Use of High Throughput SPR to Characterize Antibodies To Common Cancer Biomarkers

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Abstract

As a disease of misregulation, cancer is characterized both genetically and proteomically, requiring high quality tools to dissect the change difference between cell states. Among the reagents used to characterize biological systems at the protein level, antibodies are routinely applied, and engineered versions have proven useful for treating multiple therapeutic indications. We have generated a human proteome wide toolbox of polyclonal antibodies, identifying three antigenic 14mer sequences within each of the 20,000+ open reading frames at N-terminal, middle and C-terminal regions. Using a high throughput SPR system, Carterra® LSA™, we are able to define average on-rates, off-rates, and K_D's for pAb's generated to important cancer regulators and biomarkers, including KRAS, STAT3, and Gli1. Due to the small size of the peptide targets, the kinetics of the observed interactions with polyclonal antibodies exhibit remarkable 1:1 fit typically observed when measuring monoclonal antibody affinities. The platform is a sensitive system capable of both identifying multiple antibody-antigen interactions with K_D in the nanomolar to picomolar range, but also as a mass screening tool for developing de novo antibody pairs. We define the kinetic characteristics of these antibody reagents relative to performance in western blotting specifically. Improving the characterization of such widely used tools is critical for advancing translational opportunities into use in the clinic.

Methods

Two strategies were used to attach monoclonal and polyclonal antibodies to the surface of SPR chips. Due to the large difference in molecular mass between the ligands (~150 kDa antibodies) and analytes (~1,500 Da peptides), the ratio of the ligand on the surface to measured value of analyte is proportional to the ratio of molecular masses.

Direct amino-coupling surface preparation. A high density H200M sensor chip was primed into 0.05M MES buffer pH 5.5 and the sensor chip was preconditioned with 1-minute pulses of each of three solutions: 50 mM NaOH, 1M NaCl, and 10 mM Glycine pH 2.0. After preconditioning, the chip was activated with a 10-minute injection of a freshly prepared 1:1:1 (v/v/v) mixture of 0.4M EDC + 0.1M S-NHS + 0.1M MES buffer pH 5.5. The sodium azide protecting the polyclonal antibodies was removed by buffer exchange into 10mM Sodium Acetate pH 5.0 using Bio-Rad micro bio-spin 6 columns. The antibodies were placed on the chip using Carterra LSA multichannel printhead at several different concentrations – 50 µg/µL, 5 µg/µL, 0.5 µg/µL reaching maximum value of 10,000 RU after the 5-10 minutes application. The surface was quenched by 7-minute injection of 1M Ethanolamine pH 8.5, followed by short 0.05M MES buffer pH 5.5 injections to clean the prepared surface.

Protein A/G capture surface preparation. A protein A/G surface high density sensor chip PAGH200M was primed into 1xHBSTE buffer, and the antibodies were placed on the chip using the multichannel printhead achieving the maximum value of 10,000 RU, similar to the amino-coupling method. Cell culture supernatants were diluted in 1x HBSTE buffer with added 0.5 µg/µL bovine serum albumin (BSA) prior to multichannel printhead loading. Same filtered and degassed 1x HBSTE buffer with BSA was used for priming the LSA before the sequential addition of analytes using the single flow cell for both surface preparations.

Analysis of the results was performed using the Carterra LSA Kinetics application, which allows various types of referencing algorithms, X and Y alignment, spike removal and various mathematical models for data fitting and kinetics constants calculation.

Western Blot Characterization. 25 µg of the indicated protein whole cell or tissue extracts was loaded onto SDS-PAGE gels of varying percent concentrations, transferred to PVDF, and processed for immunoblotting with primary antibodies at the indicated dilutions and secondary detection using 1:10,000 anti-Rabbit IgG HRP, followed by washing and western blot detection and imaging.

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Results

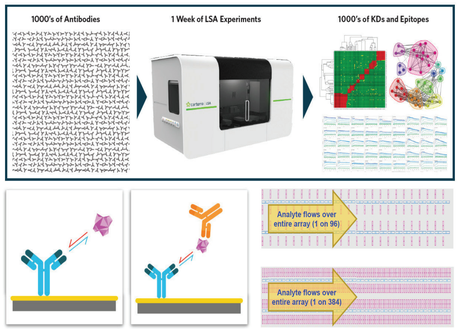


Figure 1. High-throughput Surface Plasmon Resonance. 96 or 384 antibodies can be screened vs multiple antigens to generate affinity curves and epitope maps on the Carterra LSA platform.

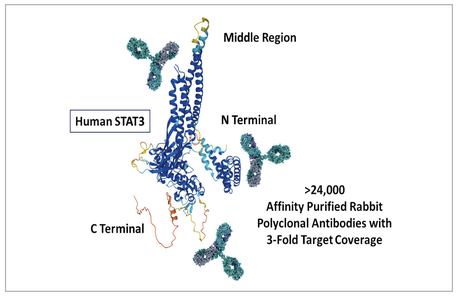
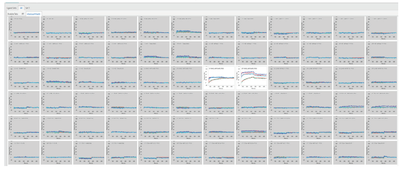


Figure 2. 24,000+ library of affinity purified polyclonal antibodies. A library of >24,000 polyclonal antibodies was generated against 14-mer peptides designed to most targets in the human genome with 3-fold coverage, including amino-terminal, carboxy-terminal and middle region designs. Many of these antibodies cross-react with model organism sequences as well, and the precise affinity purification allows clear epitope mapping of the collection.

KRAS ARP55409_P050—K_D = 500 pM



Notch 1 ARP39295_P050—K_D = 4 pM

Figure 3. Carterra Kinetic Specificity Analysis of 36 antibody/peptide combinations. Purified candidate antibodies (aka ligands) were spotted in duplicate at 25 µg/mL concentration and challenged with the cognate immunogen peptide sequences as analytes (3.3 nM-333 nM). For the two antibodies shown, KRAS and Notch1, both the specificity of the interactions and the associated K_D values were in the picomolar range demonstrating both high specificity as well as high affinity to corresponding target antigens.

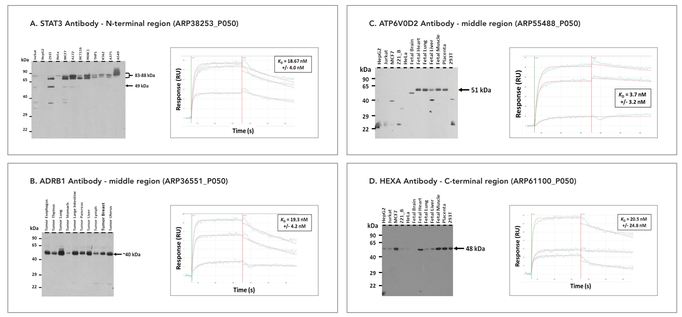


Figure 4. Evaluation of antibody affinity via SPR and specificity in immunoblot performance.

A. STAT3 N-terminal Region Characterization. STAT3, is a crucial mediator of tumor cell progression and tumor-associated immunosuppression. Immunoblot results from 1 µg/mL STAT3 antibody on 25 µg of whole cell lysate per lane of the indicated cell lines. Corresponding kinetic profiles and average K_D values were determined using antigenic peptides.

B. ADRB1 Middle Region Characterization. ADRB1, Beta-1 adrenergic receptor, has been identified as a prognosis related immune gene marker by mutational analysis in breast cancer. Immunoblot results from 1 µg/mL ADRB1 antibody on 25 ug of whole cell lysates or tissues per lane of the indicated cell lines. Corresponding kinetic profiles and average K_D values were determined using antigenic peptides.

C. ATP6V0D2 Middle Region Characterization. Differential expression of proton pumps such as ATP6V0D2 been implicated in carcinogenesis and metastasis. Immunoblot results from 2 µg/mL ATP6V0D2 antibody on 25 µg of whole cell tumor lysate per lane of the indicated tumor types. Corresponding kinetic profiles and average K_D values were determined using antigeneic peptides.

D. HEXA C-Terminal Region Characterization. HEXA and HEXB mRNA levels are upregulated in GBM patient samples. Immunoblot results from 1 µg/mL HEXA antibody on 25 µ g of whole cell lysates from cell lines or fetal tissues as indicated. Corresponding kinetic profiles and average K_D values were determined using antigenic peptides

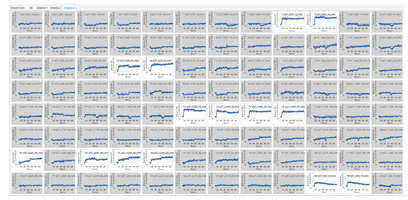


Figure 5. High throughput K_D measurements of recombinant antibody candidates in cell culture supernatants. Candidate supernatants were spotted as ligands on the surface of the chip, Antigen was flowed across the chip to probe specificity and affinity. Crude supernatants from 293 cells contain sufficient antibody to screen for specific interactions on the array surface.

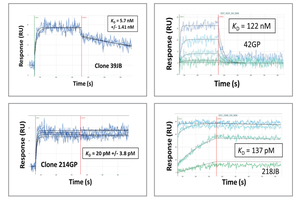


Figure 6. Diverse Candidates Identified in Supernatant Screening. Four candidates from Figure 5, clones 39JB (A), 214JB (B), 42GP (C), and 218JB (D), were used for curve fit and kinetic analysis using LSA Kinetic software. High, medium, and low affinity binders are identified and display nanomolar to picomolar affinities for the cognate targeted ligand.

SUMMARY

1. The high throughput 384 sample Carterra LSA SPR system was used to perform kinetic and specificity analysis on hundreds of antibodies related to cancer pathways and biomarkers.
2. SPR can be utilized for polyclonal antibody characterization using both larger antigens and 14-mer peptides.
3. Feasibility for detailed kinetic analysis of protein:protein interactions including on-rate, off-rate, and epitope differences was performed and supported by detection methods.
4. Western blot analysis of key cancer regulators using epitope specific antibodies correlate with SPR affinity and specificity analysis.
5. The high throughput platform can be used effectively to evaluate antibody candidates from culture supernatants for affinity measurements of both drug targets and biomarker proteins.
6. The system is capable of high throughput epitope binning of binders to facilitate development of antibody pairs for clinically relevant immunoassay development.