



Assay Linearity and Spike-Recovery Assessment in Optimization protocol for the analysis of Serum Cytokines by Sandwich ELISA Platform

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Abstract

Background: A sandwich Enzyme-linked immunosorbent assay (sELISA) can be used as a diagnostic tool in medicine, plant pathology, and biotechnology, as well as quality control checks in industries. To generate quality and reliable data using this technique, it is essential to optimise the conditions of carrying out sELISA. The optimisation protocol for sELISA includes but not limited to the determination of the right dilution of the sample type, the optimal concentration for the primary antibodies, the use of correct diluent to mention a few.

Aim of study: This study provides a practical approach to establish the right working dilution of serum samples for cytokine sELISA.

Study volunteers and methods: We carried out Linearity of Dilution (LD) assay to measure sELISA accuracy and reliability for interleukin (IL)-4, IL-6, IL-8, IL-9, FGF-basic and granulocyte-macrophage-colony-stimulating factor (GM-CSF) using serially diluted serum samples from healthy volunteers and controlled by human group AB serum (hABs). Also, we undertook spike and recovery (SAR) assay to evaluate for interfering factors in the sample matrix.

Results: Optimum sELISA signals were as follows: IL-4 = Neat, IL-6 = 1:4, IL-8 = Neat, IL-9 ≥ 1:100, FGF-basic = 1:8 and GM-CSF = Neat. The Spike & Recovery assay (SAR) data revealed higher per cent recovery rates for IL-8 and IL-9. Conversely, the remaining cytokines (IL-4, IL-6, FGF-basic and GM-CSF) showed low per cent recovery show the acceptable recovery rate of 80-120%. Our study showed that serum cytokines exhibit different signal strengths on the sELISA platform to varying dilutions of serum samples.

Conclusion: It is therefore recommended that before embarking on serum cytokine sELISA analysis, multiple serial dilutions of serum samples must be used to determine the optimum working dilution for the respective cytokines to be adopted for the rest of the study.

Keywords: sELISA; Spiked & recovery; Hook effect; Assay linearity; Sample matrix

Introduction

Enzyme-linked immunosorbent assay (ELISA) is an immunological test technique that explores the principle of antigen-antibody reaction to identify and quantify substances such as antigens, cytokines, antibodies, glycoproteins and proteins in biological samples [1,2]. The approach employs the use of a solid phase, usually, an enzyme immunoassay (EIA) to accurately

detect the presence of its target, normally an antigen in a biological sample, which is the liquid or wet phase. The analyte (antigen) would typically be absorbed to the solid surface and still participate in the specific high binding affinity reaction with the antibody [3]. The absorption process also facilitates the separation of free from the bound analyte, which gives ELISA an advantage over

radioimmunoassay (RIA) technique. ELISA technique comprises three main analytical approaches; the direct ELISA, indirect ELISA, and sandwich ELISA [4]. The sandwich ELISA (sELISA) approach is based on the detection of the interaction between two antibodies; the capture and detection antibodies. The capture antibody functions to immobilize protein or targets onto the solid plate, meanwhile the secondary antibodies (detection antibodies), which are enzyme-linked engage the immobilized targets in a catalyzed substrate transformation reactions yielding detectable signals [5,6].

The sELISA has two main advantages over the standard approaches. It can bind the target antigen in impure samples selectively. It also has a higher specificity, as the antibodies used are against different epitopes of the target antigen [6]. ELISA signal generation could be influenced by several factors grossly outline as follows; assay plate, coating buffer, capture antibody, blocking buffer, target antigen, detection antibody, enzyme conjugate, washes, substrate and signal detection. Here, we will focus on variable characteristics related to the target antigen, such as optimum dilutions and matrix effects. These variables have been shown to have some influence on the detection levels of some analytes [7]. Hook effect is a circumstance whereas a high dose of antigen leads a remarkably lower signal strength than the actual level present in a sample. This gives the impression that the assay is saturated by a very high concentration of sample antigen binding to all available sites on both the solid phase antibody as well as the detection antibody and thereby preventing the sandwich-formation. The antigen-saturated detection antibodies in solution will be washed off giving a falsely low signal. A "hook" is observed in the curve when data is plotted as a signal versus antigen concentration [8,9].

On the other hand, Spike and Recovery (SAR) assessment is a procedure that can be used for the analysing and evaluation of the accuracy of the sELISA method for particular sample types [10]. This assessment can be used to determine whether analyte detection can be affected by the differences between diluent used for the preparation of samples and the experimental sample matrix [10]. Complex sample matrices, such as serum and plasma, may contain interfering factors that may affect the ability of an assay to quantify the target analyte accurately. Recovery experiments are used to determine if test results are affected by interfering factors [10].

Cytokines perform critical roles in the control of fundamental pathways of the immune system [11]. Hence, the expression levels of certain cytokines have been shown to associate with the prognosis of a variety of diseases, including hematological cancers. For instance, high frequencies of several cytokines and angiogenic factors have been documented as biomarkers for utility in the diagnosis and prognosis of Hodgkin and Non-Hodgkin's Lymphomas (NHL) [12-14]. Adopting reliable methods of quantifying cytokines from diverse sample types can help in disease diagnosis and can provide prognostic value. Therefore, this study is aimed to provide a practical approach for optimizing optimum dilutions of serum samples for cytokine sandwich ELISA.

Materials and Methods

Study location

This experiment was carried out in a Good Clinical Laboratory Practice (GCLP) laboratory at the Royal Liverpool University Hospital, Liverpool.

Sample collection

Whole blood samples were collected from healthy volunteers (HV) into plain glass vacutainers, and serum samples were separated by spinning down the cellular components. Serum samples were stored in a minus 80 Freezer (-86 Freezer) until ready to use.

Ethical considerations and informed consent

No separate ethical clearance was obtained for this experiment since it was part of a bigger study, the PACIFICO phase 3 clinical trial that had been granted ethical clearance. The PACIFICO trial has approvals of the European Union Drug Regulating Authorities Clinical Trials (EudraCT) on a unique number 2008-004759-31 and the International Standard Randomized Controlled Trial (ISRCTN) number ISRCTN99217456. Written informed consent to donate blood for this experiment was obtained from volunteers.

Laboratory methodologies

We carried out sELISA linearity (assay linearity) on serially diluted serum samples from healthy volunteers to establish optimum working dilutions of serum samples for the serum cytokine analysis. The linearity assessment and the spike and recovery (SAR) assay employed to evaluate matrix effect on sELISA, especially for serum samples that yielded reduced ELISA signal.

Assay Linearity for optimum working dilution

Assay Linearity or Linearity of Dilution (LD) assay is an essential measure of ELISA accuracy and reliability [15]. Using serum samples from healthy volunteers and patients, we undertook assay linearity for serum IL-4, IL-6, IL-8, IL-9, FGF-basic and GM-CSF to determine the best dilutions that can be used for serum cytokine sELISA. The choice of cytokines for the experiment was not based on stringent criteria; however, these were part of the 27 cytokines analyzed by Luminex assay in the bigger project (PACIFICO). To assess for assay linearity and to determine the optimal working dilutions of the serum samples. Sandwich ELISA was carried out using serially diluted serum samples (neat to 1:100) from healthy volunteers (HV1 – HV5) and controlled by human AB serum (hABs).

Spike and Recovery (SAR) assessment

The Spike and Recovery (SAR) assessment is essential for the analysis and accuracy evaluation of the ELISA method for particular sample types [10]. Spike and recovery assay is used to determine whether the detection of an analyte is affected by biological sample matrix and differences in the standard curve diluent [16]. For the SAR assessment, healthy volunteer sera and hABs were used for the sELISA to evaluate the effect of sample matrix on serum sELISA. Serum samples were spiked with different concentrations of standard IL-4, IL-6, IL-8, IL-9, FGF-basic and GM-CSF plus unspiked control serum (0) respectively.

Data management and Statistical analysis

Results of Assay Linearity are presented as line graphs, which is the mean of the individual biological triplicates. All Line graphs were generated using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA). In determining the per cent (%) recovery, the concentration of the mean of Expected is calculated by adding the concentration of spiked level to the assay value of the un spiked serum. The per cent recovery is calculated by dividing the mean of Observed sELISA value of the spiked serum by the theoretically determined mean of Expected value [17]. Ideally, the SAR per cent recovery should not be less than 100 with results from 80 to 120% considered satisfactory [18].

Results

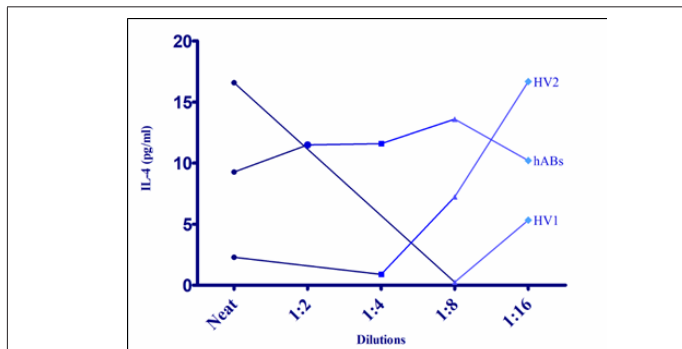


Figure 1: Line chat showing linearity of dilution for serum IL-4 of volunteer samples HV1, HV2 and human AB serum (hABs) as a control on sandwich ELISA.

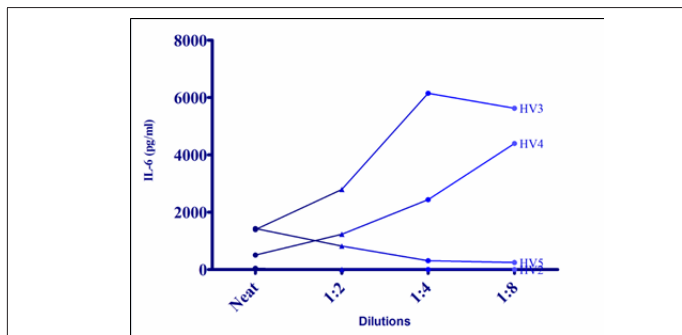


Figure 2: Line chat showing linearity of dilution for serum IL-6 of volunteer samples HV2, HV3, HV4, HV5 and human AB serum (hABs) as a control on sandwich ELISA.

Line Charts have been used to display the trends of the levels of expression or signals of the various cytokines (IL-4, IL-6, IL-8, IL-9, FGF-basic and GM-CSF) in picogram per millilitre (pg/ml) (y-axis) against the dilutions in X-axis as determined by sandwich ELISA. Higher signals of IL-4 can be seen to be expressed at 1:16 for volunteer serum samples HV1 and HV2, but hABs control showed a slight decline in the expression of IL-4 compared to the value at neat as in Figure 1. Therefore, IL-4 can be better demonstrated using undiluted (neat) serum sample. Figure 2 represents the line chat for serum IL-6 expression levels at different dilutions of the volunteer serum samples HV2, HV3, HV4 and HV5, as well as the human AB serum control. HV3 has shown a hook effect at 1:4 dilution, therefore a dilution of 1:4 of serum samples is recommended and could be adopted for IL-6 sELISA. Sandwich

ELISA signals of serially diluted patients sera pac0085, pac0001 and a volunteer serum HV5 along the hABs are depicted in Figure 3. The signal trends indicated that IL-8 could best be analysed by an sELISA method using undiluted serum samples.

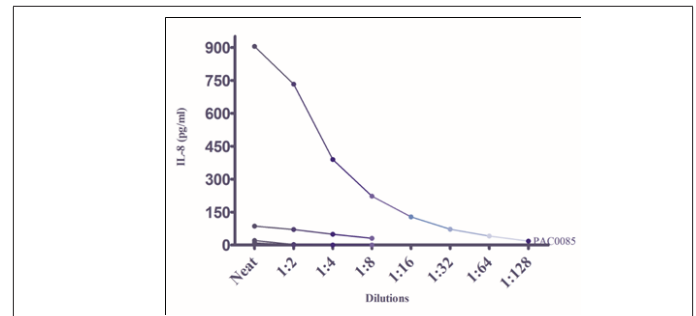


Figure 3: Line chat showing linearity of dilution for serum IL-8 of patient samples pac0085, pac0001, volunteer HV5 and human AB serum (hABs) as a control on sandwich ELISA.

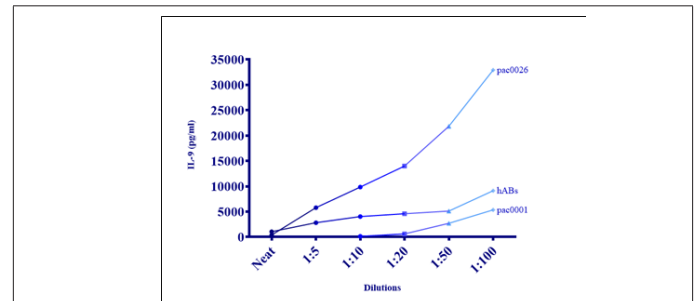


Figure 4: Line chat showing linearity of dilution for serum IL-9 of patient samples pac0001, pac0026 and human AB serum (hABs) as a control on sandwich ELISA.

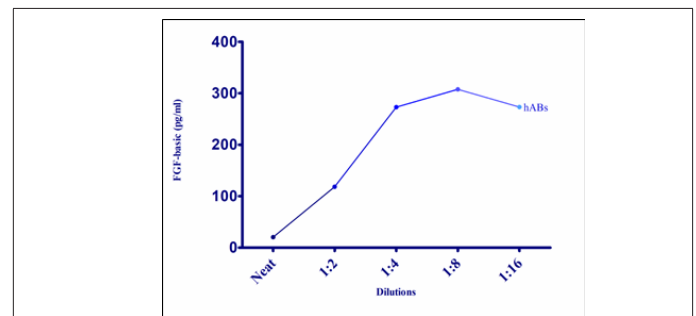


Figure 5: Line chat showing linearity of dilution for serum FGF-basic of human AB serum (hABs) control on sandwich ELISA.

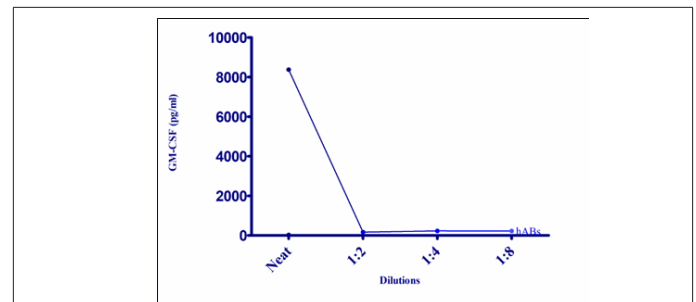


Figure 6: Line chat showing linearity of dilution for serum GM-CSF of volunteer serum samples HV1, HV2 and human AB serum (hABs) control on sandwich ELISA

As shown in Figure 4, Researchers are advised to make higher dilutions (of about 1:100) of serum samples for the analysis of IL-9 by sELISA technique. Figure 5 shows a line chat of serum FGF-basic from human AB serum (hABs) control on sandwich ELISA. A hook effect is noted at 1:8 which showed that the optimum dilution for the analysis of serum FGF-basic by sELISA is 1:8. Meanwhile, an undiluted (neat) serum sample is recommended for the analysis of serum GM-CSF for the best signal as in Figure 6. The results of Spike & Recovery assay (SAR) for IL-4, IL-6, IL-8, IL-9, FGF-basic and GM-CSF are presented in Table1. Serum samples from healthy

volunteers (HVs) were used, except for IL-9 that employed patient samples and controlled by human AB serum (hABs). In most cases, the per cent recovery rate of the serum cytokines assayed on sELISA platform appears poor. However, higher per cent recovery rates are reported for IL-8 and IL-9. All the remaining cytokines (IL-4, IL-6, FGF-basic and GM-CSF) showed low per cent recovery below the acceptable recovery rate of 80-120% [18]. This result suggests that there is possible interference in the sample matrix that could be responsible for masking the detection of these cytokines.

Table 1: The Spike & Recovery assay –evaluating matrix effects on cytokine ELISA.

| Cytokine | Spike level (pg/ml) | Expected Mean (pg/ml) | Observed Mean (pg/ml) /(SD) | Recovery (%) |
|-----------|---------------------|-----------------------|-----------------------------|--------------|
| IL-4 | 500 | 505.564 | 346.298(17.45) | 68.497 |
| | 250 | 255.564 | 146.899(0.40) | 57.480 |
| | 125 | 130.564 | 76.703(1.95) | 58.747 |
| | 62.5 | 68.064 | 45.816(3.60) | 67.313 |
| | 0 | | 5.564(1.76) | |
| IL-6 | 750 | 1,384.821 | 724.956(12.61) | 52.350 |
| | 375 | 1,009.821 | 617.713(16.21) | 61.117 |
| | 187.5 | 822.321 | 676.335(12.89) | 82.247 |
| | 93.75 | 728.571 | 636.987(6.18) | 87.430 |
| | 0 | | 634.821(6.18) | |
| IL-8 | 500 | 550.967 | 676.779(28.51) | 122.835 |
| | 250 | 300.967 | 227.125(10.12) | 75.465 |
| | 125 | 175.967 | 150.101(3.97) | 85.301 |
| | 62.5 | 113.467 | 100.891(2.10) | 88.917 |
| | 0 | | 50.967(1.24) | |
| IL-9 | 750 | 1,675.669 | 2,242.517(227.53) | 133.828 |
| | 375 | 1,300.669 | 1,582.897(126.74) | 121.699 |
| | 187.5 | 1,113.169 | 1,380.720(163.16) | 124.035 |
| | 93.75 | 1,019.419 | 1,191.819(173.39) | 116.912 |
| | 0 | | 925.669(28.26) | |
| FGF-basic | 1000 | 1000 | 361.715 | 36.172 |
| | 500 | 500 | 86.440 | 17.288 |
| | 250 | 250 | 0 | 0 |
| | 125 | 125 | 0 | 0 |
| | 0 | | 0 | |
| GM-CSF | 500 | 500 | 258.665(36.76) | 51.733 |
| | 250 | 250 | 107.610(17.41) | 43.044 |
| | 125 | 125 | 58.624(28.81) | 46.899 |
| | 62.5 | 62.5 | 14.477(2.59) | 23.163 |
| | 0 | | 0 | |

Note: Normal recovery range: 80 %-120% [18].

However, lower concentrations (higher dilutions) of IL-6, IL-8 and IL-9 can be seen to show acceptable per cent recovery rates at the lower limits. Also, this observation could be interpreted as caused by the sample matrix effect which gets diluted out in higher dilutions and yielding better recovery rates.

Discussion and Conclusion

The basic laboratory protocol commonly adopted for the optimization of sELISA technique for the validation of cytokine data include the determining of preferred working dilutions (sensitivity) of antibodies, Spike & Recovery assay and linearity

of dilution assessment to check for sample matrix effects using dilutions. However, this study is designed to provide a simple, practical approach to determine optimum working dilutions for serum cytokine sandwich ELISA. It focuses on the linearity of dilution assessment and spike and recovery assay. The validation protocol targeted mainly six cytokines which include IL-4, IL-6, IL-8, IL-9, FGF-basic and GM-CSF which were selected randomly and are part of the Bio-Plex Human Cytokine 27-plex panel. The linearity of dilution assays for adoption in the quantification of cytokines in serum samples revealed that serum IL-4, IL-8 and GM-CSF can be analysed on sELISA platform using undiluted (neat) serum samples. The study further showed that optimum signals for quantification could be obtained when serum samples are diluted by 1:4 for serum IL-6, 1:100 for IL-9 and 1:8 for FGF-basic using appropriate diluents.

However, an unexpected unending rise in signal strength (pg/ml) along the increasing serial dilutions of serum samples was observed for IL-9 as in Figure 4. Interestingly, an apparent hook effect is reported in IL-6 (HV3) at 1:4 dilution in Figure 2 and FGF-basic (hABs) at 1:8 dilution in Figure 5. The sharp increase in IL-6 levels following serial dilutions as depicted in Figure 2 suggests that sample dilution might have probably enhanced detection by diluting out the effect of interfering factors in the sample matrix that could undermine the accuracy of sELISA results. This observation can be seen in IL-9 and FGF-basic. Assay linearity for cytokines under study by sELISA could be described as generally poor. The gross inconsistencies in the sensitivity and linearity of dilution of the serum sELISA could pose difficulty in determining optimum working dilutions for the serum cytokine sELISA. Interfering agents are capable of changing the concentration of the determinable intensity of the target analyte in the serum sample [19,20] and could be responsible for this trend. Assay interference has been shown to comprise analyte-dependent or analyte-independent with a resultant increase (positive) or decrease (negative) concentration of analytes. These interferences can cause erroneously elevated or lowered analyte number in a sample depending on the type of the interfering substance or the test design [20].

One of the most common interferences in sELISA is cross-reactivity which exerts a nonspecific impact on elements in a test sample that have a structural resemblance to the analyte. Consequently, these elements may be having comparable or the same epitopes as the analyte and hence struggle for the binding site on the antibody, leading to false exaggerated or underestimated analyte concentration [21]. Additional features that are capable of influencing the results of sELISA due to elements within the sample can be grouped under pre-analytical factors such as sample collection, sample type, haemolysis, sample stability and storage [20]. Furthermore, the presence of proteins such as albumin, complement, lysozyme, fibrinogen and paraprotein in the samples has been shown to interfere with the results of immunoassays (sELISA) by affecting the antigen-antibody binding ability [19,20]. It is believed that albumin may interfere with to sELISA due to its elevated quantity and the capacity to bind or release large proportions of ligand [22-24]. However, our investigation of

possible protein interference suggests that protein concentration in the serum sample could not account for the incredible high cytokine signals (IL-9) in sELISA observed in higher dilutions (unpublished data).

The characteristic unexpected low signals in higher concentrations (lower dilutions) and high signals in lower concentrations of cytokines as observed in the serum cytokine sELISA for IL-6, and IL-9 shown in Figures 2 & 4 are more agreeable with high-dose hook effect phenomenon. This phenomenon is centred on the saturation curve of an antibody with antigen, which is due to an extremely high number of analyte concurrently saturating both capture and detector antibodies giving a decrease in the signal at very high concentration of the analyte [20,25]. In immunoassays (such as sELISA) of very high analyte frequencies in a sample; the antigen-antibody interactions can result in antigen surplus; producing misleadingly reduced signals with consequential misrepresentations. Importantly, to improve the sensitivity and quality of sELISA and data, the occurrence of high-dose hook effect must be prevented by either increasing the quantity of the reagent antibodies or reducing the amount of sample (analyte) required for the analysis by sample dilution [26]. The variably poor recovery rates observed in the serum cytokine spike-and-recovery experiments, mostly seen outside the acceptable lower limit of the normal 80 %-120% suggests the effect of interference within the sample matrix.

Other forms of interferants such as cross-reactivity needed to be validated in further validation experiments to help in the process of determining optimal working dilutions of serum samples for adoption in sELISA. In conclusion, our study showed that different serum cytokines show different signal strengths on the sELISA platform to varying dilutions of serum samples. Sandwich ELISA on multiple serially diluted serum samples is, therefore, the first activity before embarking on serum cytokine sELISA analysis for the rest of the study. This first step will help in the determination of the optimum working dilutions for the respective cytokines of interest. Therefore, further studies on the other cytokines to determine the best serum dilutions for the best signals on the sELISA platform is recommended. We further recommend a more rigorous optimisation protocol to be carried out to set the best dilution for adoption in the analysis of multiple serum cytokines on Multiplex platforms.

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