize** from the menu **Tools: Working Tools**. Prepare fresh sanitizing solution by mixing 1.5 ml BIAdisinfectant solution with 20 ml filtered deionized water. For best result, run **Desorb** before **Sanitize**.

Checking system performance

- Run **System Check** from the menu **Tools: Test Tools**, according to the instructions in the wizard.

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