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Appraisal of State-of-the-Art

Comparison of the NIDS® rapid assay with ELISA methods in immunogenicity testing of two biotherapeutics

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ABSTRACT

Introduction: Rapid lateral flow immunogenicity assays for the detection of anti-drug antibodies (ADAs) to two biotherapeutic antibodies, an anti-HER2 antibody and an anti-TNF-α antibody, were developed using ANP Technologies, Inc.'s proprietary Nano-Intelligent Detection System (NIDS®) and compared to their ELISA counterparts. **Methods:** Biotin and hapten-labeled drugs are incubated with the patient serum sample to allow ADA to form a bridge complex with each drug conjugate. The reaction mixture is then added to a test strip with an anti-hapten capture zone which captures the mixed bridge complex. The bridge-complexed biotinylated drug then reacts with streptavidin-labeled gold particles *in situ*. The signal developed at the capture zone, which is directly proportional to ADA in the sample, is then quantitatively measured with a handheld reader. The counterpart ELISAs were run using the same reagents. Dose–response, specificity/free drug depletion, and screening cut-point assays were performed using both methods. **Results:** The rapid assays' performance compare very closely to their ELISA counterparts'. Both types of assays identified the same positive samples in screening a limited population of 50 normal serum samples for the anti-HER2 antibody. In the case of anti-TNF-α, both assays identified the same positive samples out of 50 normal and 20 rheumatoid arthritis patient serum samples but differed in the assessment of two others. The rapid assay correctly identified as negative an ELISA false positive sample, and correctly tested as positive an ELISA false negative sample. Positive results were verified with a specificity/free drug depletion assay. **Discussion:** The NIDS® rapid immunogenicity assay offers distinct advantages over current methods in simplicity, low cost, and short time to result. More importantly, the method requires no sample dilution and no washing steps which can perturb fragile complexes formed by low-affinity ADAs. Thus, the assay can potentially detect ADAs with various affinities.

1. Introduction

Assays to detect circulating anti-drug antibodies (ADAs) have become a necessary tool in biotherapeutics development since such therapies carry with them the potential to elicit an immune response. The potential immunogenicity of such large molecules have been minimized by humanizing their structures, cloaking immunogenic sites or developing chimeric constructs of non-immunogenic human sequences with active sites derived from the original animal sequences. In spite of these measures, the potential still exists for patients or a subset of patients to develop an immune reaction to the administered drug, rendering it ineffective or potentially life-threatening (Koren, Zuckerman & Mire-Sluis, 2002; Porter, 2001). Immunogenicity testing is therefore a critical step in the regulatory acceptance of these new therapies.

Anti-drug immune responses can result in antibodies with a wide range of affinities and avidities. Both low and high affinity ADAs must be detectable by immunogenicity assays since an immune patient's ADAs are typically low in titer, affinity and avidity in the initial phases of treatment before developing into stronger binding immunoglobulins of higher titer. Microtiter plate-based enzyme-linked Immunosorbent assays (ELISAs) are most widely used to measure ADA responses. ELISAs are not only labor-intensive and time-consuming, but also require both sample dilution and washing steps, which can detach low affinity anti-drug antibodies from drug molecules making them undetectable. Thus, ELISAs are less useful to detect low affinity antibodies in clinical settings.

Recently, electrochemiluminescence assays based on Mesoscale Discovery's (MSD) platform have gained greater attention. While the

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single step format is not unique to MSD's technology, potentially lower background and reduced wash steps compared to ELISA has made it a method of choice for immunogenicity screening in spite of its requirement for costly instrumentation.

Surface Plasmon Resonance (SPR)-based assays have also gained use for the detection of anti-drug antibodies. Requiring no wash steps or reagent labeling, this method is recognized to be superior to ELISAs in detecting low-affinity binding events (Lofgren et al., 2007)]. However, the SPR-based assay often exhibits much lower assay sensitivity (i.e., about 1 μg/mL) and low throughput with significantly higher instrument acquisition and operating costs. In addition, the SPR assay still requires sample dilution, which may result in the indication of a negative response when patients may actually possess low, but clinically relevant, levels of antibodies.

In order to address these technical challenges, a rapid immunogenicity bridging assay format that does not require any sample dilution and washing steps has been developed. This assay method has been demonstrated to successfully detect ADAs to several large molecule drugs including peptides and antibodies, as well as ADAs to polyethylene glycol (PEG) moieties of PEGylated drugs (Pan, Small, Qin, Vallejo & Yin, 2010; Pan, Pauley et al., 2010). These novel rapid immunogenicity assays are based on the Nano-Intelligent Detection System (NIDS®), a nanomanipulation technology platform for the detection of various protein, oligonucleotide, pathogen, and small molecule targets in different liquid matrices for both biodefense and rapid medical diagnostic applications (Hydutsky et al., 2005; Knapp, Gibbs, Pan & Yin, 2005; Knapp, Odom et al., 2005; Li et al., 2008). Nanomanipulation refers in this case to the ability to orient capture and detector portions of the molecule at nanoscale, thus enabling the highest signal to noise ratio. A conceptual illustration of the technology applied to an immunoassay is shown in Fig. 1, where the Fab region of the immobilized capture antibody is oriented to an optimal configuration for antigen–antibody interactions. In addition, this method also allows the antibodies to undergo a self-assembly process that creates a dense array of oriented antibodies on surfaces, thus significantly increasing the assay sensitivity and linear dynamic range, as well as reducing the false positive responses from non-specific binding (Hopkins et al., 2008).

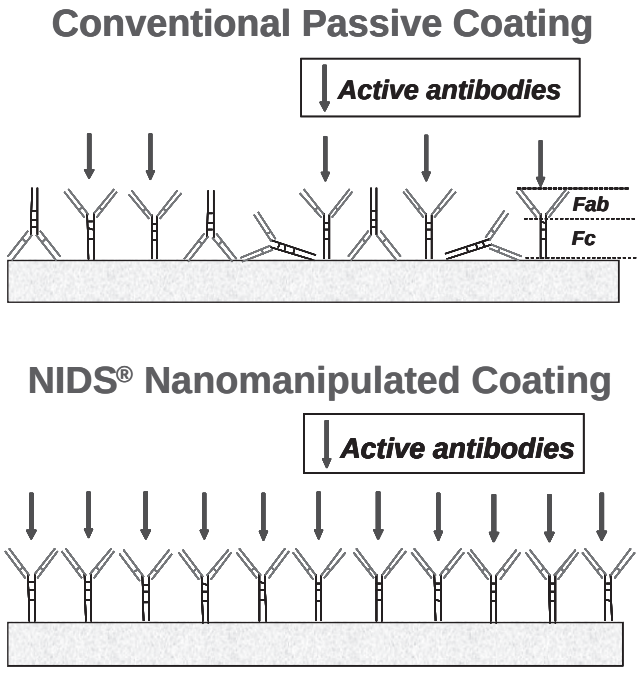

Fig. 1. Orientation of antibodies is random in conventional coating and optimally oriented with NIDS® nano-orientation.

The basic elements of a NIDS® rapid immunogenicity test consist of a test strip and a handheld reader to measure the signal produced on the test strip. The use of a reader allows quantitative data to be generated. Due to the use of nanomanipulation, the high dose hook effect often seen in traditional lateral flow assays has been significantly reduced, thus making the NIDS® assay format ideal for immunogenicity testing. To remove operator bias in the interpretation of lateral flow tests, compact handheld and desktop readers have also been developed for NIDS® rapid assays to measure the intensity of test lines based on image analysis using grayscale contrast to deliver a positive or negative answer or a quantitative result. These readers are shown in Fig. 2.

The NIDS® rapid assay requires no sample dilution and no wash steps, thus enabling it to avoid the pitfalls and shortcomings of existing immunogenicity assay methods such as ELISA and SPR-based assays. With no sample dilution required, rapid assays can deliver improved sensitivity and with no washing steps, there is no disruption of weak immune complexes created by low affinity and low avidity ADAs, particularly at low concentrations. Rapid assays achieve test results with less user manipulation and at lower instrument and reagent cost than ELISAs or SPR-based assays. In order to demonstrate that rapid immunogenicity assays match the level of accuracy and utility of accepted methods, rapid assays were compared to their ELISA counterparts for the detection of ADAs against two established therapeutic antibodies, the first against human epidermal growth factor receptor 2 (HER2) and the second against tumor necrosis factor alpha (TNF-α).

The anti-HER2 antibody is a humanized monoclonal antibody against human epidermal growth factor receptor 2, which promotes the proliferation of cancer cells in 20% of breast cancer patients (Hudis, 2007). Anti-TNF-α antibody is a mouse-human chimeric monoclonal antibody which inhibits TNF-α, a key cytokine in autoimmune disease (Van den Brande et al., 2003). Anti-TNF-α causes apoptosis of TNF-α-expressing activated T-lymphocytes, an important cell type mediating inflammation.

Immunogenicity assays in ELISA format were developed concurrently as the rapid immunogenicity assays for anti-HER2 and anti-TNF-α. In each case, the same positive control antibodies and the same drug conjugates were used in the ELISA and rapid immunogenicity assays.

2. Methods

2.1. Materials

Hapten- and biotin-conjugated target molecules (anti-HER2 antibody, and anti-TNF-α antibody), streptavidin-coated gold particles, rapid assays and the handheld reader were all produced by ANP Technologies, Inc.

Anti-HER2 antibody and affinity purified rabbit polyclonal antibodies against anti-HER2 were supplied by Merck & Co. Calibrators were prepared by serial dilution of affinity purified rabbit antibody to anti-HER2 in a normal human serum pool at 2000, 1000, 500, 250, 125, 62.5, 31.3, 15.6 and 0 ng/mL.

Anti-TNF-α and affinity purified rabbit polyclonal antibody against anti-TNF-α were acquired from Merck & Co. Calibrators were prepared by serial dilution of affinity purified rabbit polyclonal antibodies to anti-TNF-α in a normal human serum pool at 2000, 1000, 500, 250, 125, 62.5, and 0 ng/mL.

For the Immunogenicity ELISAs, horseradish peroxidase (HRP)-conjugated mouse anti-hapten (digoxigenin) was prepared by ANP Technologies, Inc. Standard HyperBind® streptavidin-coated plates and assay diluents were manufactured ANP Technologies, Inc. Tetramethylbenzidine/Peroxide Substrate Reagent (TMB) was purchased from Moss Inc. Stop solution (Sulfuric acid, 2.0 N) was obtained from VWR, Inc.

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Fig. 2. The NIDS® handheld (left) and medical (right) readers for the quantitative measurement of rapid IM assays.

2.2. Rapid assay method optimization

The rapid assay method for each target was optimized by testing two assay configurations, the first with the drug directly coated on the capture line, the second with the hapten-labeled drug captured *in situ* by an anti-hapten antibody coated on the capture line. In both cases, the biotinylated drug which forms the other half of the bridging complex would be labeled *in situ* by streptavidin-coated gold particles used. The best sensitivity and dynamic range were achieved with the second design. The serum sample and the hapten-labeled and biotinylated drugs were allowed to react in a separate receptacle prior to adding an aliquot of the reaction mixture to the test strip which contains mobilazable streptavin-labeled gold particles on a pad and the immobilized mouse anti-hapten antibody on the capture zone. The concentrations of the labeled drugs were also optimized to attain the best sensitivity and signal dynamic range. The reaction time was also optimized for the pre-incubation step with the labeled drug conjugates and the sample.

2.3. ELISA method optimization

The ELISA method for each of the targets was also optimized by testing two possible capture configurations, with the drug directly coated on the plate or the biotin-labeled drug coated on a streptavidin-activated plate. The signal dynamic range obtained with the latter was superior and the streptavidin plate coated with the biotinylated drug was used for both targets. The hapten (digoxigenin)-labeled drug which forms the other half of the bridging complex is reacted with an HRP-labeled anti-digoxigenin monoclonal antibody in a subsequent step. The concentrations of the labeled drugs and assay incubation times were also optimized to attain the best sensitivity and signal dynamic range. The assay method wherein the serum sample was preincubated with hapten-labeled and biotinyalted drug then added to a well coated with either streptavidin or the mouse anti-hapten antibody did not yield the best sensitivity and dynamic range.

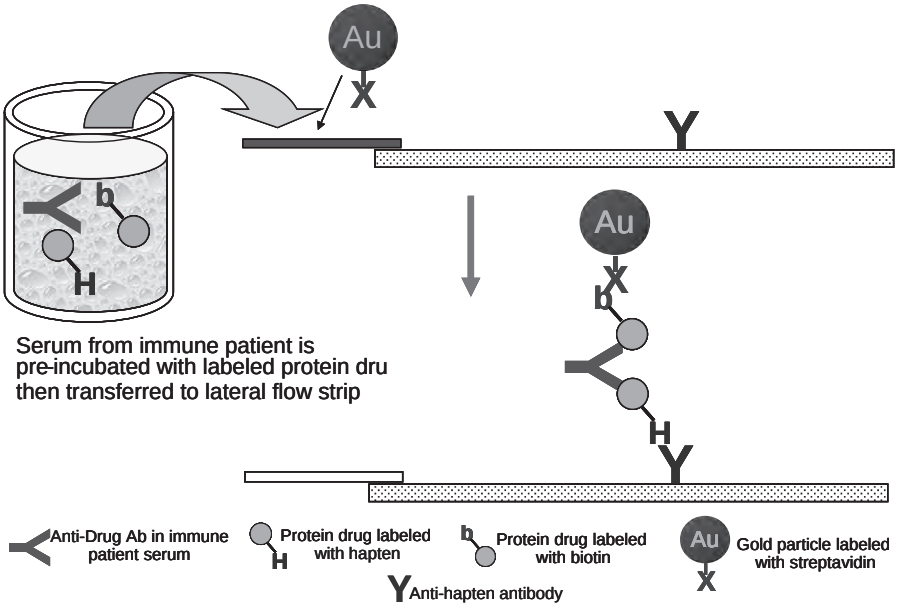

Fig. 3. NIDS® double antigen bridging immunogenicity assay with preincubation. This format will measure the total antibody response in the patient serum which includes both low and high affinity and avidity ADAs without any sample dilution and washing steps.

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2.4. Rapid immunogenicity assay procedure

The assay uses a preincubation step to allow the ADA in serum samples to react with drug covalently linked to biotin (b) and with drug covalently linked to an organic hapten (H) (Fig. 3). Both labeled reagents are allowed to react with samples in liquid phase resulting eventually in ADAs bridging the two labeled drug reagents. The preincubation step can be of several hours duration, preferably until equilibrium conditions are achieved.

The reaction mixture is then added to a NIDS® test strip containing releasable streptavidin-coated gold particles dried on an attached pad at one end and a fixed test zone consisting of anti-hapten antibodies immobilized on the membrane. The reaction mixture reconstitutes and releases the dried gold particles and migrates by capillary action with these particles down the length of the membrane to the test zone.

The anti-hapten antibodies in the test zone will bind the hapten attached to the mixed bridged complexes formed by the ADA. The biotin attached to the captured bridge complex will then be labeled by the streptavidin-coated gold particles *in situ*. The result is a visible red line formed at the test zone. The intensity of this line is directly proportional to the level of ADA in the test sample and is measured by the reader. This signal is compared to a screening cut-point value to determine if the test sample is positive or negative.

To perform a rapid assay, each of the two drug conjugates (hapten- and biotin-labeled) was spiked into calibrators, controls, and samples to a final concentration of 0.1 μg/mL of each and incubated at 4 °C for 20 min. A 100 μL aliquot of each spiked reaction mixture was applied to an assay strip. Each assay strip was allowed to develop and was read on the reader at the 25th minute.

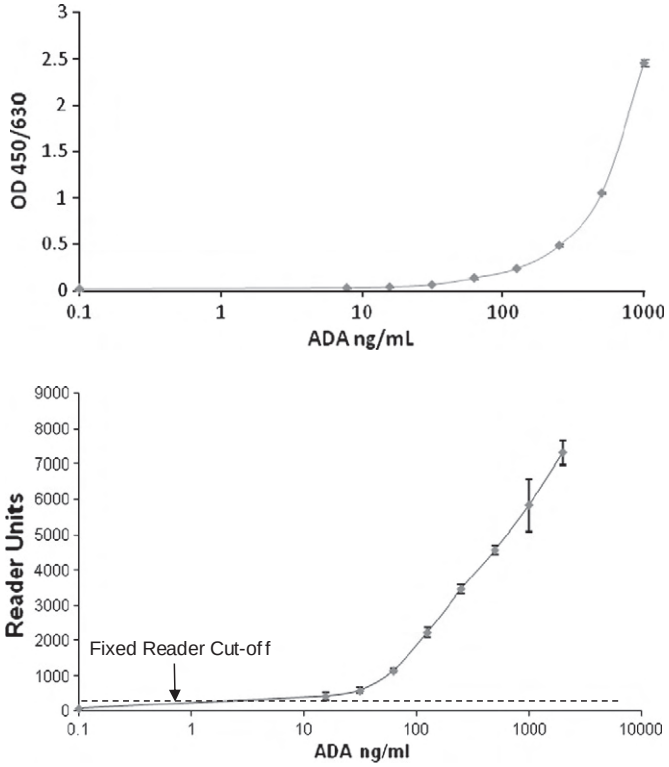

Fig. 5. Comparison of dose–response curves of immunogenicity assays in ELISA (top) and rapid assay (bottom) formats for the detection of ADA to anti-HER2 antibody.

2.5. ELISA procedure

The ELISA methods developed for the detection of ADAs to anti-HER2 and anti-TNF-α antibodies use a conventional double antigen bridging format. The same labeled reagents used in the rapid assay were utilized, the protein drug covalently linked with biotin (b) and the same drug covalently attached to an organic hapten (H). The biotinylated drug was directly coated onto a HyperBind® standard streptavidin-coated 96-well microplate. ADAs in the patient serum form bridging complexes wherein one binding site binds the coated biotinylated drug, while the other binds the hapten-labeled drug, thus immobilizing it. A horseradish peroxidase (HRP) conjugated mouse anti-hapten antibody was then allowed to react with the bridging complexes containing the hapten-labeled drug followed by a development step with (TMB). The intensity of the color developed by the immobilized HRP was directly proportional to the concentration of ADA in the patient sample. The design of these ELISAs is shown in Fig. 4.

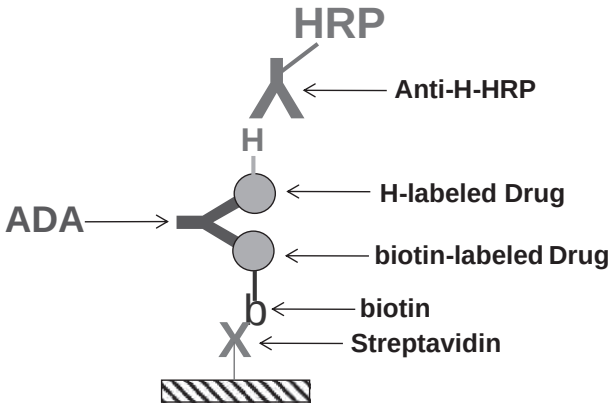

Fig. 4. Assay schematic for the immunogenicity ELISAs. H = Hapten, HRP = horseradish peroxidase.

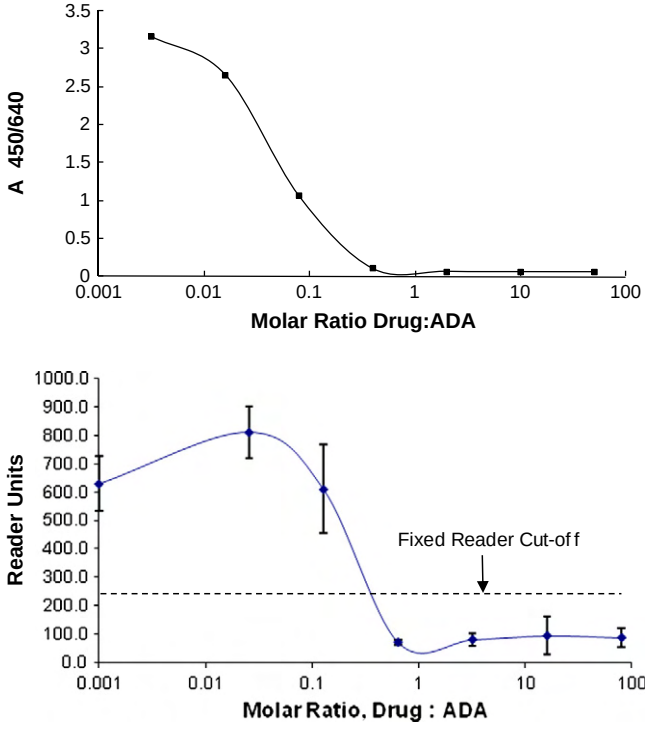

Fig. 6. Comparison of drug depletion profiles of immunogenicity assays in ELISA (top) and rapid assay (bottom) formats for the detection of ADA to anti-HER2 antibody.

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To perform the immunogenicity ELISA, equal volumes of calibrators, QC and patient samples and 1.0 μg/mL H-Drug conjugate solution were mixed and incubated at 4 °C for 30 min. Biotinylated Drug conjugate (100 μl) at 0.5 μg/mL (in IM assay diluent) was added to the streptavidin-coated plate and incubated with shaking (150 rpm) at RT for 30 min. The plate was then washed with phosphate buffered saline + 0.05% Tween20 (PBST) three times. Mixtures from step 1 (100 μL) were added to each well of the plate, and incubated at 4 °C for 1 h. The plate was then washed with PBST wash buffer another 3 times. Anti-H1-HRP conjugate (100 μL) at 1:5000 (in IM assay diluent) was added to each well of the plate, and incubated for 30 min. The plate was then washed with PBST wash buffer another 3 times.

TMB (100 μL) was added to each well and incubated for color development for 20 min. Sulfuric acid (2 N, 100 μL) was added to each well to stop the color development. Absorbance (A) of each well was measured at 450 nm subtracted by the absorbance at 630 nm.

2.6. Free drug depletion assays

To determine the specificity of dose–response curve for the immunogenicity assays for each of the drugs, a free drug depletion assay was performed by incubating increasing concentrations of each free drug with a chosen concentration of the positive control anti-drug antibody prepared in a normal human serum pool. The mixtures of the free drug and anti-drug antibody were incubated for 1 h at room temperature. The samples were then tested on the rapid immunogenicity assay according to the previously described procedures. The chosen concentrations of anti-drug antibody for each drug target used in the free drug depletion studies were as follows: anti-HER2 antibody, 62.5 ng/mL (rapid assay) and 1000 ng/mL (ELISA); anti-TNF-α, 62.5 ng/mL (rapid assay) and 2000 ng (ELISA).

2.7. Screening cut-point with normal and target population human serum samples

Fifty normal human sera were tested along with a negative control (NC) human serum pool and positive controls (PCs) comprised of the human serum pool spiked with detectable levels of the anti-drug positive control antibody. In the case of anti-TNF-α antibody, 20 target population samples of rheumatoid arthritis patients were also tested. A floating screening cut-point was determined for each of the populations screened with each of the

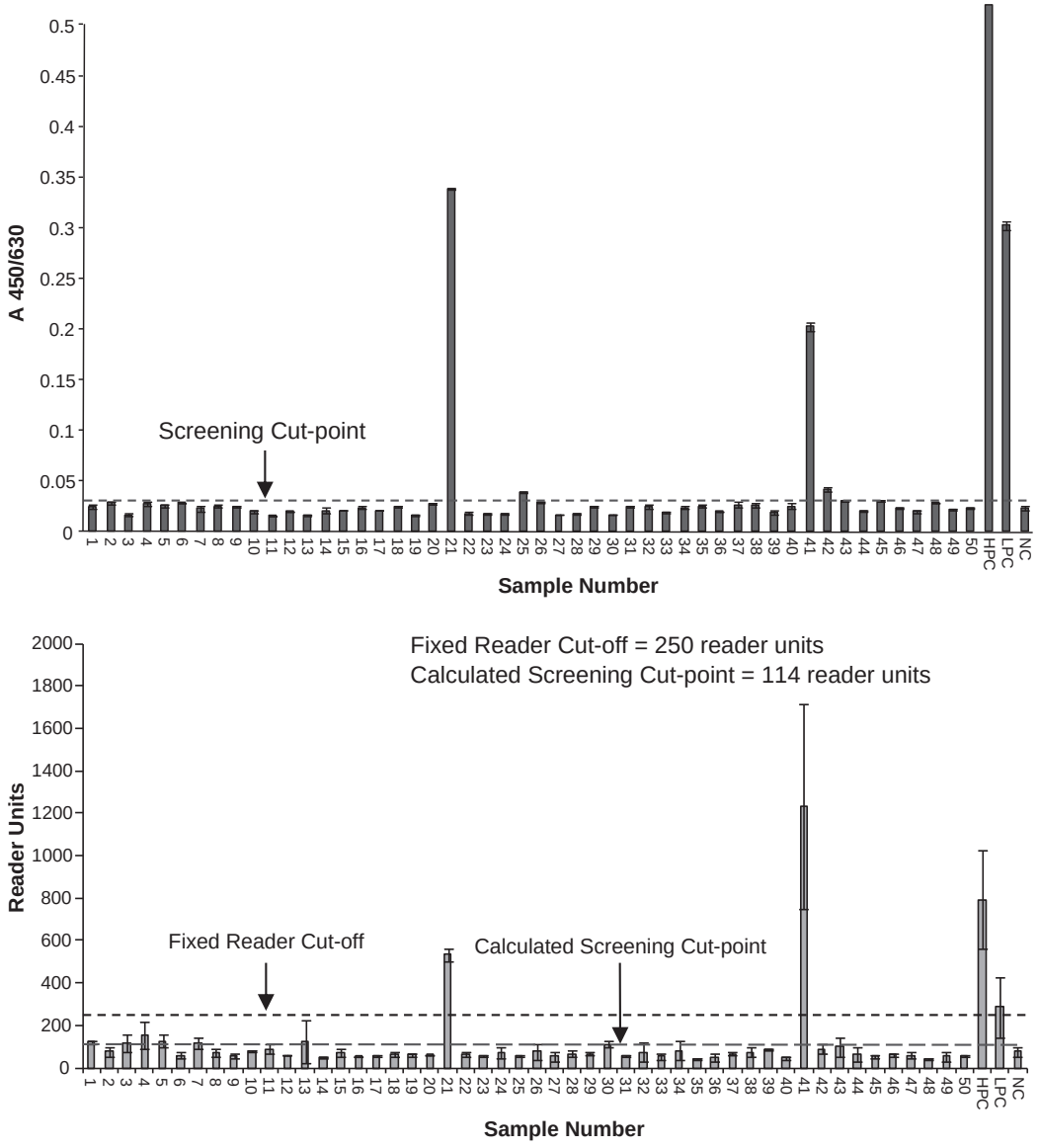

Fig. 7. Comparison of screening results with 50 normal human serum samples using immunogenicity assays in ELISA (top) and rapid assay (bottom) formats for the detection of ADA to anti-HER2 antibody. Samples 21 and 41 were true positives.

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immunogenicity assays. These cut-points were calculated using the following equation:

Screening Cut−point = Mean of signal *of all samples
+ 1.645 Standard Deviation of signal* of all samples.

*signal = A450 / 630 for ELISA, reader values for the rapid assay

The screening cut-points for the rapid assays were determined using the same calculation. The handheld reader has been designed to use a fixed cut-off of 250 reader units to separate a positive signal from a negative result. In the pattern recognition algorithm programmed into the reader, any detected signal below this value may be viewed as the noise level of the assay. The fixed reader cut-off is also presented along with the calculated screening cut-point for reference purposes.

2.8. Assay reader operation

The NIDS® handheld reader measures grayscale contrast on the capture line of the rapid assay test strip. An on-board camera captures a grayscale image of the test strip which is analyzed according to an algorithm that converts contrast to a reader unit. The reader is calibrated using control test strips containing pre-printed lines corresponding to various intensity levels likely encountered in rapid assays. A fixed reader cut-off of 250 reader units has been established whereby any assay signal below this cut-off is presumed to be within the signal noise of the assay and is therefore a negative result. To perform an analysis, the test device containing the test strip is inserted into the reader and the result displayed on the screen. The result can also be uploaded to a computer.

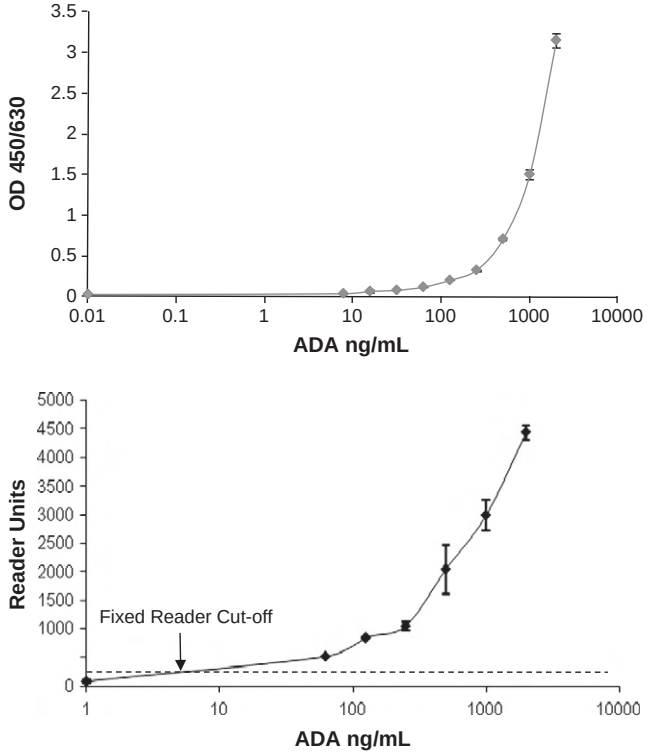

Fig. 9. Comparison of dose–response curves of immunogenicity assays in ELISA (top) and rapid assay (bottom) formats for the detection of ADA to anti-TNF-α antibody.

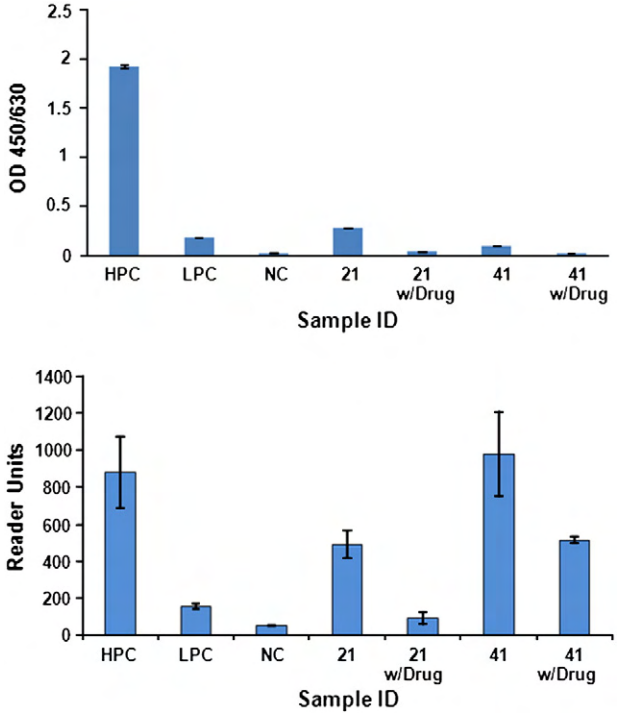

Fig. 8. Comparison of drug depletion assay results with 2 normal human serum samples found positive using immunogenicity assays in ELISA (top) and rapid assay (bottom) formats for the detection of ADA to anti-HER2. The ELISA assay used 10 μg/mL anti-HER2 antibody, while the rapid assay used 5 μg/mL of the drug. HPC = high positive control, LPC = low positive control, NC = negative control.

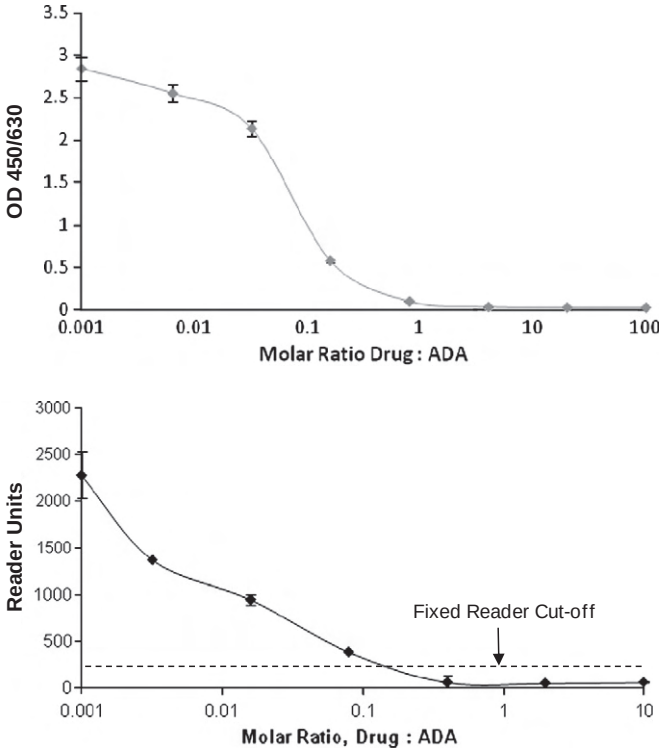

Fig. 10. Comparison of drug depletion profiles of immunogenicity assays in ELISA (top) and rapid assay (bottom) formats for the detection of ADA to anti-TNF-α antibody.

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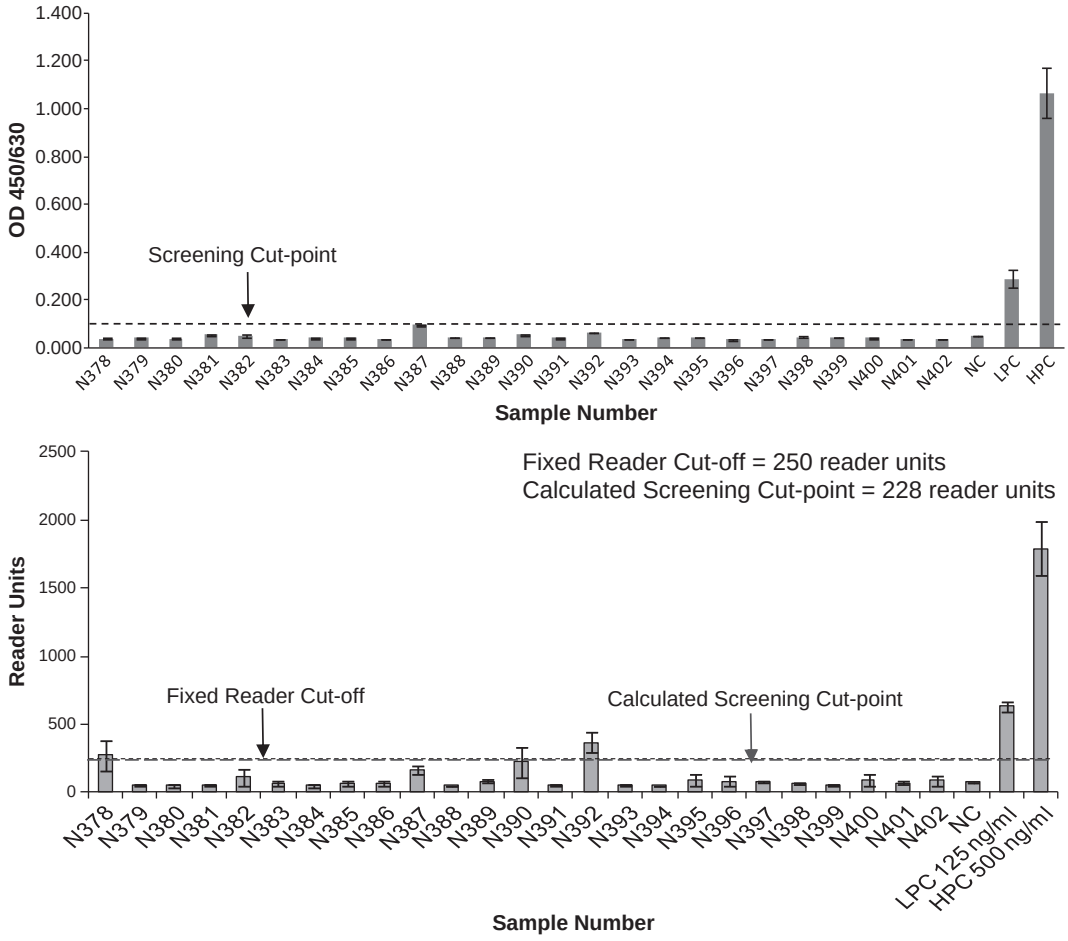

Fig. 11. Comparison of screening results with 25 normal male human serum samples using immunogenicity assays in ELISA (top) and rapid assay (bottom) formats for the detection of ADA to anti-TNF-α antibody.

3. Results

3.1. Comparison of anti-HER2 antibody ELISA and rapid immunogenicity assays

The dose–response curves of the ELISA and rapid immunogenicity assays (Fig. 5) showed a limit of detection of 62.5 ng/mL for ELISA, and 15.6 ng/mL ADA for the rapid assay. A comparison of the free drug depletion profiles obtained from each type of assay showed similar performance for the two assays (Fig. 6). In both assays, complete signal depletion appeared to occur at similar drug:ADA molar ratios of 0.4. The ELISA ADA positive control was at 1000 ng/mL while the rapid assay control was at 62.5 ng/mL.

A comparison of screening data for the same population of normal human serum samples also shows agreement between both methods (Fig. 7). The screening cut-point for the ELISA immunogenicity assay was empirically derived from the standard deviation of the absorbance measurements generated by the population tested (i.e., the calculated floating cut-point = mean + 1.645 * SD). The screening cut-point for the rapid assay was similarly determined using the same calculation with the reader units obtained for each patient sample tested. The fixed reader cut-off is shown for reference purposes in all these figures.

Both formats yielded positive results for the same two human serum samples 21 and 41. Initial false positive rates for both methods were therefore calculated as 2/50 or 4%. The confirmation testing by free drug depletion assays in each format indicated that both samples were true positives (Fig. 8). The ELISA assay used 10 μg/mL anti-HER2 antibody, while the rapid assay used 5 μg/mL of the drug for these depletion studies.

The false positive rate of the ELISA after these two true positive samples were excluded and the screening cutpoint adjusted was 2/48 or 4.2%. Using an adjusted calculated screening cut-point for the rapid assay with the two positive samples excluded a false positive rate of 6.25% was determined for the limited population tested.

3.2. Comparison of anti-TNF-α antibody ELISA and rapid immunogenicity assays

The dose–response curves of the ELISA and rapid immunogenicity assays for anti-TNF-α are compared in Fig. 9. The limit of detection for the ELISA was 125 ng/mL, while that of the rapid assay was 62.5 ng/mL.

A comparison of the free drug depletion profiles obtained from each type of assay showed similar performance for the two assays (Fig. 10). The ELISA immunogenicity assay used 2000 ng/mL of ADA to construct its free drug depletion profile while the rapid assay only used 62.5 ng/mL of ADA. When comparing the drug molar concentrations at which complete depletion occurs, the rapid assay appears completely depleted at a drug:ADA molar ratio of 0.2, while the ELISA is completely depleted at a molar ratio of 1. Screening of 50 normal human (25 male, 25 female) and 20 rheumatoid arthritis human patient serum samples gave similar results for the two assays (Figs. 11–13). The ELISA screening of all 70 samples was performed in a single batch run, while the screening of the same samples with the rapid assay was accomplished in three separate batches. The comparison of results is thus presented for three separate batches

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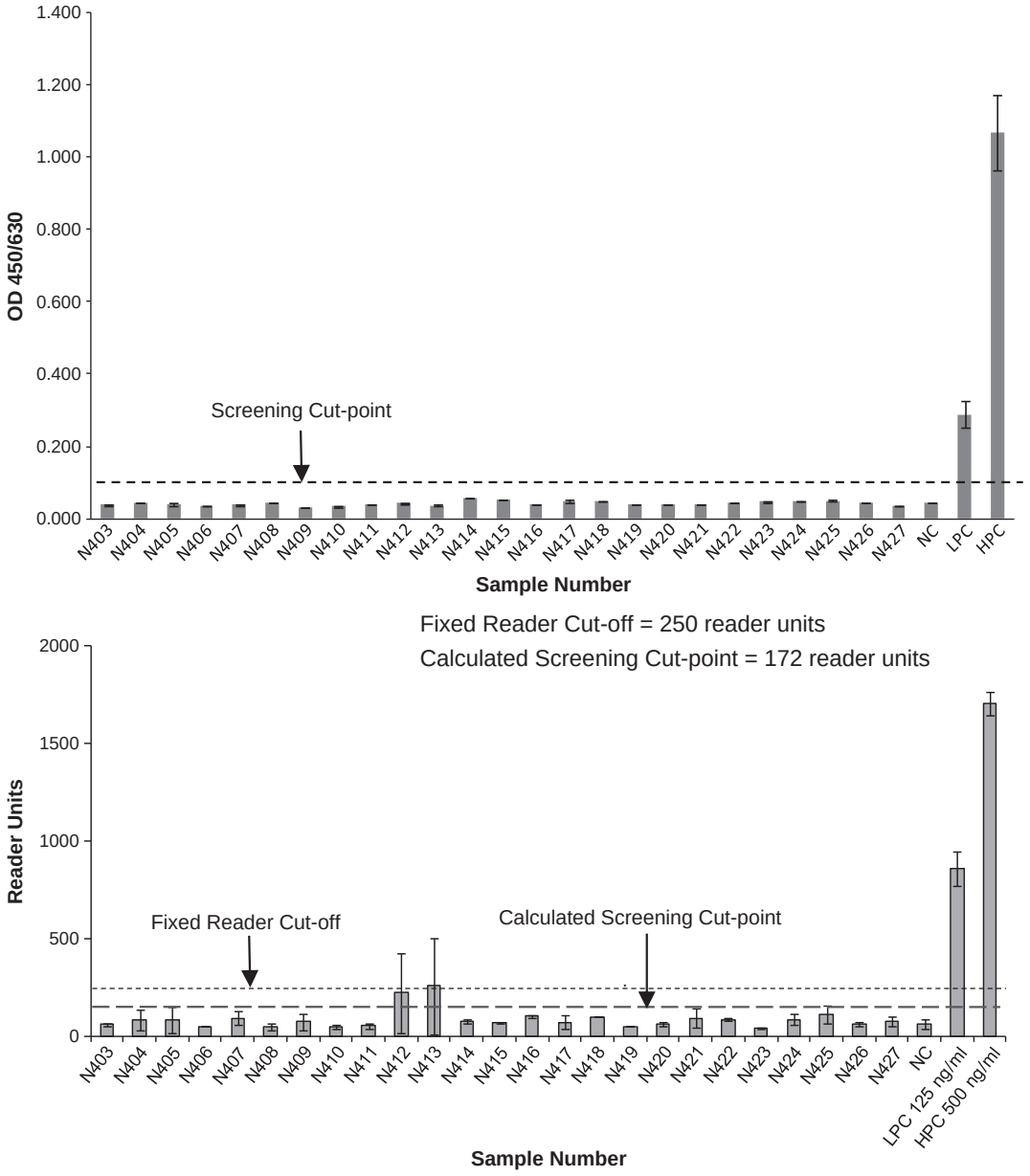

Fig. 12. Comparison of screening results with 25 normal female human serum samples using immunogenicity assays in ELISA (top) and rapid assay (bottom) formats for the detection of ADA to anti-TNF-α antibody.

(Figs. 12–14) using the same ELISA screening cut-point calculated for the entire population of 70 samples tested. The ELISA identified 6 positives out of 70 samples tested for an initial screening positive rate of 9%, while the rapid assay found 7 positives from the same population for an initial screening positive rate of 10%.

In the ELISA, Samples N387, RA80, RA83 and RA87 were weakly positive with signal at or near the screening cut-point, while samples RA82 and RA88 were strongly positive. Upon retesting and undergoing free drug depletion (Fig. 14, top), N387, RA83, and RA87 were below the screening cut-point and were determined to be negative. RA82 and RA88 showed significant inhibition in the free drug depletion assay and thus, are considered true positives. RA sample 80 did not show significant depletion and is therefore a confirmed false positive. After confirmation testing with the free drug depletion assay, the confirmed false positive rate for ELISA was calculated to be 4/68 or 5.9%.

With the rapid assay, normal samples N378, N390, N392, N412 and N413, as well as sample RA82 generated positive signals above the calculated screening cut-point, while sample RA88 was a strong positive. All these samples were retested and subjected to a free drug depletion assay using 5 μg/mL of anti-TNF-α. The results are presented in Fig. 14, bottom. Normal samples N378, N390, N412, and N413 were found to be negative upon retesting and free drug depletion assay, while sample N392 and RA samples RA82 and RA88 were retested and confirmed as true positives in the free drug depletion assay. Therefore, out of 7 positive samples found in the screening of 70 samples, 3 were proven to be true positives while 4 were confirmed as true negatives, for a false positive rate of 4/67 or 6%.

Whereas most samples that were found negative in ELISA were also negative in the rapid assay, the rapid assay did identify one ELISA-negative sample, N392 to be a true positive as confirmed by the free drug depletion assay. A sample found to be false positive by ELISA, RA80, was tested on the rapid assay as a true negative. The same two positive samples were identified by both tests. These results are summarized in Table 1.

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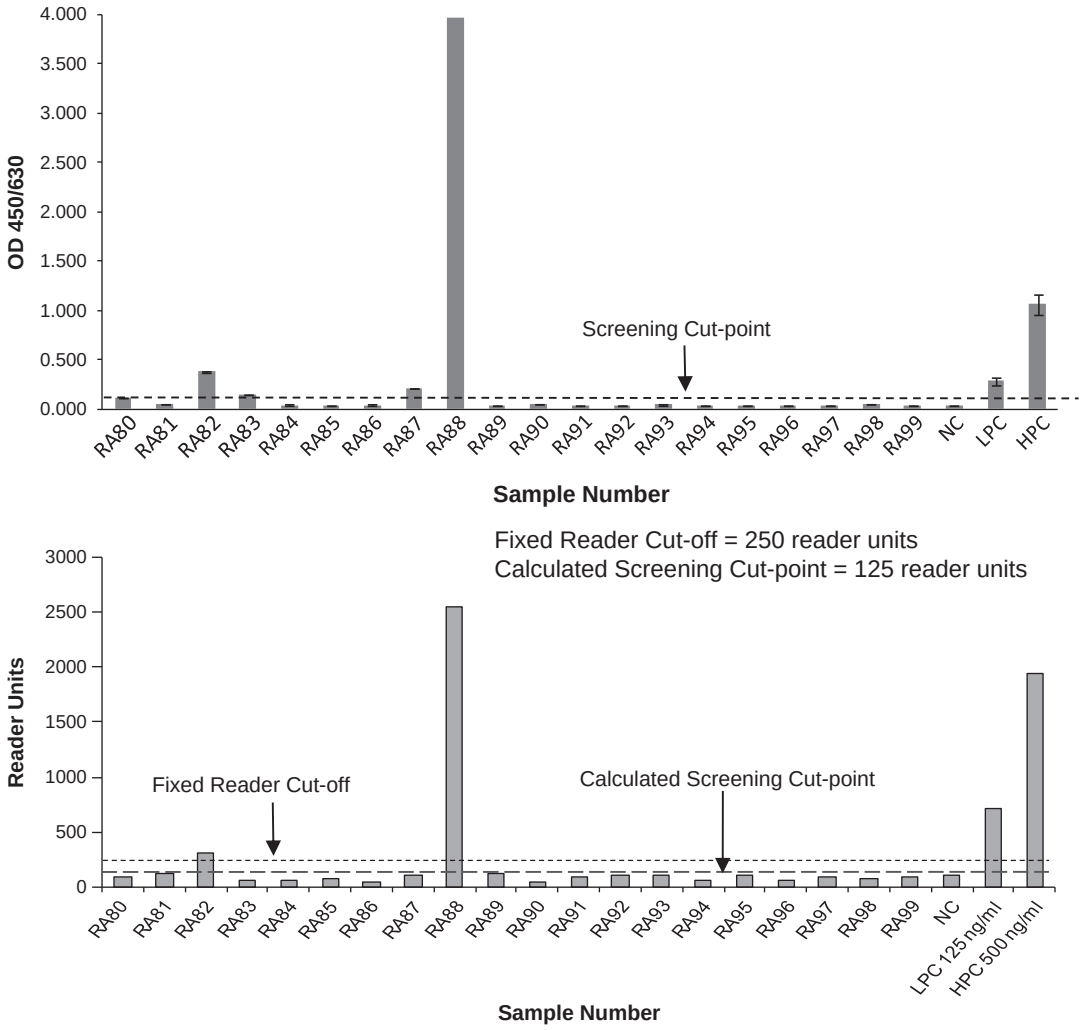

Fig. 13. Comparison of screening results with 20 rheumatoid arthritis human serum samples using immunogenicity assays in ELISA (top) and rapid assay (bottom) formats for the detection of ADA to anti-TNF-α antibody.

4. Discussion

In order to evaluate the NIDS® rapid assay technology, side-by-side comparison studies with traditional ELISAs were conducted with the same set of reagents (i.e., antibody drugs and positive control antibodies) as well as the same normal and target population samples for each of two biotherapeutic antibodies. In these studies, the performance of the rapid immunogenicity assays compares favorably with that of the conventional ELISA-based immunogenicity format. Results show a close correlation of both formats with a few interesting exceptions. In the two comparison studies undertaken for the detection of ADAs to anti-HER2 and anti-TNF-α antibodies, both assays gave equivalent results for most but not all human serum samples tested. For anti-HER2, both methods identified the same two samples as positive. However, the anti-TNF-α antibody immunogenicity study demonstrated disagreement between the ELISA and rapid immunogenicity assays with two samples among all 70 samples tested (Table 1). A short comparison summary is presented in Table 2.

Although using a less sensitive colored particle label as compared to the enzyme label (HRP) for the ELISA, the rapid assays actually showed better assay sensitivity (Table 2) and a longer linear dynamic range than ELISAs for both anti-HER2 and anti-TNF-α antibodies (Figs. 5 and 9). A possible explanation for this difference is that the rapid assay can detect larger populations of anti-drug antibodies with a wider range of affinities, avidities, as well as concentrations which can be expected in the polyclonal antibody-based positive controls used in this study.

For the anti-TNF-α immunogenicity assay evaluation, the rapid assay correctly identified one sample as negative, for which the corresponding ELISA showed a false positive result according to confirmation testing with a free drug depletion assay. The rapid assay also identified another sample as positive, while ELISA clearly indicated it as a negative sample. Repeat testing along with specificity/confirmatory testing by the addition of free drug using the rapid assay confirmed the contrary positive result for this discrepant sample. It can be speculated that perhaps this sample had either low affinity/avidity ADAs or low levels of ADAs that could not be detected by ELISA. In addition, both methods identified the same two rheumatoid arthritis patient samples as positive.

ELISA methods have difficulties in detecting bridge complexes formed by ADAs of a wide range of affinities due to their need for sample dilution and multiple washing steps. With no requirement for sample dilution and washing steps, the NIDS® rapid assays can potentially detect a wider range of ADAs and those of weaker affinity and avidity which ELISA may not detect. The successful demonstration of the ability to detect ADAs to antibody biotherapeutics such as anti-HER2 and anti-TNF-α antibodies has proven the potential utility of the NIDS® rapid assay technology for immunogenicity testing. In addition

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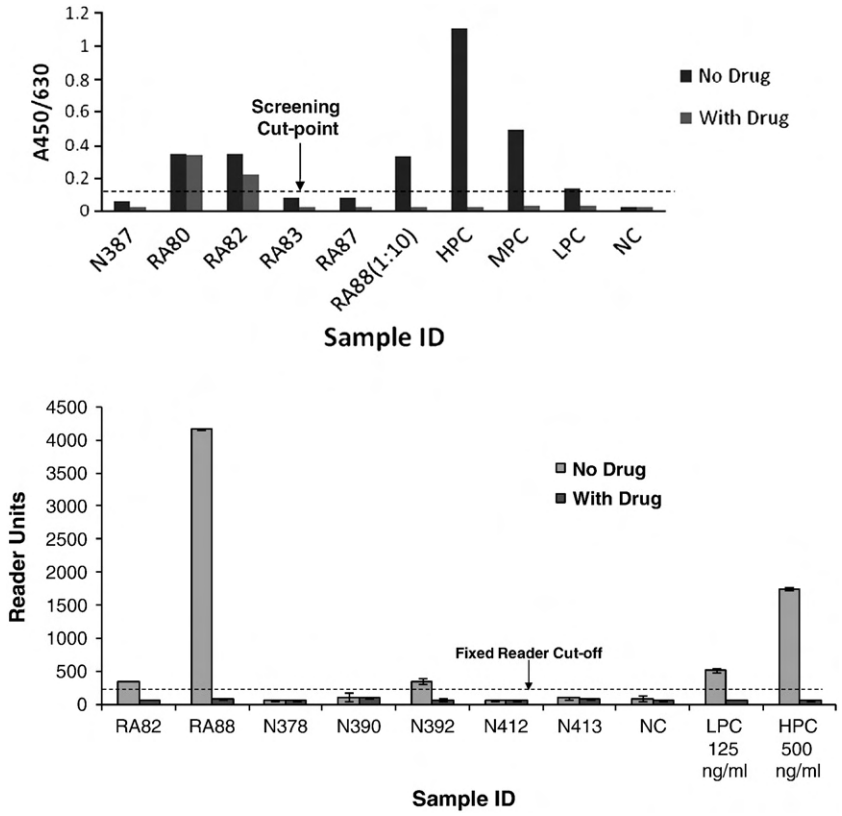

Fig. 14. Comparison of drug depletion assay results with human serum samples found positive using immunogenicity assays in ELISA (top) and rapid assay (bottom) formats for the detection of ADA to anti-TNF-α antibody. HPC = high positive control, MPC = mid-range positive control, LPC = low positive control, NC = negative control.

Table 1
Comparison of ELISA and rapid IM assay for anti-TNF-α.

Sample ID	ELISA	Rapid assay
N392	Negative	Positive
RA80	False positive	Negative
RA82	Positive	Positive
RA88	Positive	Positive

Table 2
Comparison of ELISA and rapid IM assays.

Target/performance parameter	ELISA	Rapid assay
Anti-HER2		
Limit of detection of ADA (ng/mL)	62.5	15.6
100% drug depletion (Drug:ADA ratio)	0.4	0.4
False positive rate[a]	4.2%	6.3%
Anti-TNF-α		
Limit of detection of ADA (ng/mL)	125	62.5
100% drug depletion (Drug:ADA)	1	0.2
False positive rate[a]	5.9%	6%

[a] Limited population tested.

to clinical sample testing, NIDS® rapid assays are also ideally suited for post-approval drug monitoring applications, especially in point of care settings.

References

Hopkins, S. L., Qin, D. J., Pan, J., Milunic, D., David, J. M., Kimos, M. C., et al. (2008). Surface nanomanipulation of bioactive reagents for enhanced microplate assay performance. *Clinical Chemistry*, *54*, A207.

Hudis, C. A. (2007). Trastuzumab–mechanism of action and use in clinical practice. *The New England Journal of Medicine*, *357*, 39—51.

Hydutsky, R., Street, R., Odom, M., Qin, D., Pan, J., Daniel, R., et al. (2005). Multiplexed Assay for the Detection of Biowarfare Agents in Water-Borne Samples. Baltimore MD: Oak Ridge Conference.

Knapp, H. S., Gibbs, S., Pan, J., & Yin, R. (2005). *Multiplexed cytokine assay for examining the TH1/TH2 paradigm.* Baltimore MD: Oak Ridge Conference.

Knapp, H. S., Odom, M., Gibbs, S., Pan, J., Jensen, J., Chang, C., et al. (2005). *Development of Quantitative Lateral Flow Assays.* Baltimore MD: Oak Ridge Conference.

Koren, E., Zuckerman, L. A., & Mire-Sluis, R. A. (2002). Immune response to therapeutic proteins in humans–Clinical significance, assessment and prediction. *Current Pharmaceutical Biotechnology*, *3*, 349—360.

Li, J., Small, T. J., Qin, D. J., Pan, J., Knapp, H. S., David, J. M., et al. (2008). Development of the NIDS® quantitative whole blood lateral flow for high sensitivity C-reactive protein. *Clinical Chemistry*, *54*, A214.

Lofgren, J. A., Dhandapani, S., Pennucci, J., Abbott, C. M., Mytych, D. T., Kaliyaperumal, A., et al. (2007). Comparing ELISA and Surface Plasmon Resonance for assessing clinical immunogenicity of Panitumutab. *J. Immunology*, *178*, 7467—7472.

Pan, J., Small, T., Qin, D., Pauley, C., Kaplanski, C., Verch, T., et al. (2010). NIDS® Rapid Assays for the Detection of Anti-Drug Antibodies to Various Polyethylene Glycol (PEG) Polymers. San Francisco, CA: American Association of Pharmaceutical Scientists National Biotechnology Conference.

Pan, J., Small, T., Qin, D., Vallejo, Y., & Yin, R. (2010). NIDS® Rapid Assays for the Detection of Anti-Drug Antibodies to Peptide Drugs. San Francisco, CA: American Association of Pharmaceutical Scientists National Biotechnology Conference May 2010.

Porter, S. (2001). Human immune response to recombinant human proteins. *Journal of Pharmaceutical Sciences*, *90*, 1—11.

Van den Brande, J. M., Braat, H., van den Brink, G. R., et al. (2003). Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease. *Gastroenterology*, *124*, 1774—1785.

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