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Enhancing assay performance using nanoscale detection

By: Yli Remo P. Vallejo, Jing Li, and Ray Yin
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A detection technology can enhance the sensitivity of ELISAs and lateral flow assays while reducing the total amount of reagents used.

Most immunodiagnostic and nucleic acid detection methods have in common the use of a solid surface onto which a bioactive reagent is coated, such as an antibody, antigen, or oligomeric probe. Such biomolecules are complex long-chain structures possessing optimal three-dimensional conformation with specific loci for binding sites or epitopes. However, the conventional deposition of bioactive molecules onto solid surfaces is an inefficient and random process. For example, while antibodies can easily be coated onto a plastic surface, only a subset of the deposited molecules will have the Fab binding regions available for reaction (see Figure 1). Such randomness limits both the assayís binding capacity and sensitivity that depend on conventional coating technology.

The Nano-Intelligent Detection System (NIDS) technology by ANP Technologies Inc. (Newark, DE) has been designed to control the orientation of bioactive molecules at the nanoscale level by using a polymeric scaffold covalently conjugated to a binder such as an antibody.1 Within this conjugate structure, the polymeric scaffold preferentially attaches itself to the solid surface and extends the antibody away from the surface for an advantageous immunoreaction. The conjugate lends itself to a self-assembled array of nano-oriented antibody molecules on the surface. The result is a coated surface that exhibits greater binding efficiency. The benefits of such orientation control and self-assembly can be extended to any bioactive molecule.

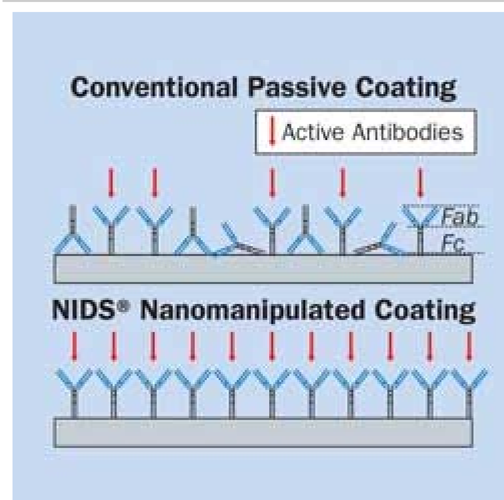

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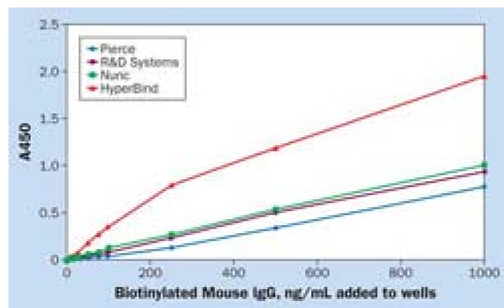
Figure 1. Orientation of antibodies is random in conventional coating and optimally oriented with the NIDS nano-orientation technology (click image to enlarge).

The NIDS technology has been applied to the precoating of microwell plates to create a series of highly sensitive tools that are available for use in drug discovery and preclinical and clinical studies. ANP Technologies has developed NIDS-activated streptavidin (SA)- and neutravidin (NA)-coated plates called HyperBind. Biotinylation of antibodies and other proteins is a common procedure that, when combined with HyperBind plates, delivers to users assays with greater sensitivity and reduced reagent usage.2 This enables researchers to receive the benefits of NIDS nanomanipulation without performing the required complex synthetic procedures. HyperBind plates are available in clear, black, and white formats for colorimetric, fluorometric, and chemiluminescent assay formats, respectively.

HyperBind plates have been compared to many other commercial plates. The nanoscale orientation achieved by conjugating streptavidin or neutravidin to the anchoring polymeric scaffold has led to performance enhancements.

Direct Binding of Biotinylated Proteins



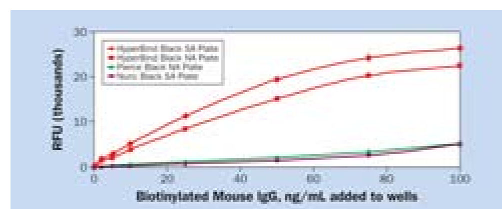
[2]

Figure 2. The binding efficiency of HyperBind clear plates is greater than other commercial plates (click image to enlarge).

HyperBind SA plates have demonstrated greater binding efficiency for biotinylated proteins than other commercial SA plates (see Figure 2). In a representative experiment, 100 µL of increasing concentrations of biotinylated mouse IgG antibody were added to the wells of a HyperBind plate, a Nunc Immobilizer, a Pierce HBC Reacti-Bind, and an R&D Systems EvenCoat plate, and incubated for one hour on a platform shaker. After washing, 100 µL of rabbit anti-mouse IgG conjugated to horseradish peroxidase (HRP) were added to the wells and allowed to incubate for one hour and a half on a platform shaker. Following the wash step, a substrate/chromogen reagent consisting of hydrogen peroxide and tetramethylbenzidine(TMB) was added to the wells. Color was allowed to develop for 20 minutes and stopped with 2N sulfuric acid, and the absorbance was measured at 450 nm.

A similar comparison was performed with the black HyperBind SA and NA plates, which are used for fluorescence-based assays. Raw polystyrene black microplates were coated with streptavidin and neutravidin using the NIDS chemistry optimized for the clear plates. The binding efficiency of the HyperBind plates was shown to be better than the Pierce Reacti-Bind and Nunc Immobilizer black plates when using the test procedure with biotinylated mouse IgG that was previously described and a fluorescent enzyme substrate reagent (see Figure 3).

White plates were also treated with NIDS-activated streptavidin for use in chemiluminescence-based assays. The binding efficiency of the HyperBind plates was also shown to be better than the Nunc Immobilizer white plate when using the test procedure with biotinylated mouse IgG that was previously described and a chemiluminescent substrate reagent.



[3]

Figure 3. Binding efficiency of HyperBind black SA and NA plates is better than commercial plates (click image to enlarge).

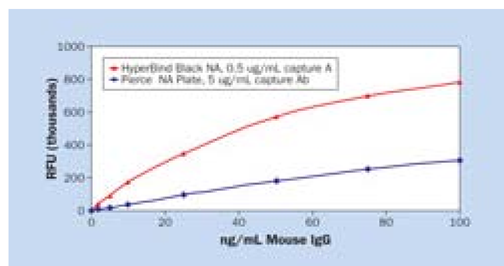
The conclusion derived from the above experiments is that for a given concentration of biotinylated antibody added to the microwells, more protein can be bound by the HyperBind clear, black, and white plates than the Pierce, R&D Systems, and Nunc plates. This finding can translate to better assay performance and reductions in reagent use and cost.

Performance in Sandwich ELISAs

To determine the effect that nano-orientation of antibodies has on the sensitivity and signal range of a sandwich assay, HyperBind plates were compared to commercial plates using a model sandwich assay. In the following example, HyperBind black NA plates were compared to commercial black Reacti-Bind NA plates by Pierce Chemical using a fluorometric sandwich enzyme-linked immunosorbent assay (ELISA) to detect mouse IgG.

In a preliminary experiment, the Pierce plate achieved the highest level of direct binding of biotinylated antibody when coated at a concentration of 5 µg/mL. The HyperBind plateís optimal direct binding concentration was 0.5 µg/mL, or 10-fold less than the Pierce plate.

Therefore, a biotinylated sheep anti-mouse antibody was coated on each plate at their optimal concentrations: 5 µg/mL for the Pierce plate and 0.5 µg/mL for HyperBind. The assay was performed by adding 100 µL of increasing concentrations of mouse IgG in PBS/0.1% BSA to each well and incubating all samples for one hour at room temperature on a shaker, followed by a wash step. A goat anti-mouse-horseradish peroxidase (GAM-HRP) detector reagent was added to each well and allowed to incubate for half an hour. After washing, QuantaBlu substrate reagent by Pierce Chemical was added to all wells, and the fluorescence was measured after one hour at an excitation wavelength of 325 nm and emission wavelength of 420 nm.



[4]

Figure 4. Sandwich ELISA for mouse IgG using HyperBind and the Pierce Neutravidin (NA) plates. HyperBind black plates were coated at 0.5 µg/mL capture Ab and the Pierce black plate at 5.0 µg/mL. HyperBind plates generated greater signal and sensitivity while using only 10% of the antibody required by other commercial plates (click image to enlarge).

Even at its optimal coating concentration, the Pierce NA plate generated lower signal and sensitivity compared with HyperBind plates (see Figure 4). The higher direct binding efficiency of HyperBind plates can result in assays with greater sensitivity and increased signal dynamic range compared to conventional plates.

Specific ELISAs

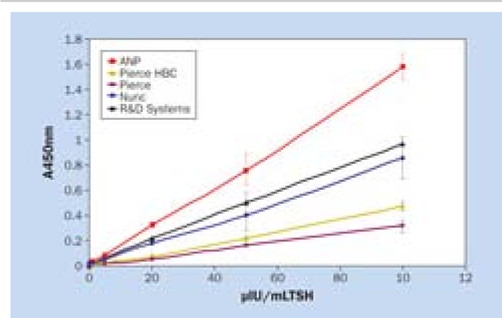
A number of ELISAs for various physiological markers have been developed using HyperBind SA-coated plates. For example, an ELISA for thyroid stimulating hormone (TSH) was performed using the biotinylated capture antibody coated on HyperBind and four commercial SA plates. The same anti-TSH detector antibody-HRP conjugate and the same incubation times were used for all of the plates. The assay that used HyperBind SA plates developed the best signal range and sensitivity among all five assays (see Figure 5).

HyperBind plates have also been successfully used in nucleic acid detection assays.

Double-Antigen-Bridging Immunogenicity Assays

The therapeutic use of protein drugs such as recombinant proteins, peptides, antibodies, binding receptors, and nucleic acids such as DNA and RNA/RNAi has proliferated during the last two decades. Such large molecules have created the need to determine if such inherently immunogenic agents generate adverse host reactions and side effects.

Developers of such large molecule therapies have minimized their potential immunogenicity by humanizing their structures, cloaking immunogenic sites, or developing chimeric constructs by combining nonimmunogenic human sequences with the active sites. However, despite such measures, patients or a subset of patients could still develop an immune reaction to the administered drug, rendering it ineffective or potentially life threatening.3,4



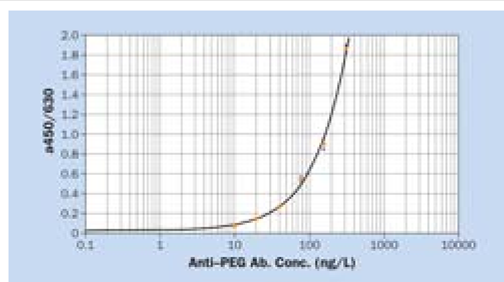
[5]

The degree to which large protein or nucleic acidñbased drugs can elicit an antibody response in patients is a major consideration in the acceptance and approval of these new therapies. Extensive immunogenicity studies are therefore required in the course of drug development.

Immunogenicity assays for detecting circulating anti-drug antibodies (ADA) have become a necessary tool in drug development. Many immunogenicity assays have been developed using HyperBind plates for therapeutic peptides and proteins ranging in molecular weight from 3ñ200 kDa. Such immunogenicity assays are serological double

antigen bridging immunoassays in which the ADA form bridge complexes with two drug molecules. One drug molecule acts as a capture and is immobilized on the microplate. The other drug molecule acts as a detector and is conjugated to a signal-producing moiety, such as an enzyme.

Figure 5. Comparison of ELISAs for thyroid stimulating hormone (TSH) using HyperBind and other commercial plates. The HyperBind assay showed better signal range and sensitivity (click image to enlarge).



[6]

Figure 6. Dose response curve for a double antigen bridging immunogenicity assay to detect PEG-specific anti-drug antibody (click image to enlarge).

Figure 6 presents an example of a dose-response curve of an immunogenicity assay for polyethylene glycol (PEG) polymer, which is often attached to biologic drugs to improve in vivo stability and delivery.5 The assay curve was generated using an anti-PEG mouse monoclonal IgM antibody diluted to the levels shown. In this assay design, the capture drug molecule is labeled with biotin and is immobilized on the streptavidin-coated HyperBind plate. The detector drug molecule is labeled with HRP.

Other Applications

At its inception, the NIDS technology was initially applied to lateral flow assays. NIDS conjugates of antibodies have been coated on various particles such as gold, latex, magnetic beads, and various other commercial particles. In most cases, improvements in sensitivity of up to 100-fold have been observed. A small sample of such improvements is presented in Table I. [7]

Target	NIDS Lateral Flow Assay Sensitivity	Conventional Lateral Flow Assay Sensitivity
Salmonella	1 x 10[illegible] cfu/mL	5 x 10[illegible] cfu/mL
E. coli	6 x 10[illegible] cfu/mL	5 x 10[illegible] cfu/mL

Table I. Sensitivity of NIDS lateral flow assays compared to conventional lateral flow assays.

An additional benefit of the NIDS technology is its ability to accommodate multiplexing in lateral flow assays. Although many multiplexed lateral flow assays are available for detecting drugs of abuse, these assays all use competitive assay formats that require limiting the amounts of reagents. For example, in the case of sandwich assays in which sensitivity is achieved with high concentrations of capture and detector antibodies, multiplexing is more challenging since the capacity of the lateral flow assay pads for on-board reagents is very limited. Multiplexing using conventional methods is achieved by using less labeled detector antibody per target, thereby reducing the sensitivity attainable by each sandwich assay in the multiplex and generating a significant high-dose hook effect (a false negative result in the presence of high concentrations of the target analyte).

ANP Technologies has combined assays for biological agents and other analytes in 5-plexed and 10-plexed formats, which maintain each assayís sensitivity but more importantly avoid hook effects at high concentration ranges. This is an important advantage for first responders dealing with suspicious biowarfare-related white-powder events. To remove operator bias in interpreting lateral flow tests, ANP has developed compact handheld readers that measure the intensity of test lines based on image analysis using grayscale contrast to deliver a positive or negative answer, or a quantitative result (see Figure 7).

[8]

Figure 7. The NIDS handheld reader (left) for on-site rapid

A Quantitative Lateral Flow Assay

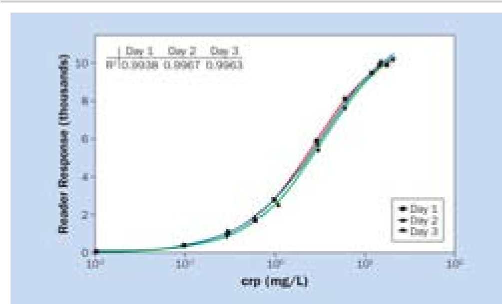
C-reactive protein (CRP) is an acute-phase plasma protein that is produced by the liver and adipocytes as a non-specific response to infectious and non-infectious inflammation. A wide-range CRP test is useful in detecting non-specific inflammation and to differentiate between viral and bacterial infections. Baseline CRP levels from individuals who are not suffering from inflammation or infection are used to assess the risk of future atherosclerosis and other cardiovascular diseases in such asymptomatic individuals. Recent studies have generated clinical data that support CRP as the best

qualitative lateral flow assay testing and the medical reader (right) for point-of-care quantitative lateral flow assays (click image to enlarge).

prognostic risk factor for heart disease. For cardiac health risk assessment, a high-sensitivity CRP (hsCRP) assay is required.6,7

Lateral Flow Assay Design. The NIDS hsCRP rapid test is a sandwich lateral flow immunoassay using mouse monoclonal antibodies to CRP as capture and detector reagents. A test-valid control line consisting of a goat anti-mouse antibody is sprayed above the test line. The intensity of the test line, which is measured with the NIDS medical reader ten minutes after adding 100 µL of sample to the test strip is directly proportional to the concentration of CRP in fingerstick whole blood (see Figure 8). The assay is calibrated against international standard CRM 470.

The medical reader uses a test-strip lot-specific standard curve that is uploaded to and stored in its memory. Using this standard curve, a quantitative result is generated for each sample that is tested based on the intensity of the test line.



[9]

Figure 8. Reproducibility of the NIDS hsCRP lateral flow assay standard curve over a three day period (click image to enlarge).

Stored Standard Curve Stability and Precision. The stability and reproducibility of the standard curve are demonstrated in Figure 8. A standard curve was run on three separate days, with each standard run in triplicate. The results showed good reproducibility of the standard curve for each day.

The precision of the assay during a 21-day period was evaluated by running three human serum sample pools twice a day. These pools contained 0.5, 1, and 3 mg/L CRP, respectively. The results of this study are summarized in Table II.

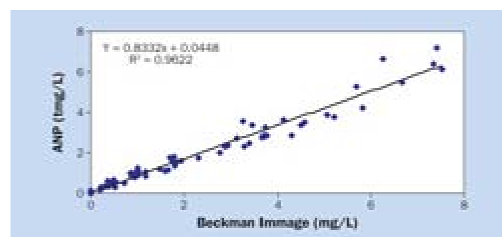
Human Serum Pool	CRP (mg/L)	%CV
A	0.5	8.6
B	1.0	7.8
C	3.0	9.8

Table II. 21-day interassay precision of the NIDS hsCRP quantitative lateral flow assay

[10] **Assay Accuracy.** The NIDS CRP quantitative lateral flow assay showed good correlation with the Beckman Immage CRP assay for centralized laboratories. The results are shown in Figure 9. Good dilution linearity was also observed with this assay.

Interfering Substances. The susceptibility of the hsCRP test to potential interferences that were found in circulation was tested by spiking each of these substances to a final concentration of double their normal maximum concentration into three human serum pools. These pools nominally contained 0.03 (a blank-stripped serum), 1, and 3 mg/L CRP, respectively. Each spiked sample was run in duplicate. No significant interference was found with bilirubin, triglycerides, hemoglobin, or lactate. Glucose, ascorbic acid, uric acid, urea, creatinine, potassium, albumin, and gamma globulins were at twice their maximum normal concentrations in circulation.

Hook Effect. No high-dose hook effect was observed up to an extreme concentration of 500 mg/L CRP in serum.



[11]

Figure 9. Correlation of the NIDS CRP quantitative lateral flow assay

Conclusion

The NIDS technology is based on the premise that biological binding agents such as antibodies will work optimally if their active sites are properly oriented. Its success in accomplishing this simple but challenging task has led to the development of new tools for researchers and product developers. The HyperBind line of activated microwell plates is an improvement over current commercial products, reducing the amount of reagents needed for assays by more than

with the Beckman Immage centralized laboratory test (click image to enlarge).

90% while still enhancing sensitivity. The versatility of these plates extends to sandwich and competitive ELISAs in colorimetric, fluorometric, and chemiluminescent formats, and nucleic acid detection assays. The versatility of the NIDS technology encompasses ELISAs, quantitative lateral flow assays, and any assay that uses a solid surface.

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Yli Remo Vallejo, PhD, is vice president for research and product development at ANP Technologies Inc. (Newark, DE). He can be reached at yli@anptinc.com [12]

Jing Li, PhD, is director for assay development at ANP Technologies Inc. (Newark, DE). She can be reached at lijing@anptinc.com [13]

Ray Yin, PhD, is the founder, president, and chief technical officer at ANP Technologies Inc. (Newark, DE). He can be reached at ray@anptinc.com [14].

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