Immunoglobulin Capture, Quantitation and Affinity Analysis

Summary

SKi Pro, a label-free biomolecular interaction analysis platform, is used to capture and quantify an immunoglobulin and to characterize kinetics of an antibody-antigen interaction. Using a standard 96-well microtiter plate and the 8-well SKi Sensor strips, the kinetics of antibody-antigen interaction can be characterized in a higher troughput mode compared to flow cell-based measurements.

An anti-mouse IgG antibody is immobilized on 8-well strips of SKi Sensor carboxyl biochips and various concentrations of IgG are then allowed to interact with this surface. The amount bound is monitored in real time by measuring the change in optical path difference (OPD). Resulting traces are analyzed by SKi Report software. Maximum binding ΔOPD values are used to quantify immunoglobulin content and to measure low limit of detection (LLOD). A global kinetics fit is performed to determine k_{on}, k_{off} and K_D.

Introduction

As antibodies increase in importance as both analytical reagents and therapeutics, there is growing need for research and diagnostic tools with ever increasing ease of use, sensitivity and economy. To meet that need the new dual mode SKi Pro system is presented here (Figure 1). SKi Pro platform uses NanoPore Optical Interferometry technology (NPOI) to monitor biomolecular interactions without the use of protein labels. SKi Pro X1000 instrument is designed for both precise kinetic measurements in flow cell mode and higher throughput screening applications in plate reader mode. Both modes allow for fully automated walk-away operation. Here the instrument has been used in a plate reader configuration to capture IgG from individual wells and to measure affinity of an antibody-antigen interaction.

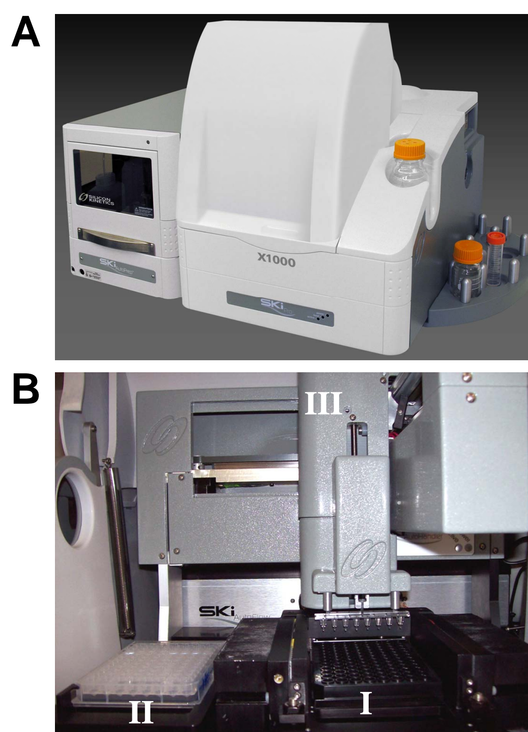


Figure 1: **A**. The SKi Pro X1000 dual mode flow cell / plate reader system used in this work. **B**. A standard 96-well microtiter plate (**I**) is placed into the instrument optical unit. A second utility plate (**II**) is used to store 8-well SKi Sensor strips and for off-line incubations. A robotic arm (**III**) picks and moves sensor strips according to the programmed method. The instrument monitors biomolecule interaction in real time.

Method

Polyclonal affinity purified anti-mouse IgG Fc-specific antibody and total mouse IgG were purchased from Thermo Scientific, Rockford, IL. Carboxyl coupling

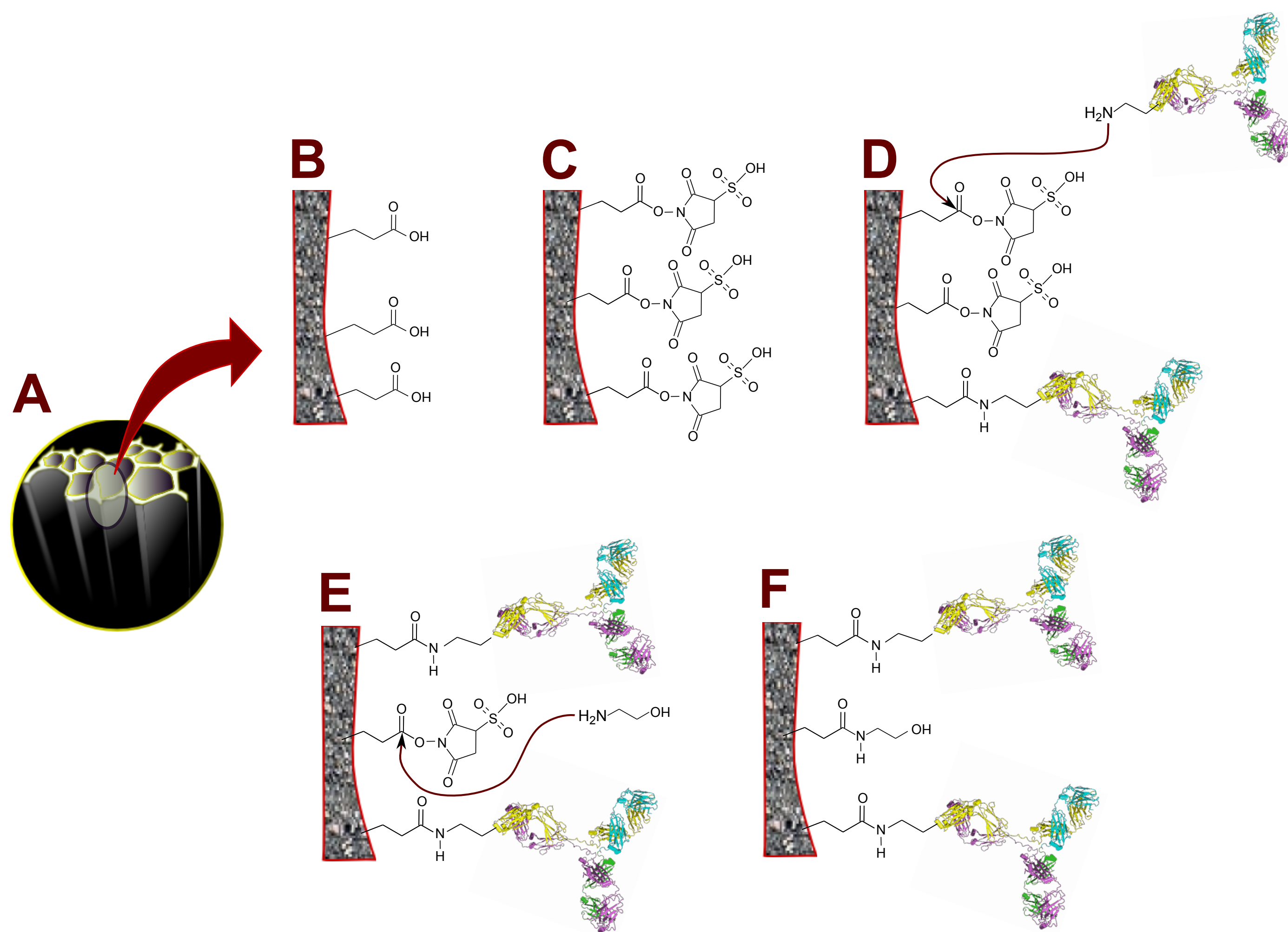


Figure 2: Biomolecules are bound to the insides of nano-pores (**A**) through their exposed carboxyl groups (**B**). These carboxyl units are actived using 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) in the presence of sulfo N-hydroxy succinimide (sulfo-NHS) to form succinimide esters throughout the pores (**C**). Primary amines on proteins from e.g. lysines, will react with the succinimide esters to form stable peptide bonds (**D**) covalently linking the protein to the surface. Unreacted succinimide esters which are blocked by reacting with ethanolamine (**E**). The resulting surface is ready for monitoring specific binding events (**F**).

reagents and sensor strips are from Silicon Kinetics. All other reagents were purchased from Sigma-Aldrich, St. Louis, MO.

The carboxyl groups on the insides of the pores (Figure 2) are modified as sulfo-succinimide esters using EDC chemistry during an initial activation step (**C**). 60 μL of activation mix (100 mM EDC, 25 mM sulfo-NHS solution in water) is dispensed into each well. The chips are then activated for 7 minutes in this solution and washed twice. Next, strips are transferred into wells containing 60 μL of antibody in immobilization buffer (20 mM acetate pH 5.0) at 50-150 μg/mL. The immobilization then proceeds for 30 minutes (**D**). Following immobilization, blocking buffer (100 mM ethanolamine-HCl, pH 8.5) is applied for 5 minutes to deactivate any unreacted succinimide esters in preparation for specific target binding (**E**). The resulting surface is ready for affinity monitoring (**F**). The strips are then transferred into wells containing a concentration series of the antigen in binding buffer (100 mM PO_4^{3-} pH 7.2, 150 mM NaCl, 0.05% Bovine serum albumin) where binding is monitored. Following antigen binding, the strips are transferred into wells with buffer only where dissociation is monitored. In order to strip bound antigen, the strips are incubated in regeneration buffer (20 mM Glycine-HCl, pH 2.0) for 5 minutes, washed briefly in water and equilibrated in binding buffer for 3 minutes.

Results

The data presented below are real-time binding curves that SKi Report software automatically generates and corrects for reference (no receptor immobilized on a chip) and/or blank (zero analyte concentration).

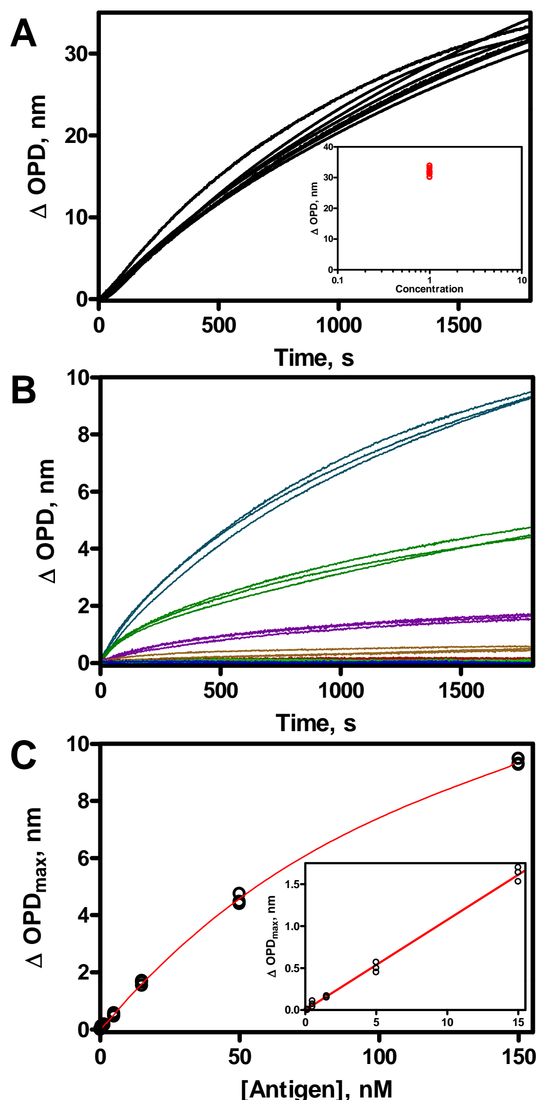


Figure 3: Antibody immobilization and antigen binding results. **A**. Anti-IgG is immobilized for 30 minutes at 1.3 μM. **B**. IgG at 0.15–150 nM is binding to the anti-IgG surface. Results of three binding-regeneration cycles are shown. **C**. Calibration curve was generated based on maximum ΔOPD values. Low concentration range is shown in the insert.

The immobilization curves of anti-IgG antibody in Figure 3 **A** show covalent binding of the receptor protein to the pore surface. Between 30–35 nm of OPD shifts are seen for the immobilization which is a value consistent with about 18% of a monolayer.[1] Experiments have shown that this amount of antibody immobilization is high enough to detect and quantify antigen with maximum sensitivity and in a wide concentration range.

For binding experiments half-log serial dilutions of the antigen at 0.15-150 nM concentrations were prepared in microplate wells. The binding-regeneration cycle was conducted 3 times in a single automated run (Figure 3 **B**). The recorded OPD changes show robust binding that comes close to saturation at the highest concentration. Maximum ΔOPD values are used to generate a calibration curve (Figure 3 **C**). As expected, the calibration curve is close to linear only at lower concentrations when the binding surface is far enough from saturation.

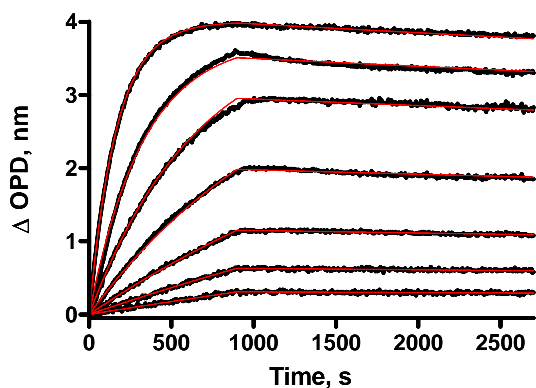
In order to estimate LLOD of antigen detection, all data points for the last 10 seconds of each run were used to calculate mean maximum signal values and their standard deviations (Table 1). The results show that measured signals are significant and reproducible at concentrations 0.5 nM (75 ng/ml) and higher.

Table 1: Statistical analysis of maximum ΔOPD values for all runs shown in Figure 3**B**. Data points for the last 10 seconds of each run were used to calculate mean and standard deviation values.

[Antigen]	Run 1		Run 2		Run 3	
nM	Avg	Std	Avg	Std	Avg	Std
150	9.47	0.01	9.26	0.01	9.30	0.01
50	4.74	0.01	4.47	0.01	4.39	0.01
15	1.64	0.01	1.53	0.02	1.70	0.01
5	0.50	0.01	0.45	0.02	0.57	0.01
1.5	0.17	0.01	0.15	0.01	0.15	0.01
0.5	0.11	0.01	0.04	0.01	0.07	0.01
0.15	0.01	0.01	0.00	0.01	-0.01	0.01

Kinetic characterization of antibody-antigen interaction was carried out in a separate experiment (Figure 4). Antibody was immobilized at a lower concentration and for a shorter time (not shown). That yielded a strip with 4.5–5.5 nm of immobilized antibody. Kinetic measurements are expected to be more accurate at lower immobilization levels when mass transfer limitation effect is minimized. Antigen binding was monitored at two-fold serial IgG dilutions in 7.8–500 nM range. Both association and dissociation phases were recorded.

[1] Based on calculation using the expected characteristic diameter and refractive index of anti-IgG together with the SEM measured pore size of the chips.



$k_{on} = 1.20 \pm 0.03\ 10^{4}\ M^{-1}\ s^{-1}$

$k_{off} = 3.08 \pm 0.04\ 10^{-5}\ s^{-}$

$K_D = 2.57 \pm 0.04\ 10^{-9}\ M$

Figure 4: Kinetic characterization of anti-IgG / IgG binding. Strip with 4.5–5.5 nm of anti-mouse IgG antibody was incubated with 2-fold serial dilutions of total mouse IgG at 7.8–500 nM. Binding for 15 minutes was followed by 30 minutes dissociation in wells with buffer only. Kinetic parameters were calculated based on a global fit with a single binding site (two-state) model.

The data was analyzed with SKi Report software. The binding-dissociation traces were globally fit using a single binding site (two-state) model and a non-linear least squares algorithm.

Discussion

The current work aims to characterize an anti-IgG antibody and to establish LLOD for IgG detection by a NPOI-based SKi Pro instrument in a plate reader configuration. IgG capture in a multi-well mode reflects a common experimental design when hybridoma clones are screened for antibody production and antigen binding.

LLOD is defined primarily by the instrument sensitivity and background noise. It also depends on the affinity of biomolecular interaction and on sizes of binding partners on the surface and in solution. The measured signals in experiments presented here are well above noise levels for IgG concentrations 0.5 nM (75 ng/ml) and higher. This is the industry-best demonstrated sensitivity of IgG detection with label-free technology in a multi-well setup. Importantly, this LLOD is sufficient for antibody capture and analysis for even low producing hybridoma clones. Results of 3 consecutive runs show good reproducibility. They also demonstrate that the binding surface with covalently immobilized capture antibody is stable and can be regenerated several times without significant loss of binding capacity.

These results can be used to quantify IgG in samples with unknown antibody content. ΔOPD_{max}-based calibration curve is linear in a low concentration range but shows some surface saturation at higher concentrations. More accurate quantitation can be achieved by calculating observed association rates (k_{obs}).

Antibody immobilization shown on Figure 3**A** have been done at a high protein concentration for a long time. That resulted in a large binding surface desired for efficient antigen capture in a wide concentration range. Kinetic characterization of the antibody was conducted in a separate experiment with 6–7 times lower immobilization levels and, therefore, much less interference of mass transfer limitation effect. Obtained association-dissociation curves allowed global kinetic fit with low uncertainty levels. The resulting kinetic parameters k_{on}, k_{off} and K_D are typical for a good analytical antibody. The measured association rate can still be limited by passive diffusion of analyte in microplate wells. However, these results are sufficient for antibody ranking in screening applications and for qualification of a capture antibody. Interestingly, binding curves fit well with a two-state model even though we used a potentially heterogeneous polyclonal antibody. That attests to a high quality of the antibody likely achieved by affinity purification.

Silicon Kinetics, Inc.
10455 Pacific Center Ct
San Diego, CA 92121-4339
www.siliconkinetics.com
+1 [858] 646-5444 (tel)
+1 [858] 646-5401 (fax)

Silicon Kinetics ©2010. SKi Pro™, SKi Sensor™& SKi Report™are registered trademarks of Silicon Kinetics.