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APPLICATION NOTE

Generating High Quality Kinetic Data from Crude Periplasmic Extracts Using High Throughput SPR

The Carterra® LSA™ enables in-line enrichment of crude antibodies available in low titers to enhance assay sensitivity relative to other methods.

1. Introduction

Therapeutic antibodies are commonly formatted as full-length immunoglobulin G (IgG) molecules, but the *in vitro* libraries from which they are often selected are routinely generated as recombinant antibody fragments such as single-chain variable-fragment (scFv) or single-domain variable heavy fragment (VHH nanobody) as available from camelids to facilitate phage display, engineering, and bacterial expression. However, the low expression level of these small antibody fragments within the complex milieu of a crude periplasmic extract (<1 µg/ml) can hinder the ability to perform accurate binding kinetics on early-stage phage display libraries, and oftentimes necessitates their enrichment or reformatting, which can be a tedious and resource-intensive exercise. Here we demonstrate how highly sensitive high throughput SPR (Surface Plasmon Resonance) can be used to measure the binding kinetics of scFv's in high throughput using libraries supplied as crude periplasmic extracts (PPE's).

Defining Assay Sensitivity for mAb Screening and Characterization

Antibodies are large proteins with molecular weights of approximately 150 kDa that bind specifically to their target antigens, typically proteins, with molecular weights varying from 2-4 kDa (peptides) to 150 kDa (other antibodies, in the case of anti-idiotypic antibodies) and higher. So, when screening antibodies (as ligands) for binding to their target antigens (as analytes), the defining measure of sensitivity when using SPR is not the size of the molecule that can accurately be detected on the surface. Although SPR detection sensitivity is based on the relative molecular weight of analyte:ligand, not on the size of the ligand itself, and since the antibodies' target antigens are usually proteins, the molecular weight ratio of analyte:ligand is not a limiting factor in mAb detection. As such, SPR detection sensitivity is not an issue in mAb screening and characterization, as it is with small molecule applications, where detection is often based on analytes that are 100-fold smaller than their specific ligands, such as CBS (or other sulfonamides) binding to CAII[1]. Rather, the critical measure of sensitivity for mAb screening is governed by the amount of material that can be immobilized on the surface from a low concentration antibody sample in order to generate a measurable signal to its target antigen. For biologics, this requires extended immobilization times to enable sufficient capture levels to be attained. Low molecular weight detection, *per se*, does not address this challenge, as it is mAb pre-concentration onto the surface from a limited but highly complex sample that is the driver for sensitivity. Therefore, the ability to measure low mAb concentrations is a function of fluidics capability, not SPR detection sensitivity.

Key Benefits of LSA

Higher Throughput

- Up to 384 immobilized spots within the SPR detection area, plus 48 local reference spots
- Potential to run 1152 kinetic interactions in a single unattended experiment
- Run 384x384 binning assay in a single unattended run

Broad Applicability

- Flow print antibodies, proteins, peptides, DNA and more, for use as ligands directly from crude mixture onto selective capture surfaces

Increased Sensitivity

- Bidirectional or multi-pass flow enables extended contact times during immobilization to concentrate material onto the surface
- Increased sensitivity for detection of low concentration clones
- Enables ligand concentrations 1,000x more dilute to be printed than other technologies
- Reduced sample volume requirement

Fully Enclosed Independent Micro-channels

- Defined spot shape
- Prevents drying and denaturation of ligands during immobilization
- Deliver multiple reagents in series across each spot
- Highly reproducible spot-to-spot immobilization

Conventional SPR Limitations

Standard SPR instruments i.e. Biacore® 8K (GE Biacore) and Sierra 32 (Bruker), utilize a uni-directional flow system that limits association times which impose restrictions on kinetic studies and increase sample usage. Crucially, for mAb studies, this type of flow system is also limited in terms of both volume and interaction time to a single sample push across the detection surface (or single pass). This fundamentally limits the immobilization time and volume available, and hence the amount of material that can be captured/coupled during the restricted timeframe available. This makes the screening of low concentration clones particularly difficult, if not impossible, because the observable signal is low due to the minimal mAb concentration immobilized, as dictated by the syringe volume's capacity. As already noted, low concentration clones are the norm rather than the exception, so the only way these can be successfully analyzed on a standard SPR system is to undertake expensive and time-consuming enrichment prior to testing. Due to the cost and time implications of this added enrichment step, the number of clones that can be screened is greatly limited, which in turn significantly reduces the chances of identifying any high value candidates. In essence, researchers have to ignore most clones due to the technical limitations of their SPR platforms.

High Throughput SPR Sensitivity Advantage: Multi-Pass Sample Delivery

The LSA™ high throughput SPR instrument overcomes these immobilization limitations through the use of Carterra®'s proprietary Continuous Flow Microspotting (CFM) technology which enables both uni- and bi-directional (back-and-forth) flow capability, here referred to as multi-pass sample delivery. By turning the orientation of the flow cell so it sits perpendicular to the chip surface, the CFM technology enables the same flow characteristics required for high quality kinetics studies, but with significant benefits in terms of fluidic performance, immobilization capacity, throughput and SPR data output, thereby expanding applications.

The LSA's integrated CFM technology uses 96 parallel micro-channels to flow and cycle ligand solutions *via* a printhead over a defined surface enabling the simultaneous immobilization of 96 individual protein spots in a single step. Each of the 96 micro-channels creates an independent fixed loop for every sample so that by utilizing multi-pass flow, each sample can move back-and-forth across the surface for a user defined time period, thereby in-line enriching the concentration of low expressing clones during immobilization (using affinity-capture or coupling chemistry), hereby termed flow printing. Proteins are maintained in a stable liquid environment throughout the immobilization step, producing highly defined active spots with coverage that can approach saturation. This overcomes the immobilization sensitivity issue, enabling very low expressing PPEs to be kinetically screened with confidence without the need for prior enrichment.

In addition, as the LSA can flowprint 3 x 384 well plates within a single integrated experiment, it is possible to screen up to 1152 samples for kinetics per automated run. As such, there is now no need to triage clones in advance, so all clones from a campaign can be screened thereby increasing the likelihood of discovering high-value candidates and gaining the true value of the high throughput expression systems now commonplace.

2. Methods

Surface Preparation of the Capture Lawn (anti-V5 mAb, Capture Reagent on the LSA)

ScFv constructs were expressed with a V5 epitope tag to enable their in-line oriented capture *via* immobilized anti-V5 mAb, which was coated as a lawn onto an HC30M or HC200M sensor chip (Carterra) using standard EDC/sulfo-NHS-mediated amine coupling method in a run buffer of HBS-ET (10 mM Hepes pH7.4, 150 mM NaCl, 3 mM EDTA, 0.01% Tween-20 (Carterra)). The LSA single flow cell was used to prepare the lawn, which involved (1) activating the chip with a 10-min injection of 1:1:1 v/v/v mixture of 0.4 M EDC + 0.1 M sulfo-NHS + 0.1 M MES pH 5.5 (Carterra), (2) coupling 50 ug/ml mouse anti-V5 mAb (clone #SV5-Pk1 Abcam cat# ab27671) in 10 mM sodium acetate pH 4.5 (Carterra) for 15 min and (3) blocking excess reactive esters with a 7-min injection of 1 M ethanolamine HCl pH 8.5 (Carterra). This yielded final coupled levels of about 4,000 RU (HC30M chip) or 7,000 RU (HC200M chip).

Surface Preparation of the Array (scFv Library)

A library of up to 384 unique-sequence scFv clones was supplied as plates of crude PPE's expressed on small-scale (100 ul/well) and diluted 2-fold in HBS-ET run buffer. Samples were flow printed for 15 min in batches of 96 PPE's in parallel using the 96 channel printhead, which passed a fixed volume of sample (200 ul) back-and-forth across the chip surface, thereby enriching the capture of PPE clones onto discrete spots. An entire 384-array of spotted PPE's was prepared by serially docking the printhead onto each of the four nested print block locations.

Capture Kinetics

The library's target antigen was supplied as a purified recombinant monomer and prepared in run buffer of HBS-ET + 0.5 mg/ml BSA as a 3-fold series with final concentrations spanning 0.5 nM – 3 µM to provide the analyte series. Analytes were delivered in the single flow cell in ascending concentration, allowing a 5 min association time and a 15 min dissociation time, without any regeneration in between them. Several binding cycles of buffer alone were injected before the antigens samples to provide blanks for double-referencing the data. Binding data were analyzed in Carterra's Kinetic Data Analysis Software to determine kinetic rate and affinity constants by fitting a simple Langmuir model to the double-referenced data.

3. Results

High Throughput, High Quality Kinetics

High quality kinetics were produced from direct capture of scFv clones from crude PPE's, using Carterra's flow printing, which efficiently extracts low titer antibodies from crude sources. **Figure 1** shows the results of a global analysis of a panel of kinetically diverse scFv clones selected as representative examples from a larger panel of 192 clones. By titrating the antigen (as analyte) across a broad concentration range, we could characterize a broad range of affinities (around 500-fold, with K_D values from single digit nM to single digit µM) within a single array.

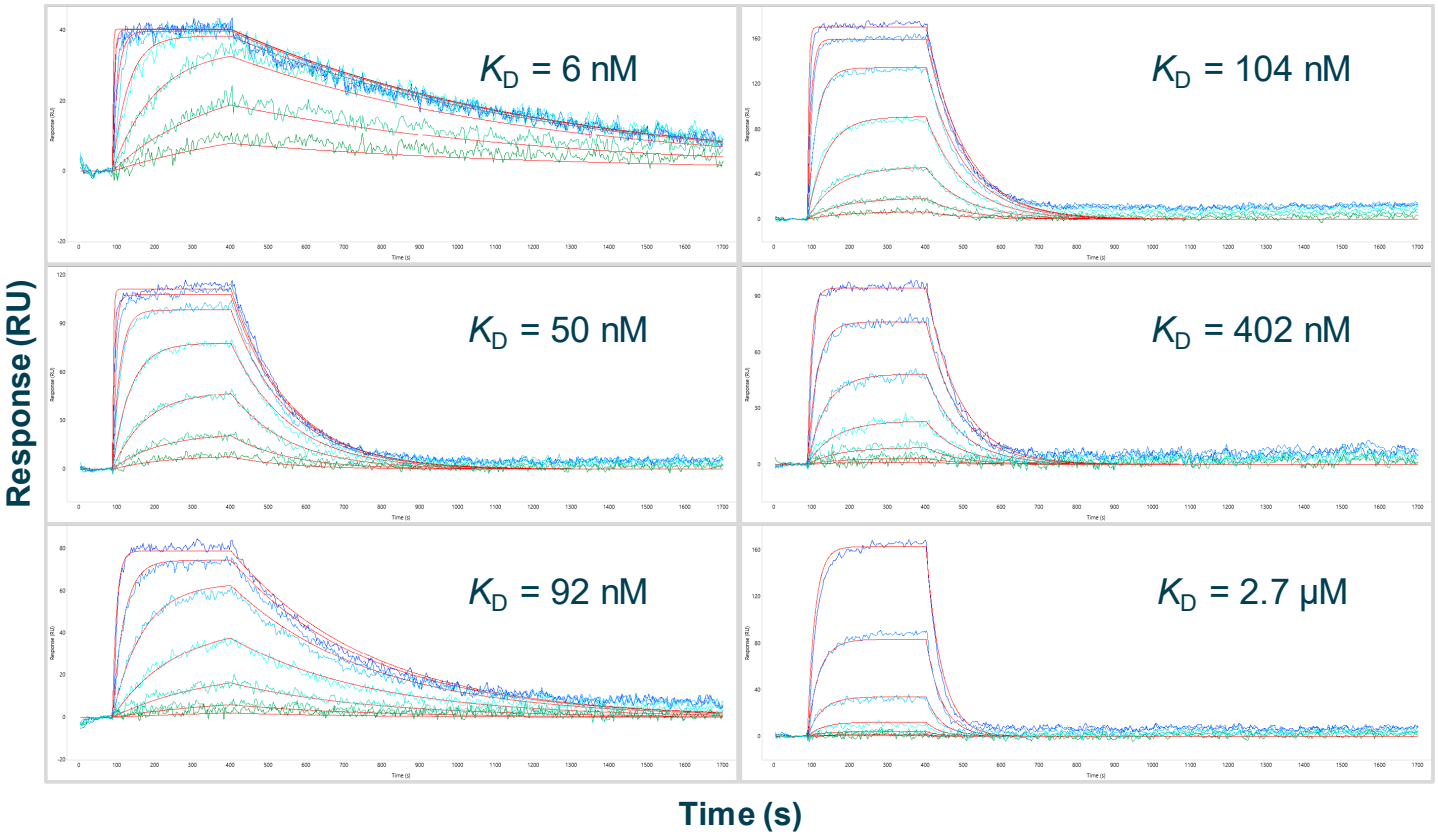

Figure 1: Kinetic diversity observed within the scFv library for clones captured directly from crude PPE via a coupled lawn of anti-V5 mAb. The colored lines (green to blue palette, representing ascending analyte concentration) and global fits (red lines) show excellent agreement, indicating that the binding kinetics were well-described by a simple Langmuir mode. Each tile depicts a single clone on a discrete spot, with K_D values reported.

High throughput SPR's highly parallel one-on-many format enables both a high-throughput and high-resolution analysis of the binding kinetics of up to 384 interactions in parallel. **Figure 2** shows an analyte-on-ligand tile-view representation of the kinetic analysis of a library of 192 scFv clones analyzed in parallel, where each tile represents the data for a single clone, captured onto a discrete spot within a 192-mAb array.

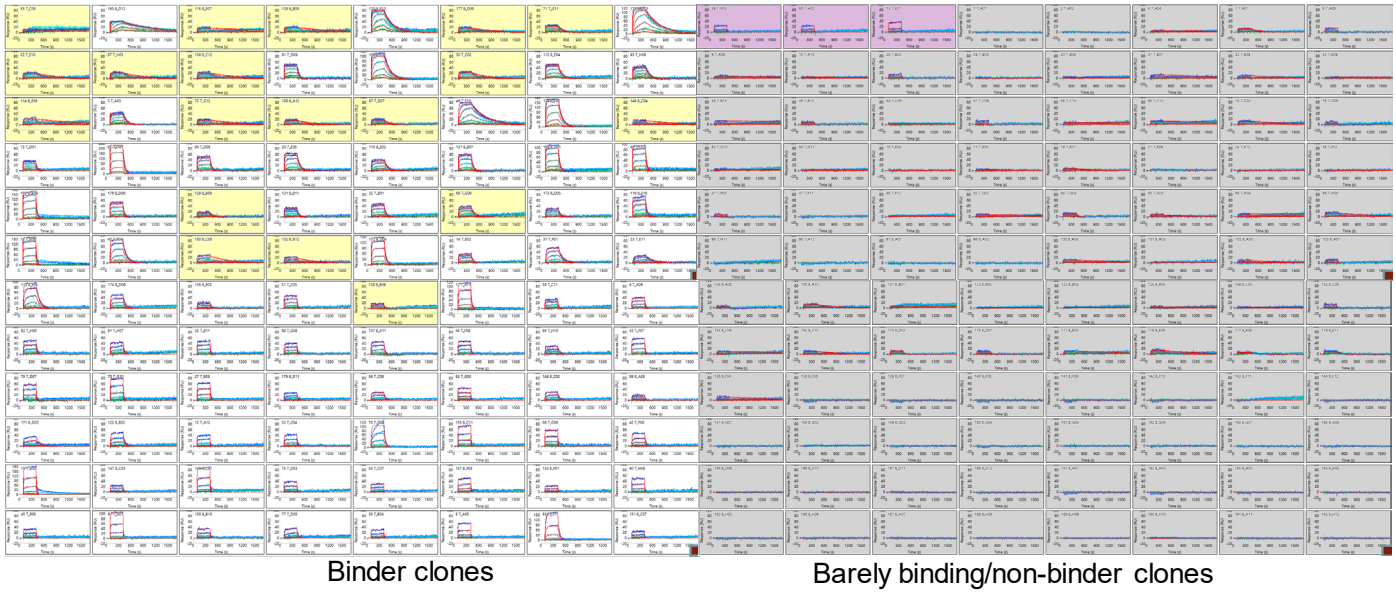

Figure 2: High throughput kinetic analysis. Each tile represents the global analysis of a specific analyte binding to a scFv clone captured onto a single spot. within a 192-array. Carterra's analysis software automatically flags spots with non-ideal behaviors and displays them as colored tiles; yellow (poor chi-squared value), purple (insufficient onrate information, due to over-estimated R_{max}) and grey (inactive or barely-binding).

High Throughput SPR Correlates with FACS Analysis in Accurately Discriminating Binder and Non-binder Clones

The scFv library was also analyzed by FACS to determine binding to the native target on cells. **Figure 3** shows an example of (A) a binder clone and (B) a non-binder clone that were identified by both FACS and high throughput SPR. These data further demonstrate the reliability of high throughput SPR to generate both accurate and information-rich data from scFv's directly from crude PPE's.

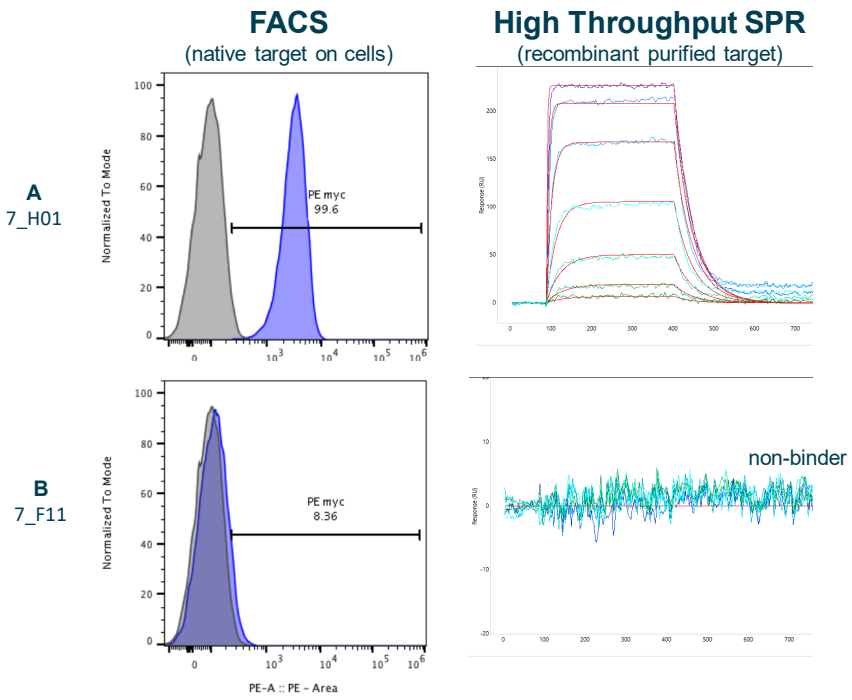

Figure 3: Identifying (A) positive and (B) negative binder clones to cell-surface expressed tumor target, as measured by FACS and to the recombinant target, as measured by high throughput SPR.

High Throughput SPR can be used to Verify that Reformatted IgGs Recapitulate the Binding Kinetics of their scFv Parent Clones

The binder clones identified from the anti-V5-based capture kinetics screen of crude scFv PPE by high throughput SPR (**Figures 1-2-3**) were reformatted into full-length IgG and retested for antigen binding using a similar capture kinetics format, this time using a coupled anti-human IgG Fc lawn as the capture reagent. **Figure 4** shows that we obtained excellent agreement across the two assay formats, namely the binding kinetics obtained from anti-V5-captured scFv and its anti-human IgG-Fc captured IgG reformatted analog, further demonstrating the reliability and accuracy of high throughput SPR in producing high quality kinetics data directly from crude samples. This is due to the highly efficient in-line enrichment that occurs during flow printing that enable crude samples to be effectively in-line purified.

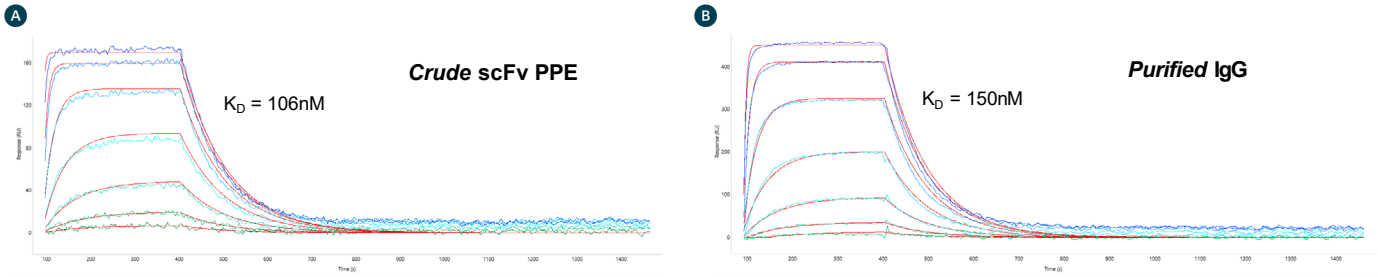

Figure 4: example of excellent agreement between kinetics obtained for the same clone when provided as (A) crude scFv PPE captured via anti-V5 lawn and (B) reformatted purified IgG counterpart captured via anti-human-IgG-Fc lawn.

Highly Reproducible Spot-to-spot Analysis Within an Array

We tested the spot-to-spot reproducibility of Carterra's flow printing technology by capturing a duplicate set of scFv PPE sups onto discrete spots (in a blinded fashion). **Figure 5** show that we achieved excellent agreement across duplicate clones that were expressed in independent campaigns and captured onto discrete spots.

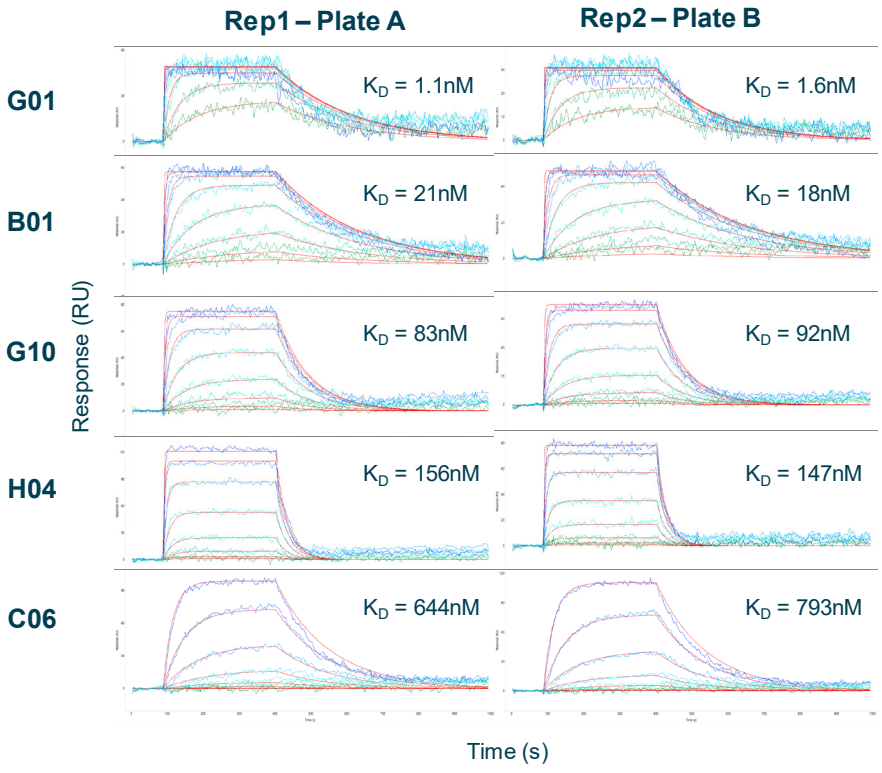

Figure 5: Duplicate analysis of binding kinetics of anti-V5-captured scFv PPE's. Reps 1 and 2 represent different spots of the same clone, produced from two plates representing independent expression campaigns of the same set of clones.

4. Conclusions

High throughput SPR is both a high-throughput and high-resolution method for determining the binding kinetics of hundreds of antibody interactions at once. Here we show that high quality data can be obtained directly from crude antibody preparations, such as scFv PPE's, owing to the highly efficient extraction that is achieved by Carterra's patented CFM technology that uses bi-directional sample delivery across spots on a chip surface, generating multiple passes to enrich capture levels beyond what is possible by other methods, even from low titer antibody preparations.

We also showed that in complex media the binder and non-binder clones identified by high throughput SPR correlate well with FACS results, providing confidence in the array method, even from crude antibody preparations.

Further, we show that binding kinetics obtained from crude antibodies can accurately predict those from their purified counterparts, increasing the reliability in high throughput SPR as a screening tool that deserves to be shifted upstream in the drug discovery process, thereby changing the paradigm from SPR's traditional role as a secondary screening step due to its low throughput.

High throughput SPR is a disruptive technology that shifts the paradigm and enables mAb screening and characterization to be the same step. High throughput SPR can generate high quality and reliable kinetic data directly from low titer crude antibody sources, making it amenable to the early stage screening where samples are numerous but available only in crude form and in low quantity.

This Application Note was developed in collaboration with Distributed Bio, South San Francisco, CA, USA.

References

1. Anal Biochem. 2004 Jun 15;329(2):316-23. Analysis of small-molecule interactions using Biacore S51 technology. Myszka DG

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