Fragment Screening of Massively-Parallel Ligand Arrays Using the Carterra® Ultra® SPR Platform

Key Highlights

- **Carterra Ultra® offers the enhanced speed and sensitivity necessary to support Fragment-Based Lead Discovery (FBLD).**
- **Ultra's large ligand array allows for many targets, off targets, mutants, and multiple species to be measured in parallel; a 100-fold enhancement over other SPR systems.**
- **Utilise ready-made panels of biotinylated-proteins to standardise the assay parameters so you can move directly to finding hits and drive your medicinal chemistry programmes.**

Fragment-Based Lead Discovery (FBLD) has emerged as a core approach to early-stage hit finding in drug discovery programmes.[1] This form of drug discovery is distinguished by the screening of libraries of very small chemical compounds (heavy atom count < 17), that bind with low affinity (double digit micromolar to millimolar KD) to therapeutic targets. Screening small compounds allows for an efficient sampling of the chemical space relevant to medicinal chemistry. The identified binders, in concert with X-ray co-crystal or cryo-electron microscopy structures, serve to provide a map of how chemical matter can interact efficiently with the targets.

The first step in FBLD is the screening of a fragment library using one or more hit-finding technologies capable of detecting the low-affinity transient interactions of very small compounds with drug targets. Surface Plasmon Resonance (SPR) has found widespread use in industry and academia for initiating and supporting FBLD programmes from hit finding through lead optimisation. SPR offers a broad dynamic range (mM to pM), sensitivity (100 kDa proteins), applicability to a wide range of target classes including membrane proteins, cost effectiveness, flexibility (direct binding and competition formats), and throughput.[2,3] The throughput of various commercial SPR platforms has improved with successive generations of instruments through a combination of reduced sample injection cycle times and increased ligand capacity allowing the testing of a single compound against multiple proteins simultaneously. This capability is frequently used to increase the target throughput of an SPR lab in supporting screening and hit-to-lead campaigns, provide information about mutants and off targets, and compile data on target ligandability.

To further capitalise on the benefits of larger ligand capacity in an SPR platform we developed and launched Carterra Ultra®, a high-throughput SPR (HT-SPR) instrument with >10-times the ligand capacity of any other commercial SPR platform. Ultra divides a single sensor chip surface into 192 regions of interest (ROIs) using two successive ligand deposition steps via Carterra's multi-channel manifold comprised of 96 independent fluidic paths (Figure 1). In typical operation, 96 of these ROIs are used to couple ligands of interest in one round of deposition, and the other 96 as in-line references during the second deposition, that provide for the highest quality reference subtraction. It is possible to extend the ligand capacity up to 191 ligands, maintaining a single spot for referencing. After the ligand deposition step, Ultra removes the multi-channel manifold from the chip and covers the ROIs with a single-channel flow cell (SFC) where analytes are directed across the entire array allowing collection of binding data from all 192 ROIs simultaneously using an enhanced version of Carterra's SPR-imaging array technology.

Ultra has numerous enhancements over prior platforms to provide the sensitivity and speed necessary to support FBLD. Several hardware changes and internal process updates have resulted in a dramatically reduced injection overhead time. Our previous instrument, the LSA^{XT}, requires ~12 minutes to perform an analyte injection cycle. In Ultra this has been reduced to ~3.5 minutes providing for much faster experiment setup and execution. In two days of unattended run time Ultra can efficiently process 768 analytes from two 384-well plates. The thermal range and performance have been significantly enhanced. The new Advanced Single Flow Cell technology does not contain pneumatic valves for more robust performance and enables automated washing of the fluidics with 50% DMSO. This design also reduced the required volume of analyte solutions from 270 µL to 200 µL. Ultra's optics have been improved and along with advances in ROI integration, ROI size, placement, and flowcell geometry, the short-term noise has been reduced to < 0.25 RU RMS and the data collection rate has been increased to 2 Hz for each of the 192 ROIs. At that noise level, Ultra is able to detect weak binding by small analytes with molecular weights as low as 100 Da.

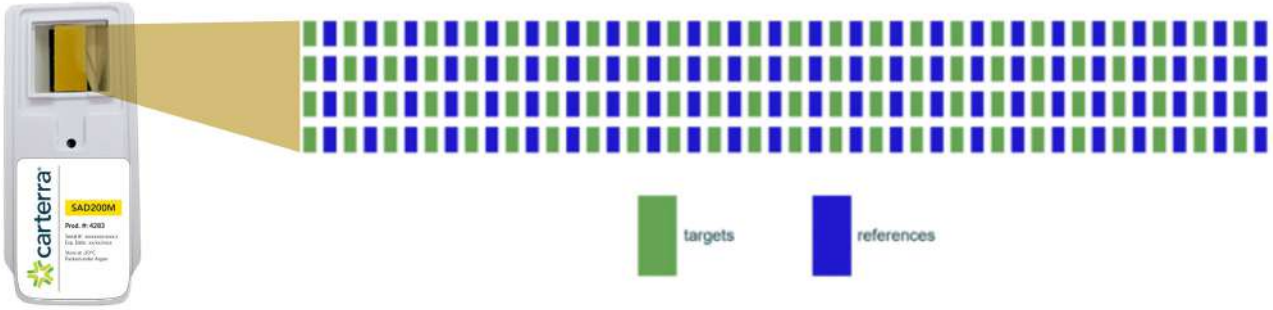


Figure 1: Ultra's ligand array. Target ROIs are interspersed with reference ROIs to give the best possible referencing. If desired, some reference ROIs can also be made into target ROIs to increase throughput.

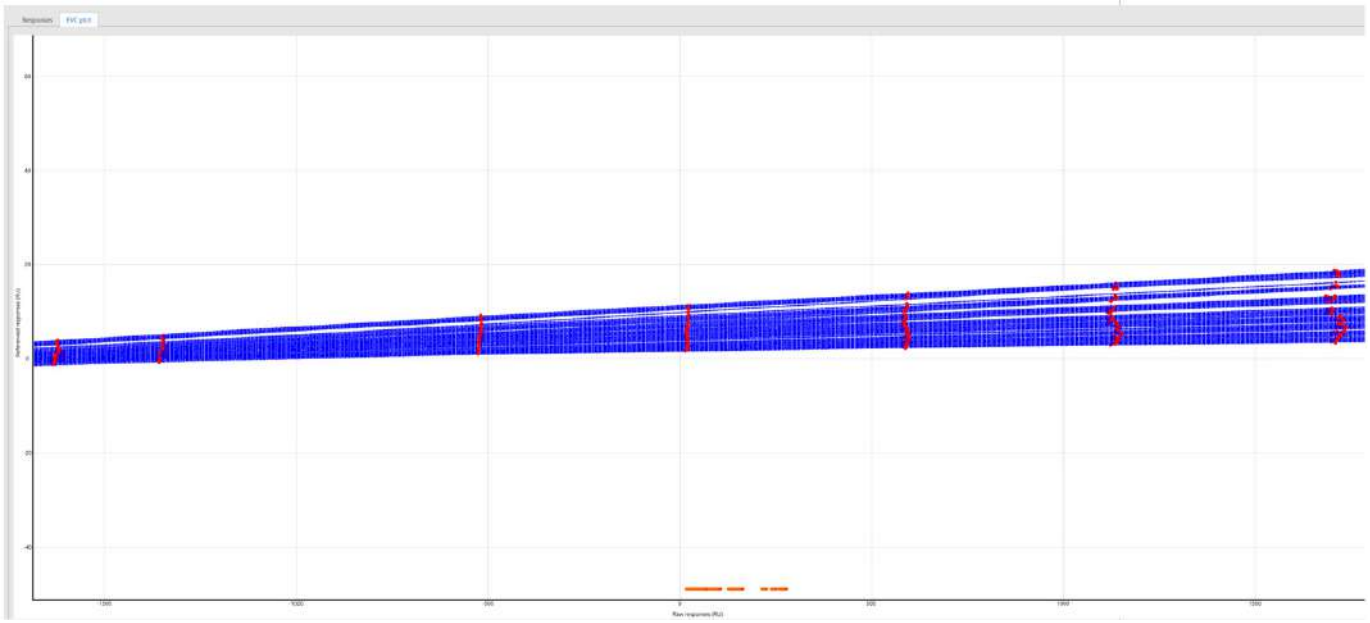


Figure 2: Example of applying the Excluded Volume Correction with Ultra and Kinetics 2.0. Seven calibration solutions with higher and lower DMSO concentrations than the running buffer are plotted in red resulting in 96 points per solution. The raw response on the reference ROI is plotted versus the difference between the reference ROI and ligand ROI for each standard. The orange circles along the X-axis represent the raw response on the reference ROI for each analyte injection. The amount of correction needed for each analyte injection is determined from the calibration curve and applied to the analyte sensorgrams.

Ultra's control and analysis software have been updated to support the Excluded Volume Correction (EVC) (sometimes referred to as the DMSO correction or solvent correction), which is required when working with ligand surfaces coupled to high density. This removes the signal differences between ligand and reference ROIs that arise due to the presence of high levels of coupled ligand. (Figure 2). Ultra also employs a calibration routine that calibrates the RU scale across all ROIs. A user simply provides two solutions with a higher and lower refractive index than the running buffer and, at the end of the experiment, the instrument will automatically generate a 12-point titration curve and apply the calibration curve across the entire experiment (Figure 3). Numerous other hardware and software changes have been incorporated including washing between cycles with 50% DMSO, software tools to support screening and hit identification, and improvements in software speed and performance. These changes allow for an instrument capable of determining KD values from low picomolar to low millimolar in full support of FBLD from hit-to-lead, and/or antibody programmes.

Screening a Panel of Kinases Against a Fragment Library

To demonstrate Ultra's capabilities in FBLD we performed a fragment screen using off-the-shelf biotinylated human kinases provided by Carna Biosciences. To demonstrate the ability to go beyond the standard 96 ligand-array format we selected 125 high-value therapeutic kinases targets and their relevant off-targets for parallel screening against the Maybridge 1000 fragment library. This library has been well-curated for chemical diversity and solubility and is a good representative of fragment collections that have been assembled in various industrial and academic groups. Kinases were shown to be active and sufficiently stable once captured to a sensor chip through testing to the non-hydrolysable ATP analog AMP-PNP. After a small amount of condition optimisation the full 1000 fragment library was tested at a single concentration against all 125 kinases over three days. Control AMP-PNP injections, along with blank buffer injections for double referencing, were injected at regular intervals through the screen to monitor surface binding stability for each kinase. No surface rebuilds were necessary and the entire dataset was collected using a single chip.

Over the three days of data collection approximately 210,000 sensorgrams (ligand & reference ROIs included) were obtained. To our knowledge, this represents the largest single fragment-screening endeavour yet reported and, notably, was executed by an individual scientist. We estimate that a similar screen utilising the next-highest throughput commercial SPR system on the market would take approximately six months of business days to complete. Thus, Ultra represents an enormous value proposition in time savings as well as reagent and chip use.

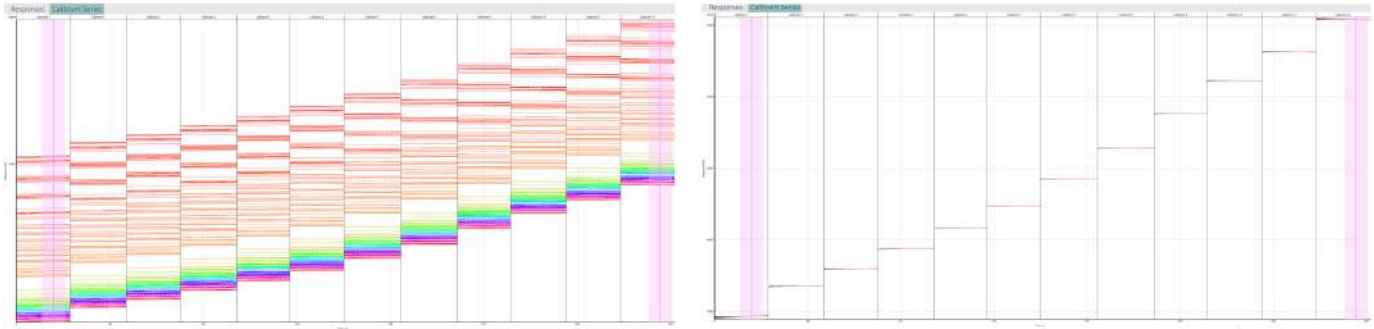


Figure 3: The raw injection data for the ladder of calibration injections automatically mixed and injected by Ultra from provided high- and low-RI solutions bracketing the RI of the running buffer. The ligand ROIs are colored red through orange and the reference ROIs are colored green, blue, and purple. After application of calibration, the response signals from all surfaces are aligned to the same scale.

Methods and Materials

A panel of 125 singly biotinylated kinases with verified purity and enzymatic activity was kindly provided by Carna Biosciences. The Maybridge 1000 library was purchased from Thermo. All binding studies were performed using a Carterra Ultra HT-SPR platform set for 10 °C. For these studies, a single Carterra SAD200M sensor chip was used. This chip has a carboxymethyl dextran hydrogel pre-derivatised with sufficient streptavidin protein to achieve high capture levels necessary for FBLD activities. The chip was thawed, docked, and thermally equilibrated for 30 minutes prior to automatic application of an air-normalisation routine. After priming into capture buffer (HBS + 0.005% Tween-20, 5% glycerol, 0.5 mg/ml BSA, pH 7.4) the chip was preconditioned with four pulses of 20 mM NaOH and two pulses of 10 mM sodium acetate pH 5.5 for 60 seconds each using the SFC. This treatment minimises baseline drift due to changes in hydrogel volume by pre-swelling the dried matrix. The SFC was automatically undocked from the chip and replaced with the mutlti-channel head creating 96-individual chambers, each with an independent flow path to the 96-needle manifold. Kinases were diluted into 96-well plates and cycled over the chip surface for ~90 minutes (Figure 4). After the first 96 kinases were captured the multichannel head automatically lifted, moved over, and resettled on the chip to create the second 96-channel array. The remaining kinases were captured as in the first step. Finally, the multichannel head was automatically undocked and the single channel side redocked. Any remaining free biotin binding sites on streptavidin were blocked with two 30 second pulses of amino-peg-biotin (0.0025 mg/mL) over the surface. This is important as fragments can bind to the biotin site on streptavidin and be recorded as false positives.

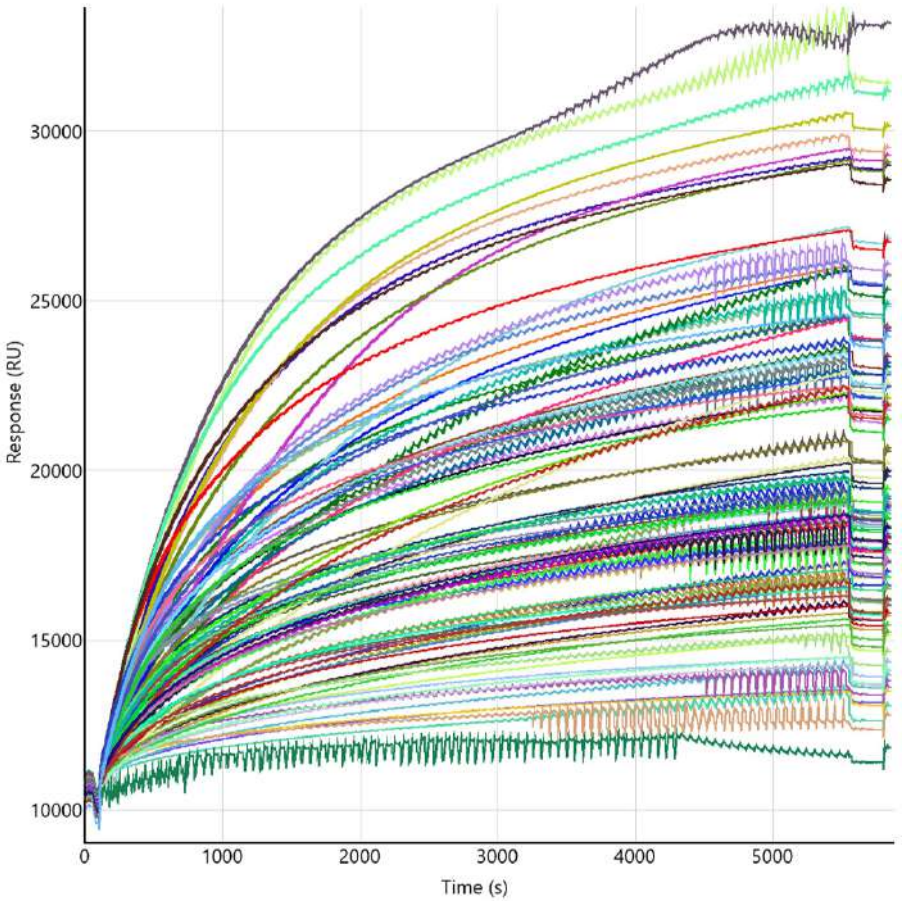


Figure 4: Capture of 96 biotinylated kinases to a SAD200M chip. Capture levels varied from 1,000 to 35,000 RU. 200 µL of each protein solution was taken from source plates and cycled back-and-forth over the surface. This allows for longer captures without using larger volumes of protein samples. The regular vertical oscillations are indicative of this cycling behavior and allow for very dilute samples to be captured to high levels. This process is non-destructive since the samples are returned to the source plate and may be used for other experiments or additional captures.

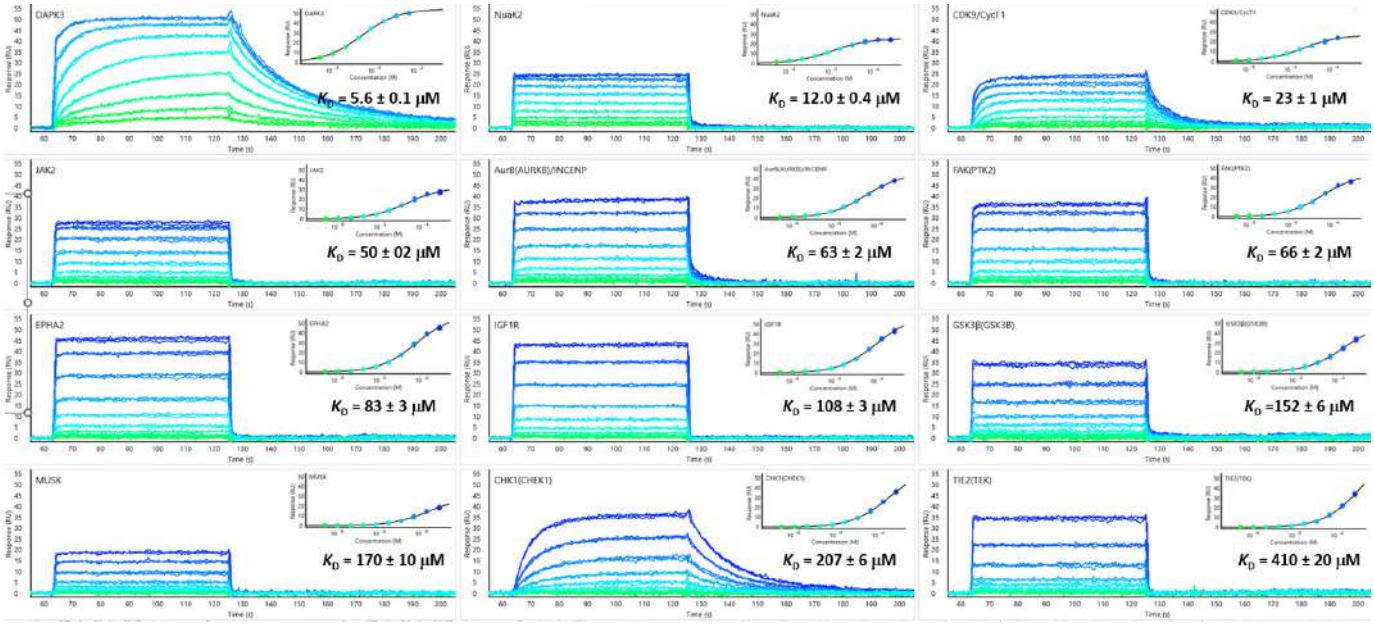


Figure 5: Affinity determination for AMP-PNP against captured kinases. Triplicate dose-responses are overlayed. The last 5 seconds of data points from the association phases were averaged and plotted on the Y-axis against the log of the analyte concentration (X-axis) in the insets. Fits to these curves yielded the equilibrium KD values and standard errors as reported by Kinetics and shown for each panel.

The system was primed into assay buffer (HBS + 0.005% Tween-20, 5% glycerol, 1 mM DTT, and 5 mM MgCl2, 3% DMSO at pH 7.4). Surface activity of all kinases was assessed by triplicate injections of AMP-PNP from 5–400 µM in a 2-fold dilution series to determine the affinity for every kinase to this control compound (Figure 5).

Fragments were plated in 384-well plates at 300 µM. After every 20 fragment injections an AMP-PNP control injection and a buffer blank injection for double referencing were collected. After priming the instrument in running buffer 10 startup cycles were run to equilibrate the instrument followed by fragments and controls. The injection of EVC correction solutions and calibration solutions completed the experiment. Data reduction and analysis was performed with the Carterra Kinetics 2.0 software package. After applying the calibration, the data were zeroed, EVC and referencing were applied, and the data was double referenced by subtracting the nearest buffer blank injection. Sensorgrams were also normalised for the molecular weight of each analyte. Screening performance was evaluated in the new Screening tab in Kinetics 2.0 to assess protein stability and apparent hit rate. Report-point data were created by averaging the last 5 seconds of association phase for each injection and exported from Kinetics into Excel®. These were assembled in Datawarrior (www.openmolecules.org) and merged with the SMILES strings representing each compound for a comprehensive cheminformatic analysis of all 125 screens simultaneously.

Results

Over the course of three days, over 125,000 fragment/protein interactions were characterised (Figure 6). This amount of data stands in stark contrast to that typically acquired in pharmaceutical fragment screening campaigns where 1,000–10,000 fragment/ligand interactions for 1–3 targets are typically collected over a course of 1–2 weeks utilising one or more instruments simultaneously. Analysis of the hits selected for individual kinases frequently exhibit the hydrogen bond donor/acceptor 'hinge' motif typical of kinase binders. An example of an aminothiazole compound is highlighted binding to CHK1 kinase in figure 5, lower right corner. Since this represents the largest fragment screen ever reported, a comprehensive analysis of the complete dataset is beyond the scope of this application note, but was consistent with expected hit rates. Figure 7 shows representative report-point analysis from the Screening tab in Kinetics 2.0 for seven kinases. Hovering over the individual points shows the sensorgram allowing the user to quickly perform quality control on their binding data and remove any data-points that come from badly-behaved samples such as those exhibited by promiscuous binders.[4]

While many kinases showed no significant loss in control binding capacity throughout the multi-day experiment, others showed some degree of decay. While the apparent affinity of the control remained unchanged, the total binding capacity decreased. This is a typical feature of SPR-based fragment screens where proteins are required to be stable for at least a day or more, which is a high bar compared to biochemical assays that usually require only a few hours of stability. If a protein doesn't decay more than 50% in 24 hours it is possible to model this drift, apply data corrections and normalisations for control binding and baseline drift, and recover reliable binding data for the fragments that were tested later in the run.[3] Virtually all the tested kinases in this panel met that requirement and hit selection can be robustly applied across the screen.

Discussion and Takeaways

The combination of increased sensitivity, speed, and the > 10x larger ligand capacity relative to other commercial biosensors makes Ultra a valuable tool in the pursuit of FBLD from screening through hit-to-lead activities. The large array can support endeavours to study on- and off-target binding, compound selectivity across all the members of a signalling pathway, and

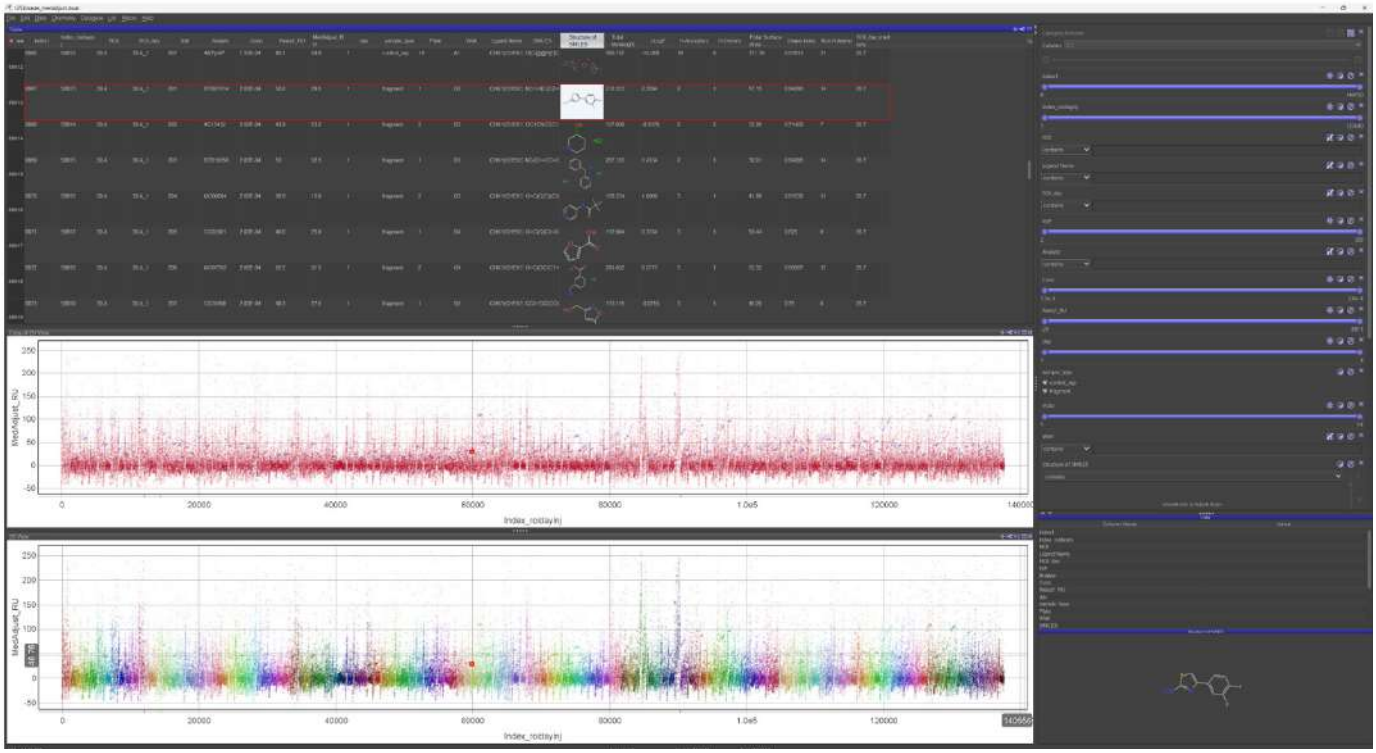


Figure 6: Compiled data for the 125,000 interaction fragment screen. Individual rows with data and chemical structure are shown at the top. The upper graph shows binding levels of fragments (red) and AMP-PNP controls (blue). The lower panel shows all data coloured by individual kinase. Each colour represents a single 1,000 compound screen on a single kinase over three days. Prior to graphing each kinase/day, data had adjustments applied for control and baseline drift including a vertical adjustment so negatives evenly scatter around zero RU.[3]

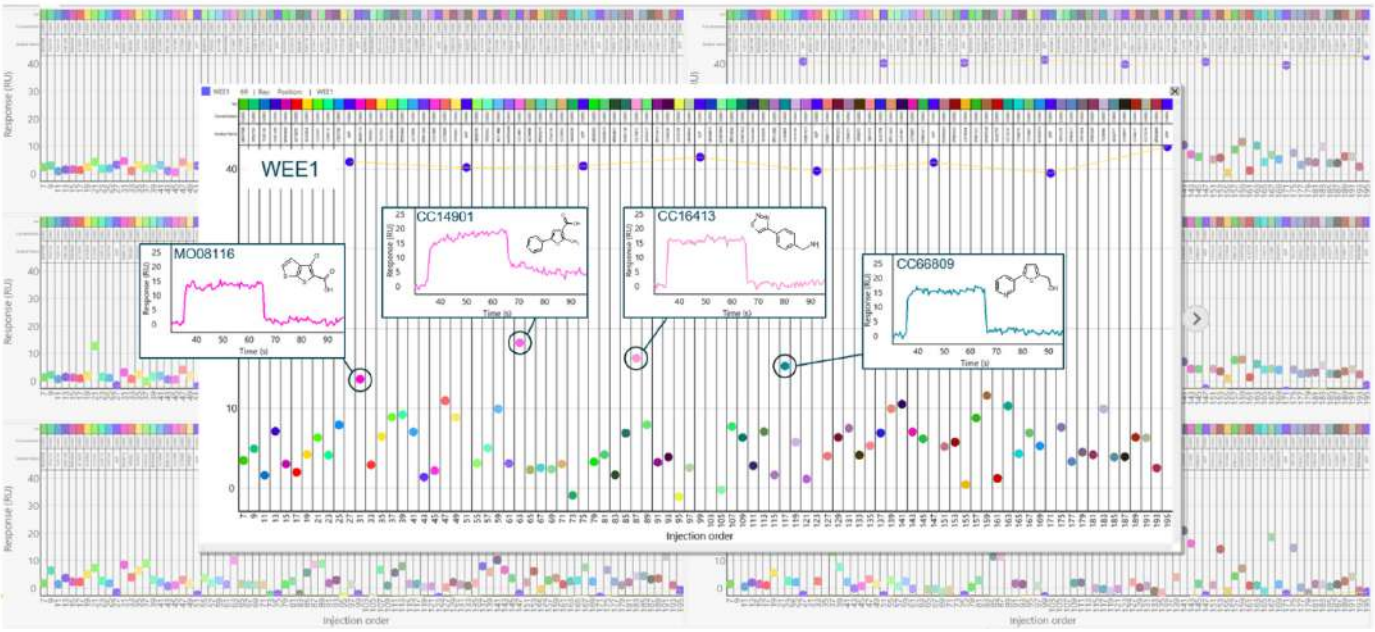


Figure 7: Example data taken from the new screening tab in Kinetics 2.0. The binding levels of analyte replicates (purple) are shown vs. injection order for easy assessment of on-chip target stability. Hovering over individual data points will trigger a pop-up showing the injection cycle that generated the point allowing for fast sensorgram quality control and false-positive elimination. Structure view is added for clarity here and not present in the analysis software.

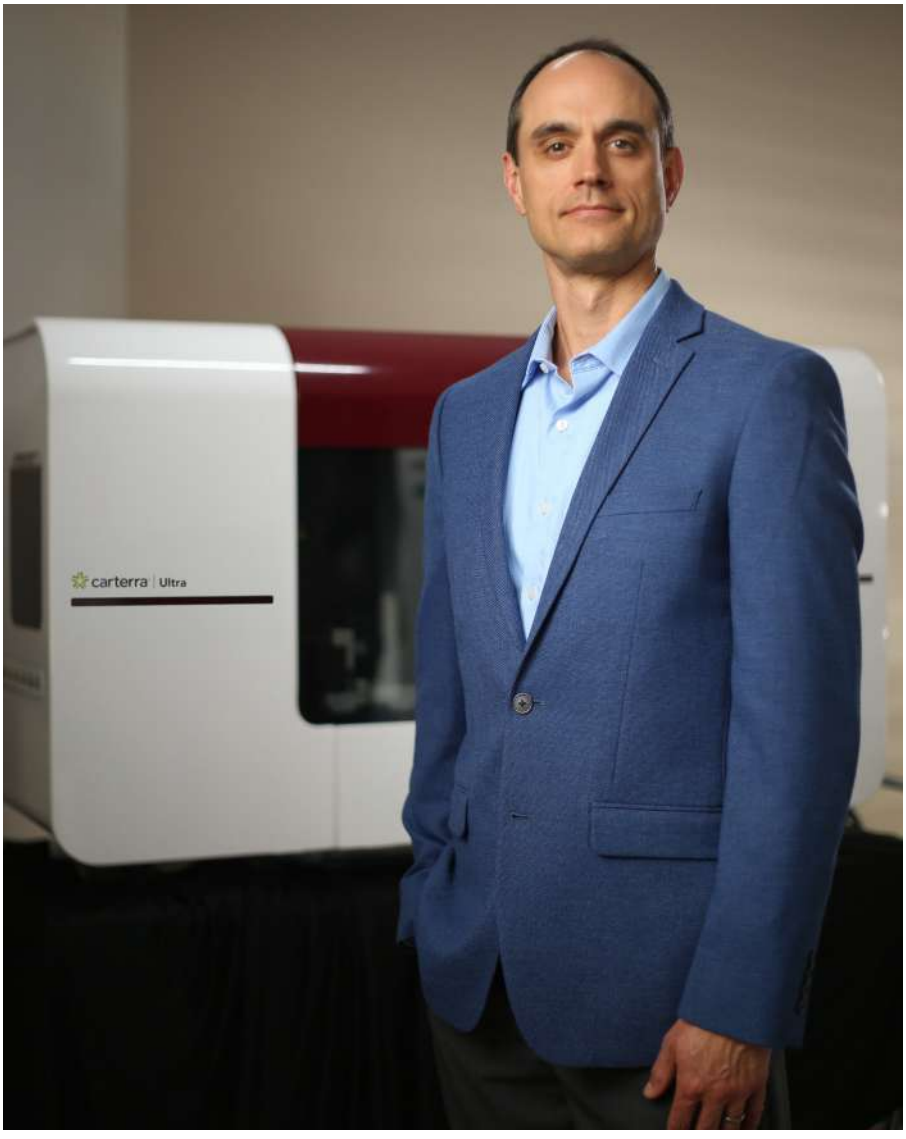
provide ligandability information on more than 100 proteins simultaneously for triaging target entry into discovery portfolios. The improvements in hardware and software described here will help scientists apply the best practices in small-molecule SPR at more than 10-times the scale of other instruments easily and efficiently helping ensure that every assay produces quality decision-making data. Ultra's ability to run for several days unattended to collect up to 768 single-point fragment tests, or 96 six-point dose-response curves for accurate KD determinations, allows users to spend less time setting up experiments and more time on analysing the vast amounts of data that can be generated in parallel using the large ligand array. While Ultra represents Carterra's first instrument intended to fully support small-molecule applications, it was also designed to carry out all the same large-molecule workflows that have typified our prior instruments, including large-scale epitope binning, but now with higher sensitivity and speed.

Acknowledgments

Thanks to Carna Biosciences for providing the kinases, and Drs. Anthony M. Giannetti and Daniel Bedinger for developing this work.

REFERENCES

1. Kang C, Weijun X. J Med Chem. 2024, Feb;68(5):5000-5004
2. Giannetti AM Methods in Enzymology, V493:169-218
3. Giannetti AM, Gilber, HN, Huddler DP, Reiter M, Strande C, Pitts KE, Bravo BJ RSC Drug Discovery Series Fragment-Based Drug Discovery, 2015:19-48
4. Giannetti AM J Med Chem. 2008 Feb;51(3):574-80



Josh Eckman, Carterra CEO, standing in front of the Carterra® Ultra™ platform