

## Screening and Characterization of Human Immunoglobulin G Binding Peptides Using the ProteOn™ XPR36 Protein Interaction Array System

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### Introduction

Human immunoglobulin G (hIgG) is a major immunoglobulin in serum that plays a central role in immune response. There is great interest in the use of IgGs in basic biomedical research and for the diagnosis and treatment of various diseases. Immunoglobulins (Ig) are usually purified by Protein A affinity chromatography. However, a high level of purification, including the removal of all contamination by endotoxins and by leaching Protein A, is required for pharmaceutical use of Igs. The development of new purification procedures for antibodies that do not present these inconveniences is much needed. We investigated the use of antibody-binding peptides to build a new IgG purification system by screening a random peptide library constructed in the bacteriophage T7 display system (Yuji et al. 2008).

The ProteOn XPR36 protein interaction array system is a surface plasmon resonance (SPR) device that can measure 36 interactions between 6 ligands and 6 analytes in a single injection (Figure 1). This system is amenable to high-throughput screening since the measurement of 36 interactions can be completed within 30 minutes.

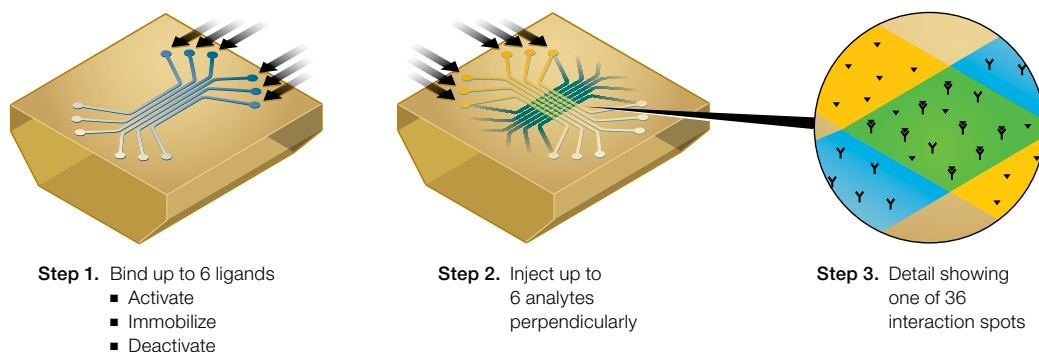
Here we report the screening of active phage clones obtained by phage panning from random peptide libraries constructed using a T7 phage display system. We subsequently analyzed the interaction of synthetic peptides with antibodies using the ProteOn XPR36 system (Sakamoto et al. 2009). We also describe a convenient method for the immobilization of human IgG on the ProteOn sensor chip.

### Methods

#### Experiment 1: Screening of human IgG antibody-binding T7 phage clones from a T7 phage peptide library

To identify peptides with high binding affinity to hIgG we constructed a random peptide library, cyclized via cys-cys disulfide bridges — using the T7Select 10-3 vector (Novagen), and isolated several hIgG-binding phages by biopanning the library. Based on the peptide sequence obtained from the initially isolated clones, secondary and tertiary libraries for affinity maturation were constructed, and the clones with the highest binding affinity were isolated. Instead of using ELISA, we employed the ProteOn XPR36 system to screen the phage-infected lysed bacteria.

ProteOn GLM sensor chips were activated by a combination of sulfo-NHS and EDAC in the vertical direction. Goat IgG, rabbit IgG, mouse IgG, and two hIgG1s were dissolved in 10 mM acetate buffer (pH 5.0) at a concentration of 5 µg/ml and immobilized using a five minute injection; one channel received only buffer. The two hIgG1s were tocilizumab



**Fig. 1. ProteOn XPR36 protein interaction array system.** Simultaneous surface binding of 6 ligand (targets) is possible. Analytes can then be injected in 6 perpendicular channels. Interaction between ligands and analytes can be measured at the 36 intersection points.

(trade name MRA) and trastuzumab (trade name Herceptin) which is an anti-HER2 human IgG. Approximately 2,000 to 6,000 response units (RU) of ligand were coupled to the chip. The chip surface was blocked using 1 M ethanolamine (pH 8.5). The lyzed bacteria and phage solution were centrifuged, filtered through a 0.22  $\mu\text{m}$  filter, and injected in the horizontal direction. For each clone, a report point was taken at 255 sec after injection. See Figure 2 for an outline of the experimental design.

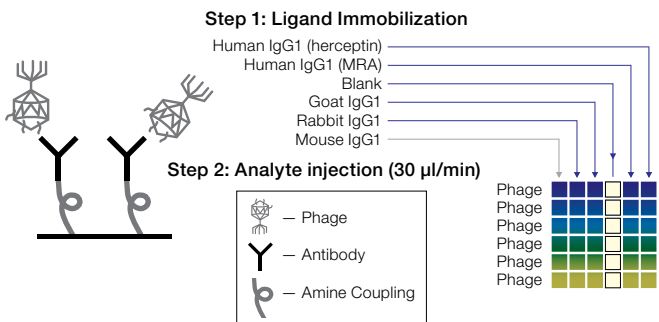


Fig. 2. Screening of human antibody-binding phage 7 clones with ProteOn XPR36 biopanning of T7 phage clones. See experiment 1 for full details.

### Experiment 2: Analysis of the kinetic interactions between human antibody-binding synthetic peptides and human IgG antibody – peptides as ligands

Based on the peptide sequences displayed by the T7 phages, several peptides (A–E) were prepared. The peptides were synthesized, N-terminally biotinylated, and oxidized to form an intramolecular disulfide bridge. The purified peptides, dissolved to 50  $\mu\text{M}$  in HBS-EP buffer, pH 7.4, were immobilized in the vertical direction on NLC (NeutrAvidin-conjugated) sensor chips by injection at 30  $\mu\text{l}/\text{min}$  for 5 minutes. Responses between 500 and 1,500 RU were achieved. As a blank reference flow cell, 50  $\mu\text{M}$  biotin solution in HBS-EP buffer was injected. Herceptin was dissolved in HBS-EP buffer at concentrations ranging from 6.3 to 100 nM and injected across the peptide surface. The interaction was monitored for 180 sec during the association phase and 600 sec during the dissociation phase using ProteOn Manager™ software. See Figure 3 for an outline of the experimental design.

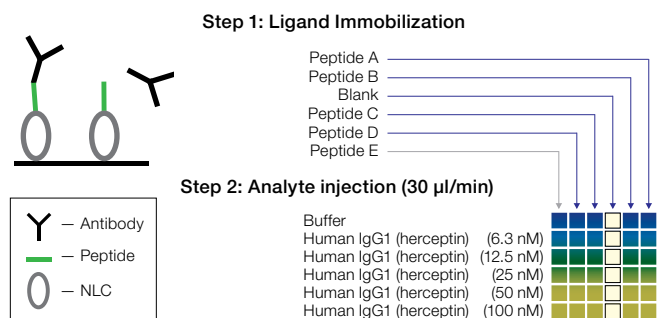


Fig. 3. Assay design for synthetic peptides–human IgG antibody binding kinetic analysis. See experiment 2 for full details.

### Experiment 3: Analysis of the kinetic interactions between human antibody-binding synthetic peptides and human IgG antibody – hlgGs as ligands

To confirm the kinetic parameters collected in experiment 2, we performed the same experiment in reverse, exchanging the ligand and analyte. Five antibodies (herceptin, MRA, goat IgG, rabbit IgG, and mouse IgG) were immobilized on a sensor chip, in the vertical direction, and peptides A–E were injected as analytes in concentrations ranging from 30 to 1,000 nM in the horizontal direction. See Figure 4 for an outline of the experimental design.

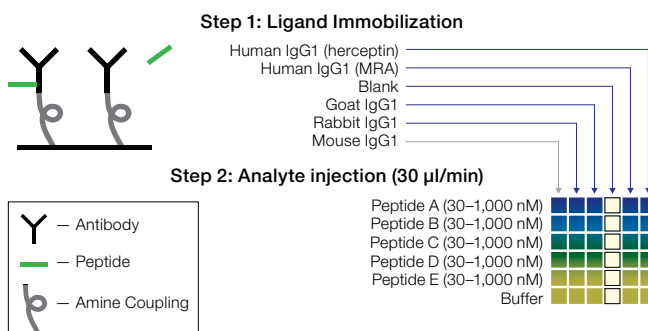


Fig. 4. Assay design for confirming kinetic parameters collected in experiment 2. See experiment 3 for full details.

### Experiment 4: Example of capture using human antibody-binding peptides

We performed this experiment to demonstrate the noncovalent capture of hlgG using these new hlgG-binding peptides, which were immobilized on a ProteOn sensor chip. Peptide A was chosen to capture the hlgG because it had the strongest binding affinity to and highest specificity for hlgG of the peptides tested. Biotinylated peptide A was noncovalently immobilized to a ProteOn NLC sensor chip as previously described. Herceptin was injected as an antigen and captured by peptide A. HER2 protein, the herceptin antigen, was injected to confirm the activity of the captured herceptin. Binding was measured in triplicate over a range of HER2 concentrations. See Figure 5 for an outline of the experimental design.

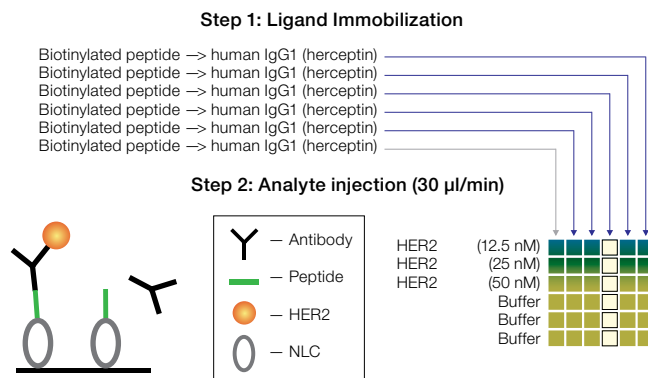
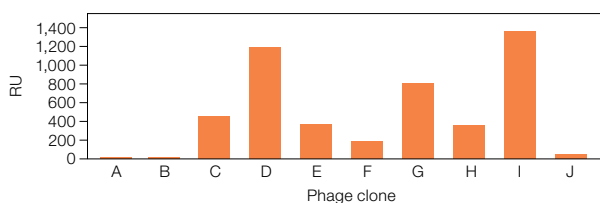


Fig. 5. Assay design for confirmation of interaction of peptide A with herceptin. HER2 was injected as an analyte and the interaction with the captured herceptin was monitored.

## Results and Discussion

### Experiment 1: Screening of human IgG antibody-binding T7 phage clones from a T7 phage peptide library

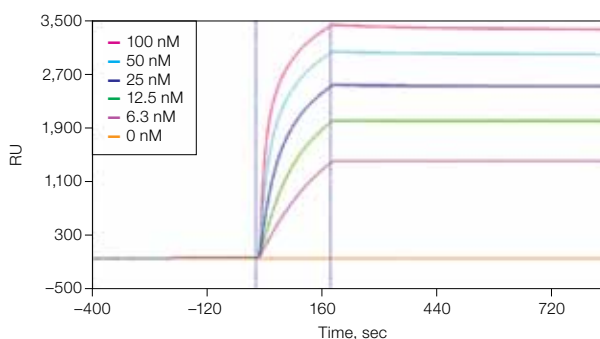
The SPR signals, reflecting the binding strength of each clone, are plotted in Figure 6. These data correlate well with ELISA binding data (data not shown) indicating that the ProteOn system may be used in place of ELISA in this workflow. Additionally, the ProteOn system is able to measure six samples within 15 minutes and, therefore, up to 30 samples within 75 minutes. Therefore, this system is suitable for rapidly screening large numbers of samples and provides binding interaction data in real time.



**Fig. 6. Screening of 10 human antibody-binding phage clones with the ProteOn system.** Comparison of SPR binding response (RU) in 10 phage clones with the IgG antibody at 255 seconds.

### Experiment 2: Analysis of the kinetic interactions between human antibody-binding synthetic peptides and human IgG antibody – peptides as ligands

Interaction sensorgrams are shown in Figure 7. Association ( $k_a$ ), dissociation ( $k_d$ ), and equilibrium ( $K_D$ ) constants for the binding of peptides A–E to Herceptin are summarized in Table 1. From these results, we identified that peptide A had the highest binding affinity with an apparent  $K_D$  of 0.4 nM. Utilizing the One-shot kinetics approach we collected all the kinetic parameters for one antibody with all ligands in a single analyte injection using the ProteOn system, and all data were collected within 30 minutes. This type of multi-affinity analysis makes it possible to save both time and effort in interaction research. In cases where the ligand may be easily denatured or inactivated, this system has the unique advantage of obtaining all necessary kinetic parameters in a single analyte injection without a regeneration step. Research is further streamlined by ProteOn Manager™ software, which can efficiently process and analyze the collected data without significant user effort or time-consuming operations.



**Fig. 7. Interaction sensorgram between the synthetic peptide A and (hlgG) herceptin.** Results were obtained after reference subtraction using a blank channel and double referenced to the blank analyte injection.

**Table 1. Kinetic constants for the binding of hlgG (herceptin) to the 5 synthetic peptides (A–E).** Results were obtained after reference subtraction using a blank channel and double referenced using a blank analyte injection.

Peptide	$k_a$ , $M^{-1} s^{-1}$	$k_d$ , $s^{-1}$	$K_D$ , nM
Peptide A	$1.4 \times 10^5$	$5.8 \times 10^{-5}$	0.4
Peptide B	$1.1 \times 10^5$	$1.9 \times 10^{-4}$	1.8
Peptide C	$1.3 \times 10^5$	$8.9 \times 10^{-3}$	68
Peptide D	$6.3 \times 10^4$	$60 \times 10^{-3}$	1,320
Peptide E	$1.1 \times 10^4$	$20 \times 10^{-3}$	2,260

### Experiment 3: Analysis of the kinetic interactions between human antibody-binding synthetic peptides and human IgG antibody – hlgG's as ligands

The kinetic values obtained from the reverse interaction, with the hlgG immobilized to the sensor chip surface as ligand and the peptides as analytes, are shown in Table 2. There are noticeable differences between these results and the results from the inverse experiment previously described. For example, The  $K_D$  of peptide A was approximately 20-fold greater in the assay orientation of experiment 2 than experiment 3. Similar decreases in affinity were seen for the other peptides in experiment 3 as well. One possible explanation for this discrepancy is bivalent binding of the peptides and antibodies in the orientation where the peptides are immobilized to the surface. The avidity effect caused by bivalent binding would largely influence the perceived binding affinity. Furthermore, antibodies used as analytes have the potential to rebind to the peptides concentrated on the chip surface. Decreasing the density of immobilized peptides could potentially improve the accuracy of the results obtained from experiment 2 to make them comparable with the results from experiment 3.

Ultimately, the binding affinity results generated in experiments are more likely to accurately reflect the true interaction than the results generated in experiment 2. In this experiment a total of 216 sensorgrams were produced from the interaction of five peptides (A–E) and one blank with the five antibodies (two human IgG, rabbit IgG, mouse IgG and goat IgG) and one blank ligand channel. These sensorgrams were obtained within two hours.

**Table 2. Binding kinetic parameters between hlgG (herceptin) immobilized to sensor chips and 5 synthetic peptides (A–E).**

Peptide	Antibody	$k_a$ , $M^{-1}s^{-1}$	$k_d$ , $s^{-1}$	$K_D$ , nM	Remarks
Peptide A	Herceptin	$6.3 \times 10^5$	$5.4 \times 10^{-3}$	8.6	
Peptide B	Herceptin	$2.6 \times 10^5$	$5.6 \times 10^{-3}$	21.4	
Peptide C	Herceptin	$2.5 \times 10^5$	$60 \times 10^{-3}$	227	
	Herceptin	—	—	150	Equilibrium analysis
Peptide D	Herceptin	—	$5.4 \times 10^{-3}$	8.6	Equilibrium analysis
Peptide E	Herceptin	n.d.	n.d.	n.d.	Unmeasurable

n.d. = not determined.

#### Experiment 4: Example of an application of human antibody-binding peptides

The binding analysis results from this experiment are depicted in Table 3. The data obtained in this experiment were almost identical to the data obtained when herceptin was covalently immobilized to GLM sensor chips (refer to experiment 3).

**Table 3. Interaction of HER2 with herceptin.**

	$K_D$ , nM
Herceptin amine coupled (experiment 3)	1.3
Herceptin captured by peptide A	0.9

This method of noncovalent capture of herceptin hlgG using a peptide has several advantages: 1) The immobilization of antibodies to a sensor chip is performed simply by a single injection. 2) The immobilized antibodies are all active because the peptide binds to the Fc portion of the IgG and does not interfere with antigen binding. 3) The sensor chip may be reused because the chip can be completely regenerated using the regeneration buffer.

#### Conclusions

The ProteOn XPR36 system enables multi-affinity analysis within a short time making it a powerful tool for general screening of drugs, evaluating of cross reactivity, analyzing binding of mutants/variants, and epitope mapping of proteins/antibodies.

We demonstrated the usefulness of the ProteOn system in the screening of specific phages from a random peptide library and in the evaluation of hlgG-binding peptides. This utilization of the system allowed us to develop a new method for the purification of antibodies using antibody-binding peptides. The ProteOn system has applications in several important research areas including antibody screening and drug discovery.

#### References

- Sakamoto K et al. (2009) Discovery and characterization of a peptide motif that specifically recognizes a non-native conformation of human IgG induced by acidic pH conditions. *J Biol Chem* 284, 9986–9993.
- Yuji I et al. (2008) Molecular design using phage display — from antibodies to low molecular peptides. *Seibutsu butsuri* 48, 294–298.

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