Application Note

Affinity Ranking of Small Molecules SPD304, Evans Blue, and Trypan Blue Against TNFα Using Agile R100

Savannah Afsahi; Jolie Nokes, PhD; Deng Pan, PhD; Angela Shue; and Francie Barron, PhD

- While affinity ranking is crucial in the hit validation stage of drug discovery, current assay techniques have limitations such as the inclusion of labels that can cause unwanted interactions, limited dynamic range to gain accurate analysis, or a large amount of background noise caused by solvents.
- Agile R100 uses the breakthrough electrical technique FEB, an orthogonal label-free sensing mechanism that enables a wide dynamic range and is unaffected by optically impure samples such as solvents. This **reduces the assay development time needed for affinity ranking studies, while providing additional kinetic binding rate information.**
- In this application note, we provide kinetic characterization of 3 small molecule inhibitor compounds, SPD304, Evans Blue, and Trypan Blue, interacting with target TNFα, a cytokine, as measured with Agile R100. We show that **rank ordering using Agile R100 K_D values matches the rank order achieved with IC_{50} values from published studies.**
- We further share standard curves for SPD304 interacting with TNFα in assay buffer containing a range of 0% to 10% DMSO. **All K_D values are within a factor of 1.6, displaying the platform's impartiality to optical impediments.**

ABSTRACT

Affinity ranking is a vital component of the drug discovery hit validation phase. However, many assay techniques suffer from drawbacks including the requirement of labels which complicate assay development, a lack of adequate dynamic range to accurately characterize the interaction, or background noise caused by optical impediments such as solvents. Agile R100 uses Field Effect Biosensing (FEB), a proprietary electrical technique that is label-free, has an 11-log dynamic range, and is unaffected by complex samples that are difficult to measure with optical biosensors. In this application note, we share affinity and kinetic binding rates of SPD304, Evans Blue, and Trypan Blue interacting with Tumor Necrosis Factor alpha (TNFα), a cytokine involved in the body's inflammatory response and a common therapeutic target. The small molecules SPD304, Evans Blue, and Trypan Blue inhibit the activity of TNFα by interfering with trimerization of the protein to yield its active form. The K_D of each interaction as measured by Agile R100 rank orders the same as previously-published peer-reviewed half-maximal inhibitory concentration (IC_{50}) values,[1,2] while also providing kinetic binding rates. In addition, Agile R100 provides precise data for SPD304 binding with TNFα in measurements of up to 10% DMSO, showcasing the label-free platform's impartiality to optical impediments.

INTRODUCTION

Once a target has been determined in early stage drug discovery, chemical libraries are screened to identify compounds that modulate the activity of that target, a.k.a. hits. In the hit validation and medicinal chemistry phases of discovery, interactions are often quantified and optimized through an iterative process. Different orthogonal techniques may be chosen so that results are not affected by the technical limitations of any one platform. In these phases, affinity ranking is a critical analytical method to prioritize hits by IC_{50}/EC_{50} values or binding affinity (K_D).

Spectroscopy-based techniques (e.g. fluorescence resonance energy transfer [FRET] and enzyme immunoassays [EIA]) require labels to characterize interactions. These assays yield IC_{50}/EC_{50} values that can be used for rank ordering purposes. The interference of labels with activity measurements combined with the time required to develop appropriate labels motivate researchers to find label-free solutions.

Label-free optical biosensing techniques (e.g. surface plasmon resonance [SPR] or Bio-Layer Interferometry [BLI]) are also used to provide binding affinity data for rank ordering purposes, while additionally providing information-rich kinetic binding rate data. The rank order generated with these techniques is comparable with the rank order generated by IC_{50}/EC_{50} values from spectroscopy-based techniques. However, the dynamic range of these instruments is limited by lack of sensitivity to low molecular weight molecules as well as the fast on- and off-rates indicative of interactions with low affinity. Additionally, compounds at the hit validation stage tend to have high micromolar affinities. At these concentrations, dimethyl sulfoxide (DMSO) is required to solubilize the compounds. On optical platforms, DMSO can cause background noise that is magnitudes larger than the signal generated by the target and compound interaction, and thus must be accounted for by time-consuming and often

error-prone solvent correction.[3,4] The addition of solvents limits the sensitivity and dynamic range of optical techniques.

Agile R100 offers an orthogonal label-free option that streamlines hit validation, providing highly-sensitive kinetic characterization of small molecules over an unprecedented 11-log dynamic range.[5–7] The platform uses FEB, an electrical technique that characterizes biomolecular interactions by measuring the current across a sensor surface on which the target is immobilized. Any interaction or binding that occurs causes a change in conductance that is monitored in real-time, enabling accurate binding affinity and kinetic measurements.[8–13] Because FEB is an electrical technique, optical challenges such as solvents do not cause the large amounts of background noise that hinder optical platforms.[14] Due to its wide dynamic range, impartiality to solvents, and label-free sensing mechanism, Agile R100 provides an exceptional orthogonal methodology to rank order compounds with less time and resources.

TNFα is a cytokine involved in the body's inflammatory response. Dysregulation of this response is involved in many diseases, such as rheumatoid arthritis and plaque psoriasis. TNFα is primarily produced by activated macrophages but can also be produced by a variety of other immune cells, even neurons. The active form of TNFα is a trimer (see Figure 1 for the trimeric structure). Physiological consequences of TNFα signaling include fever, apoptosis, and inflammation. Despite clinical success of protein-based drugs against TNFα, development of small molecule inhibitors is stalled by challenges in drug screening.[13-15] Several small molecule inhibitors that interact directly with the TNFα trimer have low affinity, poor activity, are cross-reactive, or inhibit TNFα indirectly.[2]

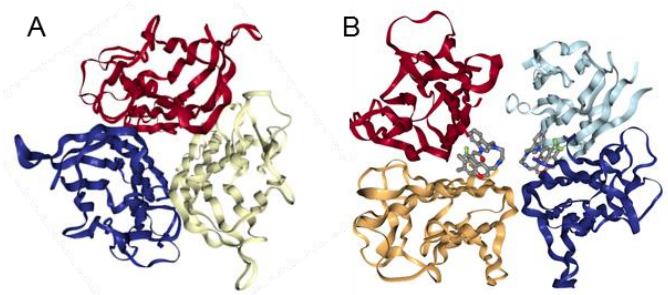

Figure 1: The structure of TNFα is pictured. A) Human TNFα is biologically active as a trimer, and inhibitors of TNFα reduce activity by inducing deoligomerization of the trimeric structure. SPD304 binds to the core of the trimer to dislodge a subunit. Evans Blue and Trypan Blue disrupt the quaternary structure of TNFα, causing deoligomerization. B) The inhibitor/dimer complex then reoligomerizes as a tetramer.[1,2] PDB ID: 1TNF.[18]

SPD304, Evans Blue, and Trypan Blue are small molecule inhibitors of TNFα, and their structures are shown in Figure 2. SPD304 (620.53 Da) directly inhibits active trimeric TNFα by binding between 2 TNFα subunits and displacing the third, causing deoligomerization of the active form.[19] Additionally, while SPD304 binds in the TNFα binding pocket through hydrophobic interactions, SPD304 can also bind to both the membrane-bound or soluble form of TNFα.[19] Since SPD304 is large enough to contact 2 TNFα subunits simultaneously, SPD304 can block the third TNFα subunit from joining and prevent formation of the active trimer.[19] Thus, SPD304 can both deoligomerize a formed TNFα trimer and prevent trimer formation. The IC_{50} value for this interaction has been reported as 22 µM.[2] The mechanisms of action (MOA) for Evans Blue (960.81 Da) and Trypan Blue (960.81 Da) have also been elucidated. Evans Blue and Trypan Blue bind to the core of the TNFα trimer, causing deoligomerization of the TNFα trimer by modifying the quaternary structure. The IC_{50} of Evans Blue inhibiting TNFα has been reported to be 750 µM while the IC_{50} of Trypan Blue interacting with TNFα has been reported to be 1000 µM.[1] According to these previously-published IC_{50} values,[1,2] the ranking of the 3 small molecule compounds from highest to lowest potency are: SPD304, Evans Blue, and Trypan Blue.

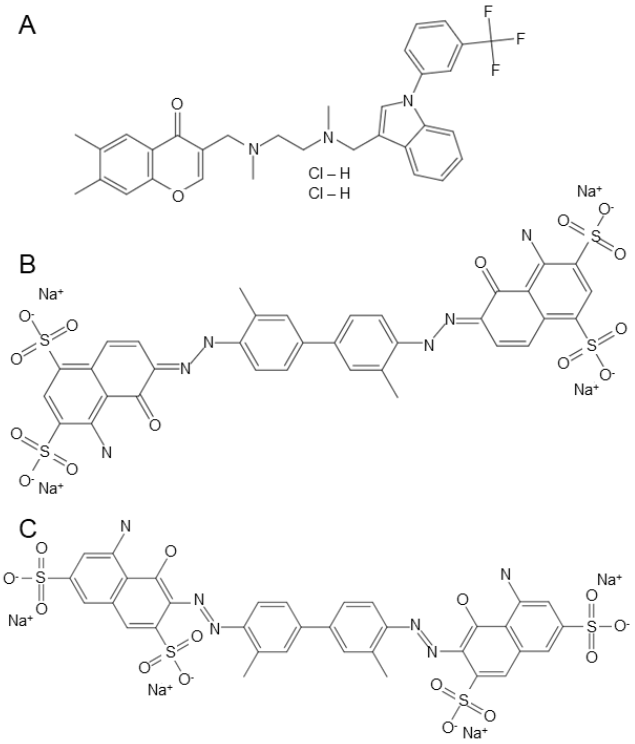

Figure 2: The small molecules studied are A) SPD304, B) Evans Blue, and C) Trypan Blue. SPD304 binds to the core of the TNFα trimer, disrupting the quaternary structure and causing dissociation into dimeric and monomeric forms. Evans Blue and Trypan Blue both induce deoligomerization of the TNFα trimer by modifying the quaternary structure.[1,20]

MATERIALS AND METHODS

TNFα Immobilization

Recombinant human tumor necrosis factor alpha (TNFα) (17.5 kDa) was used as the target protein. Immobilization was

performed at room temperature with a 75 µL drop of sample. To covalently immobilize TNFα to an Agile R100 COOH biosensor chip, standard carbodiimide crosslinker chemistry (N-Ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-Hydroxysulfosuccinimide (sNHS)) in biotechnology grade 2-(N-morpholino) ethanesulfonic (MES) pH 6.0 buffer was added for 15 minutes. Next, 179 ng/mL TNFα in immobilization buffer (cell culture grade 1X phosphate buffered saline [PBS] pH 7.4) was added to the activated graphene surface for 15 minutes. To block the exposed graphene to nonspecific binding and to inactivate surface binding sites, Quench 1 (3 mM polyethylene glycol [PEG]-amine in 1X PBS pH 7.4) and Quench 2 (1 M ethanolamine in water pH 8.5) were sequentially added to the biosensor chip for 15 minutes each. Assay buffer was added to the biosensor chips to equilibrate prior to addition of the small molecule.

Kinetic Characterization

Small molecules SPD304 (620.53 g/mol), Evans Blue (960.81 g/mol), and Trypan Blue (960.81 g/mol) were investigated as drug compounds interacting with the target protein. The assay buffer for the kinetic characterization interactions was 1X PBS pH 7.4. A 75 µL drop of sample at room temperature was added to the biosensor chip for all measurements.

Fresh assay buffer was added to the Agile R100 biosensor chip to calibrate a baseline measurement for 5 minutes, followed by the addition of the compound in assay buffer for 10 minutes to measure association. After the association measurement was complete, assay buffer was added to measure dissociation of the interaction, and kinetic binding rates were calculated using integrated Agile Plus software. The tested concentrations are shown in Table 1 below.

Table 1: The concentrations of SPD304, Evans Blue, and Trypan Blue interacting with TNFα.

Compound	Concentrations (µM)
SPD304	0.004, 0.04, 0.2, 0.4, 2, 4, 20, and 40
Evans Blue	10, 100, 250, 750, 1000, 5000, 75000, 10,000, 15,000, and 20,000
Trypan Blue	0.1, 1, 10, 30, 100, 250, 500, 750, 1000, 1300, 2000, 3000, 4000, 5000, and 10,000

To release the target protein and compound interaction between dilutions of compound, assay buffer was used as regeneration buffer. To accomplish regeneration, 5 quick rinses followed by a 5-minute wash step in regeneration buffer was performed between each measurement cycle. Repeated cycles of regeneration were completed until release of the TNFα target was successful (characterized by the sensor response reaching >80% return to baseline). Following regeneration, assay buffer was added to calibrate prior to the next concentration measurement of compound. The calibration, association, dissociation, and regeneration steps were repeated for all compound concentrations. Each concentration series was performed on the same Agile R100 biosensor chip, and concentrations were measured from low to high.

DMSO Measurements

The assay buffer for the TNFα and SPD304 interaction in DMSO was 1X PBS pH 7.4 containing 0% DMSO, 1% DMSO, 3% DMSO, or 10% DMSO. A 75 µL drop of sample at room temperature was added to the biosensor chip for all measurements. The measurement conditions in DMSO were the same as the conditions for the kinetic characterization experiment outlined above. To reiterate briefly, calibration was performed for 5 minutes in assay buffer, association was performed for 10 minutes with SPD304 in assay buffer, and dissociation was performed for 5 minutes with assay buffer. The concentrations of SPD304 ranged from 0.04 µM to 4 µM, and concentrations were tested on the same Agile R100 biosensor chip from low to high. Regeneration was performed with assay buffer until the sensor response returned >80% to baseline.

Analysis

For both kinetic characterization of SPD304, Evans Blue, and Trypan Blue and the SPD304 DMSO measurements, the on-rate (k_{on}) and off-rate (k_{off}) were determined by Agile Plus software with a single exponential curve fit to the association and dissociation sensor responses.[21] The sensor response is the magnitude of the I-Response caused by the biomolecular interaction, and a standard curve was created from the sensor responses with respect to the compound concentration. The K_D was determined with a Hill-Langmuir fit of the standard curve, automated in Agile Plus software. Sensor response magnitudes vary for each compound, and the sensor response magnitudes in the standard curve were graphed by the software as normalized to the maximum sensor response found from the Hill-Langmuir curve fit. The mean and standard deviation are reported for all kinetic binding values, and error propagation of mean and standard deviation was used to carry through the variation. The data for mean and standard deviation come from the average and variation between the n = 3 independent replicate biosensor chip measurements. Here, k_{on} is reported in $M^{-1}s^{-1}$, k_{off} is reported in x 10^{-3} s^{-1} (perseconds), and K_D is reported in µM.

RESULTS AND DISCUSSION

Kinetic Characterization

The sensorgrams used to calculate kinetic binding rates of SPD304, Evans Blue, and Trypan Blue interacting with TNFα are shown in Figure 3. As the compound interacts with the target, the sensorgram is recorded in real-time, and k_{on} and k_{off} are calculated by Agile Plus software (Table 2).

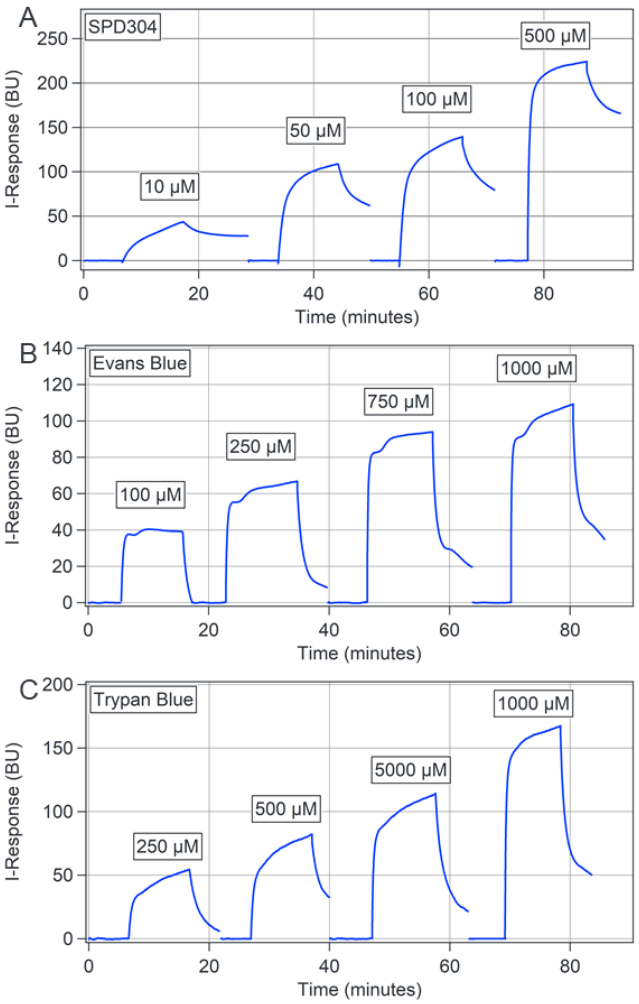

Figure 3: Sensograms taken from Agile R100 show the dependence of the sensor response on the concentration of the inhibitor compound. As the concentration of compound A) SPD304, B) Evans Blue, and C) Trypan Blue increases, the magnitude of sensor response correspondingly increases.

Table 2: The kinetic binding rates for SPD304, Evans Blue, and Trypan Blue interacting with TNFα as measured by Agile R100.

	k_{on} ($M^{-1}s^{-1}$)	k_{off} ($x\ 10^{-3}\ s^{-1}$)
SPD304	2960 ± 855	9.63 ± 1.98
Evans Blue	44.9 ± 10.8	49.4 ± 10.8
Trypan Blue	10.7 ± 2.55	26.2 ± 4.43

Figure 5 shows 3 standard curves, plotted as the sensor response versus concentration of each compound as it interacts with target protein TNFα, and the K_D values are calculated from these. The results show that **the binding affinity ranking generated with Agile R100 is the same as the rank order determined from previously-published IC_{50} values (Table 3).[1,2] Agile R100 provides additional kinetic binding rate data which gives users more information earlier in the drug discovery process to make better decisions.**

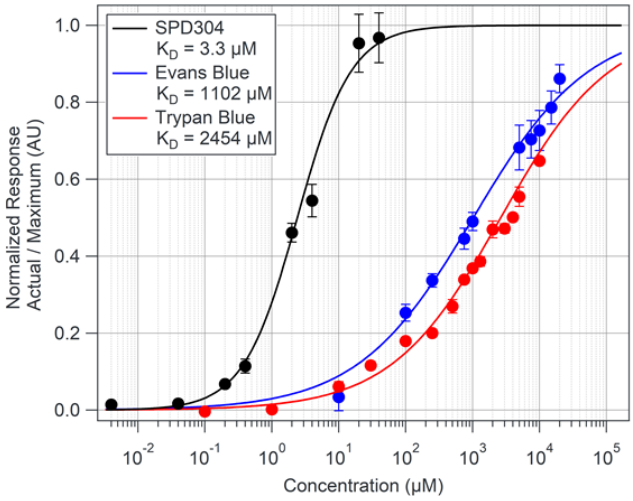

Figure 5: Standard curves generated by Agile R100 for TNFα interacting with SPD304 (black; K_D = 3.25 ± 0.659 µM), Evans Blue (blue; K_D = 1102 ± 107 µM), and Trypan Blue (red; K_D = 2454 ± 415 µM). The IC_{50} of each inhibitor respectively is 22 µM, 750 µM, and 1000 µM.[1] The combined standard curves demonstrate that the affinity ranking of the TNFα and inhibitor interactions generated with Agile R100 replicate the previously-published IC_{50} rank order.

Table 3: The K_D values for SPD304, Evans Blue, and Trypan Blue interacting with TNFα as measured by Agile R100 are shown below. For comparison versus K_D, previously-reported IC_{50} values are also shown below.

	K_D (µM)	IC_{50}[1,2] (µM)
SPD304	3.25 ± 0.659	22
Evans Blue	1102 ± 107	750
Trypan Blue	2454 ± 415	1000

DMSO Measurements

The TNFα and SPD304 interaction is further evaluated using a standard curve with assay buffer containing 0% DMSO, 1% DMSO, 3% DMSO, and 10% DMSO (Figure 6). **The K_D values determined in buffer containing DMSO are within a factor of 1.6 of the K_D value without DMSO.** Agile R100 reproducibly senses interactions in buffers containing DMSO because solvents do not impact the electrical FEB technique or cause large background noise on the system.

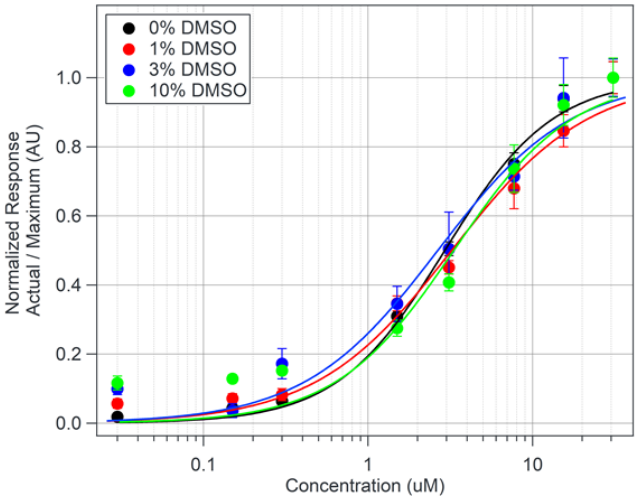

Figure 6: Standard curve generated by Agile R100 for TNFα interacting with SPD304 in assay buffer containing 0% DMSO (black), 1% DMSO (red), 3% DMSO (blue), and 10% DMSO (green).

CONCLUSIONS

In this application note, we demonstrate that Agile R100 is a powerful platform for affinity ranking studies during hit validation. The system delivers kinetic binding rates (k_{on} and k_{off}) in real-time and generates affinity data that rank order in line with published studies, without interference from labels or background noise from solvents. FEB provides a next-generation mechanism to gather comprehensive kinetic binding data over a large dynamic range (nM to mM), enabling complete and cost-effective characterization of drug candidates. As a result, Agile R100 shows promise as a groundbreaking orthogonal sensing platform to streamline and improve drug discovery efforts.

REFERENCES

1. Song Y, Buchwald P. TNF Superfamily Protein–Protein Interactions: Feasibility of Small-Molecule Modulation. *Curr Drug Targets*. 2015;16(4):393-408. doi:10.1038/nbt.3121.ChIP-nexus.
2. He MM, Smith AS, Oslob JD, et al. Small-Molecule Inhibition of TNF- a. *Sci Reports*. 2005;310(5750):1022-1025. doi:10.1126/science.1116304.
3. GE Healthcare Life Sciences. Biacore Assay Handbook. *GE Healthc Bio-Sciences AB*. 2012:1-78.
4. Lerner MB, Nokes J. Solvent Correction versus In-line Reference Measurement. 2018:1-5.
5. Xu G, Abbott J, Qin L, et al. Electrophoretic and field-effect graphene for all-electrical DNA array technology. *Nat Commun*. 2014;5:1-9. doi:10.1038/ncomms5866.
6. Lerner MB, Pan D, Gao Y, et al. Large Scale Commercial Fabrication of High Quality Graphene-Based Assays for Biomolecule Detection. *Sensors Actuators B Chem*. 2016;(Cvd). doi:10.1016/j.snb.2016.09.137.
7. Lerner MB, Matsunaga F, Han GH, et al. Scalable Production of Highly Sensitive Nanosensors Based on Graphene Functionalized with a Designed G Protein-Coupled Receptor. *Nano Lett*. 2014;14(5):2709-2714. doi:10.1021/nl5006349.
8. Goldsmith BR, Coroneus JG, Khalap VR, Kane A a, Weiss G a, Collins PG. Conductance-controlled point functionalization of single-walled carbon nanotubes. *Science*. 2007;315(5808):77-81. doi:10.1126/science.1135303.
9. Bergveld P. The development and application of FET-based biosensors. *Biosensors*. 1985;2(1):15-33. doi:10.1016/0265-928X(86)85010-6.
10. van der Schoot BH, Bergveld P. ISFET based enzyme sensors. *Biosensors*. 1987;3(3):161-186. doi:10.1016/0265-928X(87)80025-1.
11. Kergoat L, Piro B, Berggren M, Horowitz G, Pham MC. Advances in organic transistor-based biosensors: From organic electrochemical transistors to electrolyte-gated organic field-effect transistors. *Anal Bioanal Chem*. 2012;402(5):1813-1826. doi:10.1007/s00216-011-5363-y.
12. Viswanathan S, Narayanan TN, Aran K, et al. Graphene-protein field effect biosensors: Glucose sensing. *Mater Today*. 2015;18(9):513-522. doi:10.1016/j.mattod.2015.04.003.
13. Allen BL, Kichambare PD, Star A. Carbon nanotube field-effect-transistor-based biosensors. *Adv Mater*. 2007;19(11):1439-1451. doi:10.1002/adma.200602043.
14. Nokes J, Afsahi S, Pan D, Barron FE. Detecting Interactions of Small Molecules in DMSO Using Agile R100. 2016:1-7.
15. Nero TL, Morton CJ, Holien JK, Wielens J, Parker MW. Oncogenic protein interfaces: small molecules, big challenges. *Nat Rev Cancer*. 2014;14(April):248-262. doi:10.1038/nrc3690.
16. Hoelder S, Clarke PA, Workman P. Discovery of small molecule cancer drugs: Successes, challenges and opportunities. *Mol Oncol*. 2012;6(2):155-176. doi:10.1016/j.molonc.2012.02.004.
17. Arkin MR, Wells J a. Small-molecule inhibitors of protein-protein interactions: progressing towards the dream. *Nat Rev Drug Discov*. 2004;3(4):301-317. doi:10.1038/nrd1343.
18. Eck MJ, Sprang SR. The structure of tumor necrosis factor-a at 2.6A resoolution: Implications for receptor binding. *J Biol Chem*. 1989;264(29):17594-17605.
19. Leung C, Chan DS, Kwan MH, et al. Structure-Based Repurposing of FDA-Approved Drugs as TNFa Inhibitors. *ChemMedChem*. 2011;6:765-768. doi:10.1002/cmdc.201100016.
20. Papaneophytou CP, Mettou AK, Rinotas V, Douni E, Kontopidis GA. Solvent Selection for Insoluble Ligands, a Challenge for Biological Assay Development: A TNF- α /SPD304 Study. *ACS Med Chem Lett*. 2013;4:137-141. doi:10.1021/ml300380h.
21. Pollard TD. A guide to simple and informative binding assays. *Mol Biol Cell*. 2010;21(23):4061-4067. doi:10.1091/mbc.E10-08-0683.

For questions, please contact techsupport@nanomedical.com.