

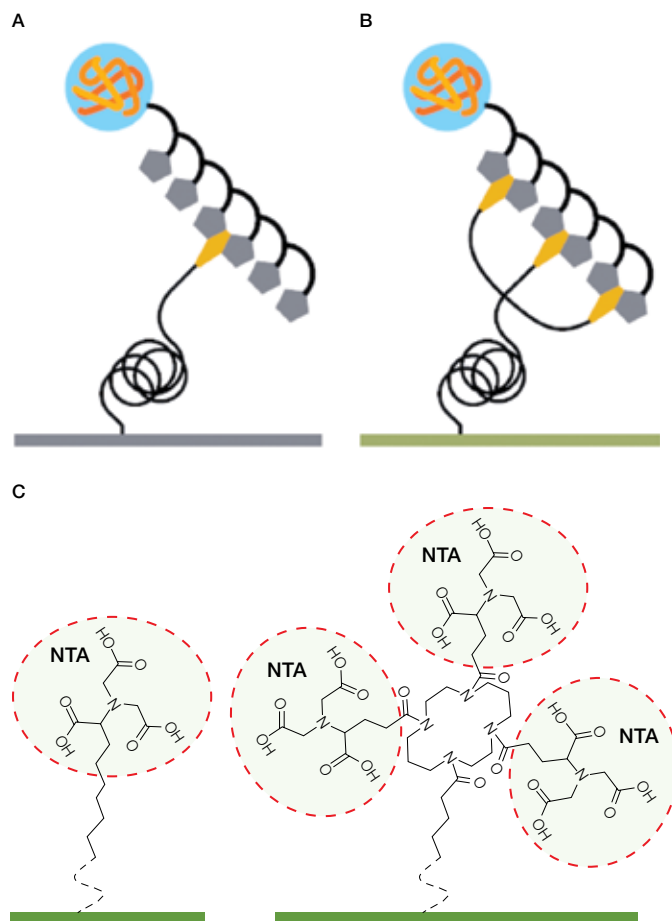
## The ProteOn™ HTG Sensor Chip: Novel Surface for Stable Capture of Histidine-Tagged Proteins for Protein-Protein Interaction Analysis

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### Introduction

In the past two decades, the use of recombinant proteins for research, drug discovery, and therapeutic applications has increased dramatically. A critical step in the production of recombinant proteins is their isolation from cell culture supernatants or cell lysates. A common strategy for this purification step includes the use of tagged proteins. The most widely used tag, the histidine tag, is a sequence of six or ten histidine residues fused at the amino or carboxyl protein terminus. This tag exhibits affinity to divalent metal ions, such as nickel (II). Based on this affinity, histidine-tagged proteins can be purified by immobilized metal affinity chromatography (IMAC) using active nickel (II)-nitrilotriacetate (Ni-NTA) complexes that are prepared from nickel (II) activation of nitrilotriacetic acid (NTA).

This property of histidine-tagged proteins can also be utilized for protein immobilization on the surface of sensor chips used with surface plasmon resonance (SPR) biosensors. A histidine-tagged protein is captured on a Ni-NTA coated chip surface as a ligand for subsequent interaction analysis with an analyte. This method offers selective, oriented, and reversible binding of proteins to the chip surface. However, in many cases, the affinity of the histidine-tagged proteins to the Ni-NTA surface is not strong enough to achieve stable ligand immobilization. Thus, ligand release from the surface is frequently observed, causing an unstable baseline in SPR experiments. The kinetic analysis of the ligand-analyte interaction is consequently inaccurate or even impossible to obtain in some cases (Figure 1A).



**Fig. 1. Tris-NTA technology: comparison of mono-NTA and tris-NTA surfaces.** The mono-NTA or tris-NTA groups are activated by nickel (II) to form Ni-NTA or 3 x Ni-NTA complexes, respectively. These complexes are able to capture polyhistidine-tagged proteins. **A**, the mono-NTA surface is the traditional approach for capturing histidine-tagged proteins but achieves weak binding and results in ligand decay, baseline drift, distorted kinetic results, and inaccurate fitting to kinetic models; **B**, the ProteOn trivalent tris-NTA (3 x NTA) surface improves binding stability and increases binding selectivity to histidine-tagged proteins. Ni-NTA (●) binding to histidine (●) occurs after activation with nickel(II); **C**, structure of surface bound mono-NTA molecule (left) and tris-NTA molecule (right). Each individual NTA group is circled.

The ProteOn HTG chip is a new SPR sensor chip for use with the Bio-Rad ProteOn XPR36 protein interaction array system. An innovative technology is used to enable strong and selective capture of histidine-tagged proteins allowing stable baseline and accurate kinetic analysis. The HTG chip is coated with tris-NTA (3 x NTA) groups that can be easily activated to form a complex of three Ni-NTA moieties (called tris-Ni-NTA or 3 x Ni-NTA, Lata et al. 2006a, Lata and Piehler 2005) held together in an optimal orientation for binding of histidine-tagged proteins. Since each Ni-NTA moiety interacts with two histidine residues, the 3 x Ni-NTA complex is ideally suited to bind six histidine residues (Figures 1B and C). The significantly higher affinity of 3 x Ni-NTA over Ni-NTA for histidine-tagged proteins has been demonstrated (Andre et al. 2009, Lata et al. 2006b, Reichel et al. 2007).

In this report, we use different models to show the high stability of surface-bound ligand proteins, surface regeneration for chip reuse, and the ability to capture non-purified histidine-tagged proteins. The kinetics of three protein-protein interactions were analyzed and high quality results were obtained showing the potential applications of the HTG chip, including high-throughput screening for mutagenesis research and online purification of crude samples.

## Methods

### Instrument and Reagents

Experiments were performed using the ProteOn XPR36 system with HTG sensor chips. ProteOn GLC sensor chips were used for comparison. Unless otherwise stated, the running buffer was ProteOn PBS/Tween (phosphate buffered saline, pH 7.4 with 0.005% Tween 20). Histidine-tagged protein A and protein A/G were from BioVision, histidine-tagged ubiquitin was from R&D Systems, and human IgG1 was from Sigma-Aldrich. Histidine-tagged antizyme (Az), ornithine decarboxylase (ODC), interferon receptor 1 (IFNAR1), interferon  $\alpha 2$  (IFN $\alpha 2$ ), and interferon  $\alpha 2$  mutant YNS proteins were kindly provided by Professor Gideon Schreiber of the Weizmann Institute of Science in Rehovot Israel.

### Ligand Capture on HTG Chip—General Protocol

All ligand proteins were diluted in running buffer. All ligands were captured on HTG chips with the microfluidic multichannel module (MCM) of the ProteOn system oriented vertically. Ligand capture requires three steps: conditioning, activation, and ligand injection. The chip conditioning protocol provides rapid stabilization of the baseline and verifies that the tris-NTA surface is free of metals. Chip conditioning was performed on all six channels according to the recommended HTG protocol, which includes horizontal and vertical injections of 0.5% SDS, 50 mM NaOH, 100 mM HCl, and 300 mM EDTA pH 8.5 at a flow rate of 30  $\mu$ l/min. Immediately after EDTA injection, the surface was activated with 10 mM NiSO<sub>4</sub> for 2 min prior to ligand injection. The conditioning and activation protocol of the HTG chip is used in all other experiments in this report.

### Ligand Capture Stability and Regeneration Assays

Three histidine-tagged proteins (protein A, protein A/G, and ubiquitin) were captured on the HTG sensor chip and sensor chip NTA (for use with GE's Biacore 3000). Protein concentrations were in the range of 10 to 100  $\mu$ g/ml, and were adjusted so that similar binding levels would be reached for each protein on the two chips.

For the regeneration assay, 150  $\mu$ l protein A/G (25  $\mu$ g/ml) was injected in an activated channel. Chip regeneration was performed by injection of 400  $\mu$ l EDTA 300 mM pH 8.5 at a flow rate of 30  $\mu$ l/min. Nine consecutive cycles of regeneration, activation, and ligand capture were performed on all six channels in a vertical MCM orientation.

### Capture of Histidine-Tagged IFNAR1 and Binding of Interferon $\alpha 2$ /YNS

A volume of 300  $\mu$ l histidine-tagged IFNAR1 (300 nM) was injected on an activated HTG chip at a flow rate of 30  $\mu$ l/min. An additional channel was activated with NiSO<sub>4</sub> for referencing. For analyte injection, six concentrations of wild type or mutants of IFN were injected in a horizontal orientation (volume of 120  $\mu$ l at a flow rate of 60  $\mu$ l/min). For IFN $\alpha 2$ , a series of concentrations from 8000 to 81 nM in fourfold dilutions were used and for IFN-YNS, a series of concentrations from 300 to 9.3 nM in twofold dilutions were used. No regeneration was required between the two analyte injections since the analyte dissociation was fast.

### Capture of Histidine-Tagged Protein A and Binding of IgG Antibody

In order to compare the kinetics obtained on a 3 x Ni-NTA capture surface and a regular amine-coupling surface, the experiment was implemented on both an HTG chip and a GLC chip. For capture of the ligand to an HTG chip, 100  $\mu$ l of histidine-tagged protein A (2  $\mu$ g/ml) was injected on an activated HTG chip at a flow rate of 30  $\mu$ l/min. For amine coupling of the ligand to a GLC chip, 30  $\mu$ l of 10 mM EDC and 2.5 mM sulfo-NHS solution was injected to activate the chip surface, followed by 100  $\mu$ l of protein A (10  $\mu$ g/ml in 10 mM Na-acetate buffer pH 4.5) and deactivation with 120  $\mu$ l of 1 M ethanolamine pH 8.5, at a flow rate of 30  $\mu$ l/min. The analyte injection was done similarly on both chips: after MCM rotation to a horizontal orientation, 75  $\mu$ l of human IgG was injected in five channels at concentrations ranging from 100 nM to 6.25 nM in twofold dilutions at a flow rate of 50  $\mu$ l/min. In the sixth channel, running buffer was injected to enable double referencing.

Regeneration of only the human IgG analyte, without removing the histidine-tagged protein A ligand, was accomplished by injecting 100  $\mu$ l of 50 mM NaOH at a flow rate of 100  $\mu$ l/min in a horizontal orientation. Then a second analyte injection was performed, followed by an additional analyte regeneration injection. Overall, three cycles of analyte regeneration and rebinding were performed on the same ligand surface.

### Capture of Histidine-Tagged Antizyme and Binding of Ornithine Decarboxylase

For ligand capture, 150  $\mu$ l of purified histidine-tagged Az or crude *E. coli* extracts were injected in three different channels at a flow rate of 30  $\mu$ l/min. Purified Az (19  $\mu$ g/ml) was injected in the first channel. Crude *E. coli* extracts, diluted 250-fold in PBST with or without 15 mM imidazole, were injected in the two other channels. For analyte injection, running buffer was set to HBS with 1 mM DTT and 0.01% Tween. A volume of 300  $\mu$ l ODC was injected at a flow rate of 70  $\mu$ l/min in a horizontal MCM orientation using a five concentration series from 100 nM to 1.23 nM in threefold dilutions (in running buffer). One channel was left with running buffer for double referencing.

### Sensorgram Acquisition and Data Analysis

For each of the kinetic models studied, a single injection of five or six analyte concentrations over the captured ligand surface provided the full kinetic profile without the need for regeneration between different concentrations. Channel reference was achieved by subtracting the signal from an empty ligand channel or interspots, which corrected analyte

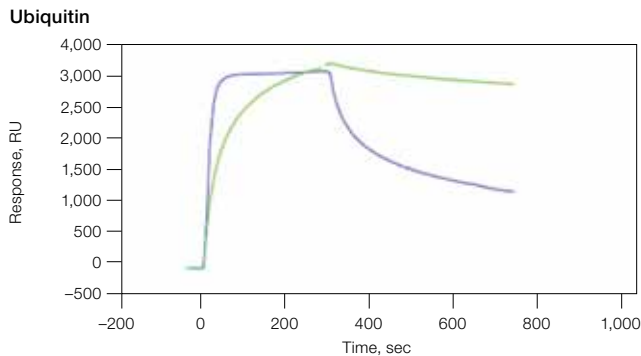
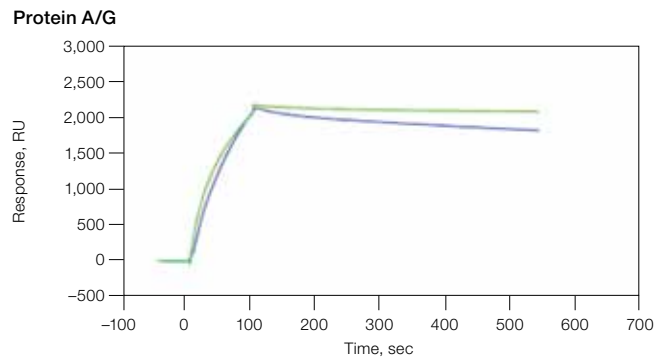
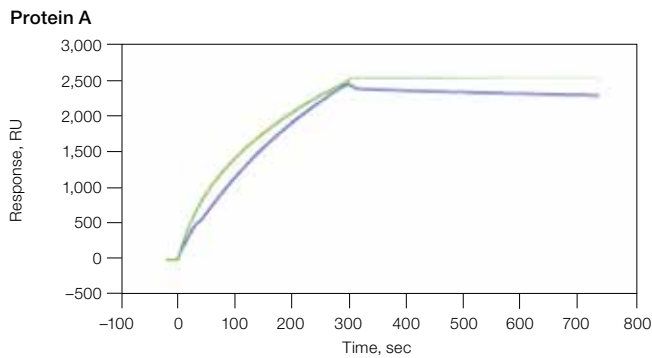
bulk effect and nonspecific binding. In most cases, a blank sample (running buffer with no analyte) was injected in the sixth channel, which allowed for real-time double referencing. Any slow dissociation of the captured ligand could be measured at the same time as the interaction itself for accurate and reliable double referencing without the need to perform a separate blank injection.

Data were analyzed with ProteOn Manager™ software version 3.1. Each set of five or six reference-subtracted and aligned sensorgrams was fitted to a Langmuir 1:1 homogenous bimolecular reaction model. Global kinetic rate constants –  $k_a$  and  $k_d$  were derived for each reaction.  $K_D$  was calculated from the  $k_d/k_a$  ratio or by equilibrium analysis.  $R_{max}$  value, the maximal analyte signal at ligand saturation, was also globally fitted from the sensorgrams.

## Results and Discussion

### High Stability of Histidine-Tagged Protein Captured on the HTG Chip

The use of tris-NTA instead of traditional NTA (mono-NTA) enables high ligand stability, a requisite for analysis of interaction kinetics. Ligand drift can lead to distorted kinetic results or make the fitting process to a kinetic model inaccurate or even impossible. To demonstrate this, we examined the binding stability of three histidine-tagged proteins, protein A, protein A/G, and ubiquitin, to Bio-Rad's HTG chip and to GE's NTA chip (a mono-NTA coated chip). Both chips were similarly washed with EDTA and activated with NiSO<sub>4</sub> prior to ligand capture and the ligands were captured to similar final densities. Figure 2 shows the stability of the captured histidine-tagged proteins in a 7 min long dissociation after being captured on both the HTG chip and the NTA chip. The remaining quantity of histidine-tagged proteins was measured at 5 min of the dissociation for comparison. The results indicate that proteins have different capturing stability on the two chips. Ubiquitin, the protein that showed the lowest capturing stability, was much more stable on the HTG chip, with 92% protein left compared to only 45% on the NTA chip. Protein A and protein A/G also exhibited better stability on the HTG chip compared to the NTA chip. These results show that, as anticipated from the chemical structure, the 3 x Ni-NTA complexes (activated tris-NTA groups) on the HTG chip surface capture histidine-tagged proteins more strongly than the traditional Ni-NTA complexes (activated mono-NTA groups), leading to stable binding of the proteins to the chip surface. Also, on the same chip, the binding stability varied between different proteins, which is possibly related to the binding energetics of each protein.



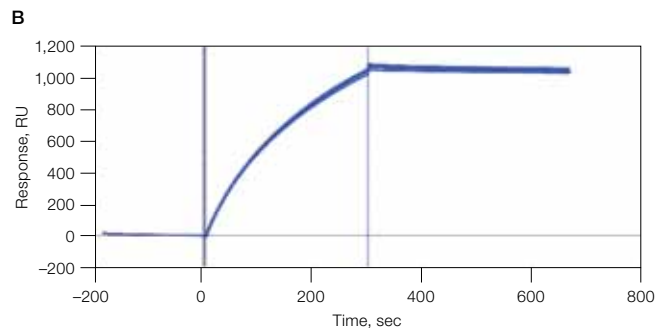
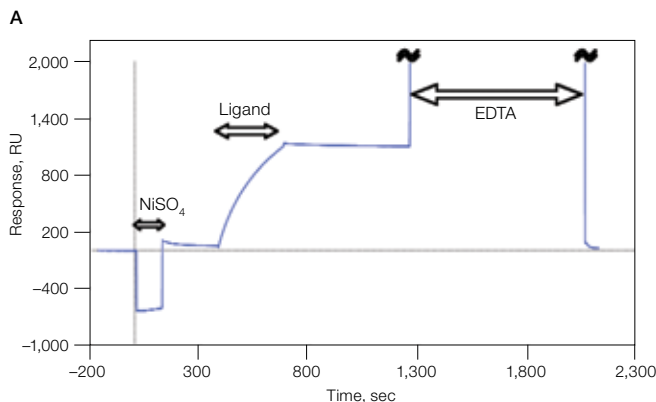
	HTG Chip	NTA Chip
Protein A	100%	97%
Protein A/G	96%	88%
Ubiquitin	92%	45%

**Fig. 2. Capture stability comparison of different histidine-tagged proteins to HTG (—) vs. NTA (—) chips.** The proteins were captured to similar binding levels on both chips. The table shows the percentage of remaining bound protein at 5 min after the start of dissociation.

### Reproducible Surface Regeneration and Ligand Capture of the HTG Chip

The ability to regenerate the tris-NTA surface is another important feature of the HTG chip, allowing reutilization of the chip surface for multiple ligand capturing cycles. In the regeneration, EDTA is used to chelate the nickel (II) ions and break the binding between the histidine-tagged protein and the 3 x Ni-NTA complexes (activated tris-NTA groups). By this means the ligand can be completely removed from the tris-NTA surface. Nine consecutive cycles of ligand capture

and regeneration were applied to the chip as described in the Methods section. Figure 3A shows a regeneration cycle and demonstrates full regeneration of the chip surface and complete ligand removal after EDTA injection. Figure 3B shows an overlay of the nine ligand capture steps which exhibit high uniformity between all cycles with densities ranging from 1030 to 1070 RU. These results demonstrate the high regenerability of the HTG tris-NTA surface, allowing repetitive experiments to be performed without a reduction of ligand density between cycles.



**Fig. 3. HTG chip regeneration performance. A,** each capturing and regeneration cycle included activation with  $\text{NiSO}_4$ , histidine-tagged Protein A/G ligand capture, and EDTA injection. The EDTA step demonstrates full regeneration of the chip surface. For simplification only one interaction spot from one ligand channel is presented. Similar results were obtained for all other ligand channels; **B,** overlay of nine consecutive Protein A/G ligand capturing steps on the same interaction spot.

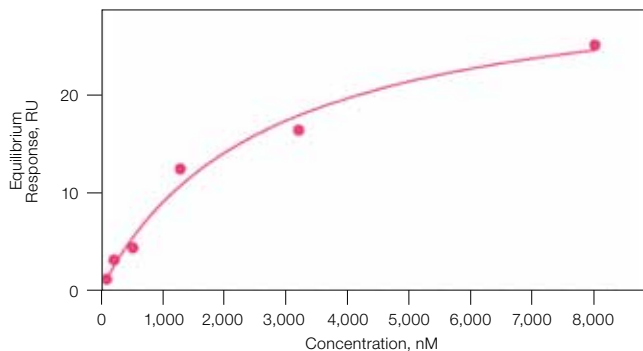
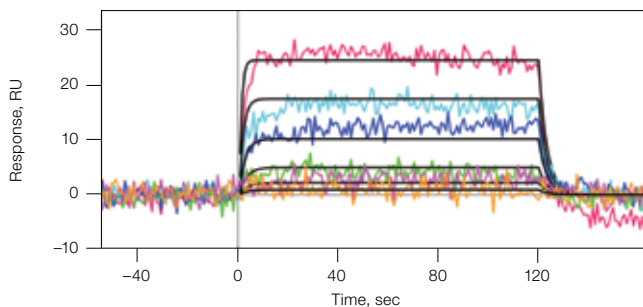
### Protein-Protein Interaction with a Histidine-Tagged Ligand

This experiment analyzed the interaction between IFNAR1 and IFN $\alpha$ 2 or its H57Y-E58N-Q61S mutant (YNS). Type I interferons (IFNs) orchestrate many immunological and cellular processes and are used as therapeutic agents for viral infections and some malignancies. All IFNs share two common IFN receptors (IFNAR1 and IFNAR2) and carry out their biological activity upon receptor binding. The IFN $\alpha$ 2 YNS mutant was designed to enhance the binding of IFN $\alpha$ 2 to its IFNAR1 receptor (Kalie et al. 2007).

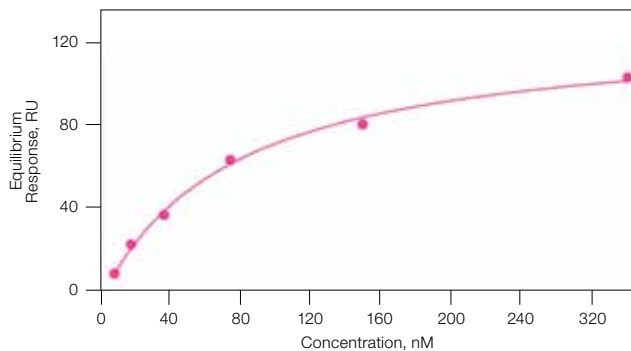
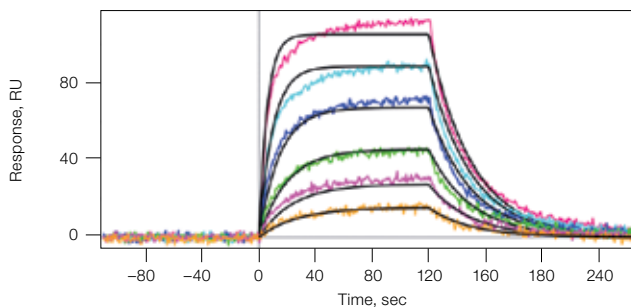
The binding kinetics of histidine-tagged IFNAR1 to wild-type IFN $\alpha$ 2 and its YNS mutant were examined by capturing the histidine-tagged IFNAR1 on the HTG surface, and using

One-shot Kinetics™ analyte injection (Figure 4 and Table 1). Conditioning and activation followed by histidine-tagged IFNAR1 capturing yielded a ligand density of 575 RU with a baseline drift of ~1 RU/min. This stable ligand capture allowed two analyte injections without the need for ligand recapture (Figure 4). The data obtained for both analytes fit well with a 1:1 binding model, and the  $K_D$  values calculated from kinetic and equilibrium analyses were consistent (Table 1). The affinity of the YNS mutant to IFNAR1 is 40-fold stronger than the affinity of the wild-type IFN $\alpha$ 2, which is in agreement with previous results (Kalie et al. 2007). As can be seen from the kinetic analysis, the higher affinity of the mutant is mainly a result of a slower dissociation rate compared to the wild-type IFN $\alpha$ 2 binding.

#### IFN $\alpha$ 2 WT



#### IFN $\alpha$ 2 YNS mutant



**Fig. 4. Detailed kinetic and equilibrium analysis of IFN $\alpha$ 2 wild type and YNS mutant to histidine-tagged IFNAR1.** IFNAR1 was captured on HTG chip to 575 RU density. Capture was followed by One-shot Kinetics injections of IFN $\alpha$ 2 wild type and YNS mutant. The top panels show the sensorgrams for the six analyte concentrations with the overlaid 1:1 model fit in black. The bottom panels show the plot of equilibrium response versus analyte concentration.

**Table 1. Kinetic and equilibrium constants derived from the plots in Figure 4.**

Analyte	Analysis	$k_a$ , 1/Ms	$k_d$ , 1/s	$K_D$ , nM	$R_{max}$ , RU	Chi2, RU
IFN $\alpha$ 2 WT	Kinetic	$1.0 \times 10^5$	0.30	$2.9 \times 10^3$	34	4.8
	Equilibrium			$2.7 \times 10^3$	33	1.9
IFN $\alpha$ 2 YNS mutant	Kinetic	$4.8 \times 10^5$	$3.3 \times 10^{-2}$	69	129	7.7
	Equilibrium			73	137	6.6

### Histidine-Tag Capturing Provides Higher Ligand Activity than Amine Coupling

This experiment analyzed the interaction between protein A and human IgG. Protein A is a member of the immunoglobulin binding protein group isolated from a bacterial wall. Protein A binds to the immunoglobulin Fc region and is commonly used as an antibody capturing agent for antibody screening and purification.

Kinetic analysis of human IgG binding to captured histidine-tagged protein A allowed comparison of the kinetic constants and ligand activity to those of amine-coupled protein A on a ProteOn GLC sensor chip. Histidine-tagged protein A was captured on an HTG chip to a final density of 58 RU; this was followed by the injection of human IgG. The data obtained were consistent with a 1:1 binding model with subnanomolar affinity (Figure 5 and Table 2). Comparing the captured histidine-tagged protein A and the amine-coupled protein A reveals similar kinetic constants for the two binding methods, yet the histidine-tagged protein A ligand shows almost twofold higher activity compared with the amine-coupled protein A ligand. The higher activity of captured ligand molecules results from a higher proportion of appropriately oriented captured histidine-tagged proteins compared to the randomly bound amine-coupled protein molecules. Higher ligand activity leads to higher analyte signals and thus higher assay sensitivity.

Table 2. Kinetic constants derived from the plots in Figure 5.

Chip	Ligand density, RU	$k_a$ , 1/Ms	$k_d$ , 1/s	$K_D$ , nM	$R_{max}$ , RU	Chi2, RU	Activity*, %
HTG chip	58	$2.1 \times 10^5$	$1.5 \times 10^{-4}$	0.74	358	22.7	32%
GLC (amine-coupled)	101	$2.3 \times 10^5$	$1.0 \times 10^{-4}$	0.45	355	3.3	18%

\* % Activity = Fitted  $R_{max}$  / Theoretical  $R_{max}$   
 Theoretical  $R_{max}$  = (Analyte MW / Ligand MW) x Ligand density x Binding stoichiometry.  
 The binding stoichiometry is 5 for recombinant Protein A.

### Regeneration of the Ligand Surface by Analyte Removal from Captured Histidine-Tagged Ligand

The high stability of ligand binding on the HTG chip allows, in many cases, researchers to use regeneration conditions that remove only the bound analyte, leaving the ligand free for another round of analysis. In this study, the histidine-tagged protein A/human IgG model was used to demonstrate this capability. After screening several regeneration conditions (not shown), it was found that using 50 mM NaOH can remove all of the analyte (human IgG) while the captured ligand (histidine-tagged protein A) is not affected and remains captured on the HTG surface in a very stable manner. An overlay of three consecutive analyte injections (Figure 6) shows complete superimposition of the sensorgrams, indicating that all of the analyte was completely removed from the ligand by the regeneration solution, and at the same time, the ligand remained fully active and was not removed from the surface. The ability to remove only the analyte makes the HTG chip highly reusable, allowing multiple analyte injections on the same surface even if the analytes are slowly dissociating from the ligand. Such analyte-only regeneration conditions are model specific, and optimization is needed as in amine-coupled ligand regeneration.

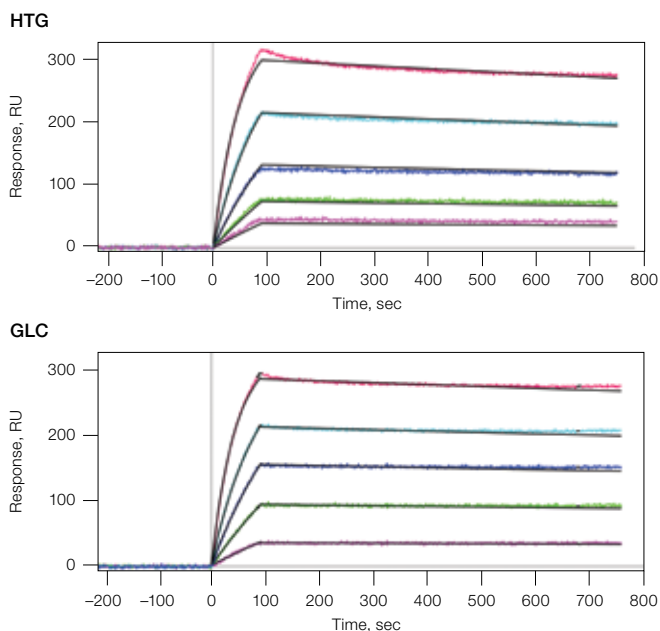


Fig. 5. One-shot Kinetic analysis of human IgG to captured and amine-coupled histidine-tagged protein A. The top panel shows results for HTG captured protein A, and the bottom panel for GLC immobilized protein A. The sensorgrams for the five analyte concentrations with the 1:1 fit overlaid in black are shown.

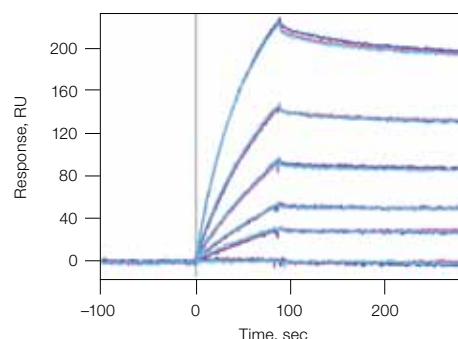


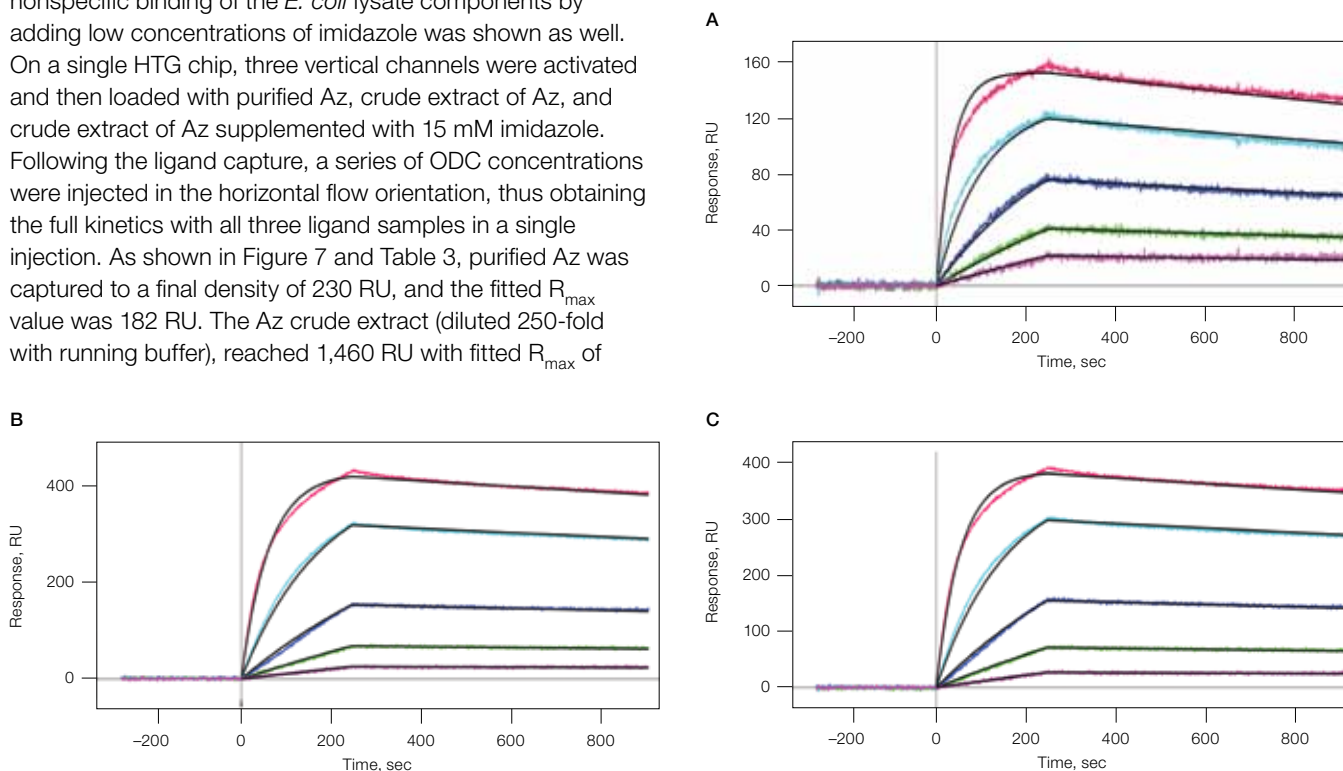
Fig. 6. Analyte-only regeneration of human IgG from captured histidine-tagged protein A. Overlay of three consecutive human IgG One-shot Kinetics injections on HTG captured protein A. Between each two analyte injections, ligand regeneration by 50 mM NaOH was done. The sensorgrams shown are of unreferenced data, and the sixth channel in each injection was of running buffer. First injection (—), Second injection (—), Third injection (—).

### Kinetic Analysis with Histidine-Tagged Protein Ligands Captured Directly from Crude Extracts

This experiment analyzed the interaction between antizyme (Az) and ornithine decarboxylase (ODC). ODC is a metabolic enzyme, that catalyzes the first step of polyamine synthesis in cells. ODC activity is regulated through inhibition by binding of regulators, such as the Az protein.

The capability to capture and carry out kinetic analysis of nonpurified histidine-tagged proteins was demonstrated in this experiment using the Az/ODC model. Control of nonspecific binding of the *E. coli* lysate components by adding low concentrations of imidazole was shown as well. On a single HTG chip, three vertical channels were activated and then loaded with purified Az, crude extract of Az, and crude extract of Az supplemented with 15 mM imidazole. Following the ligand capture, a series of ODC concentrations were injected in the horizontal flow orientation, thus obtaining the full kinetics with all three ligand samples in a single injection. As shown in Figure 7 and Table 3, purified Az was captured to a final density of 230 RU, and the fitted  $R_{max}$  value was 182 RU. The Az crude extract (diluted 250-fold with running buffer), reached 1,460 RU with fitted  $R_{max}$  of

471 RU. The addition of imidazole to a similar sample reduced the final density 1.5-fold to 935 RU, but the fitted  $R_{max}$  was reduced only 1.1-fold to 430 RU, implying that most of the ligand density reduction by imidazole came through removal of irrelevant proteins, not from removal of the Az ligand. For all three ligand samples, similar kinetic constants were obtained, indicating that the presence of the nonspecifically bound crude extract components did not affect the binding kinetics of the captured histidine-tagged Az on the HTG chip surface.



**Fig. 7. One-shot Kinetics analysis of ornithine decarboxylase (ODC) binding to purified and nonpurified captured histidine-tagged antizyme (Az).** **A**, ODC binding to purified Az; **B**, ODC binding to crude extract of Az; **C**, ODC binding to crude extract of Az supplemented with 15 mM imidazole in order to reduce nonspecific binding of irrelevant proteins. For all panels, the sensorgrams of the five analyte concentrations are shown with the 1:1 fit overlaid in black. The table lists the kinetic constants derived from the plots.

**Table 3. Kinetic constants derived from the plots in Figure 7.**

Ligand	Ligand density, RU	$k_a$ , 1/Ms	$k_d$ , 1/s	$K_D$ , nM	$R_{max}$ , RU	Chi2, RU
Purified Az	230	$2.7 \times 10^5$	$2.4 \times 10^{-4}$	0.86	178	10.6
Az crude extract without imidazole	1460	$1.6 \times 10^5$	$1.4 \times 10^{-4}$	0.88	471	18.4
Az crude extract with 15 mM imidazole	935	$1.8 \times 10^5$	$1.4 \times 10^{-4}$	0.76	430	19.4

## Conclusions

The ProteOn HTG chip provides an optimal surface for easy binding of histidine-tagged proteins. The very stable ligand capturing and the option to completely regenerate the ligand and/or the analyte make the HTG chip the ideal choice for reliable, accurate, and reproducible biomolecular interaction analysis with histidine-tagged proteins. The high quality of results comes from the outstanding binding stability and regenerability of the novel tris-NTA surface.

The option to capture histidine-tagged proteins directly from their crude production medium (without prepurification) is valuable for bio-drug development and discovery screening workflow. This capability significantly reduces the workup labor and cost in the sample preparation process. When combined with the One-shot Kinetics approach in the ProteOn XPR36 system, the HTG chip offers unprecedented throughput to save time and effort in biomolecular interaction research.

## Acknowledgements

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