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A Comparative Evaluation of Four Commercially Available ELISA Kits for Measuring Adalimumab and Anti-adalimumab Antibodies.

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Ethics

The study was conducted with oversight by the local research ethics committee (HREC/11/LPOOL/227)

ABSTRACT

Background: Therapeutic drug monitoring of tumor necrosis factor inhibitors, such as adalimumab, is increasingly being carried out for the management of autoimmune diseases. However, there can be significant variation in drug and antibody concentrations obtained by different assay methods. The aim of this study was to compare the performance of four ELISA kits for measuring adalimumab and anti-adalimumab antibodies.

Method : Dilutions of adalimumab or anti-adalimumab spiked sera were assessed for recovery rate and precision using the following four kits: LISA-Tracker (Theradiag, Croissy-Beaubourg, France), Promonitor (Grifols, Barcelona, Spain), Ridascreen (R-Biopharm, Darmstadt, Germany), and Shikari (Matriks Biotek, Gölbaşi/Ankara Turkey). Interference samples were also assessed.

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Results:At the therapeutic concentration, adalimumab detection was comparable among the four ELISA kits. Lisa-Tracker and Shikari kits produced low-range false positive results in normal sera. Infliximab and etanercept caused false positives in Lisa-Tracker and Shikari kits. Anti-adalimumab antibody ELISA kits performed differently with spiked samples due to different measuring units and ranges. Ridascreen and Shikari kits were dose-responsive across the entire standard curve and correlated well with each other ($r^2 = 0.997$). Cross-reactivity was observed in rheumatoid factor positive sera tested on the Promonitor anti-adalimumab kit.

Conclusion: All adalimumab kits tested were dose-responsive within the therapeutic range and correlated well. The significance of observed low-range false positives and cross-reactivity with infliximab in LISA-Tracker and Shikari kits is dependent on the indications received for testing in the laboratory. Anti-adalimumab ELISA kits produced varied results for spiked sera; however, they showed good precision. Inter-kit variability suggested that anti-adalimumab levels should be compared only when using the same method.

Key Words: adalimumab, ELISA, tumor necrosis factor, anti-drug antibodies, inflammatory bowel disease

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Adalimumab (ADM) (Humira, AbbVie, North Chicago, Illinois, USA), a recombinant human immunoglobulin G (IgG) monoclonal antibody against tumor necrosis factor-α (TNF-α), is a biological agent used for the treatment of autoimmune disorders, including inflammatory bowel disease (IBD) and rheumatoid arthritis (RA). ADM is increasingly being used as an alternative to infliximab (IFX) in cases of loss of response (LOR) and due to its ease of use (available as a self-administered subcutaneous injection). ADM binds specifically to soluble TNF and contains only human peptide sequences [1, 2]. Following the failure of previously used conventional therapies, ADM is effective in inducing and maintaining remission in different patient groups, ranging from those having moderate to severe Crohn's disease (CD) [3-6].

Studies have demonstrated that higher serum trough concentrations of ADM are associated with improved clinical remission rates, patient outcomes, and mucosal healing in CD and ulcerative colitis (UC) [6-9]. Aguas Peris et al. showed that serum concentrations of ADM were predictive of clinical remission, and normalization of inflammatory markers at a mean serum trough concentrations of 9.2 µg/mL was useful in the development of clinical treatment protocols, such as dose de-intensification [9]. These results were consistent with previous findings, which demonstrated that serum ADM concentrations of 10.1 µg/mL, 7.4 µg/mL, and 4.5 µg/mL were associated with clinical remission, mild disease, and moderate disease activity, respectively, in CD [10]. Additionally, one group found that a 'cut off' serum trough concentration of ADM 4.85 µg/mL was associated with clinical remission, above which clinical remission could be reliably predicted [8].

The measurement of anti-drug antibodies (ADAs) plays an important role in the 'treat to target' approach [8, 11]. This is because the development of antibodies against TNF is commonly linked with secondary LOR, which occurs in up to 60% of the patients with IBD within three years, and increased levels of ADAs correlate with decreased serum trough

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concentrations of TNF inhibitors and increased disease activity [8, 11-13]. Various studies have also shown that monitoring of serum levels of anti-adalimumab antibody (AAA) is beneficial in ADM treatment protocols. A study reported that detection of AAA is a predictor of the lack of mucosal healing [8]. In addition, the development of AAA is associated with negative outcomes, increased rates of discontinuation of treatment, and higher levels of disease activity [14]. The development of AAA was observed in two-thirds of the patients within 28 weeks of treatment. Patients with AAA rarely achieved clinical remission[14].

Several studies have reported the benefits of therapeutic drug monitoring (TDM), which uses a combination of serum drug concentrations and the measurement of AAA levels, in improving patient outcomes [11, 13, 15]. However, data regarding the optimal targets for various indications is limited. Furthermore, the variability in assays used in different clinical studies adds to the complexity and makes it difficult for the clinician to accurately interpret the results. Previously this group conducted a comparative evaluation of commercial ELISA kits for the detection of IFX trough concentrations [16]. While several commercial ELISA kits are available in the Australian market, to date there has been no published study evaluating and comparing the kits for the testing of ADM for clinical diagnostic use. The aim of this study was to compare the performance of four commercially available ELISA kits for the assays of ADM and AAA, three of which are approved for *in-vitro diagnostic* use in Australia.

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MATERIALS AND METHODS

Reagents and Sample Preparation

Analysis was performed using four commercial ELISA kits: LISA-Tracker (LT) (Theradiag, Croissy-Beaubourg, France), Promonitor (PROM) (Grifols, Barcelona, Spain),

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Ridascreen (RIDA) (R-Biopharm, Darmstadt, Germany), and SHIKARI (SHIKARI) (Matriks Biotek, Gölbeşi/Ankara, Turkey), according to the manufacturers' instructions. All assays were performed using a Triturus (Grifols, Barcelona, Spain) auto-analyzer.

Pooled normal human serum (NHS) was donated by five healthy volunteers, consisting of both males and females, with no known medical conditions. The antinuclear antibody (ANA) screens of these volunteers were found to be negative, as detected by immunofluorescent staining. Pooled patient samples, positive for rheumatoid factor (RF), IgG kappa paraprotein (IgGkPP), and IgM kappa paraprotein (IgMkPP), were used to assess interference. All samples were confirmed negative for other monoclonal antibody therapies and other possible immunological cross-reactants. Samples were excluded if previous medication and health history was considered incomplete. All samples were analyzed in duplicate, on at least two separate assay runs, using each kit

ADM, IFX (Remicade, Janssen-Cilag, Beerse, Belgium), and etanercept (ETN) (Enbrel, Pfizer, New York City, New York, USA) were purchased from Liverpool Hospital Pharmacy. High-affinity AAAs (0.5 mg/mL) were purchased from Bio-Rad Laboratories (Hercules, California, USA), HCA-204 (neutralizing) and HCA-232 (non-neutralizing). Reference samples, provided by Grifols Australia, were run on each ELISA kit in triplicate. Aliquots of the prepared samples were stored at -20 °C and were single-used to avoid repeat freeze-thaw processes. Dilution curves were prepared in duplicate and interference samples in triplicate, unless specified otherwise.

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ADM Experimental Design

NHS spiked with ADM were run on each kit, at a concentration range of 0.5-20 µg/mL. To determine intra-assay percent coefficient of variation (%CV), seven replicates of ADM (1.0, 2.0, and 10.0 µg/mL) were run on each ELISA kit. Inter-assay %CV was calculated by measuring 7-10 replicates across three runs on each ELISA kit.

Pharmacological interference by other TNF-α inhibitors was tested by spiking IFX or ETN into ADM (5.0 µg/mL) and NHS to achieve a final concentration of 5.0 µg/mL of the interferent. RF, IgMk PP, and IgGk PP were each spiked with ADM to achieve a final concentration of 5.0 µg/mL. Pooled samples of RF, IgMk PP, and IgGk PP samples (negative for ADM) were also analyzed. NHS spiked with ADM 5.0 µg/mL was subjected to five freeze thaw cycles at -20 °C. Samples were run in triplicate.

NHS spiked with ADM (2.0 µg/mL) was used to dilute AAA (both neutralizing and non-neutralizing) to achieve a dilution curve of antibody concentrations, ranging from 10 ng/mL to 2.5 µg/mL.

Anti-adalimumab Antibody Experimental Design

Neutralizing AAA were run on each kit at a concentration range of 0.1-20 µg/mL. To determine intra-assay %CV, seven replicates of AAA (0.5, 1.0, and 2.5 µg/mL) were run on each ELISA kit. An antibody concentration of 2.5 µg/mL was selected for interference samples. Samples positive for IFX or ETN, both in NHS and in the presence of AAA. were assessed. Pooled samples of RF, IgMk PP, and IgGk PP were spiked with AAA to achieve a final concentration 2.5 µg/mL AAA. Pooled samples of RF, IgMk PP, and IgGk PP samples, negative for AAA, were also analyzed in this experiment. NHS spiked with 2.5 µg/mL AAA

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were subjected to five freeze thaw cycles at -20 °C. Pooled patient samples, positive for anti-infliximab (ATI), were used to assess immunological interference. This pooled serum was first assessed using LT anti-IFX kit, with a mean measured concentration of 76.3 ± 5.3 ng/mL (n = 15). In addition, reference samples positive for ATI, supplied by Grifols Spain, were run on the AAA kits to test for cross-reactivity.

NHS spiked with AAA 2.5 µg/mL was used to dilute ADM, at concentrations of 0.01-5 µg/mL.

Statistical Analyses

All statistical analyses were performed using PRISM software version 8.4.3 (GraphPad Software, La Jolla, CA, USA). Linear regression analysis was performed. Inter- and intra-assay %CV were calculated using the root mean square approach. Recovery rate was expressed as a percent of spiked concentration. Limit of quantification (LOQ) was calculated as the limit of detection by which %CV and percent deviation from target (%tar) was <20%.

RESULTS

Comparison Among ADM Elisa Kits

Comparison of the dilution series data obtained from the four ELISA kits showed a statistical difference ($P < 0.01$). Bland Altman analysis showed a positive bias for the LT kit as compared to that of the other ELISA kits (Bias = 1.695) (Fig. 1).

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However, the assay measurements using each kit correlated well with each other and with the prepared ADM standard of known concentrations (Fig. 2a). PROM kit produced results closest to the known ADM standard (Fig. 2a). Linear regression analysis was performed on all ELISA kits compared to the spiked concentration of ADM (see Table 1, Supplemental Digital Content,http://links.lww.com/TDM/A428). All kits had good overall inter- and intra-assay precision (<20%). At lower concentrations of ADM, the LT and SHIKARI kits showed high inter-assay %CV and/or %tar (>20%) (see Table 2, Supplemental Digital Content,http://links.lww.com/TDM/A428). In addition, the LT and SHIKARI kits had a higher LOQ than indicated in their product information.

The LT and SHIKARI kits produced false low-range positive results for the samples negative for ADM (0.59 ± 0.06 µg/mL and 0.2 ± 0.06 µg/mL, respectively) when compared to the results of the PROM (0.05 ± 0.006 µg/mL) and RIDA kits (0.028 ± 0.016 µg/mL). This difference in measured concentration was significant for the LT kit ($P < 0.0001$) (Fig. 2b). These findings are in line with our LOQ calculation findings.
Evaluation of the reference samples provided by Grifols Australia showed similar results as those obtained for our prepared samples (data not shown).

Assessment of Interferents on ADM Measurement

Both PROM and RIDA kits showed no interference from IFX or ETN (Fig. 2c and d). In contrast, LT and SHIKARI kits showed strong positive cross-reactivity in samples containing IFX 5.0 µg/mL, resulting in an increase in the observed drug concentration by >3.0 µg/mL ($P < 0.001$) in ADM negative samples, and by >2.6 µg/mL in ADM 5 µg/mL samples ($P < 0.001$) (Fig. 2c).

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Similarly, the LT and SHIKARI kits also demonstrated cross-reactivity with ETN 5.0 µg/mL, resulting in an increase in the observed drug concentration by >1.8 µg/mL in ADM negative samples ($P < 0.0001$ and $P < 0.05$, respectively). In addition, in case of LT kit, ADM 5.0 µg/mL samples spiked with ETN 5.0 µg/mL ledto an increase in the observed drug concentration by >4.0 µg/mL ($P < 0.001$) (Fig. 2d).

Samples positive for RF, IgGk PP, and IgMk PP showed no interference or cross-reaction in any of the ADM kits. After five freeze-thaw cycles, no difference was observed in the measured ADM concentration in ADM 5.0 µg/mL samples

For all kits, samples of ADM 2.0 µg/mL spiked with neutralizing antibody showed a reduction in detectable ADM as the antibody levels increased. In the PROM and RIDA kits, ADM detection dropped below the known concentrations at an antibody level of 0.5 µg/ml and were completely undetectable at antibody levels >2.0 µg/mL. The LT kit readings were higher in comparison to the spiked concentration of ADM and dropped below the known concentration of ADM at > 1.0 µg/mL. The SHIKARI kit measured lower than the spiked overall concentration of ADM and showed a drop in ADM detection at 1.0 µg/mL (Fig. 2e).

Samples of ADM 2.0 µg/mL spiked with non-neutralizing antibody showed no difference in detectable concentration of ADM and were consistent with the expected results. Again, samples spiked with non-neutralizing AAA produced lower than expected results in SHIKARI (Fig. 2f). This overall inhibition of ADM detection in SHIKARI kits, in the presence of neutralizing or non-neutralizing AAA cannot be explained from our data.

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Comparison Between AAA Elisa Kits

Dilution Series on AAA ELISA Kits

Spiked antibody samples behaved differently in each ELISA kit, thus making direct comparisons between the kits difficult. The standard curve range was different for each kit, and samples with the same concentration produced different results. While the results for PROM kit are reported in AU/mL, those of LT, RIDA, and SHIKARI kits are in ng/mL. However, all kits performed in a consistent dose-responsive manner, across lower concentrations (Fig. 3 a-e).

The LT kit had a broad detection range of spiked AAA. It was able to detect samples in the concentration range of 100 ng/mL to 10 µg/mL, levelling off towards the top end of the standard curve (Fig. 3b). Spiked samples below 100 ng/mL tested negative.

The PROM kit could detect higher concentrations of AAA, with the highest concentration of AAA tested (20 µg/mL), measuring at 104.5 ± 6.5 AU/mL, which was short of the maximum detection range of the kit of 400 AU/mL. Samples in this range were less dose-responsive, as demonstrated by flattening of the curve (Fig. 3c). Spiked samples were negative at concentrations <250 ng/mL.

The RIDA and SHIKARI kits had the narrowest detection ranges of spiked AAA, and samples >5.0 µg/mL and > 3.75 µg/mL were higher than the maximum concentration of the standard curve in each kit, respectively (Fig. 3d and 3e). Both kits displayed the best dose response at the upper ranges of their standard curve. Samples <500 ng/mL and <250 ng/mL on the RIDA and SHIKARI kits, respectively tested negative. Grifols reference samples showed good dose-responsiveness up to 200 AU/mL on the PROM kit and low reactivity on LT, RIDA, and SHIKARI kits (data not shown).

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Replicates (n = 7) of samples at concentrations of 500 ng/mL, 1.0, and 2.5 µg/mL were selected to assess %CV and the values were compared between kits (see Table 3, Supplemental Digital Content,http://links.lww.com/TDM/A428). All kits produced good %CV at the lower AAA concentrations.

Assessment of Interferents On AAA Measurement

No interference was observed in any AAA kits in samples spiked with IFX or ETN. High ATI samples showed no cross-reactivity on any of the kits. Pooled samples positive for RF (1080 IU/ml) showed no cross-reaction in the LT or RIDA kits. The PROM kit showed a cross-reaction in the RF positive pooled sample (1080 IU/ml), negative for AAA. This cross-reaction increased in the presence of AAA (Fig. 4a). This was confirmed using a second pool of RF positive patient samples (589 IU/ml) with similar results. Cross-reaction with the high-level RF positive pool (1080 IU/ml) was observed in the SHIKARI kit (Fig. 4b) but not in the lower level RF positive pool. Confirmation testing on the SHIKARI kit, as per manufacturer's instructions, demonstrated false positive results for the high-level RF positive pooled sample.

Samples positive for IgMkPP showed interference of AAA detection in RIDA ELISA kit, with spiked AAA levels measuring 14 ng/mL lower in pooled IgMk PP samples vs NHS (Fig.4 c) ($P < 0.001$). No other cross-reaction or interference with IgMk PP or IgGk PP positive sera was observed on any of the kits.

The presence of ADM inhibited AAA detection in a dose-responsive manner in all the kits (Fig. 5 a-d). Measured AAA dropped below the expected levels in the presence of >0.5 µg/mL ADM in RIDA, PROM, and SHIKARI ELISA kits. In addition, the signal was

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undetectable at concentrations >2.5 µg/mL in all kits. The LT kit followed a similar dose response as other kits. However, our data showed that AAA can be detected at higher ADM concentrations, thus suggesting that it is a more drug-tolerant assay. Detection levels dropped below expected in the LT kit at ADM concentrations >1.5 µg/mL and the signal was completely undetectable at 5.0 µg/mL.

DISCUSSION

The importance of the measurement of drug and anti-drug antibodies in anti-TNF therapies has been previously described [7, 12, 13, 15, 17]. The aim of our study was to evaluate and compare four commercially available ELISA kits for the testing of ADM and AAA, three of which are approved for IVD use in Australia. In our study, with the exception of LT and SHIKARI showing low-range false positives, all kits performed well and demonstrated good precision and recovery rates.

Low-range false positives observed in LT and SHIKARI kits were above the negative cut-off value published in the kit insert and were statistically significant. The results observed with the LT ADM kit are in line with our previous study that reported that the LT IFX ELISA produced low-range false positives [16]. In a routine setting, low-range false positive results should be taken into account when determining reflex concentrations for antibody testing to ensure that all low drug concentrations are investigated for anti-drug antibodies. In addition, in some clinical cases, low-range false positives may be relevant, particularly in neonates where detection of complete drug clearance is of high importance. Previous studies have shown that IFX and ADM concentrations cleared in neonates after 12 months post-birth [18]. Low-range false positives may misrepresent drug clearance in this situation, and therefore, patient population must be considered when selecting an ELISA kit for routine analysis.

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Good precision, which was observed in all the tested kits in this study, is important to detect actual change in the drug concentration. Our data may be useful for laboratories, which may want to change kits or clinicians comparing ADM results between different assays.

Cross-reaction of IFX and ETN was observed on the LT and SHIKARI kits. The capture antigen used on the LT ADM ELISA kit is TNF, and therefore cross reactivity with other TNF agents was expected. It is unclear from the product information of the SHIKARI kit regarding the exact nature of the capture antigen, as the product names it as a 'reactant for adalimumab.' However, cross-reactivity with other TNF agents is disclosed in the specificity of this assay. Cross-reactivity could potentially cause confusion when transitioning from one agent to another before complete clearance of the other anti-TNF. Hence, the reporting laboratory needs to clarify that other anti-TNF agents that may be present could falsely elevate the ADM concentration.

Evaluation of AAA ELISA kits showed that a direct comparison was not possible, unlike ADM evaluation. Regression analysis was able to predict a relationship between kits and regression statistics were most robust in comparison between kits with similar dose response curves (LT vs PROM and RIDA vs SHIKARI). There was good precision observed for each kit, making repeat evaluations reliable, as long as they are performed using the same kit.

While PROM and LT kits offered broader detection ranges; a plateau effect was observed towards the top of the measuring ranges for the spiked AAA used in our study. Although RIDA product information also allows for further dilution with sample diluent to detect up to 1000 ng/mL, this was not done in this evaluation. The dose-responsive nature of the RIDA and SHIKARI kits allows the user to detect changes in antibody levels more accurately.

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The clinical significance of AAA quantification is not clear at this point in time. It is, therefore, uncertain whether the ability to detect low/high levels or changes at low/high levels is of clinical importance. Experience with ATI however suggests that there may be a significance that is yet to be defined.

Cross-reaction of RF in the PROM kit for both AAA negative and positive samples could falsely increase the AAA concentration and may affect clinical decisions. This is particularly relevant for individuals with RA, who are commonly treated with ADM [19]. Although the SHIKARI kit displayed a similar cross-reaction, it provides a confirmatory step to avoid this problem. Similarly, the reduced detection of AAA in patients positive for IgMk PP, using the RIDA kit, was statistically significant. However, the size of the change was unlikely to be clinically relevant. This should be investigated further.

The presence of antibodies resulted in the reduction in the detectable concentrations of ADM on all ELISA kits. The addition of >2.0 mg/mL ADM led to complete loss of detection of AAA on all kits. Similarly, the addition of >2.5 µg/ml of AAA effectively resulted in the loss of detection of ADM when measuring drug concentrations on all kits. We chose a drug concentration of 2.5 µg/ml ADM to generate automatic antibody testing to ensure antibodies were detected.

CONCLUSION

TDM of ADM is a useful tool in the clinical management of patients with various autoimmune conditions. Currently, there are four commercially available ELISA kits in Australia, for testing both ADM drug concentrations and AAA levels. Our study demonstrated that results can be compared between ADM kits. However, transition from one

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AAA assay to another requires evaluation at the routine laboratory level to ensure that clinicians can interpret results for use in patient management, and where possible longitudinal measurements of AAAs should be performed on the kit from the same brand.

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Figure Legends

Figure 1: Bland-Altman analysis comparing ELISA kits. LT showed a positive bias compared to other kits, particularly at higher concentrations.

Figure 2: Measured levels of ADM on ELISA kits compared to spiked concentrations of ADM. (a) Dilution curves of measured ADM vs spiked concentration, (b) measured ADM in ADM negative samples. Axis range is expanded in the 0-1.0 µg/mL range to demonstrate the incidence of low-range false positives in LT and SHIKARI kits. (c) Measured ADM in presence of IFX, (d) measured ADM in presence of ETN, (e) measured ADM in presence of increasing concentrations of neutralizing AAA, (f) measured ADM in presence of increasing concentrations of non-neutralizing AAA.

Figure 3: The effect of spiked antibody on ADM detection. (a) Spiked antibody analysis performed separately on all ELISA kits. (b-e) Graphs of measured AAA in spiked samples on each ELISA kit. The kits had different ranges and units and spiked antibody did not perform in the same way in all kits. LT and PROM kits appeared to plateau at higher concentrations of spiked AAA, as they approached the upper limit of the assay. Regression analysis showed good agreement with spiked values in the RIDA and SHIKARI kits (supplemental table 3,http://links.lww.com/TDM/A428). Inter and intra-assay %CV was good on all kits. Dotted lines represent upper and lower detection limits of the kits.

Figure 4: Measured AAA levels in the presence of interferents. (a) and (b) RF interference on AAA detection in PROM and SHIKARI ELISA kits. A significant difference was observed between measured AAA in RF positive and NHS samples in both the kits. There

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was no interference observed in LT or RIDA kits (data not shown). (c) IgMk PP interference on AAA detection in RIDA ELISA. Measured AAA was significantly decreased in IgM-positive samples compared to NHS. There was no interference observed in LT, PROM, or SHIKARI Elisa kits (data not shown).

Figure 5: Graphs of measured ADM interference with AAA detection in each ELISA kit. Dotted lines represent expected AAA level in spiked samples for each kit. LT and PROM were more drug tolerant, able to detect AAA up to 5 µg/mL-spiked ADM. All measured AAA levels dropped below expected levels at ADM levels >1.0 µg/mL and in RIDA and SHIKARI kits AAA was undetectable at ADM concentrations > 2.5 µg/mL

Abbreviations used in Figures (in order of appearance):- Adalimumab (ADM), LISA-Tracker (LT), Infliximab (IFX), Etanercept (ETN), Anti-adalimumab antibodies (AAA), Promonitor (PROM), Ridascreen (RIDA), Rheumatoid Factor (RF), Normal human sera (NHS), IgM kappa paraprotein (IgMk PP)

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Figure 1

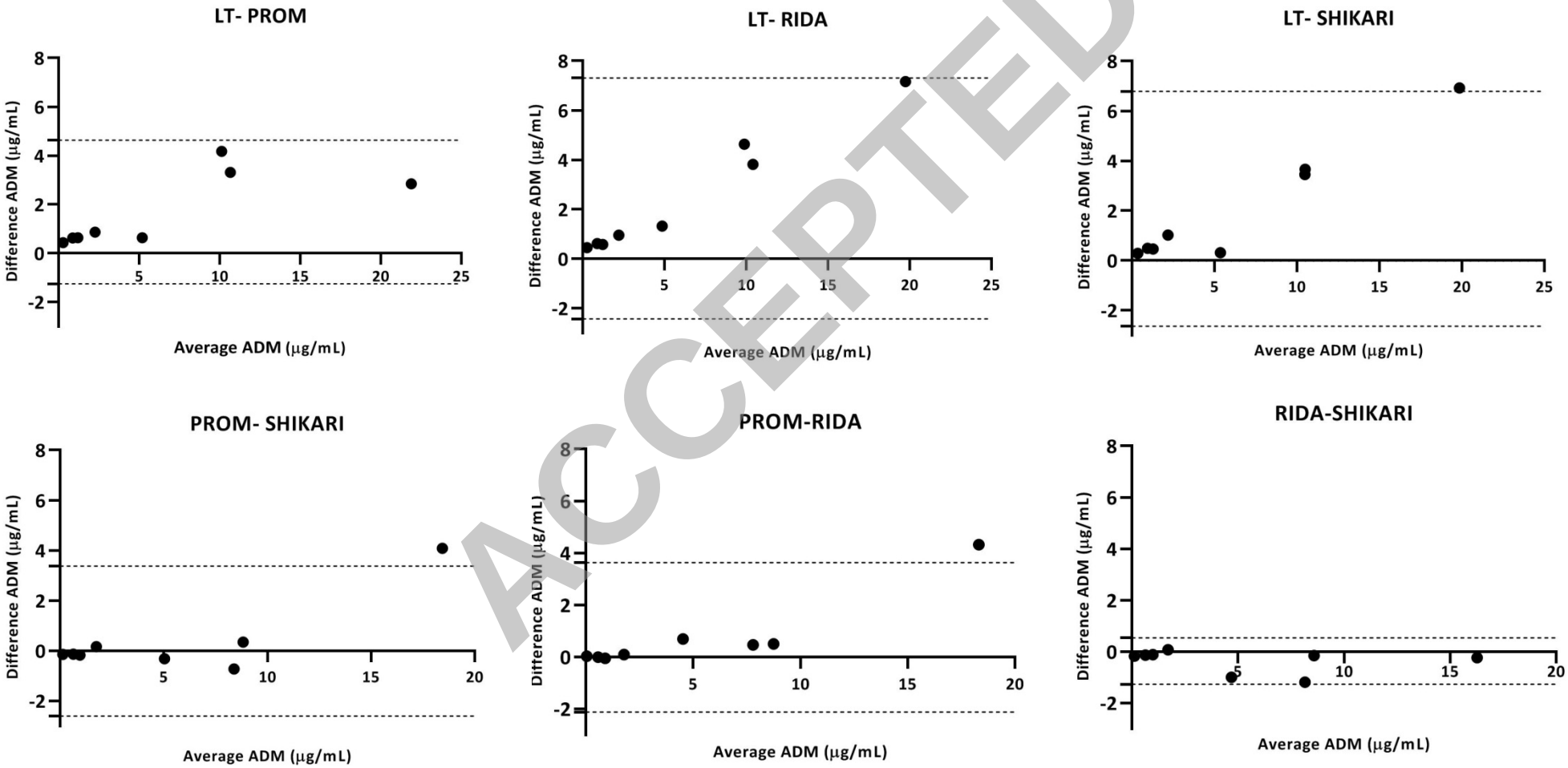

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Figure 2

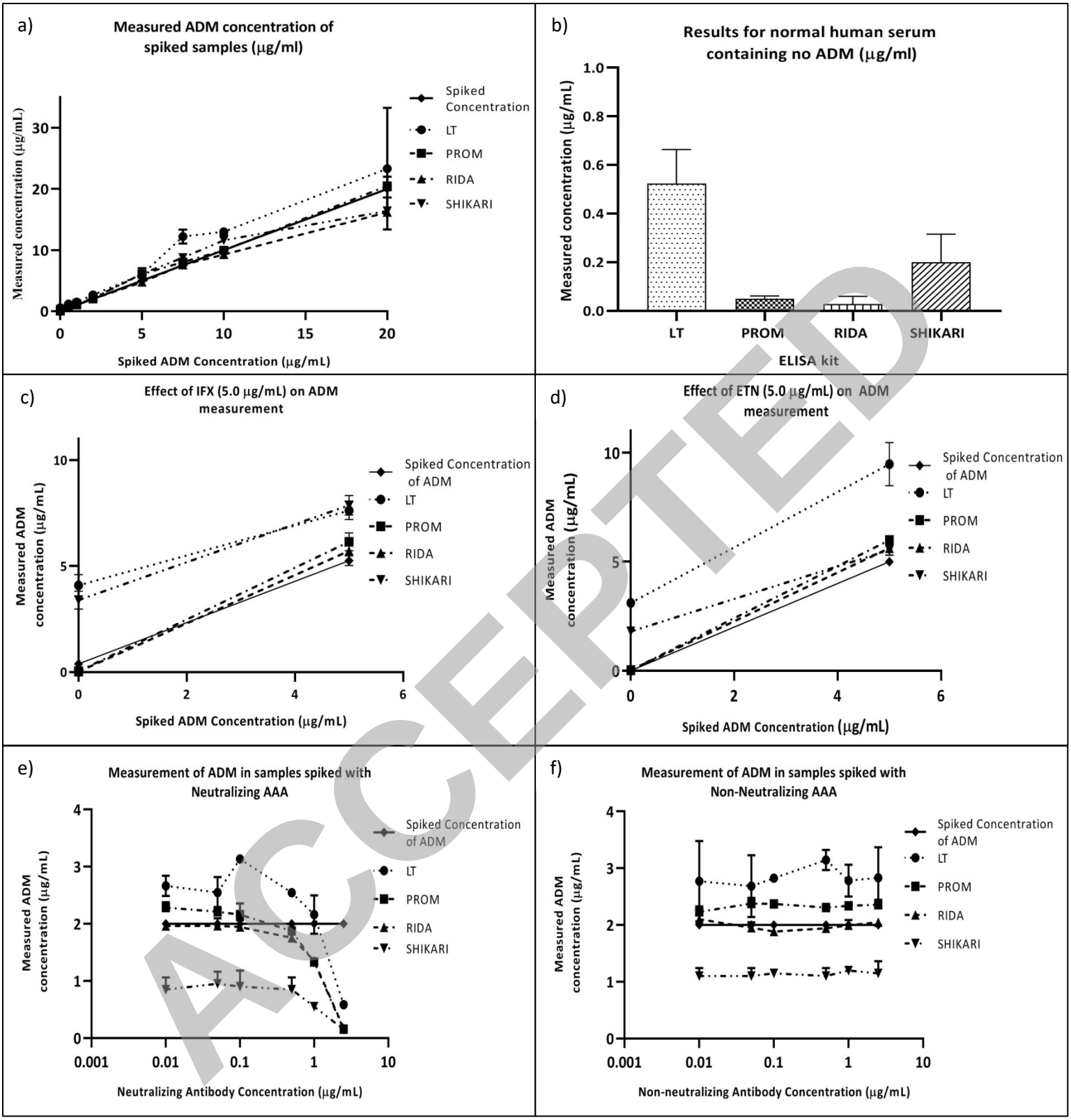

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Figure 3

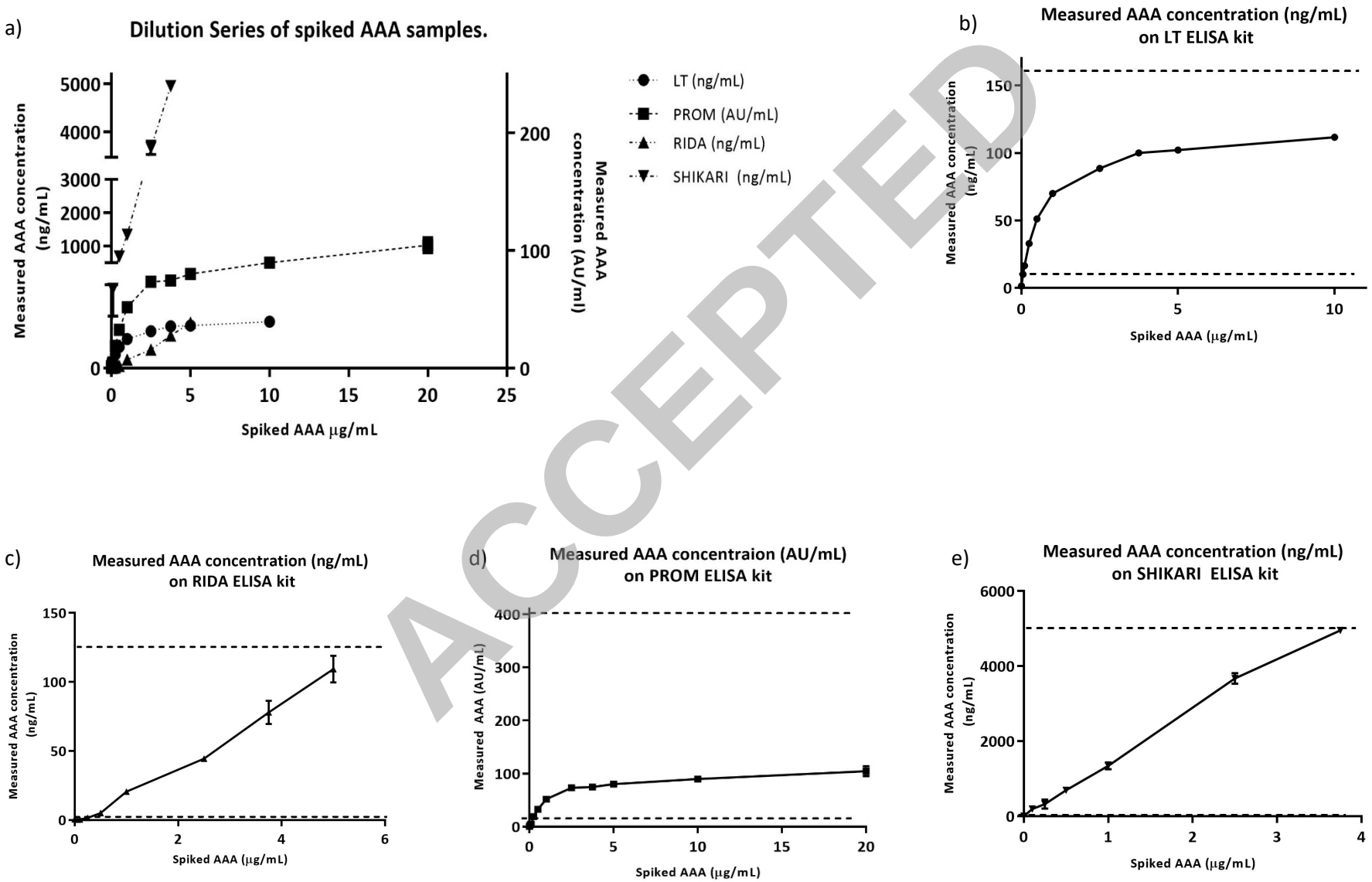

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Figure 4

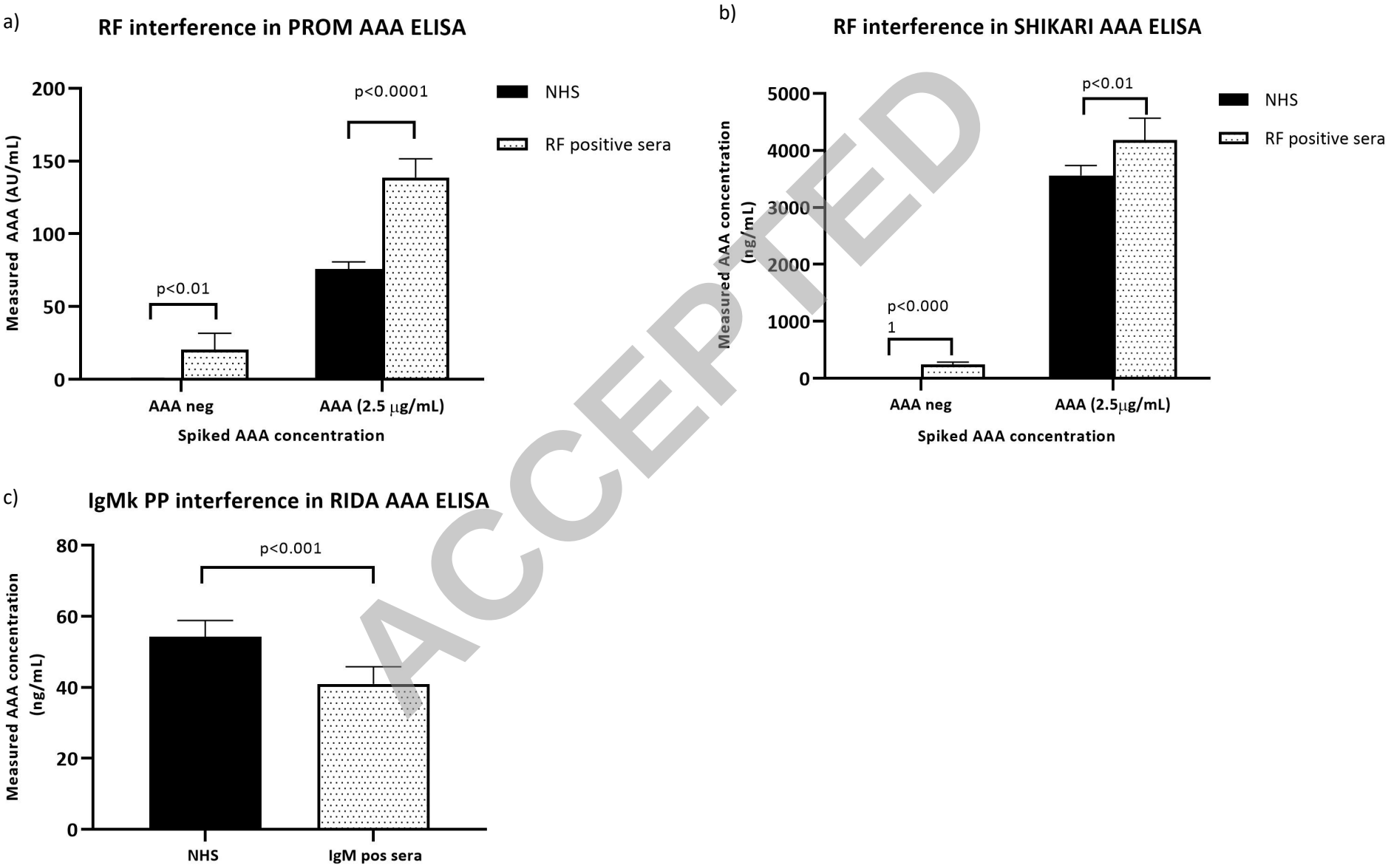

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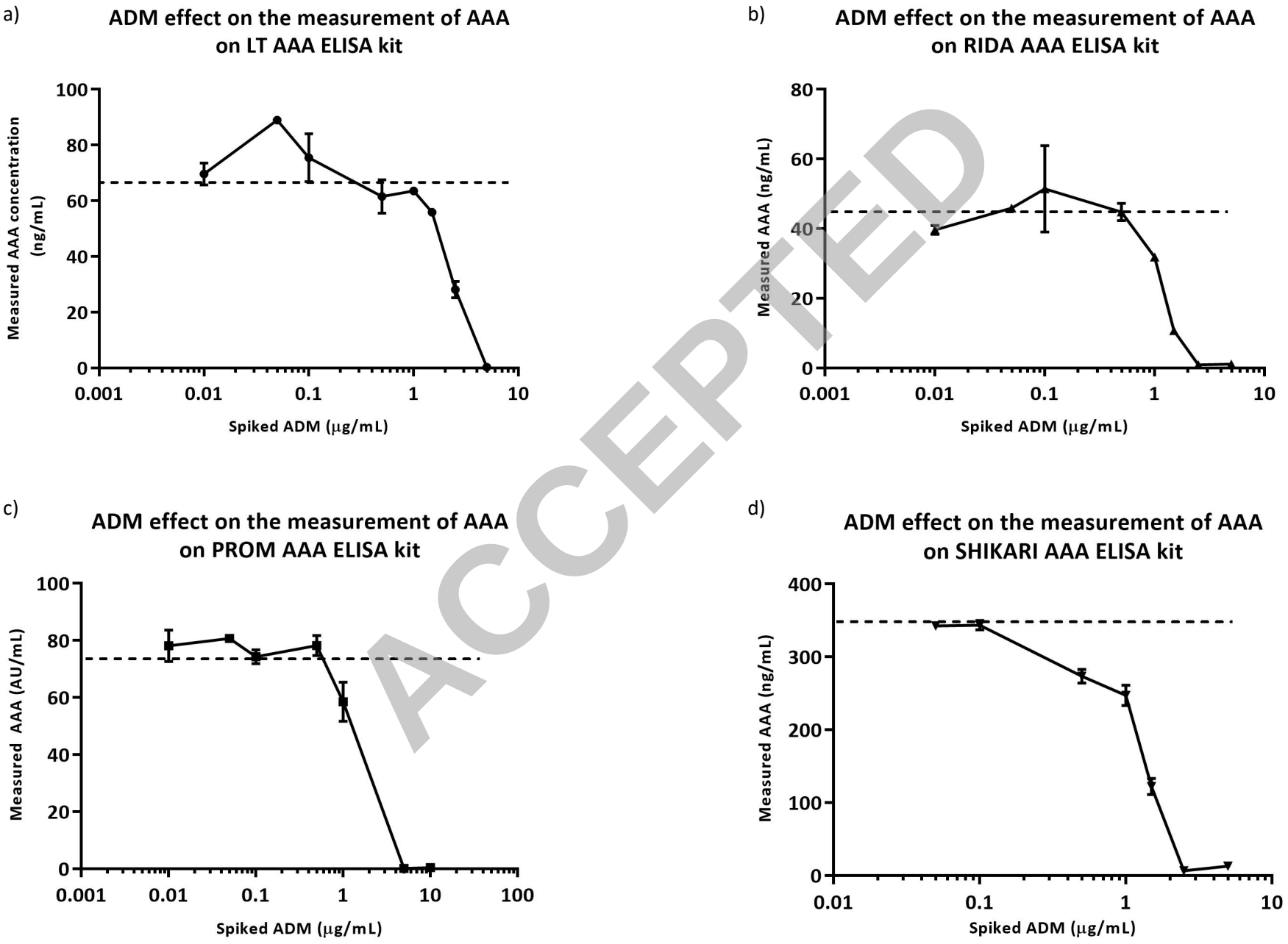
Figure 5



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