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# Assessing long-term glucose regulation by measurement of glycated hemoglobin using Biacore™ 8K

Persistently elevated levels of blood glucose lead to irreversible attachment of glucose molecules to hemoglobin in red blood cells. The longer the hyperglycemia occurs, the more glucose binds to hemoglobin. By measuring the binding of antibodies that recognize glycated hemoglobin (HbA1c), long-term serum glucose regulation is assessed. In this study, Roche Diagnostics monitored long-term glucose regulation through the binding of monoclonal antibodies (mAbs) targeted against HbA1c using the highly sensitive Biacore 8K system. In the model experiment described, a set of rabbit mAbs specific to HbA1c were characterized with respect to their binding to a human HbA1c peptide. To assess specificity, binding of rabbit mAb to derivatives of the HbA1c peptide was characterized. The results showed a large variation in binding profiles with affinities in the nanomolar range. The specificity test displayed more uniform kinetic profiles than in the kinetic characterization but larger variation in binding strength with affinities ranging from micromolar to low nanomolar concentrations. The parallel configuration and the high sensitivity of Biacore 8K enabled rapid characterization of mAbs binding to small peptides at low  $R_{max}$  levels and with significantly reduced run times compared to single-needle systems. This study demonstrates that the new Biacore 8K is an enabling tool for all kinds of screening and functional SPR assays where sensitivity, robustness, and throughput are essential.

## Introduction

Roche Diagnostics have relied on Biacore SPR technology to characterize product-relevant molecular interactions for 25 yr. During that time hormones, vitamins, peptides, small molecules, aptamers, nucleic acids, fragments, liposomes, micelles, membranes, native and recombinant proteins, enzymes, and cell lysates have been in focus. However, the dominating target from the early days and on has always been antibodies. SPR technology using

Biacore systems has become an indispensable process standard in the early development of mAbs for diagnostic and pharmaceutical applications. In particular, kinetic antibody screening is established in the diagnostic antibody production process. Kinetic screening resolves kinetic rates, which contribute to the apparent antibody dissociation constant and supports the scientist with more information than conventional ELISA delivers. Moreover, Biacore SPR technology today has found its way from an early phase research and development support into late-stage antibody product quality control, and in FDA-relevant functional SPR assays. With an increasing number of high-performance industrial SPR applications, there is a clear demand for an instrument that merges high throughput and sensitivity in a single system. Biacore 8K is a high sensitivity, eight-channel parallel system with two flow cells in each channel (active and reference). The instrument is intended for screening and characterization of both small molecules and biotherapeutics, see data file 29205902 from GE Healthcare's life sciences solutions.

Biacore 8K was used in a model kinetic study for the binding of rabbit mAbs to HbA1c. When blood glucose levels are persistently elevated, glucose molecules irreversibly attach to hemoglobin in red blood cells. The longer the hyperglycemia persists, the more glucose binds to hemoglobin. Measuring HbA1c assesses the long-term serum glucose regulation. Here, the objective was to determine antibody kinetics against relevant HbA1c epitopes.

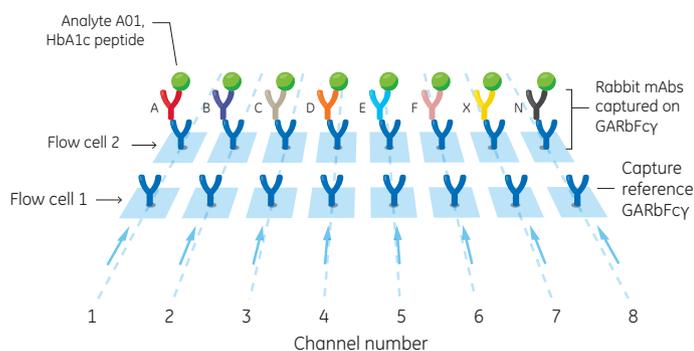
## Materials and methods

### *Kinetic characterization of rabbit mAb binding to HbA1c peptide*

Biacore 8K was used to kinetically assess the binding behavior of six rabbit mAbs (mAbs A to F) targeted against human HbA1c. Rabbit mAb X with unrelated target binding function and rabbit normal (N) antibody (Rb-N-IgG, [Sigma-Aldrich, code no. I5006]) were used as controls.

A Biacore Series S Sensor Chip CM5 was mounted into the Biacore 8K system and was normalized in instrument buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% w/v Tween™ 20) according to the manufacturer's instructions. Samples were prepared in system buffer supplemented with 1 mg/mL of carboxymethyl dextran (CMD, Sigma-Aldrich, code no. 86524). Samples were analyzed at 37°C. Approximately 12 000 RU goat antirabbit Fc fragment-specific antibody (GARbFcy, Jackson ImmunoResearch Laboratories Inc, code no. 111-005-046) was immobilized according to the manufacturer's instructions using EDC/NHS coupling in both flow cells and in all eight channels. The sensor surface was finally saturated with 1 M ethanolamine. The binding kinetics of the respective rabbit mAbs against an HbA1c peptide (analyte A01, relative molecular mass [ $M_r$ ] of 1200) were generated and evaluated. The experimental assay setup is shown in Figure 1.

Antibodies were injected at 200 nM concentration for 1 min at 10  $\mu$ L/min. After capture, the flow rate was increased to 80  $\mu$ L/min. Analyte A01 was injected for 3 min in a concentration series of 0 (buffer control), 3, 10, 30 (in duplicate), as well as 90 and 270 nM. The analyte dissociation was monitored for 5 min. After each analyte injection, the antibody capture system was fully regenerated by a 15 s injection of HBS-ET buffer at 20  $\mu$ L/min; a 20 s injection at 20  $\mu$ L/min with 10 mM glycine buffer pH 2.0; and two injections for 1 min at 20  $\mu$ L/min with 10 mM glycine pH 2.25. Kinetic signatures were evaluated using a 1:1 binding model with local  $R_{max}$ .



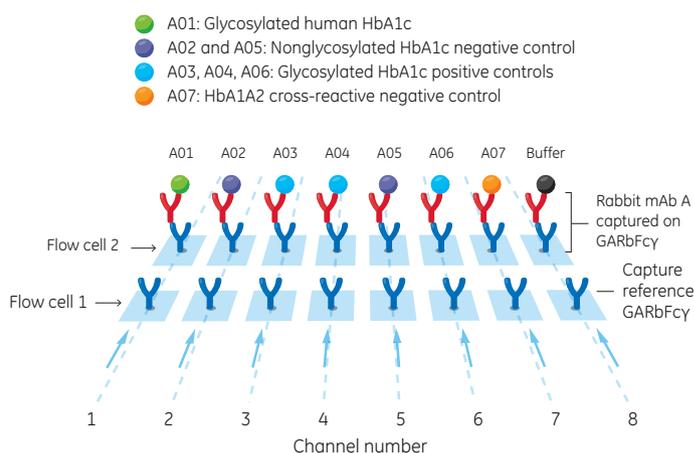
**Fig 1.** Experimental assay setup for kinetic evaluation of six rabbit mAbs (A to F) with specificity for human HbA1c (analyte A01). GARbFcy was immobilized in flow cells 1 and 2 and in all eight channels. The rabbit mAbs were captured on GARbFcy in flow cell 2. Human HbA1c was injected in both cells. Rabbit mAb X with unrelated target binding function and rabbit normal (N) IgG were used as controls.

## Binding specificity of rabbit mAb to various human HbA1c peptide derivatives

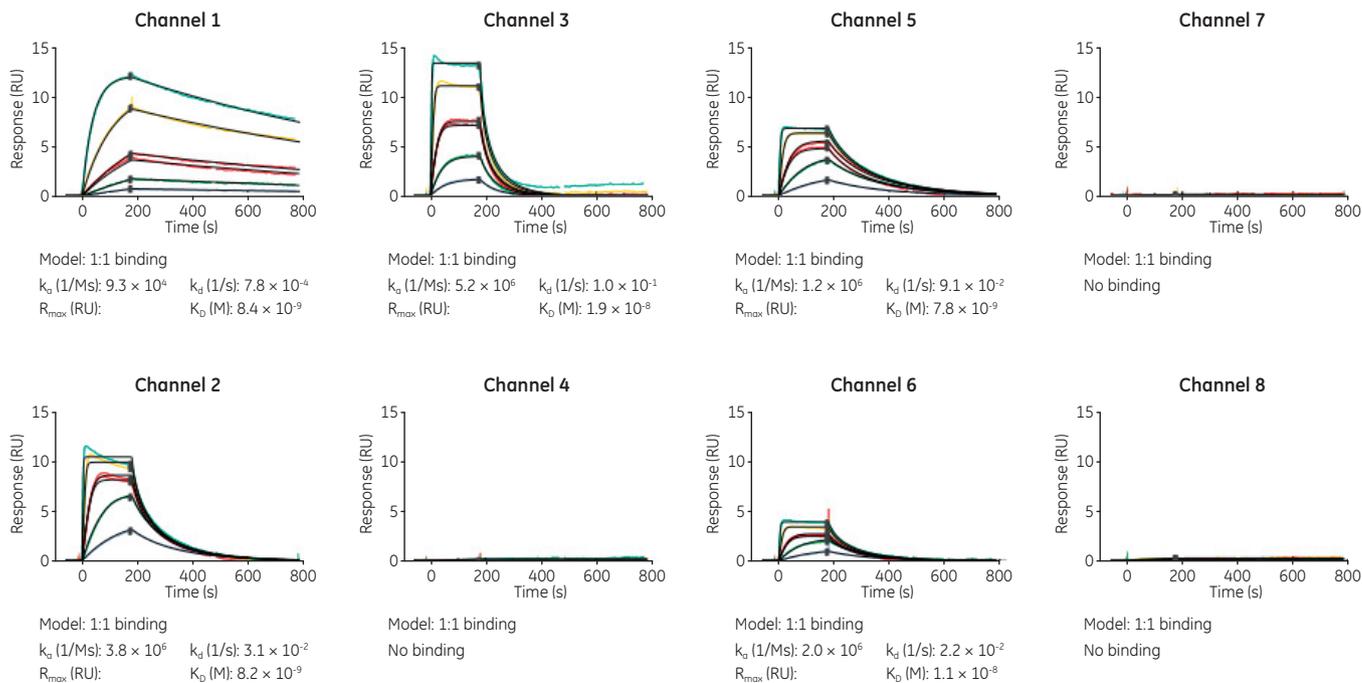
After the kinetic experiments, mAb A was selected to determine specificity against seven human HbA1c peptide analytes with  $M_r$  between 1100 and 1500 and a buffer control (Fig 2).

The peptide analytes differed in their amino acid composition, sequence length, glycosylation, or N-/C-terminal chemical modifications. For example, A01 is glycosylated HbA1c positive control peptide and A02 and A05 are nonglycosylated HbA1c peptide negative controls. A07 is the HbA1A2 cross-reactive negative control. A03, A04, and A06 are positive controls for HbA1c peptide derivatives with chemical modifications or prolonged peptide sequences to avoid N- or C-terminal antibody peptide binding.

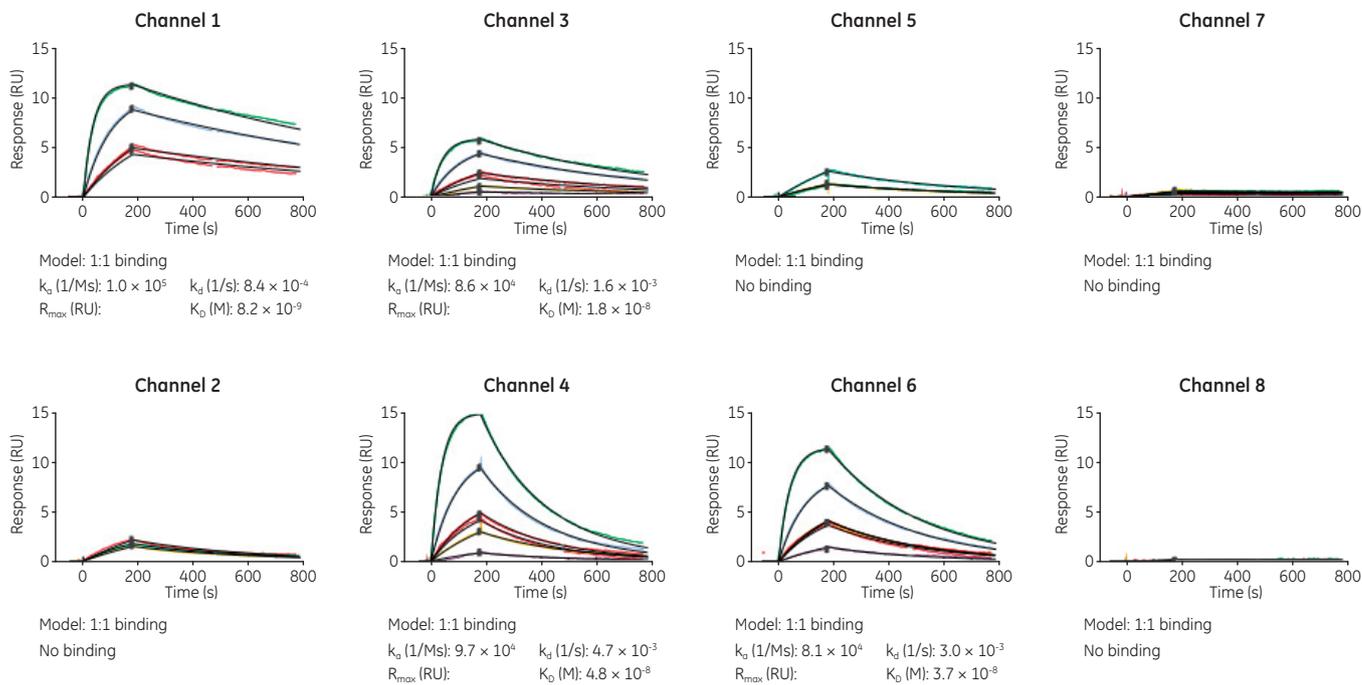
Analytes A01, A03, A04, A05, and A06 were injected in a concentration series as previously described. Analytes A02 and A07 were injected at a higher concentration series from 0, 10, 30, 90 nM (in duplicate), and 270 nM to 810 nM to better identify unwanted cross-reactive binding.



**Fig 2.** Experimental assay setup for the specificity test of mAb A against seven human HbA1c peptide analytes, A01 to A07. GARbFcy was immobilized in flow cells 1 and 2 and in all eight channels. Rabbit mAb A was captured on GARbFcy in flow cell 2. Human HbA1c peptide analyte was injected in both cells.



**Fig 3.** Multicycle kinetic analysis of six rabbit mAbs binding to glycosylated hemoglobin HbA1c.



**Fig 4.** Multicycle kinetics of HbA1c peptide derivatives binding to rabbit mAb clone A. The positive controls (channels 1, 3, 4, and 6) display concentration-dependent 1:1 kinetics. No binding was seen for the negative controls (channels 2, 5, 7, and 8). Kinetic profiles of the peptide derivatives are similar while affinities vary between micromolar and low nanomolar concentrations.

## Results

The results of concentration-dependent multicycle kinetics with glycosylated HbA1c peptide as analyte in solution are shown in Figure 3. Varying kinetic profiles were obtained for antibodies captured in channels 1 to 6. Rabbit mAb A in channel 1 bound relatively tightly to HbA1c while all other antibodies displayed faster dissociations. Running buffer was used as negative control in channels 7 and 8. The relatively slow antigen dissociation of mAb A led to the selection of this clone for further specificity testing. mAb A was subsequently captured at constant RU levels in flow cell 2 in all 8 channels.

The results of the specificity test using multicycle kinetics analysis with HbA1c peptide derivatives plus a buffer control are shown in Figure 4. The sensorgrams of the target HbA1c (channel 1), positive controls (channels 3, 4, and 6), and negative controls (channels 2, 5, 7, and 8) are shown in the figure. mAb A bound to all glycosylated HbA1c derivatives with concentration-dependent 1:1 kinetics while neither the nonglycosylated derivatives nor the cross-reactive HbA1A2 derivative displayed binding. The specificity test displayed more uniform kinetic profiles than were observed in the kinetic analysis (Fig 3) providing a larger variation in binding strength with affinities ranging from the micromolar to low nanomolar range.

Biacore 8K Control and Evaluation Software allowed a clear overview of the resulting sensorgrams.  $R_{max}$  levels between 5 and 15 RU underline the high sensitivity of the instrument.

## Conclusions

The results showed a large variety in binding profiles of the glycosylated human HbA1c with different rabbit mAbs displaying affinities in the nanomolar range. The antibody with the slowest dissociation, mAb A, was found to be of interest for further testing in subsequent detailed investigations in HbA1c immunoassays. The sensitivity of the system enabled confident measurement of multicycle kinetics with small modified peptides at low  $R_{max}$  levels. Run times were significantly reduced saving days compared with single-needle systems.

The architecture of Biacore 8K allowed rapid generation of scientifically significant data in a typical antibody development setting, as it is usually performed in daily research and development work. Biacore 8K Control and Evaluation Software, which supports the user in programming, evaluation, and reporting provided a clear overview of the resulting sensorgrams. With the four microplate capacity and the new queueing functionality, larger screening efforts were easily managed. Biacore 8K is an enabling tool for all kinds of screening and functional SPR assays, where sensitivity, robustness, and throughput are essential. The system will greatly support typical pharmaceutical specificity, screening, and characterization assays.

## Acknowledgement

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