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Development and validation of a recombinant human TNF-α based ELISA to detect and quantify adalimumab

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ABSTRACT

Adalimumab, a humanized IgG1 monoclonal antibody is currently used to treat inflammatory diseases. However, a sensitive, in-house ELISA for evaluating inter- and intra-individual pharmacokinetic variability of adalimumab remains limited. In this study, an ELISA was developed to measure adalimumab levels, using recombinant human TNF-α (rhTNF-α) as capture antibody. Initially, surface plasma resonance showed acceptable binding kinetics (K_D) of $2.38x10^{-07}$ nM for adalimumab. Next, a standard curve of adalimumab (1.54 ng/ml to 300 ng/ml), with five quality control points (5.2, 16, 27, 150, and 200 ng/ml) was evaluated for inter and intra-assay accuracy and precision, using serum matrix, by four independent validations. The linear range of the validated assay was 5.2 ng/ml to 200 ng/ml, upper limit of quantification (ULOQ) and lower limit of quantification (LLOQ) were 200 ng/ml and 5.2 ng/ml, respectively. The assay specificity was validated by testing cross-reactivity of rituximab with rhTNF-α, which was found to be non-reactive. Further, the hook effect was over-ruled by diluting the highest concentration of adalimumab tested to assay linear range, and dilution integrity was observed for entire concentrations within linear range (%RE $\leq$ 20 %), as recommended by European Medicines Agency. Collectively, this rhTNF-α binding-based ELISA method is highly sensitive, reproducible, and useful for monitoring adalimumab.

1. Introduction

Tumor necrosis factor-alpha (TNF-α) functions as a pro-inflammatory cytokine that promotes a variety of human disorders, including inflammatory diseases. Additionally, it has a role in cell proliferation, cell survival, and apoptosis [1]. TNF-α exerts its effects through binding to TNF-α receptors I and II, also known as p55 or p60 and p75 or p80, respectively [2]. TNF-α can elicit inflammatory reactions as well as apoptosis of tumor cells and possibly autoreactive T cells. In the latter instance, increased TNF-α production would result in a variety of pathologies including osteoporosis, sepsis, inflammatory neuropathies, and autoimmune illnesses [3,4].

Monoclonal antibodies (mAbs) have been extensively used for the treatment of various disorders by targeting specific antigens in humans [5,6]. These antibodies mostly contribute to antibody-dependent cell-mediated cytotoxicity, through complement-mediated cell lysis [7]. Among the currently available therapeutic mAbs, adalimumab (Humira®) being the first completely humanized IgG1 [8,9], is used for the treatment of rheumatoid arthritis, spondylarthritis, psoriasis, and inflammatory bowel diseases. Adalimumab specifically targets TNF-α by blocking its interaction with cell surface TNF-α receptors p55 and p75, which enhances health by reducing inflammation [10].

The serum levels of TNF-α inhibitor in human subjects vary depending upon the variances in absorption, distribution, and excretion of the drug, which are influenced by body weight, gender, health, and concomitant medication of methotrexate [11–13]. Around 20–30 % of

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patients on adalimumab therapy experienced the development of idiotype antibodies against the drug [14–16]which might result in a decline or variations in adalimumab concentration. Hence, a robust and sensitive method is essential to measure the adalimumab level for therapeutic drug monitoring [17,18]Although ELISA, flow cytometry and surface plasma resonance could be used to measure and monitor adalimumab levels in human sera [19–21], a reliable, robust, and sensitive method is critical to be developed and validated. ELISA has been often used to quantify therapeutic mAbs in biological fluids, due to its specificity, efficacy, and low-cost high throughput [22,23]. Therefore, this study aimed to develop and validate an indigenous in-house sensitive ELISA method using recombinant human TNF-α (rhTNF-α), to detect and quantify adalimumab and evaluate inter- and intra-individual pharmacokinetic variability of adalimumab in biological matrices.

2. Materials and methods

2.1. Materials

Nde-I and BamH-I (New England Biolabs Inc., UK), adalimumab (Humira®, AbbVie, USA), microtiter costar high binding 96-well assay plates (Corning Inc., USA), skimmed milk, tryptone type-II, 3-[(3-Cholamidopropyl) dimethylammonio]-1-propane sulfonate, LB Media and Tris-Cl (Hi-Media Laboratories, India), Tween-20, IPTG, human serum matrix (Sigma Aldrich, USA), kanamycin (Calbiochem, USA), peroxidase-conjugated goat anti-human IgG specific for Fc fragment (Jackson ImmunoResearch Cat# 109-035-008), 1X phosphate-buffered saline (1X PBS), and tetramethyl benzidine (20X TMB) (Denovo Biolabs, India), Ni-NTA column in AKTA (GE-AKTASTART, Cytiva, Sweden), surface plasma resonance (SPR) and 1-ethyl-3-(3-dimethylaminopropyl) (N-hydroxy succinimide) carbodiimide in combination with N-hydroxy succinimide (EDC-NHS) (Affinite, Canada), were procured for the study.

2.2. Cloning and expression of rhTNF-α

The rhTNF-α used as coating reagent in ELISA was commercially cloned using pET28 a (+) vector from Gene Universal Inc. USA. Human TNF-α sequence analysis was performed using UniProt and optimized for the expression in *Escherichia coli*. The complete sequence along with the restriction sites of hTNF-α is presented in Supplementary Fig. 1. The restriction digestion with Nde-I and Hind-III was used to confirm the presence of the insert and cloned hTNF-α gene in pET28a (+) *E. coli*. All cultures were maintained in LB media, and kanamycin was used for antibiotic selection. The hTNF-α protein expression was induced by treating the bacterial culture with 1 mM IPTG at 37 °C for 4 h, harvested pellet, dissolved in Tris-NaCl buffer (50 mM Tris, 150 mM NaCl, pH 8.0), and sonicated. The protein samples were then analyzed on 12 % SDS PAGE.

2.3. Purification of rhTNF-α

The purification of rhTNF-α was performed on nickel nitrilotriacetic acid (Ni-NTA) affinity resin, wherein the N-terminal His-tag of the construct aids in affinity purification. The microbial culture was scaled up to produce rhTNF-α, followed by Ni-NTA affinity purification. Briefly, the rhTNF-α culture pellet was dissolved in 10 ml Tris-NaCl buffer (50 mM Tris, 150 mM NaCl, pH 8.0), followed by sonication for 1 h, and then centrifuged at 10,000 rpm for 10 min. The supernatant was collected and filtered through a 0.2 μm syringe filter. The filtrate sample was passed through the Ni-NTA column in an AKTA purification system, followed by the wash using 20 ml of 50 mM imidazole in 50 mM Tris 150 mM NaCl, pH 8.0. Finally, the rhTNF-α protein was eluted with 250 mM imidazole in 50 mM Tris 150 mM NaCl, pH 8.0, as presented in Supplementary Fig. 2. The protein concentration was estimated by the BCA method, purity was analyzed by 12 % SDS PAGE, and the specific reactivity of purified rhTNF-α with adalimumab was further characterized on SPR.

2.4. Affinity (K_D) determination

Adalimumab drug affinity was evaluated against the purified rhTNF-α protein on SPR. The rhTNF-α protein was immobilized on a 16-mercaptohexadecanoic acid-coated gold sensor at 20 μg/ml using EDC-NHS. Adalimumab drug concentration gradient of 5 μg/ml to 40 μg/ml was used to evaluate the drug affinity (K_D; dissociation constant) against the purified rhTNF-α protein.

2.5. Assay dilutions

The adalimumab stock was prepared in a human serum matrix as per the dilutions and stored at −80 °C for the preparation of standards and quality control (QC), to mimic the clinical subject samples. The minimum required dilution of human serum matrix used for this assay was 1:200, where the signal-to-noise (S/N) ratio was found to be optimum after evaluating S/N of 1:10, 1:20, 1:100, and 1:200 dilutions. The human serum matrix at the described minimum required dilution (MRD) was used as an assay matrix. A total of fourteen standard calibrators (300, 200, 133.33, 88.89, 59.26, 39.51, 26.34, 17.56, 11.71, 7.80, 5.20, 3.47, 2.31, 1.54 ng/ml respectively) were prepared by diluting the working stock in assay matrix. QC samples (200, 150, 27, 16, and 5.2 ng/ml respectively) were used for evaluating accuracy and precision parameters. The background signal was determined by adding only a diluent buffer in the wells. The detection antibody was diluted 1:2000 in assay diluent.

2.6. ELISA

The 96-well microtiter plates were coated with 100 μl of 5 μg/ml rhTNF-α per well (prepared in the coating buffer - 1X PBS, pH 7.4) and incubated overnight at 2–8 °C. The plates were blocked using 300 μl of 2 % skimmed milk and 0.05 % Tween-20 for 1hr at room temperature (RT), followed by a wash with 1X PBS buffer (pH 7.4). Next, 100 μl of standard and QC samples prepared in assay diluent containing 1 % casein were added to the wells in duplicates. The plates were washed three times with wash buffer with 2 min of soaking time each, after incubating for 1 h at RT. Later, 100 μl of peroxidase-conjugated detection antibody specific to human IgG Fc fragment (1:2000) was added, and washed three times with a soaking time of 2 min each, after incubation for 30 min at RT. Next, 100 μl of TMB substrate was added, and the reaction was allowed to develop in the dark at RT for 20 min. Finally, the color reaction was terminated by adding 50 μl of 2 N sulfuric acid, and the absorbance was measured at 450 nm, along with 630 nm as a reference using a microtiter ELISA plate reader. The concentration of adalimumab in the samples was interpolated from the standard calibration curve. Further, the bioanalytical method was developed by optimizing ELISA parameters such as coating of rhTNF-α, blocking/diluent buffer, and detection antibody. The selection of assay format was the first step in assay development, followed by different combinations of blocking and diluent buffer for optimizing the signal-to-noise ratio. HRP conjugated-goat anti-human IgG Fc specific antibody was used as a detection antibody and optimized for the appropriate dilution to be used in this assay.

2.7. Matrix effect

Critically, the matrix effect should be evaluated to ensure the precise and reliable quantification of analyte, as per EMA guidelines for the bioanalytical method development. This test rules out the possibility of any interference from the serum matrix. Herein, the matrix effect was determined by calculating the matrix dilution factor. The matrix dilution factor was calculated by the ratio of the absorbance in the presence of

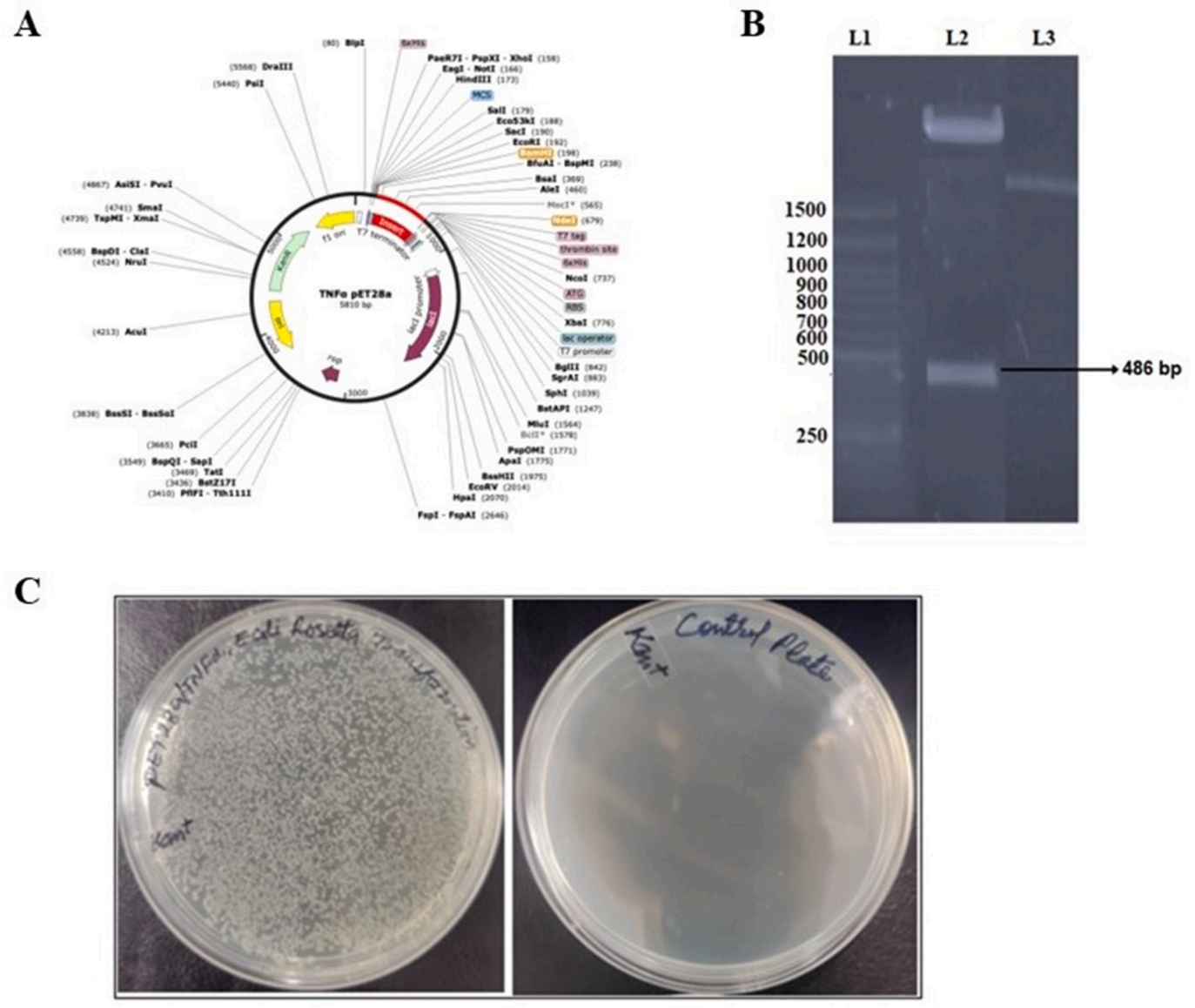

Fig. 1. Construction and expression of TNF-α in *E. coli*.
The rhTNF-α used as coating reagent in ELISA was commercially cloned using pET28 a (+) vector from Gene Universal Inc. USA. **(A)** pET28a (+) vector map with human TNF-α gene insert of 486 bp between Nde-I and Hind-III restriction sites. **(B)** 1 % agarose gel electrophoresis of pET28a (+)-TNF-α DNA construct after restriction digestion with Nde-I and Hind-III enzymes (lane 1- DNA molecular weight marker, lane 2- digested pET28a (+)/TNF-α plasmid, lane 3- undigested pET28a (+)/TNF-α plasmid). **(C)** pET28a (+) with TNF-α construct transformed into *E. coli* colonies and selected on kanamycin antibiotic.

the matrix to the absorbance in the absence of the matrix (blank).

2.8. Calibration curve

The drug calibration curve was developed using a total of fourteen calibrators ranging from 1.54 ng/ml to 300 ng/ml. The calibrators were prepared by diluting the analyte adalimumab in a diluent buffer spiked with human serum matrix. The back-calculated concentrations of the calibrators were within 20 % of the nominal value (25 % at the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ)) as per EMA guidelines, for at least 75 % of calibration standards. The anchor calibrators do not require acceptance criteria, since they are beyond the curve quantifiable range. The data analysis was performed using software generating four parameter logistics (4 PL) fit.

2.9. Repeatability - inter-assay precision

The QC samples were prepared and examined in twelve replicates on a single plate. Five different concentrations of QC samples such as ULOQ, high-level quantification (HQC), mid-level quantification (MQC), low-level (LQC), and LLOQ within the calibration range were calculated based on the following criteria. Briefly, HQC is one-third of the ULOQ of the calibration curve, MQC is near the midpoint on the calibration curve, and LQC is three times the LLOQ. The repeatability was acceptable if the relative error (%RE) of the back-calculated value for each level was <20 % (≤25 % at ULOQ and LLOQ), coefficient of variation (%CV) ≤20 % (≤25 % at ULOQ and LLOQ), and total error (sum of the %RE and %CV) ≤ 30 % (≤40 % at ULOQ and LLOQ).

2.10. Reproducibility - intra-assay precision

The same set of QC samples tested in repeatability was evaluated in

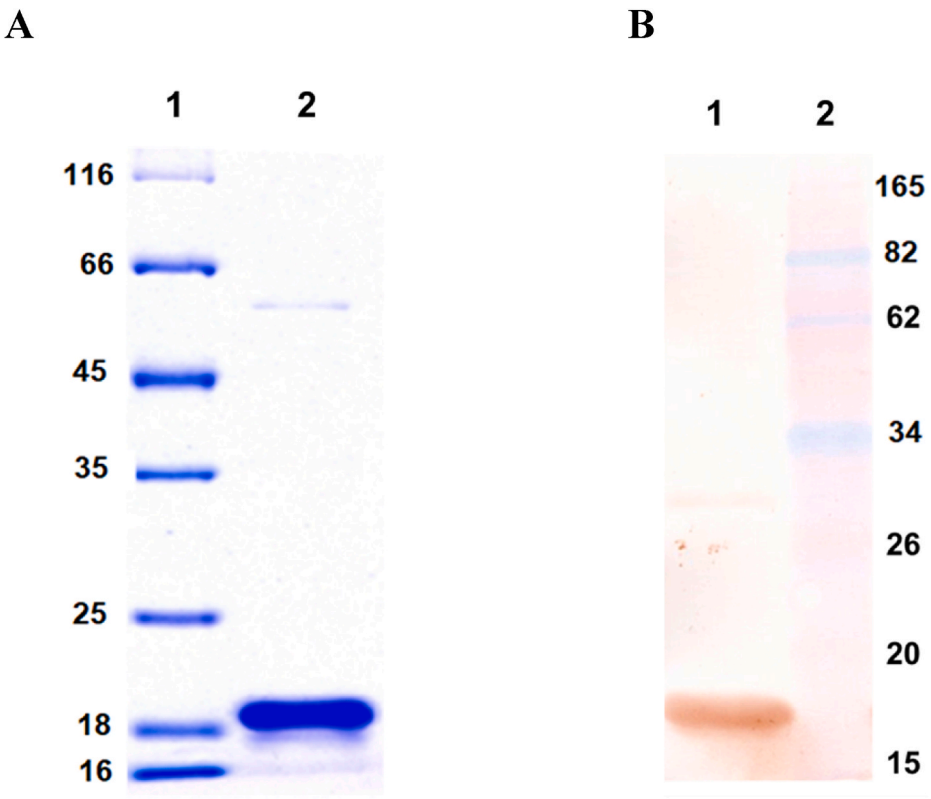

Fig. 2. Purification of TNF-α protein by affinity chromatography **(A)** SDS-PAGE (12 %) of purified rhTNF-α by affinity chromatography. Lane 1- protein molecular weight marker and lane 2- purified rhTNF-α. **(B)** Western blot analysis of purified rhTNF-α. Lane 1- Confirmation of rhTNF-α protein expression by immunoblotting, using an adalimumab antibody, and lane 2- pre-stained protein molecular weight marker.

quadruplet by two independent analysts to determine the reproducibility through intra-assay precision. The reproducibility was acceptable if the relative error (%RE) of the back-calculated value for each level ≤20 % (≤25 % at ULOQ and LLOQ), coefficient of variation (%CV) ≤20 % (≤25 % at ULOQ and LLOQ), and total error (sum of the %RE and % CV) ≤ 30 % (≤40 % at ULOQ and LLOQ).

2.11. Sensitivity

The sensitivity of the assay is to detect the minimum analyte concentration (adalimumab) reliably. The QC at LLOQ (Lower limit of Quantification) was determined by testing the twelve replicates at LLOQ concentration on four independent occasions. The coefficient of variation (%CV) and relative error (%RE) ≤ 25 % and total error (sum of the %RE and %CV) ≤40 % was acceptable at LLOQ concentration.

2.12. Selectivity

The selectivity of the rhTNF-α binding-based ELISA method was determined by testing 8 matrices by spiking adalimumab at LLOQ. A minimum of 80 % of the near-LLOQ QC samples should have assay values that are within 20 % of the nominal concentration (or within 25 % at the LLOQ).

2.13. Dilution linearity

The adalimumab was prepared at 1000X of 1000 ng/ml and diluted 10 times to the concentration within the assay range, using an assay diluent. Different concentrations of adalimumab were prepared and the Hook's effect in the assay was determined.

2.14. Specificity for TNF-α binding drug

The specific binding of adalimumab to rhTNF-α was evaluated during the method development and validated. Rituximab, a non-specific drug that does not bind TNF-α was tested against rhTNF-α-coated ELISA plate to ensure the specific binding of adalimumab and not rituximab to rhTNF-α.

2.15. Statistical analysis

The statistical data analysis was performed using GraphPad Prism 4 PL sigmoidal dose response curve with variable slope. Standard and quality control validation data were analyzed for inter and intra run accuracy, precision and sensitivity, and expressed as %CV, %RE and % total error. All the validation experiments including precision, accuracy, and specificity were performed in quadruplet by two independent analysts. The analyzed data were observed to be in line with EMA guidelines for bioanalytical method development and validation.

3. Results

3.1. Cloning, expression, and purification of rhTNF-α

The vector was constructed using hTNF-α insert in a pET28 a (+) vector (Fig. 1A) with the help of restriction sites of Nde-I and Hind-III. The digested products and undigested plasmid controls formed following the double digestion of the vector (2 μg) with Nde-I and Hind-III were analyzed on 1 % agarose gel electrophoresis. Of note, the digested product (~486 bp) confirmed the presence of insert in the vector (Fig. 1B). Next, pET28a (+)/hTNF-α transformed *E. coli* colonies were observed (Fig. 1C), and rhTNF-α protein was found to be expressed both in soluble and insoluble fraction (data not shown). However,

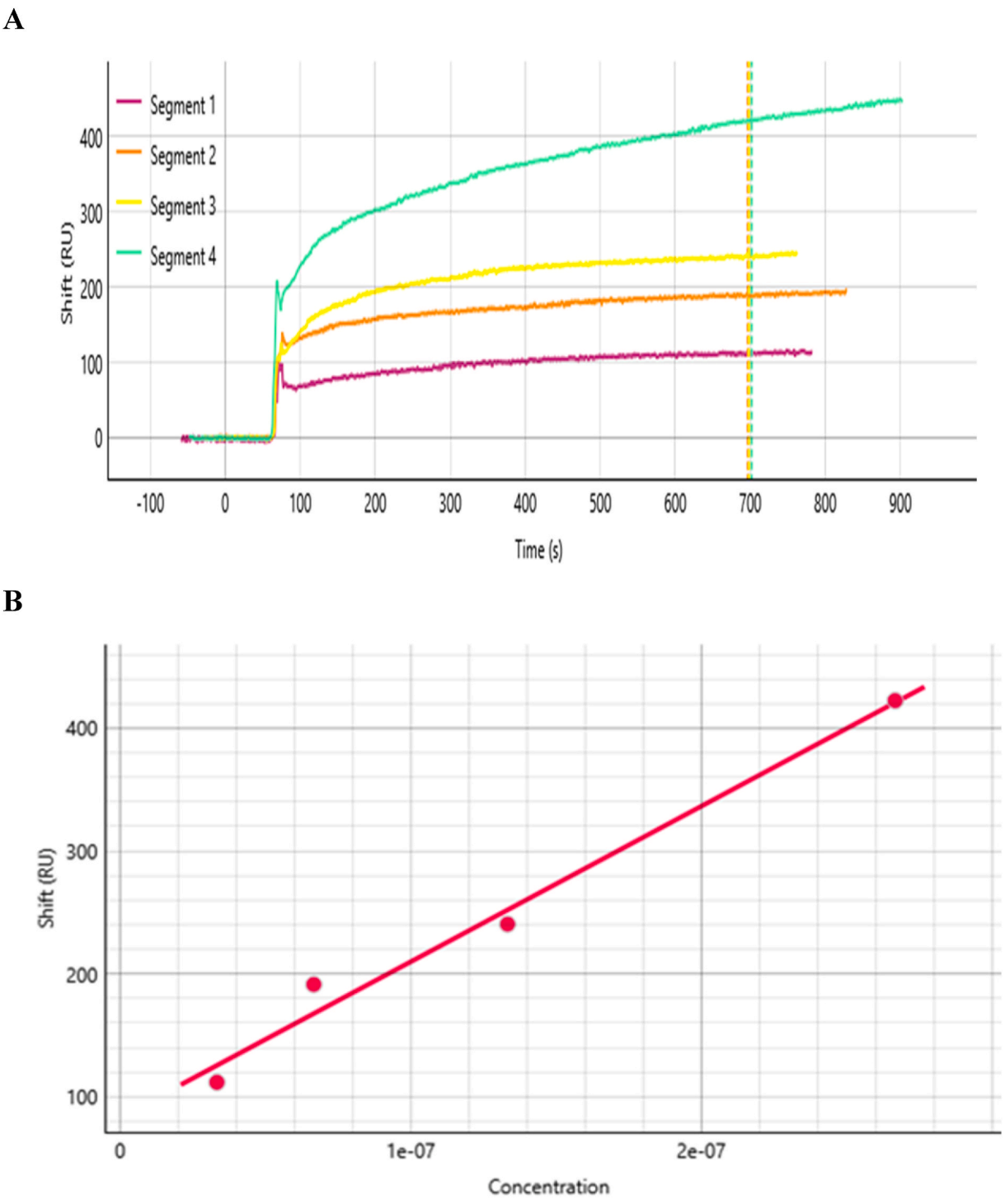

Fig. 3. Affinity of adalimumab for TNF-α **(A)** Binding of adalimumab on immobilized TNF-α protein; segment-1 (5000 ng/ml), segment-2 (10000 ng/ml), segment-3 (20000 ng/ml) and segment-4 (40000 ng/ml). **(B)** Linear fit of nM concentration of adalimumab drug *vs* RU shift.

Table 1
Determination of adalimumab's affinity (KD) binding to immobilized rhTNF-α protein on MHDA coated gold sensor by SPR.

Sl. No.	Cycle	Start cycle (Sec.)	End Cycle (Sec.)	Adalimumab Conc. (ng/ml)	Antibody Molecular weight (KDa)	Conc. (nM/ml)	RU	KD (nM)
1	Segment 1	12900	13740	5000	150	33.33	111.69	$2.38X10^{-07}$
2	Segment 2	14900	15740	10000		66.66	191.36	
3	Segment 3	16550	17340	20000		133.33	240.45	
4	Segment 4	17900	18850	40000		266.66	422.36	

Table 2
Standard calibrators linear assay range (200 ng/ml to 5.2 ng/ml) %RE calculated for the validation run using 4 PL.

Concentration of adalimumab (ng/ml)	Acceptance Criteria	Relative Error (% RE)
200.00	%RE ≤ 25 %	1.6
133.33	%RE ≤ 20 %	1.8
88.89	%RE ≤ 20 %	3.1
59.26	%RE ≤ 20 %	0.9
39.51	%RE ≤ 20 %	1.3
26.34	%RE ≤ 20 %	1.8
17.56	%RE ≤ 20 %	4.1
11.71	%RE ≤ 20 %	0.4
7.80	%RE ≤ 20 %	3.0
5.20	%RE ≤ 25 %	1.7

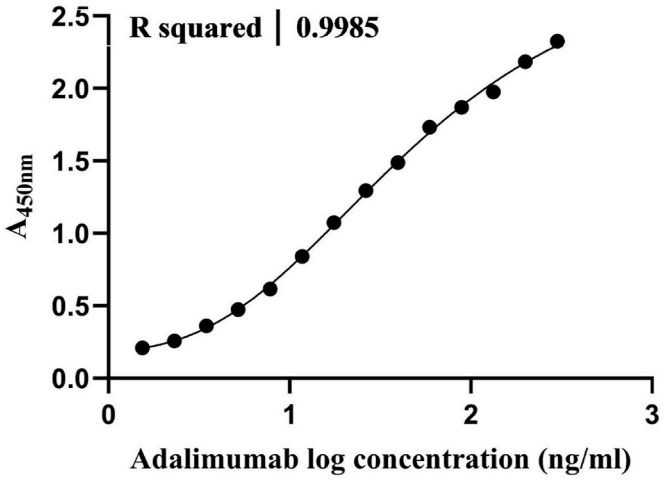

Fig. 4. Standard calibration curve of adalimumab for ELISA.
The calibration curve generated using fourteen calibrator standards was fitted using the 4 PL model. Calibration curve was prepared using calibrators ranging from 1.54 ng/ml to 300 ng/ml, by diluting the analyte adalimumab in a diluent buffer spiked with human serum matrix.

rhTNF-α expression was high in the soluble form. The rhTNF-α protein eluted on Ni-NTA affinity column at 44th min (Supplementary Fig. 2), and expression of rhTNF-α (19 KDa) was confirmed by Western blot using an adalimumab antibody (Fig. 2).

3.2. Affinity of adalimumab for rhTNF-α

Adalimumab at four different concentrations (5, 10, 20, and 40 μg/ml) exhibited concentration-dependent increases in binding to rhTNF-α immobilized on MHDA coated gold sensor chip (Fig. 3A). The R^2 value against the increasing concentrations of adalimumab was found to be 0.95 (Fig. 3B). Moreover, the data analysis by SPR software revealed a K_D value of 2.38 x 10^{-07} nM, which is in the acceptable range of drug affinity (Table 1).

3.3. Effect of serum matrix

The serum matrix dilutions with and without adalimumab (1:10, 1:20, 1:100, and 1:200) were tested. The percent recovery was found to be acceptable at an MRD of 1:200, at which there was no observed matrix interference. Hence, the MRD of 1:200 dilution is considered suitable for the assay validation. The formula (recovered concentration/spiked concentration) × 100 was used to determine the percentage recovery.

3.4. Calibration curve of adalimumab

The calibration curve generated using fourteen calibrator standards was fitted using the 5 PL model (Table 2). The regression model was accepted as the %RE of the back-calculated value for at least 75 % calibrators, within 20 % of nominal concentration. The standard curve was within the acceptance criteria as %RE and %CV of ≤20 % and total error (inter and intra assay) within 30 % for all calibrators (Fig. 4 and Supplementary Figs. 3A and B).

3.5. Precision and accuracy of rhTNF-α-binding ELISA

The precision and accuracy of rhTNF-α-binding ELISA were determined based on the data of inter-assay and pooled intra-assay precision (%CV) accuracy (%RE) of five different concentrations of QC samples. Both %CV and %RE were found to be within ≤20 %, which is acceptable for the ligand-binding assay. However, the total error analyzed was found to be acceptable and within 30 % (Fig. 5A and B).

3.6. Selectivity of rhTNF-α-binding ELISA

The selectivity of rhTNF-α-binding ELISA was determined by testing eight different matrices by spiking adalimumab at ULOQ and LLOQ points. A minimum of 80 % of the ULOQ and LLOQ samples showed assay %RE values ≤ 20 % of the nominal concentration, which showed the recovery of spiked adalimumab (Table 3).

3.7. Sensitivity of rhTNF-α-binding ELISA

The sensitivity of rhTNF-α-binding ELISA was evaluated by inter and intra-batch accuracy and precision. Notably, both inter and intra-batch accuracy and precision showed LLOQ at 5.2 ng/ml, and the %RE of the LLOQ sample was found to be ≤ 20 % range (Table 4). This confirms the assay sensitivity of adalimumab at 5.2 ng/ml.

3.8. Specificity of rhTNF-α-binding ELISA

The specificity of rhTNF-α for adalimumab was confirmed by measuring the levels of adalimumab and rituximab bound to rhTNF-α pre-coated microtiter plate. Notably, adalimumab alone bound to the rhTNF-α in a concentration-dependent manner pre-coated microtiter plate and not the rituximab (Fig. 6).

3.9. Dilution linearity of rhTNF-α-binding ELISA

The dilution linearity was expressed as %RE for QC samples tested. The QC samples were within the linear range exhibiting the acceptance criteria (≤20 %) as per EMA guidelines (Fig. 7). The Hook's effect was observed beyond the linear range of the assay, and the non-linearity was observed beyond the detection range. However, the dilution integrity was exhibited by the assay. Therefore, the assay is suitable for clinical use.

4. Discussion

The use of therapeutic antibodies and other biopharmaceuticals is constantly rising, which requires validated pharmacokinetic and pharmacokinetic-pharmacodynamic assays. Also, it is important to understand the correlations between serum levels of adalimumab, and the therapeutic efficacy and adverse effects of adalimumab. Notably, the rheumatoid arthritis subjects on adalimumab treatment have been reported to lose responsiveness to further doses of adalimumab [24]. One of the major reasons for the loss of responsiveness to adalimumab in these subjects might be the development of anti-drug antibodies [25,26]. Therefore, it is critical to develop a bioanalytical assay, to monitor the blood levels of adalimumab, and ascertain the pharmacokinetics of

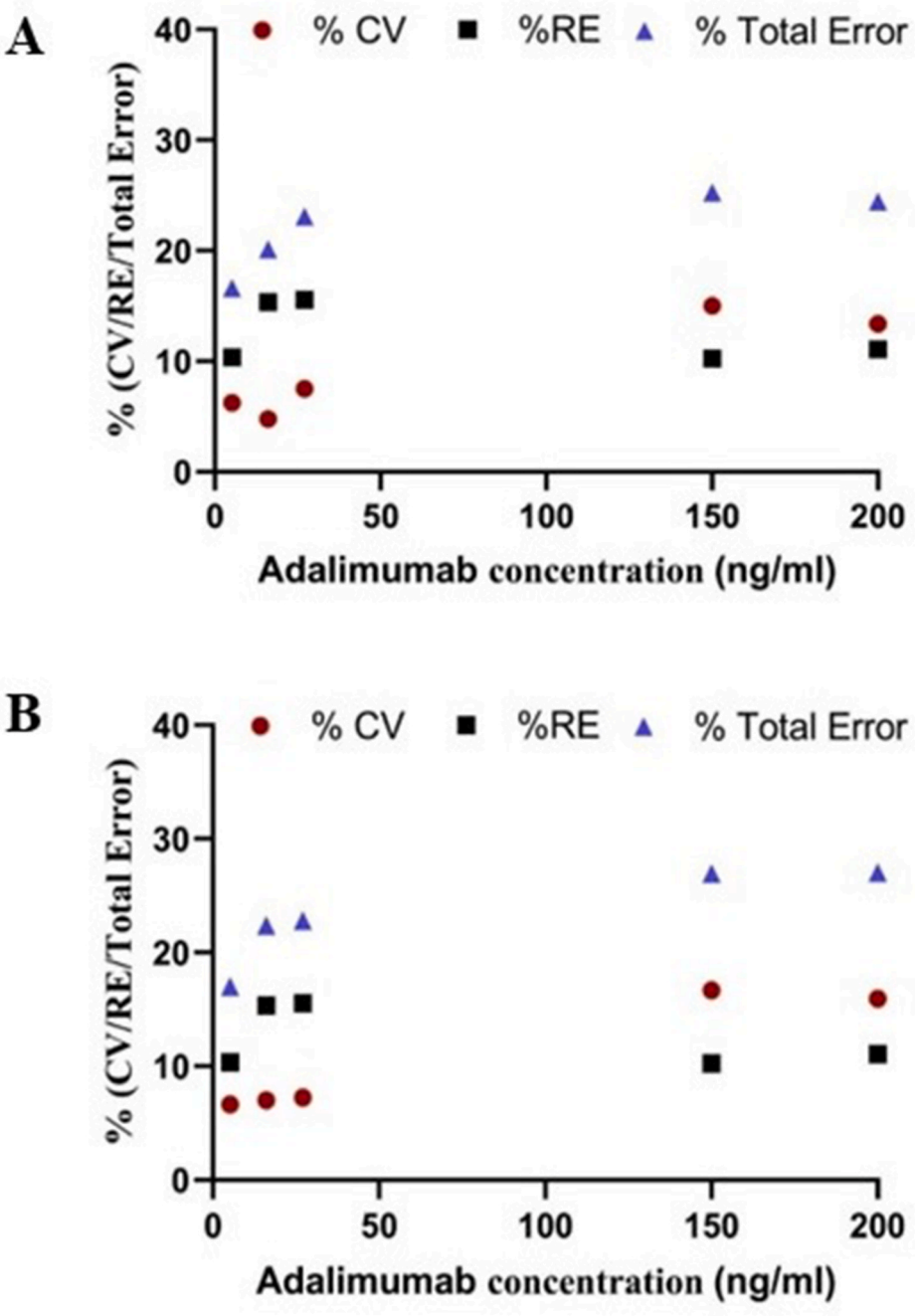

Fig. 5. Precision and accuracy of rhTNF-α-binding ELISA **(A)** Intra-assay accuracy and precision. **(B)** Inter-assay accuracy and precision analysis. Intra and inter assay %CV, %RE and %total error variance of quality controls (ULOQ, HQC, LQC and LLOQ) within and between the analyst run was analyzed using 4 PL. The QC samples were tested in twelve replicates on a single plate. Five different concentrations of QC samples such as ULOQ, HQC, MQC, LQC, and LLOQ within calibration range were calculated.

adalimumab. Previously, several studies have highlighted the critical importance of the development and validation of ligand-binding assays for pharmacokinetic evaluation [27–30]. The development and validation of ELISA to quantify adalimumab in human serum could help validate immunoassays to quantify biopharmaceuticals and their pharmacokinetics. Therefore, the present study aimed to develop a sensitive indigenous ELISA method to assess the pharmacokinetics of adalimumab in the biological matrices. The TNF-α-binding ELISA developed and validated in this study could detect adalimumab rapidly and precisely with almost ten times higher sensitivity in serum matrix when compared to the previously reported method by Desvignes et al., [31] and several-fold higher sensitivity when compared to the commercially available adalimumab ELISA kit ab237641 from Abcam.

The TNF-α-binding ELISA was validated for parameters including precision, accuracy, selectivity, sensitivity, dilution linearity, and specificity under acceptable criteria, by following the guidelines of EMA [23,28,29,32]. The precision and accuracy assays exhibited less than 20 % CV, and the method was demonstrated to be robust, accurate, sensitive, and reproducible for adalimumab concentrations between 200 ng/ml to 5.2 ng/ml. However, accuracy and precision were within 25 % of ULOQ and LLOQ. Besides, the regression model was accepted, since the percentage relative error of the back-calculated value for at least 75 % of the calibrators was within 20 % of the nominal concentration. Moreover, the sensitivity of the assay was tested and found to be 5.2

Table 3
Specificity and selectivity of QC samples, by spiking adalimumab at ULOQ (200 ng/ml and LLOQ (5.2 ng/ml) in different individual serum matrix and back-calculated %RE were observed within the acceptance range (≤25 %).

ULOQ- 200 ng/ml		LLOQ - 5.2 ng/ml	
Measured Mean Conc. Of adalimumab ng/ml	%RE	Measured Mean Conc. Adalimumab ng/ml	%RE
201.38	0.69	5.25	0.87
171.89	−14.06	5.07	−2.52
163.49	−18.26	5.01	−3.67
173.47	−13.27	5.13	−1.44
174.04	−12.98	5.02	−3.37
160.85	−19.58	5.23	0.63
152.05	−23.98	5.05	−2.96
149.55	−25.23	4.89	−5.97

Table 4
Assay sensitivity of QC samples (spiked adalimumab) was determined by analysing intra and inter assay %RE at LLOQ (5.2 ng/ml).

Validation Parameter	QC Sample	Adalimumab Concentration (ng/ml)	Parameters (% RE)
Intra-batch Precision	LLOQ	5.2	−6.26
Inter-batch Precision	LLOQ	5.2	−6.65
Accuracy	LLOQ	5.2	−10.36

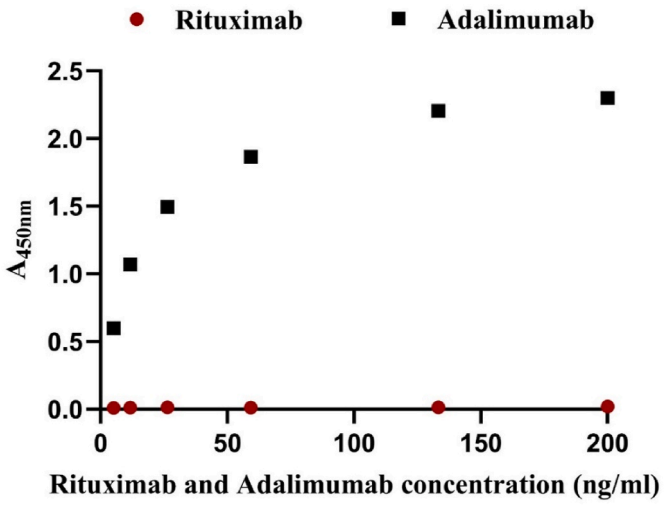

Fig. 6. Specificity of rhTNF-α-binding ELISA.
Adalimumab showed the specific binding to rhTNF-α, and the cross reactivity of rituximab with rhTNF-α was not observed. A non-specific drug, rituximab was tested against rhTNF-α-coated ELISA plate to ensure the specific binding of adalimumab to rhTNF-α.

ng/ml, which qualifies the acceptance criteria.

Of note, the Hook effect frequently observed in ligand-binding assays, is primarily due to high analyte concentration resulting in false negative results [33]. Hence, the Hook effect of the newly developed ELISA was determined by measuring rhTNF-α-bound adalimumab concentrations. Further, it was found that the adalimumab above the assay range (>200 ng/ml) displayed the Hook effect. Remarkably, reports emphasize the impact of assay matrix and dilution linearity on the quality of the antibody-based assays [34,35]. In this line, the current ELISA was also validated using various levels of adalimumab, and found that the dilution linearity was maintained between 5.2 ng/ml to 200 ng/ml. However, due to high variability in quantification below LLOQ, this assay might not be appropriate for measuring adalimumab

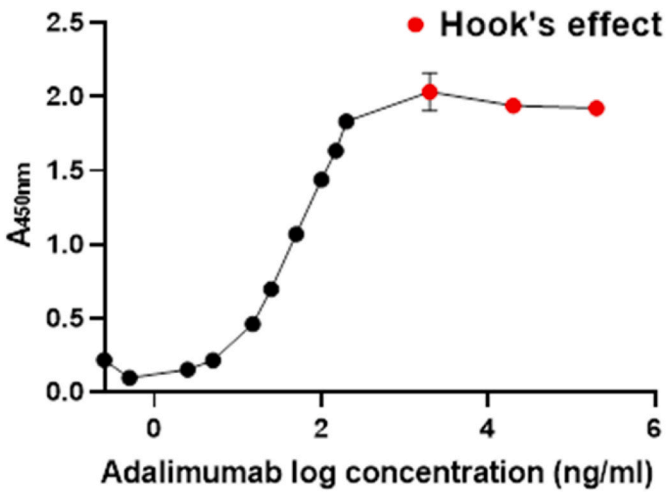

Fig. 7. Dilution linearity of rhTNF-α-binding ELISA.

concentrations below 5.2 ng/ml. Furthermore, the serum matrix did not affect the measurement of adalimumab and thus ruled out matrix interference in the assay. Moreover, LLOQ of 5.2 ng/ml for adalimumab on ELISA confirmed the sensitivity.

5. Conclusions

In this study, an indigenous ELISA method using a rhTNF-α as capture antibody was developed and validated to quantify high and low adalimumab concentrations and rhTNF- binding assay. Further, the method was validated for its analytical performance, to ensure its robustness, sensitivity, and reliability for therapeutic drug monitoring in the biological matrices. The overall outcome of this study highlights the potential use of the validated rhTNF-α-binding ELISA as a standard procedure for adalimumab's characterization, batch release assay in quality control (QC) during drug manufacturing, pharmacokinetics, and bio-comparability during biosimilar development and monitoring drug therapy.

CRediT authorship contribution statement

Dinesh Kumar Saini: Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Manjunath S. Devaramani:** Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation. **Hemalakshmi Shanmugavel:** Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Syeda Zuhin Tabassum:** Validation, Methodology, Investigation, Formal analysis. **Kiran Kumar Mudnakudu-Nagaraju:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Funding acquisition, Data curation, Conceptualization. **Jalahalli Mariswamy Siddesha:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Formal analysis, Data curation, Conceptualization. **Radhakrishna Shetty:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Ethics approval

The study did not require ethical approval.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Dilution linearity exhibited the hook effect over and above the assay linear range. The adalimumab was prepared at 1000X of 1000 ng/ml and diluted 10 times to the concentration within the assay range, using an assay diluent. Different concentrations of adalimumab were prepared and the hook effect in the assay was determined. The Hook's effect was observed beyond the linear range of the assay, and the non-linearity was observed beyond the detection range.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2025.102005.

Data availability

Data will be made available on request.

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