Sensor Probe CM5 Handbook

Edition April 1997

Sensor Probe CM5 Handbook, edition April 1997 (version AA)

Biacore AB reserves the right to make changes to the design and specification of Sensor Probe CM5 and to the content of the Handbook without prior notice.

Warranty

Biacore AB guarantees that the product delivered has been thoroughly tested to ensure that it meets the published specifications. The warranty included in the conditions of delivery is valid only if the product has been installed and used according to the instructions supplied by Biacore AB.

The technology in this product was developed in collaboration with EBI Sensors Inc., who were in part supported by the U.S. Defense Advanced Research Projects Agency (contract #DAAL01-95-C-3503).

Read the manual before using the product.

An understanding of the English language is required.

Contents

1. Introduction

BIACORE®probe detects and measures analyte on the basis of interaction with a specific user-selected detecting molecule. This interaction takes place on the surface of the sensor probe, which is an optical fibre coated at the tip with a gold layer (see Figure 2-1). The detecting molecule is immobilized directly or indirectly on the surface to provide a biospecific probe. Analyte is detected by measuring the surface plasmon resonance (SPR) response, a phenomenon which is sensitive to the refractive index in the solution (and therefore the solute concentration) at the surface of the sensor probe. The principle of SPR is described in the BIACORE®probe Handbook.

This Sensor Probe CM5 Handbook provides a description of Sensor Probe CM5 and recommended procedures for using the sensor probe. For a description of how to operate BIACORE®probe, see the BIACORE®probe Handbook.

Sensor Probe CM5 is designed for use only with the instrument BIACORE®probe, and under the terms of license may not be used with any other instrument.

2. Description

2.1 Construction

Sensor Probe CM5 (Figure 2-1) consists of an optical fibre (diameter at the sensor tip about 400 µm) with a screw attachment for fitting to BIACORE®probe at one end. The fibre is covered with a protective cladding, except for 10 mm at the tip which is exposed to the solution being measured. This tip is coated with a thin layer of gold (~ 50 nm thick) to provide conditions for SPR (see BIACORE®probe Handbook, Appendix B). The sensor probe is supplied in a capped protective tube which also serves as a holder when fitting the sensor probe to the probe holder on the instrument (see BIACORE®probe Handbook, Chapter 4).

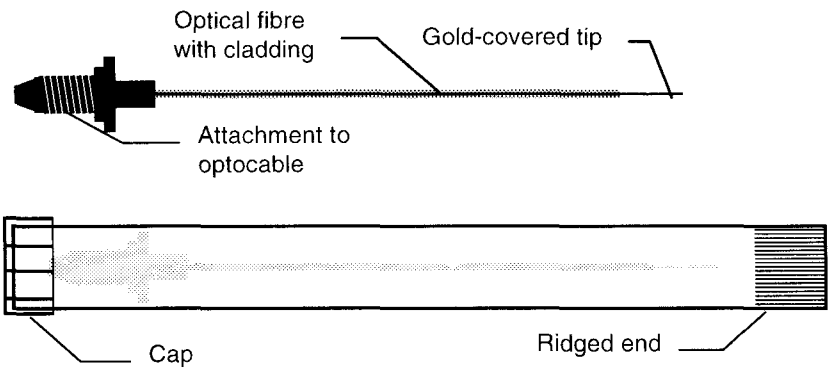

Figure 2-1. *Construction of the sensor probe (top) and protective tube (bottom) with the position of the sensor probe marked in grey.*

2.2 Surface properties

The gold film on the tip of Sensor Probe CM5 is covered with a layer of carboxymethylated dextran, covalently attached to the gold through an inert linker layer. The dextran is swollen in aqueous media, providing an extensively solvated hydrogel with a thickness of about 100 nm (Figure 2-2). The detecting molecule is attached to the dextran matrix either directly or through binding to a covalently immobilized capturing molecule (Chapter 3). Carboxymethyl groups on the dextran supply convenient sites for attachment of proteins using well-defined chemistry (see Chapter 3) .

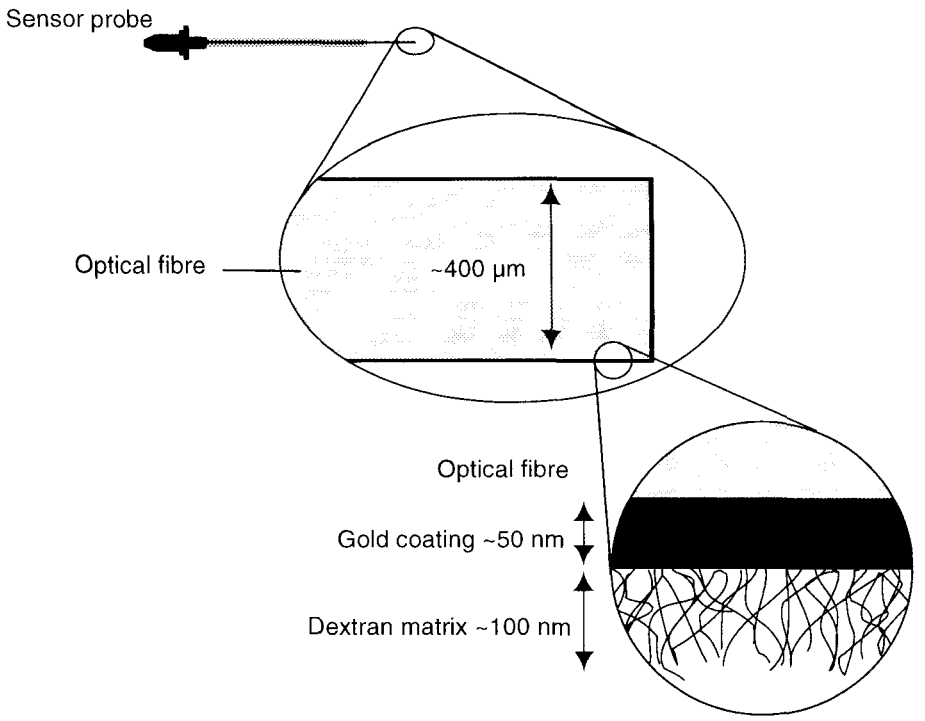

Figure 2-2. *Structure of the tip of Sensor Probe CM5, showing the relationship between the optical fibre, the gold coating and the dextran matrix.*

Attachment to the flexible dextran chains preserves a hydrophilic environment around the detecting molecules and provides a high degree of accessibility to the molecular surfaces. As a result, the biological activity of attached detecting molecules is retained to a high degree. This is a significant advantage of BIACORE®probe technology in comparison with techniques such as ELISA that rely on adsorption of proteins to plastic surfaces which can impair biological activity.

The dextran layer on Sensor Probe CM5 is chemically stable in most buffers used for preparing and studying biomolecules, and can be exposed to extremes of pH for short periods (2–3 minutes) without significant deterioration. In general, the stability of a functionalized sensor probe will be determined by the properties of the detecting molecule rather than of the dextran layer.

3. Functionalizing the sensor probe

Before use, Sensor Probe CM5 is functionalized by attaching a detecting molecule to the dextran layer on the surface of the sensor probe. This chapter gives practical guidance in attachment of the detecting molecule.

The detecting molecule may be attached either directly ("immobilized") or indirectly through high affinity binding ("captured") as illustrated in Figure 3-1. For the latter approach, the capturing molecule is immobilized directly on the dextran layer.

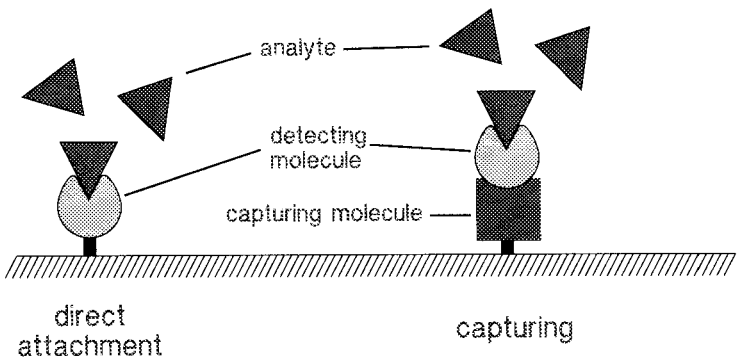

Figure 3-1. *Two ways of functionalizing the sensor probe.*

The recommended method for immobilizing biomolecules is covalent coupling via amine groups. The dextran layer may also be amenable to other methods for covalent attachment in the event that amine coupling is unsatisfactory.

Antibodies are suitable as detecting molecules for most purposes, and may be immobilized directly or captured on e.g. rabbit anti-mouse IgG1 (RAM IgG1) or Protein A. General recommended procedures for immobilizing user-selected antibodies, RAM IgG1 and Protein A, and for capturing IgG on RAM IgG1 and Protein A, are given in this chapter. These procedures can also serve as starting points for developing functionalization procedures using other detecting or capturing molecules.

3.1 Amine coupling chemistry

Covalent coupling of proteins and other biomolecules using amine coupling chemistry is based on conversion of carboxyl groups on the dextran to N-hydroxysuccinimide esters using a mixture of N-hydroxysuccinimide (NHS) and N-ethyl-N'-(dimethylaminopropyl)-carbodiimide (EDC). These esters react with uncharged amino groups and other nucleophilic groups on biomolecules to form covalent links. The steps in immobilization are illustrated in Figure 3-2.

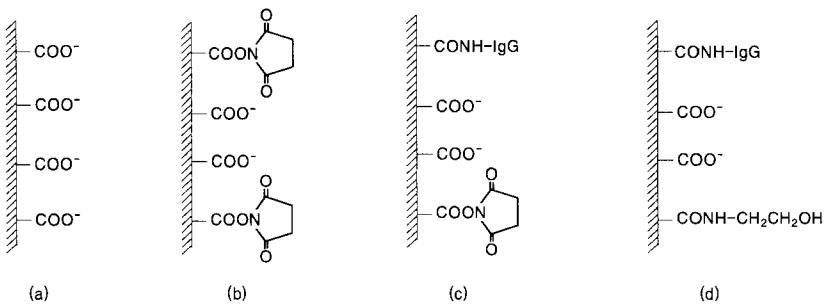

Figure 3-2. *Steps in immobilization of proteins by amine coupling.*
(1) The dextran matrix on the sensor probe surface contains non-activated carboxyl groups.
(2) Some of the carboxyl groups are converted to active NHS-esters.
(3) Amine groups on the protein (here shown as IgG) react with NHS esters, covalently linking the protein to the matrix.
(4) Excess NHS-esters are inactivated by reaction with ethanolamine.

At pH values above 3.5 (the average pI of the carboxymethyl groups), and below the pI of the protein being coupled, electrostatic attraction between the negatively charged dextran and the positively charged protein will concentrate the protein on the surface of the sensor probe and enhance the efficiency of the coupling procedure. The optimum pH for coupling is usually about 0.5-1 units below the pI of the protein (see table). Acidic proteins with pI <3.5 cannot generally be attached using amine coupling.

After coupling the detecting molecule, any NHS-esters remaining on the dextran are blocked by reaction with 1 M ethanolamine-HCl at pH 8.5 (converting the NHS-esters to hydroxyethylamides). The high ionic strength of this solution helps to remove any detecting molecule which may be non-covalently bound to the surface, leaving only the stable covalently bound molecules.

The functionalized sensor probe surface may retain charged properties depending on the nature of the detecting molecule and the degree of substitution of the carboxymethyl groups. Using the recommended procedures for attachment of detecting molecule by covalent amine coupling, approximately 20-40% of the carboxymethyl groups are modified.

3.2 Direct coupling (immobilization)

Materials required

- Amine Coupling Kit (Biacore AB) containing
  N-hydroxysuccinimide (NHS)
  N-ethyl-N'-(dimethylaminopropyl)carbodiimide (EDC)
  1 M ethanolamine-HCl pH 8.5.

- Reference buffer. Physiological buffer solutions (150 mM salt, pH 7-7.5) are generally suitable as reference buffer.[1]

- Coupling buffer. Buffer requirements are low ionic strength (≤ 20 mM), no free amine groups and pH between 3.5 and 6. The recommended buffer conditions depend on the pI of the protein being coupled and are summarized in Table 3-1.

- Detecting or capturing molecule solution, concentration at least 100 µg/ml in coupling buffer. If the solution is prepared by dilution of a stock solution, make sure that the dilution is sufficient to reduce the ionic strength to ≤ 20 mM and that the pH of the final solution is adequately buffered.

Protein pI	*Buffer pH*	*Suitable buffer*
< 3.5	Amine coupling not suitable	
3.5-4.5	0.5 units below pI	10 mM Na-formate
4.0-5.5	0.5 units below pI	10 mM Na-acetate
5.5-7.0	1 unit below pI	5 mM Na-malate
>7.0	pH 6	5 mM Na-malate
unknown	pH 5	10 mM Na-acetate

Table 3-1. *Recommended conditions for coupling buffer, in relation to the pI of the protein being coupled.*

[1] A ready-made HEPES-buffered saline (HBS buffer BIA *Certified*: 10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Surfactant P20) is available from Biacore AB.

Immobilization strategy

BIACORE®probe uses a mass detection principle, leading to the following general guidelines (see Figure 3-3):

- higher immobilization levels give a lower detection limit for the same analyte
- to attain the same detection limit for a given analyte using different detecting molecules, a higher molecular weight detecting molecule requires a higher immobilization level
- for different analytes using the same detecting molecule immobilized at the same level, the detection limit will be lower for the larger analyte.

When antibodies are used as detecting molecules, suitable immobilization level are 10-15 kRU for detection of analytes of molecular weight above 50,000 daltons. Higher immobilization levels can improve the detection limit, particularly for smaller analytes.

Under recommended immobilization conditions, electrostatic attraction concentrates the molecule being immobilized in the dextran layer on the sensor probe surface. The extent of this electrostatic binding to a non-activated sensor probe usually gives a good indication of the immobilization level that may be expected when the sensor probe has been activated using NHS/EDC. To help establish suitable immobilization conditions, you can measure the level of electrostatic binding to a non-activated sensor probe as follows:

1. Touch **Start** to start an analysis. Follow the instructions on the screen to set the zero level in reference buffer.

2. Wash the sensor probe with the coupling buffer being tested, then aspirate detecting molecule solution in coupling buffer. Wait 5 minutes, then touch **Read** to record the response.
   Important: *In this step, you measure the response in the presence of sample. The normal procedure of transferring the sensor probe to reference buffer before measuring cannot be used, since electrostatically bound detecting molecule will dissociate rapidly in reference buffer.*

3. Dispense the detecting molecule solution. If your detecting molecule is valuable, you may want to keep the dispensed solution (the amount of detecting molecule bound to the sensor probe is typically less than 1% of the amount in the 100 µl of solution used). Wash the sensor probe with 1 M ethanolamine-HCl (included in the Amine Coupling Kit) to remove all electrostatically bound detecting molecules from the sensor probe, and then wash with reference buffer.

The expected level of immobilization is 60-80% of the amount of detecting molecule that binds to the sensor probe in coupling buffer. If this level is too low, adjust the coupling conditions as follows:

- Establish the optimum pH for electrostatic binding in the pH range 3.5-6 using the buffers listed in Table 3-1. It is usually sufficient to measure binding at intervals of 0.5 pH units.
- Increase the concentration of detecting molecule if pH adjustment is not sufficient.

Immobilization procedure

A profile for standard amine coupling procedure is provided with BIACORE®probe Software. The profile name is AMINECPL. The description below assumes that you use this profile. See the BIACORE®probe Handbook, Chapter 4.8 for a description of how to define and edit profiles.

1. Prepare solutions of 0.1 M NHS and 0.4 M EDC in water as described in the Amine Coupling Kit Instructions for Use.
2. Touch **Profile** on the screen, select AMINECPL and touch **Load** to load the profile into working memory. The profile name appears at the top right of the screen when the profile is loaded.
3. Touch **Start** on the screen to start recording the analysis. Follow the instructions on the screen to set the first zero response level in reference buffer.
4. Mix equal volumes of NHS and EDC solutions as described in the Amine Coupling Kit Instructions for Use. The NHS/EDC mixture is unstable: use the solution directly after preparation. Wash the sensor probe with distilled water and aspirate the NHS/EDC mixture into the pipette tip. Touch **ACTIVATE** in the bottom button area on the screen to start a 10-minute timer for NHS/EDC activation.
5. When the timer reaches zero, dispense and discard the NHS/EDC solution. Wash the sensor probe with coupling buffer then aspirate detecting molecule solution. Touch **COUPLING** to start a 10-minute timer for the coupling reaction.
6. When the timer reaches zero, dispense the detecting molecule solution. If your detecting molecule is valuable, you may want to keep the dispensed solution (the amount of detecting molecule immobilized on the sensor probe is typically less than 1% of the amount in the 100 µl of solution used). Remember however that the coupling conditions may be unfavourable for storing the detecting molecule.

7. Aspirate 1 M ethanolamine-HCl. Touch **DEACT** to start a 10-minute timer for deactivation of remaining reactive esters.

8. When the timer reaches zero, a one minute delay period starts before the response level is recorded. During this period, dispense and discard the ethanolamine solution, wash the sensor probe with reference buffer and aspirate reference buffer.

9. Touch **Stop**.

10. Save and/or print the results.

3.3 Capturing

Direct coupling is the preferred approach for functionalizing the sensor probe. However, capturing the detecting molecule by binding to an immobilized capturing molecule can be useful in the following situations:

- When the detecting molecule is not available in a pure state. The capturing step then represents a selective extraction of the detecting molecule from the crude preparation. For example, capture on RAM IgG1 will efficiently select monoclonal antibodies from unfractionated hybridoma culture medium.

- When one sensor probe is to be used for a range of different detecting molecules (e.g. in scanning candidate antibodies for the most suitable for use as a detecting molecule).

- When suitable regeneration conditions cannot be found for the detecting molecule. With a capturing approach, regeneration involves removal of both the analyte and the detecting molecule, and can be done using standard conditions developed for the general capturing interaction.

This section describes the general experimental approach to capturing. Conditions have been tested at Biacore AB for capturing mouse IgG1 on rabbit anti-mouse IgG1 (RAM IgG1, Biacore AB) and for capturing antibodies on Protein A (Pharmacia Biotech AB). Recommended conditions are summarized in Table 3-2. For functionalization using other capturing and/or detecting molecules, the conditions for immobilization and capture should be optimized as described in the remainder of this section.

	RAM IgG1	Protein A
Source [1]	Biacore AB Order no BR-1000-55 Lot no 711	Pharmacia Biotech AB Order no 17-0872-01 Lot no 231516
Immobilization		
Coupling buffer	10 mM Na-acetate pH 4.7	10 mM Na-acetate pH 4.7
Recommended concentration	100 µg/ml	300 µg/ml
Expected immobilization level	≈10 kRU	≈5 kRU [2]
Capturing		
Capturing buffer	10 mM Na-acetate pH 5.25	HEPES-buffered saline pH 7.4
Recommended capturing level (IgG)	3-4 kRU [3]	10-12 kRU

Notes:

[1] Optimal conditions for immobilization and capturing may vary with different batches. The conditions listed have been established only for the specified lot numbers.

[2] The recommendation of a lower immobilization level for Protein A is related to the smaller molecular size (molecular weights RAM IgG1 150,000 daltons, Protein A 42,000 daltons). Since BIACORE® probe uses a mass detection principle, smaller capturing molecules require lower immobilization levels to capture the same amount of detecting molecule.

[3] At higher levels of capturing on polyclonal RAM IgG1, lower affinity interactions can contribute significantly to the capturing process, with the result that captured antibodies dissociate to some extent during analysis. Captured levels of 3-4 kRU have been found to give the best results.

Table 3-2. *Recommended conditions for using RAM IgG1 and Protein A as capturing molecules.*

Materials required

- Amine Coupling Kit (Biacore AB) containing
  N-hydroxysuccinimide (NHS)
  N-ethyl-N'-(dimethylaminopropyl)carbodiimide (EDC)
  1 M ethanolamine-HCl pH 8.5.

- Coupling buffer. See Section 3.2 for recommendations.

- Capturing buffer. Suitable capturing conditions depend on the interaction properties between the capturing and detecting molecules. See Table 3-2 for recommended conditions for capturing on RAM IgG1 and Protein A.

- Reference buffer. Physiological buffer solutions (150 mM salt, pH 7-7.5) are generally suitable as reference buffer.[1]

- Capturing molecule solution in coupling buffer. Recommended concentration at least 100 µg/ml capturing molecule. See Table 3-2 for recommendations for RAM IgG1 and Protein A.

- Detecting molecule solution, typical concentration 25-100 µg/ml in capturing buffer.

Capturing procedure

1. Immobilize capturing molecule on the sensor probe as described in Section 3.2 above.

2. Wash the sensor probe with reference buffer, aspirate reference buffer and touch **Zero**.

3. Change the pipette tip and aspirate detecting molecule solution. Wait 15-60 seconds (you may vary this contact time to regulate the amount of detecting molecule captured).

4. Dispense and keep the detecting molecule solution. Wash the sensor probe twice with reference buffer, then aspirate reference buffer, wait 30 seconds and touch **Read** to record the amount of detecting molecule captured.

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[1] A ready-made HEPES-buffered saline (HBS buffer BIA *Certified*: 10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Surfactant P20) is available from Biacore AB.

Optimizing the capturing

The performance of an assay using a capturing approach is determined mainly by the amount of detecting molecule captured, which in turn is influenced by the amount of capturing molecule immobilized. Either or both of these parameters may be adjusted to optimize the assay performance. A critical factor in assay performance is the stability of the capturing interaction. For heterogeneous capturing or detecting molecules (e.g. RAM IgG1 and other polyclonal antibodies), contributions from low affinity capturing interactions may become significant at high captured levels, which can limit the amount of detecting molecule that can usefully be captured.

In general, follow the recommendations given in Section 3.2 for optimizing the amount of capturing molecule immobilized. If possible, adjust the amount of immobilized capturing molecule so that about 10 kRU of antibody detecting molecule will be captured. Recommended immobilization levels for RAM IgG1 and Protein A are given in Table 3-2.

To control the level of detecting molecule captured:

- Adjust the concentration of the detecting molecule in capturing buffer.
- Adjust the contact time of detecting molecule with the sensor probe.

3.4 Storing functionalized sensor probes

Conditions and shelf life for storing functionalized Sensor Probe CM5 will depend largely on the properties of the attached detecting molecule. Two storage methods are available:

Wet storage

Materials required: Storage buffer
A 50 ml capped plastic tube (e.g. Falcon tube)

Immerse the sensor probe in its capped protective tube in sterile-filtered buffer so that the tip of the sensor probe is completely covered. The tip of the sensor probe is towards the ridged end of the protective tube. Do not immerse the optocable connector at the other end of the sensor probe. Store the sensor probe in a capped tube (e.g. Falcon 50 ml) at 4-8°C. Do not freeze the sensor probe.

A recommended buffer which is generally suitable for storing sensor probes functionalized with antibodies is HBS buffer *BIA Certified* (10 mM Hepes pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Surfactant P20), available from Biacore AB.

Dry storage

Materials required: Blue silica gel (humidity indicator)
A 50 ml capped plastic tube (e.g. Falcon tube)

With the sensor probe in its capped protective tube, gently blow the tip of the sensor probe dry with nitrogen or oil-free compressed air. Place the sensor probe in its protective tube in a tube (e.g. Falcon 50 ml) with about 5 g blue silica gel. Cap the tube securely and store it at 4-8°C. Do not freeze the sensor probe.

For many antibodies, dry storage is preferable to wet storage. The table below gives the analyte binding capacity as a percentage of the initial value for Sensor Probe CM5 functionalized with an anti-myoglobin antibody in test experiments at Biacore AB.

	1 week	2 weeks	3 weeks	4 weeks
Wet storage (HBS Buffer *BIA Certified*)	91%	84%	80%	77%
Dry storage	98%	93%	94%	90%

4. Analysis procedures

The analysis cycle using BIACORE®probe consists of sample measurement determination followed by regeneration of the sensor probe. Regeneration can be omitted if no response is detected (i.e. no analyte remains bound to the sensor probe after sample measurement). Operating procedures differ slightly according to whether the sensor probe is functionalized by direct coupling or by capturing. The essential differences are summarized in Table 4-1.

	Direct coupling	Capturing
Analysis procedure	Sensor probe ready for re-use immediately after regeneration (see Figure 4-1).	Detecting molecule must be re-captured after regeneration (see Figure 4-2).
Analysis conditions	Determined by the detecting molecule-analyte interaction.	Determined by both the capturing molecule-detecting molecule and the detecting molecule-analyte interactions.
Regeneration	Removes analyte. Conditions must be established for each new assay.	Removes detecting molecule and analyte. Conditions for one assay will probably work for another.
Typical performance	Detection limit ~1 µg/ml	Detection limit ~2-5 µg/ml

Table 4-1. *Essential differences between analysis using direct coupling and capturing approaches.*

4.1 General recommendations

Analysis conditions

With Sensor Probe CM5, conditions for performing analyses are determined almost entirely by the properties of the detecting molecule and analyte. The sensor probe itself is resistant to most environments used in purification and analysis of biomolecules.

In general, using physiological salt concentrations (150 mM) or higher in the sample and reference buffer will reduce non-specific binding of molecules to the functionalized sensor probe. For samples in low ionic strength buffer (e.g. fractions eluted from ion exchange or

hydrophobic interaction chromatography columns), add salt as appropriate. Make measurements in low ionic strength only if the interaction between the detecting molecule and the analyte is disrupted by physiological salt concentrations.

Oxidizing agents such as periodate, peroxides, bromine and iodine solutions should however be avoided since these may destroy the dextran layer or the linker layer. Exposure to enzymes that hydrolyze dextran (dextranases) should also be avoided.

Analysis procedures

BIACORE®probe determines the presence of analyte by measuring the amount of analyte that binds specifically to the detecting molecule on the sensor probe. When the sensor probe is in contact with the sample solution, other solutes in the sample will contribute to the measured response through bulk refractive index effects (see BIACORE®probe Handbook, Appendix B). For this reason, measurements are always made with the sensor probe in reference buffer. Allow 15-30 seconds for the signal to stabilize after transferring the sensor probe to reference buffer before recording the response.

For analysis procedures to be used regularly, create a profile with preset timer durations and automatic response reading. This will help to improve the consistency of the analysis procedure. See BIACORE®probe Handbook, Chapter 4 for a description of how to create and use profiles.

4.2 Using directly coupled detecting molecule

Follow the steps below to perform an analysis using a sensor probe functionalized by direct coupling (see Figure 4-1):

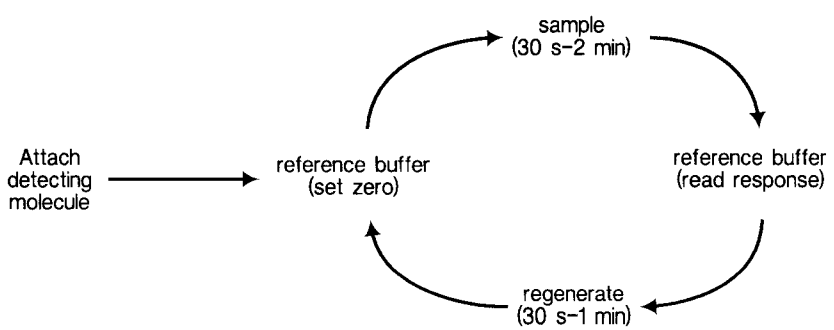

Figure 4-1. *Analysis cycle using directly coupled detecting molecule.*

1. Touch **Start** on the screen to start recording the analysis. Follow the instructions on the screen to set the first zero response level in reference buffer.

2. Change the pipette tip to avoid carry-over of reference buffer to the sample, aspirate sample and touch **Reset** on the timer.

3. Wait for a fixed time interval (typically 30 seconds-2 minutes) to allow analyte to bind to the detecting molecule.

4. Dispense the sample and wash the sensor probe twice with reference buffer. Aspirate reference buffer, wait 15-30 seconds for the signal to stabilize, then touch **Read** to record the sample response.

5. If the response is negligible (i.e. there is no analyte in the sample), regeneration is not necessary: touch **Zero** to set the zero response level for the next cycle then go to step 2 for the next sample. Otherwise continue with the regeneration steps below.

6. Dispense the reference buffer, then follow the procedure established for optimal regeneration of the sensor probe. See Chapter 5 for a general procedure for establishing optimal regeneration conditions.

7. After regeneration, wash the sensor probe with reference buffer, aspirate reference buffer and touch **Zero** to set the zero response level for the next cycle. Go to step 2 for the next sample.

8. Touch **Stop** to stop the analysis when the measurements are complete.

9. Save and/or print the results.

4.3 Using captured detecting molecule

When performing an analysis using captured detecting molecule, it is advisable to record the level of detecting molecule captured as well as the amount of analyte bound at each cycle. This provides a check on the consistency of the capturing procedure.

Follow the steps below to perform an analysis using a sensor probe functionalized by capturing (see Figure 4-2). This description assumes that capturing molecule is immobilized on the sensor probe but no detecting molecule is captured:

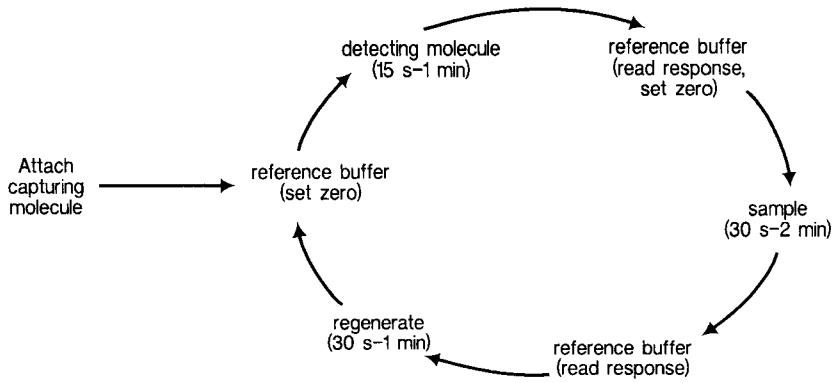

Figure 4-2. *Analysis cycle using captured detecting molecule.*

1. Touch **Start** on the screen to start recording the analysis. Follow the instructions on the screen to set the first zero response level in reference buffer.

2. Change the pipette tip to avoid carry-over of reference buffer to the detecting molecule, aspirate detecting molecule and touch **Reset** on the timer.

3. Wait for a fixed time interval (typically 15-60 seconds) to allow capture of the detecting molecule.

4. Dispense and keep the detecting molecule solution for use in the next cycle. Wash the sensor probe twice with reference buffer, aspirate reference buffer, wait 15-30 seconds for the signal to stabilize and then touch **Read** to record the amount of detecting molecule captured.

5. Touch **Zero** to reset the zero level response in preparation for sample measurement.

6. Change the pipette tip to avoid carry-over of reference buffer to the sample, aspirate sample and touch **Reset** on the timer.

7. Wait for a fixed time interval (typically 30 seconds-2 minutes) to allow analyte to bind to the detecting molecule.

8. Dispense the sample and wash the sensor probe twice with reference buffer. Aspirate reference buffer, wait 15-30 seconds for the signal to stabilize, then touch **Read** to record the sample response.

9. If the response is negligible (i.e. there is no analyte in the sample), regeneration is not necessary: go directly to step 5 for the next sample. Otherwise continue with the regeneration steps below.

10. Dispense the reference buffer, then follow the procedure established for optimal regeneration of the sensor probe. See Chapter 5 for a general procedure for establishing optimal regeneration conditions.

11. After regeneration, wash the sensor probe with reference buffer, aspirate reference buffer and touch **Zero** to set the zero response level for the next cycle. Go to step 2 for the next sample.

12. Touch **Stop** to stop the analysis when the measurements are complete.

13. Save and/or print the results.

Analysis and regeneration conditions for RAM IgG1 and Protein A

Analysis using capturing of antibodies as detecting molecules on RAM IgG1 (Biacore AB) and Protein A (Pharmacia Biotech AB) has been tested at Biacore AB. The table below summarizes conditions that can be used.

	RAM IgG1	Protein A
Analysis [1]	pH 5-9 0.1 - 1.0 M NaCl 0 - 1 M $(NH_4)_2SO_4$	pH ≥6 [2] 0.1 - 1.0 M NaCl 0 - 1 M $(NH_4)_2SO_4$
Regeneration	10 mM glycine-HCl pH 1.7 Contact time 1 minute	10 mM glycine-HCl pH 1.7 Contact time 1 minute

Notes:

[1] The conditions tested provide a general indication of the useful range of the assay and are not exhaustive. Deviations from the listed conditions may occur depending on the nature of the analyte.

[2] The captured detecting molecule tends to dissociate from Protein A at pH values below 6.

Table 4-2. *Recommended analysis and regeneration conditions for analysis using RAM IgG1 or Protein A as a capturing molecule*

Analysis using capturing of antibodies as detecting molecules on RAM IgG1 (Biacore AB) and Protein A (Pharmacia Biotech AB) has been tested at Biacore AB. The table below summarizes conditions that can be used.

	RAM IgG1	Protein A
Analysis [1]	pH 5-9 0.1 - 1.0 M NaCl 0 - 1 M $(NH_4)_2SO_4$	pH ≥6 [2] 0.1 - 1.0 M NaCl 0 - 1 M $(NH_4)_2SO_4$
Regeneration	10 mM glycine HCl pH 1.7 Contact time 1 minute	10 mM glycine HCl pH 1.7 Contact time 1 minute

Notes:

[1] The conditions tested provide a general indication of the useful range of the assay and are not exhaustive. Deviations from the listed conditions may occur depending on the nature of the analyte.

[2] The captured detecting molecule tends to dissociate from Protein A at pH values below 6.

Table 4-2. *Recommended analysis and regeneration conditions for analysis using RAM IgG1 or Protein A as a capturing molecule*

5. Regeneration

Regeneration of the sensor probe after detection of analyte is a critical step in the use of BIACORE®probe. Regeneration conditions are determined by the nature of the detecting molecule-analyte interaction (for assays using direct coupling) or by the nature of the capturing molecule-detecting molecule interaction (for assays using capturing). This chapter outlines a general procedure that may be used to establish suitable regeneration conditions without destroying the functionalized sensor probe.

5.1 Regeneration solutions

Antibodies may usually be regenerated with low (≤3) or high (≥9) pH at low ionic strength (<30 mM) solutions. Experience has shown that in general antibodies against proteins are best regenerated using low pH while antibodies against haptens often respond better to regeneration at high pH. Dilute HCl or NaOH solutions are convenient and easy to prepare for testing optimal regeneration conditions. When the best pH value has been found, a 10 mM glycine-HCl buffer (low pH) or 10 mM glycine-NaOH (high pH) may be used to provide better control of pH values between different analysis occasions.

5.2 Regeneration test procedure

Start investigating regeneration conditions using mild solutions, then increase the harshness of the treatment progressively until suitable conditions are found. In this way, you minimize the risk of damaging the detecting or capturing molecule on the sensor probe by exposure to excessively harsh conditions. For example, Table 5-2 gives a suggested sequence of solutions to test for regenerating antibodies on the sensor probe.

pH	Solution
3	1 mM HCl
2.5	3 mM HCl
2	10 mM HCl
1.5	30 mM HCl

Table 5-2. *Suggested test solutions for establishing conditions for regenerating antibodies against protein antigens. Start with the mildest solution (1 mM HCl).*

1. Set a zero response level with the sensor probe in reference buffer.

2. Bind analyte to the functionalized sensor probe using purified analyte if available. Use a response level that is representative for your samples. Wash the sensor probe with reference buffer and read the response.

3. Wash the sensor probe once with regeneration solution, then aspirate regeneration solution. Wait 1 minute, then dispense the regeneration solution, wash with reference buffer and read the response.

4. If the response is zero or negative, removal of the analyte is complete. You may want to test a shorter regeneration time (30 seconds).

   If regeneration is not complete, repeat steps 1 to 3 using increasingly harsh conditions until a negative response is obtained.

5. When you have established the mildest conditions that remove all of the analyte, repeat steps 1 to 3 three times more to check the consistency of the regeneration procedure. Triplicate cycles of binding and regeneration using the same conditions should show consistent values for both analyte response and baseline level. If the analyte response decreases, this indicates that the detecting molecule does not withstand the conditions used. Repeat the consistency tests using half the contact time for regeneration.

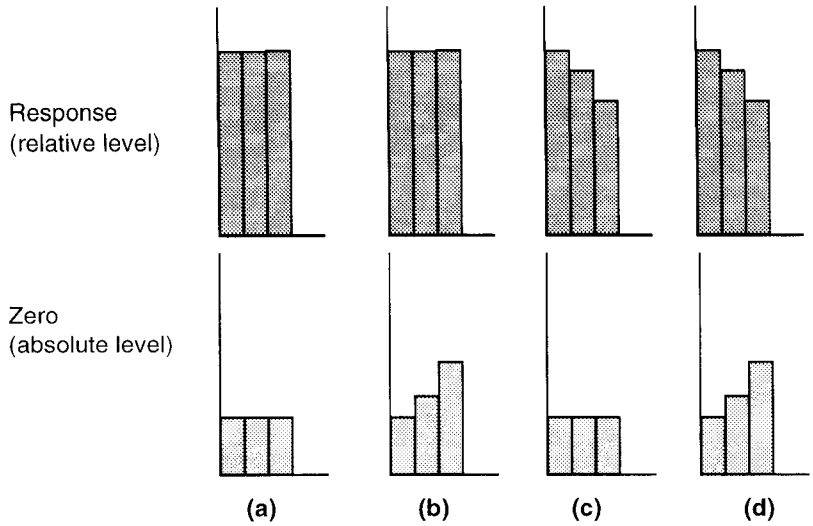

Figure 5-1. *Schematic illustration of successful and unsuccessful regeneration, showing response and zero levels over three analysis cycles.*
(a) *Successful regeneration. Reproducible sample response and consistent zero level.*
(b) *Incomplete regeneration. Reproducible sample response, but increasing zero level.*
(c) *Damage to the detecting molecule. Decreasing sample response, consistent zero level.*
(d) *Combination of (b) and (c). This situation can also arise with incomplete regeneration if the sample response represents the maximum binding capacity of the sensor probe.*

If the detecting molecule is damaged by the mildest conditions that remove all analyte, you should try alternative approaches to regeneration (e.g. high pH instead of low pH, mild denaturing agents, etc). Another alternative is to use a capturing approach, where the capturing interaction is well defined and conditions for regeneration are established.

Regeneration in practice

If BIACORE®probe is used for qualitative or semi-quantitative detection of analyte, a certain degree of inadequate regeneration can be tolerated. Figure 5-1 illustrates the effects over three analysis cycles of incomplete regeneration (increasing zero levels between cycles, Figure 5-1b) and deterioration of the detecting molecule (loss of binding capacity between cycles, Figure 5-1c). The two effects may be combined in some cases (Figure 5-1d). Provided that these effects are small in relation to the response expected from samples, the sensor probe can still be used for detecting analyte.

To compensate for small changes in the absolute response, remember to reset the zero level after each cycle of measurement and regeneration. In this way each sample response is measured relative to a correctly established zero level: the absolute response at each zero setting records the effect of each regeneration cycle.

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