Luminex®

Application Note

Measuring Antibody Binding Kinetics Using xMAP® Technology and the FLEXMAP 3D® System

Introduction

Across the drug discovery and development process, understanding the kinetics of how antibodies bind to their targets is essential. This information is important for antibody selection, quality control processes, assay development for analyte detection, data interpretation, and more.

Conventional techniques for generating kinetic data on antibodies tend to be tedious and time-consuming. Each antibody must be tested individually. Generation of kinetic data as part of antibody characterization is essential, not only during the assay development process, but also for several applications in biology and medicine. Therefore, a higher-throughput approach would be ideal for collecting this type of data.

Recently, scientists at the Fraunhofer Institute for Cell Therapy and Immunology (Branch Bioanalytics and Bioprocesses, group for Biomarker Validation and Assay Development) evaluated a multiplex method for measuring antibody kinetics. Their research spans several human health applications, tracking food contaminants, identifying disease markers, and detecting drug abuse indicators, all through analyte detection. For this project, they used different peptides, which originate in human pain-receptors, to show that the tested antibody binds to its supposed motifs, but with different affinities, depending on slight differences in the amino acid sequence. For the assay, all peptides were immobilized via an N-terminal histidine, followed by the sequence of interest. Additionally, every peptide contained a C-terminal V5-Tag which could be addressed for coupling control.

The Luminex FLEXMAP 3D® System with bead-based xMAP® Technology was used to measure seven peptides and their binding to the antibody simultaneously. This study offers the first demonstration of how xMAP multiplexing can be used to generate kinetic data on antibody binding. Results allowed the team to rank antibody targets based on their binding performance and select the best candidates for further assay development.

Methods

Peptide Sequence

To ensure the best results, the peptides were synthesized as follows. An N terminal histidine increases the probability of N terminal binding to the bead. This is followed by the original amino acid sequence of interest with a midterm antibody binding motif. Finally, at the C-terminus, a V5-Tag was added as a coupling control.

Bead Preparation

MagPlex® Microspheres of seven different regions were handled according to the xMAP® Cookbook protocol for Carbodiimide Coupling. 5 µg of peptide per 1,000,000 beads were used. The coupling was successfully confirmed by addressing the C-terminal V5-Tag of the peptide sequence.

Sample Preparation

A Phospho-PKA Substrate Antibody (Cell Signaling Technology®, RRXS*/T*, 100G7E, Rabbit mAb, catalog #9624) was RPE-labeled with the PE/R-Phycoerythrin Conjugation Kit- Lightning-Link® (abcam, catalog #ab102918).

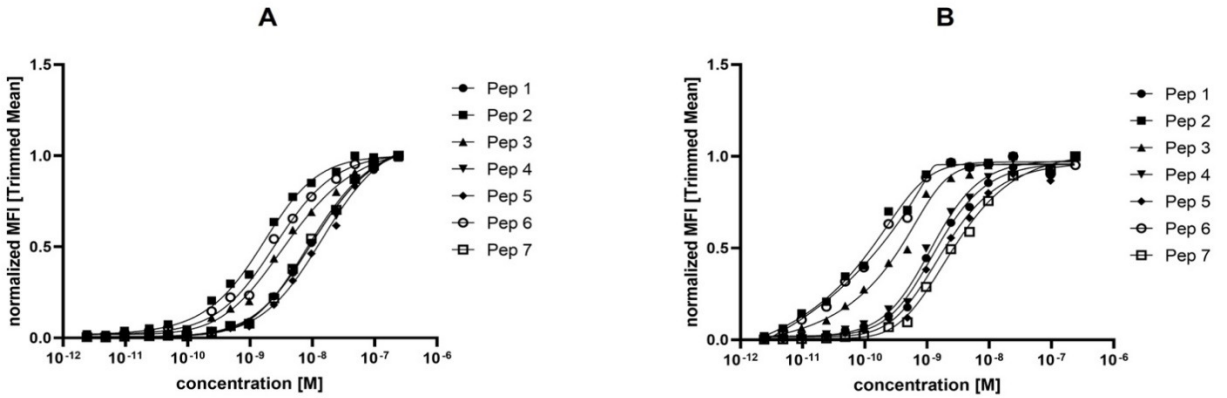
Antibody dilutions, unlabeled and labeled, ranging from 0.0001 µg/mL to 10 µg/mL in 1X PBS + 0.005 % Tween™ were prepared. Each antibody dilution was incubated under constant shaking at 600 rpm with the bead-coupled targets of interest. The unlabeled samples needed an additional incubation with RPE-labeled anti-rabbit antibody (Invitrogen™, catalog #P-2771 MP) with a fixed concentration of 1 µg/mL.

File Acquisition

Each bead region was measured in at least 100 replicate measurements per bead set, per antibody dilution. The measurement was performed on the FLEXMAP 3D System, with a gating of 5,000-20,000 DD (doublet discriminator) under the normal PMT calibration setting. The trimmed mean was used for data evaluation. The curve per target was plotted semi-logarithmically with PRISM 8.0 (GraphPad) and analyzed using a five-parameter nonlinear fit. The EC_{50} value (effective concentration, when 50 % of binding occurred) for each target was calculated automatically. Previous studies have shown that the EC_{50} roughly equals the KD value (binding affinity) in the set-up used.

Results and Conclusions

The Fraunhofer Institute researchers evaluated the xMAP-based approach by incubating different concentrations of a Phospho-PKA Substrate Antibody. To exclude the influence of the binding between primary and secondary antibody, the detection was either done by using a directly labeled antibody (**A**), or by incubating with a RPE-labeled secondary antibody (**B**). They calculated EC_{50} as the KD value and compared the outcome to values obtained through conventional methods for measuring antibody binding kinetics.



	Pep 1	Pep 2	Pep 3	Pep 4	Pep 5	Pep 6	Pep 7
A	7,254e-009	5,954e-010	1,758e-009	5,300e-009	8,796e-009	1,375e-009	3,746e-009
B	1,389e-009	1,101e-010	3,264e-010	1,162e-009	1,854e-009	1,347e-010	2,828e-009

Figure 1: Semi-logarithmically plotted values of the normalized signal to the antibody concentration. At least 100 beads per peptide per well were analyzed. In **A**, a directly RPE-labeled Phospho-PKA Substrate Antibody was used to bind to the peptides (Pep 1-7). In **B**, the peptides were incubated with a Phospho-PKA Substrate Antibody and an additional RPE-labeled anti-rabbit antibody. The EC_{50} values were calculated by using a five-parameter nonlinear fit. The values were calculated both for the direct labeling, as well as the labeling of the secondary antibody approach.

Figure 1 shows the independence of the calculated values with directly labeled antibody and secondary labeled antibody. Thus, an additional incubation step with RPE-labeled antibody didn't interfere with the measurements. By calculating the EC_{50} values of each peptide towards the antibody, a ranking of the peptide binding was possible. The lower the calculated EC_{50} (and so the KD value), the higher the affinity of the antibody towards the tested peptides.

In this study, kinetic results from the FLEXMAP 3D System were comparable to results from commonly used techniques like SPR (surface plasmon resonance spectroscopy) and other commonly used immunoassays like ELISA (enzyme-linked immunosorbent assay), indicating that xMAP multiplexing technology is an efficient, high-throughput alternative to generating essential data about antibody binding patterns. In addition to measuring the binding of one antibody towards a number of targets, it would also be possible to reverse this set-up and screen a number of different antibodies towards one target, to determine the best binder. Additionally, xMAP Technology supports extremely small sample volumes, making it simpler and more cost-effective to test antibody binding in studies like these.

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