

## RESEARCH ARTICLE

### A STUDY ON THE IMPACT OF REPEATED FREEZE-THAW CYCLES ON THE SERUM CYTOKINE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

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**ABSTRACT:** Sandwich ELISA is a modified form of the standard ELISA technique used by researchers and in routine laboratories for detecting and quantifying specific analytes in samples. This study is designed to evaluate the impact of freeze-thaw cycles on the results of serum cytokine measurement by sandwich ELISA technique. Serum samples were collected from four healthy donors and stored in 3 aliquots at -80°C for three days per thaw-freeze cycle. The concentrations of IL-4, IL-6, IL-8 and IL-9 in the aliquots of serum samples exposed to one, two and three freeze-thaw cycles respectively were determined. Data shows an increase in the cytokine levels following two and three freeze-thaw cycles compared to the expression levels obtained after one freeze-thaw cycle (baseline). The increases reported are as follows: IL-4; 6.56pg/ml (19%) and 3.52pg/ml (10%), IL-6; 103pg/ml (20.9%) and 24.6pg/ml (5%), IL-8; 105.05pg/ml (14%) and 102.76pg/ml (11%) and IL-9; 117pg/ml (17.77%) and as high as 312pg/ml (47.52%) respectively. These results suggest that the repeated thawing and freezing of serum samples can alter the values of serum cytokines measured by ELISA, in most cases with an increase. The reasons for the increase in the values of these cytokines following repeated thawing and freezing have not been investigated. To minimize the effects of thaw-freeze cycles on serum cytokine ELISA, fresh serum samples should be used for the analysis of serum cytokines. Alternatively, aliquots of serum samples can be stored in the -80°C Freezers for single use or by the application of a methodology that will subject samples to similar treatments, for instance, analysing several samples in parallel as in Multiplex assay. These approaches will minimise variations due to repetitive freeze-thaw cycles.

**KEY WORDS:** Sandwich ELISA, Freeze-thaw cycles, IL-4, IL-6, IL-8 and IL-9

### INTRODUCTION:

Historically, enzyme immunoassays were first reported in 1971 and were used to quantify antigens and for the titration of antibodies in a sample<sup>1</sup>. The principles of these immunoassays are likened to those of radioimmunoassays that

immobilise an antigen or an antibody on a solid phase (immunoabsorbent) to facilitate the separation of the free antigen or antibody from the antigen-antibody complex for detection and quantification<sup>2</sup>. This reaction forms the

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fundamental principle of the present day enzyme-linked immunosorbent assay (ELISA). Enzyme-linked immunosorbent assay (ELISA) is a plate-based 'wet lab' powerful technique used for detecting and quantifying specific analytes usually antigens such as peptides, proteins and hormones as well as antibodies within a crude sample<sup>3,4</sup>. The technique involves immobilising antigen on a solid surface of polystyrene plates and then bound to an antibody-enzyme complex<sup>4</sup>. The essential element used for the detection strategy is a highly specific antibody-antigen interaction. Four species of ELISA methods including direct ELISA, indirect ELISA, competitive ELISA and sandwich ELISA have been identified<sup>5,6</sup>.

As one of the dependable and efficient tools used in immunology, ELISA utilises the natural characteristics of antigen-antibody binding potentials to quantify interest targets by the colour emission of the reaction solution<sup>6</sup>. However, steps must be taken to ensure working with optimal working concentrations (dilutions) of the target analyte (antigen) and antibody for generation of credible data<sup>8</sup>. In all ELISA experiments, the use of standard curve is imperative as it serves to ensure patterned and genuine quantification of the target protein across different sample types<sup>9</sup>. Ironically, as the quality of ELISA results relies upon the quality of standard curves, so is the quality of the standard curve reliant on the optimal dilution of the standard reagent<sup>10</sup>. Therefore, dilution is an indispensable protocol in ELISA, which in proper sequence, plots the values of detection spread for antibody and target antigen concentrations. The standard way of interpreting ELISA results is via the Optical Density (OD) obtained at various strengths of the standard. The hyperbolic curve function is used to determine the OD to dilution correlation, and the more the linearity of the curve, the stronger the correlation between the OD and standard concentration<sup>9,11</sup>. Nevertheless, in situations where a straight-lined standard curve is not achieved, an optimal value of correlation coefficient  $r^2$  of approximate 0.98 is satisfactory<sup>12</sup>. Other factors that can influence the

quality of ELISA results may include temperature, concentrations of antigen/antibody, contact time and incubation, pH, ionic strength, enzyme treatment and polymers as well as potentiators<sup>13,14</sup>. Cytokines are large soluble proteins that mediate innate and adaptive immunity and are usually involved in the inflammatory process, bone marrow differentiation, recruitment of immune cells, cellular activation and adhesion molecule expression<sup>15</sup>. Through interactions with specific receptors on membranes of antigens and pathogens, cytokines can regulate immune response<sup>16</sup>.

Due to the discrepancies observed in an ELISA cytokine data from a previous experiment (data not shown) aimed at validating results of serum cytokine levels determined by Multiplex assay, we hypothesised that the freeze-thaw cycles experienced by the serum samples in the course of the optimisation could be responsible for the discrepancies in the results. We, therefore, aimed to examine the impact of freeze-thaw cycles on the results of ELISA of serum cytokines. The data will undoubtedly provide researchers with an understanding of the necessary precautions to be taken when carrying out serum cytokine measurement using sandwich ELISA, especially those from locations suffering from the unstable and unreliable power supply.

## **MATERIALS AND METHODS:**

### **Study population & sample size**

This pilot study aims to investigate the extent to which freeze-thaw cycles of serum samples can affect the expression levels of serum cytokines determined by ELISA. The interest lies in correlating cytokine data determined by ELISA and Multiplex assay. In this experiment, the frequent freezing and thawing process of serum samples in hospital and research laboratories was mimicked using serum samples from five healthy blood volunteers.

### **Ethical Approval & Informed Consent**

This experiment forms part of sELISA validation process in the context of the translational work of PACIFICO trial ethics application. 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 Randomised Controlled Trial (ISRCTN) number ISRCTN99217456. Written informed consent of apparently healthy blood donors to participate in the validation process was obtained before recruitment to the study as per the International Council for Harmonisation (ICH)-Good Clinical Practice (GCP) regulations.

### **Sample processing & the freeze-thaw cycles**

Samples were processed, and three lots of serum aliquots per donation were categorised and stored in a -80°C Freezer. On the third day following the freezing of the sample lots, the first set of aliquots were thawed and refrozen while the other two lots remained in the freezer. After another three days, a second thaw-freeze cycle was repeated on the first set along with the second set of aliquots. Finally, on the day of the ELISA experiment, all the three sets of serum aliquots were thawed, and ELISA was performed. By this protocol, the first set of serum aliquots experienced a 3-cycle of freeze-thaw; the second had 2-cycles while the third set had a thaw after the initial freezing.

### **The Sandwich ELISA kits and reconstitution of antibodies**

The ELISA Development Kits contains essential components for the quantitative measurement of human cytokines in a sandwich ELISA format including capture antibodies (CAs), detection antibodies (DAs), Wash Buffer, Block Buffer and Diluent were supplied by PeproTech EC Ltd, London, UK. Additional materials and reagents required and not provided in the kit are ELISA microplates, ABTS Liquid Substrates solution (Sigma Cat. #: A3219), Bovine serum albumin (BSA; Sigma Cat. #: A-7030), Dulbecco's

Phosphate buffered saline (PBS; [10 ×]) (Gibco BRL. Cat #: 14200-075), Wash Buffer, Block Buffer, Diluent and Tween-20 (Sigma Cat. #: P-7949). The ELISA assay targets included the human IL-4 (Cat. #: 900-K14), IL-6 (Cat. #: 900-K16), IL-8 (Cat. #: 900-K18) and IL-9 (Cat. #: 900-K20) (PeproTech EC Ltd, London, UK).

Following manufacturer's instructions (PeproTech, USA), CAs, DAs and standard cytokine reagents were reconstituted to stock concentrations by the addition of the respective amounts of sterile distilled water (dH<sub>2</sub>O). These form the stock working reagents. The working reagents were then prepared by diluting the stock reagents in 1 × PBS thereby producing the recommended working concentrations of the antibodies for each cytokine. The working reagents were prepared for a single 96-well ELISA experiment and were not stored.

### **Methodology for sandwich ELISA**

The vials of working CAs were gently vortexed, and 100 μL was added to each well of the ELISA plate. The microplate was sealed firmly with sealing film and incubated overnight at room temperature (RT). The plate was washed on the following day four times using 1 × Wash Buffer and blotted on clean, dry paper towel. To block for non-specific binding, 300 μL of ready-to-use Blocking Buffer was added to each ELISA well; then the ELISA plate was kept sealed at room temperature (RT) for 1 hour. Next, 100 μL of each standard concentration, samples, internal control (IC) and blank (B) were added to assay wells in triplicate. ELISA plates were sealed and incubated for 2 hours at RT. The plate layout allows for the inclusion of 8-point standards S1-S8 with a 4-fold serial dilution between each point, including one internal control (IC) or human AB serum (hABs) and two blanks (B) rows in triplicates. The blanks are essential in determining the efficacy and validity of the measurements of the samples. After the 2 hours incubation, the plate was removed and washed four times in 1 × Wash Buffer ready for the addition of working DA reagent. Enough DAs

for a single experiment were prepared fresh. Each assay well had 100  $\mu$ L added immediately before the plate was sealed and incubated for another 2 hours at room temperature. After incubation, plate content was discarded and washed four times in 1  $\times$  Diluent and 100  $\mu$ L of a 1:2000 dilution of Avidin conjugated with horseradish peroxidase (HRP) was added to each ELISA well immediately. The ELISA plate was sealed and incubated for 30 minutes at room temperature. After the 30 minutes incubation, ELISA plate was aspirated and washed four times in 1  $\times$  Diluent. Next, 100ul of already-to-use ABTS substrate (2,2'-Azinobis [3-ethylbenzothiazolone-6-sulphonic acid]-diammonium salt) was added to each ELISA well using a multichannel pipette. A SPECTRA max PLUS 384 ELISA Plate Reader (Molecular Devices, California, USA) set at 405nm with wavelength correction at 650nm was used to monitor colour development at 5 minutes intervals at RT for up to 50 minutes. Colour development was recorded by reading the optical density (OD) of the wells, and SOFTmax Pro software (Molecular Devices, California, USA) was used for endpoint reading. When readings fall between zero and the highest standard values; reliable standard curves are said to be obtained.

### The Cytokine Standard curves

The 8-point concentrations of standard cytokines were prepared by diluting each cytokine in 1  $\times$  Diluent as in Table 1. Briefly, 400  $\mu$ L of Diluent was added to standard tubes except for the first tube. Then 800  $\mu$ L of the reconstituted cytokine standard solutions were added to the first (highest) standard tube (S1), and 400  $\mu$ L was transferred to the second tube (S2). The tube content was mixed carefully, making a 1:2 dilution of S1. The serial dilution process was repeated through to the 8th tube/concentration (S8) where 400  $\mu$ L of the standard diluent solution was discarded. Each standard curve tube contains a final volume of 400  $\mu$ L for a triplicate of each point of the standard curve. Readings of the standards were taken as for the test samples and plotted.

**Table 1: 8-point Concentrations for the cytokine standards**

Cytokine (pg/ml)	IL-4	IL-6	IL-8	IL-9
Tube	1000 - 0	1500 - 0	1000 - 0	3000 - 0
1	1000	1500	1000	3000
2	500	750	500	1500
3	250	375	250	750
4	125	187.5	125	375
5	62	93.75	62	187.5
6	31	46.88	31	93.75
7	15.5	23.44	15.5	46.88
8	7.8	11.72	7.8	23.44

### Data management & statistical considerations

The respective standard cytokine curves were used to determine the concentrations of cytokines in serum samples. The respective optical densities obtained from tests and blank triplicate wells were translated into numerical values of cytokine concentrations after subtracting the average values of optical density of the blank wells. Using Excel, the average of the three measurements were reported as the concentrations of the cytokines at each number of the freeze-thaw cycle. The percentage of the differences (increases) in the concentration of each cytokine at different numbers of freeze-thaw cycles against the baseline values (one freeze-thaw cycle) were reported. Data were presented in bar-charts.

### RESULTS:

Figure 1 is a Bar-chart showing serum IL-4 expression levels of 34.50pg/ml, 41.06pg/ml and 38.02pg/ml determined by sandwich ELISA following one, two and three freeze-thaw cycles respectively. Data reveals a general increase in IL-4 expression levels by 6.56pg/ml (19%) and 3.52pg/ml (10%) consequent to two and three freeze-thaw cycles of the serum sample. The



impact of freeze-thaw cycles on sandwich ELISA result of serum IL-6 is presented in Figure 2. ELISA results show an increase in the levels of serum IL-6 of about 103pg/ml (20.9%) following two freeze-thaw cycles and 24.6pg/ml (5%) at three freeze-thaw cycles. Expression levels of 92.54pg/ml, 105.05pg/ml and 102.76pg/ml were reported for IL-8 following one, two and three freeze-thaw cycles respective as in Figure 3. An increased IL-8 expression level of about 14% and 11% were reported in two and three freeze-thaw cycles respectively. Figure 4 depicts the levels of IL-9 following one, two and three freeze-thaw cycles. It reveals an increase in IL-9 expression levels of about 117pg/ml (17.77%) and as high as 312pg/ml (47.52%) in two and three freeze-thaw cycles respectively. The percentage variation in the expression levels of cytokines as shown in Figure 5 reveals that an increase in the ELISA signals accompanies every subsequent freeze-thaw cycle. Comparing the ELISA results following two freeze-thaw cycles with three freeze-thaw cycles, a drop in the percentage ELISA signals for IL-4, IL-6 and IL-8 were observed. However, IL-9 can be seen to maintain a very high percentage increase at three freeze-thaw cycles.

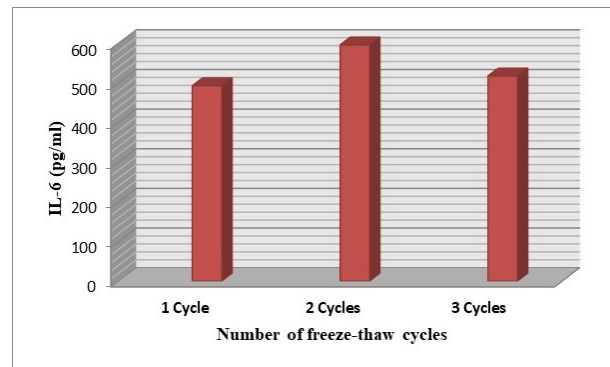


Figure 2: IL-6 expression levels measured with ELISA in serum samples after different freeze-thaw cycles

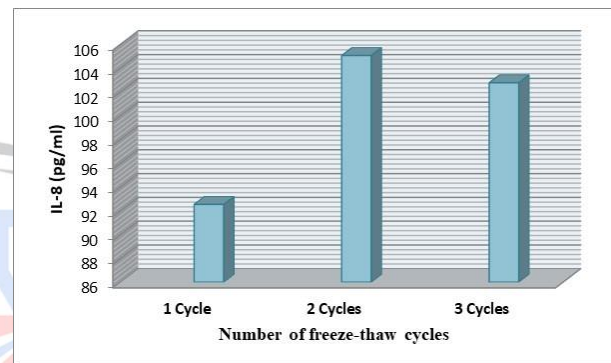


Figure 3: IL-8 expression levels measured with ELISA in serum samples after different freeze-thaw cycles

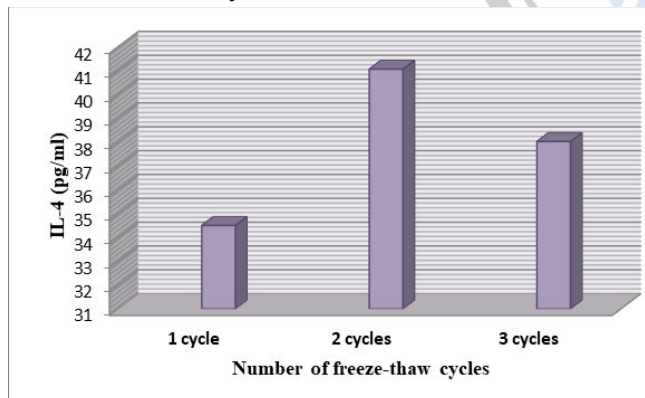


Figure 1: IL-4 expression levels measured with ELISA in serum samples after different freeze-thaw cycles

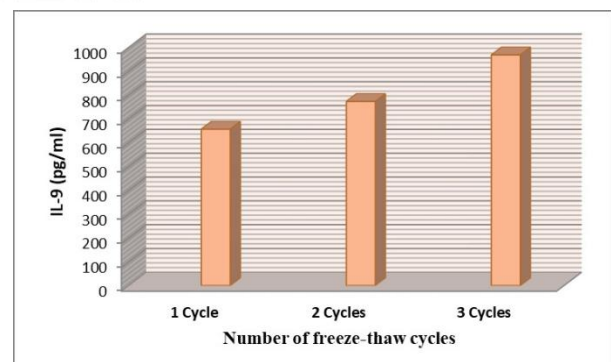
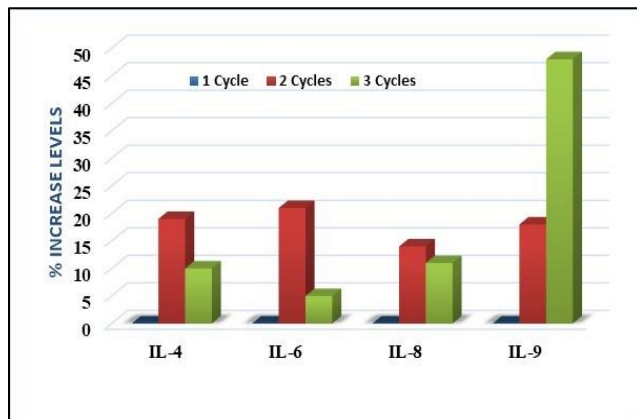


Figure 4: IL-9 expression levels measured with ELISA in serum samples after different freeze-thaw cycles



**Figure 5: The relative percentage changes in cytokine levels following freeze-thaw cycles**

## **DISCUSSION:**

This experiment was designed to examine the impact of repeated freeze-thaw cycles on the results of serum cytokines, IL4, IL6, IL8 and IL9 determined by ELISA method. The primary objective of the experiment was to test the hypothesis that states that exposing serum samples to repeated freeze-thaw cycles before analysis can cause changes in the expression levels of serum cytokines determined by sandwich ELISA technique.

The data as presented in Figures 1 to 5, column charts of cytokine expression levels revealed a marked increase in the values of serum IL-4, IL-6, IL-8 and IL-9 following repeated freeze-thaw cycles respectively. To assess the effect of freeze-thaw cycles, we considered the expression levels of cytokines reported at one freeze-thaw cycle to be the baseline values. It was observed in Figure 5 that following two freeze-thaw cycles, the percentage increase in the expression levels of IL-4, IL-6, IL-8 and IL-9 were found to be 19%, 21%, 14% and 18% respectively. These values changed to 10%, 5%, 11% and 48% following three freeze-thaw cycles to in that order. The experiment reveals that any procedure that can cause the thawing of stored serum samples in Freezers, especially in a repeated manner is capable of altering and invalidating the expression levels of serum cytokines in the sample. Although we do

not have any particular explanation for the changes observed in the expression levels of the cytokines following the repeated freezing and thawing, results may imply that specific artefacts that mimic the reactions of such cytokines may have been introduced to samples in the course of repeated freeze-thaw cycles. Also, not much is known as to how freeze-thaw cycles can affect the biological structure of the cytokines.

A similar study carried out by Lee *et al.* reported that matrix metalloproteinase-7 (MMP-7) and vascular endothelial growth factor (VEGF) levels were increased by up to >15% in stored plasma and up to 7% in stored serum after five freeze-thaw cycles respectively<sup>17</sup>. Their study showed a tendency to further increase in expression levels of analytes with the increasing number of freeze-thaw cycles. Contrary to our report, Lee *et al.* indicate that IL-8 was stable in both plasma and serum following repeated freezing and thawing. In a study that assessed the effect of multiple freeze-thaw cycles on detection of Measles, Mumps, and Rubella Virus antibodies, IgG levels and IgM activity were reduced to 25% below pre-freezing levels<sup>18</sup>.

A group evaluated serum C-reactive protein (CRP), an acute-phase reactant whose levels increase in response to a variety of inflammatory stimuli using highly sensitive CRP enzyme immunoassay and the report of their study showed that CRP levels were unaffected by up to seven freeze-thaw cycles<sup>19</sup>. It shows that unlike the cytokines we studied, CRP may be stable even after several cycles of freeze-thaw, meaning the results of the analysis of CRP from stored serum samples may be more reliable than those of cytokines. Also, a study by Wang and his colleagues revealed that CD163, NGAL (neutrophil gelatinase-associated lipocalin (NGAL), high mobility group box-1 protein (HMGB1) and macrophage inflammatory protein 2 (MIP-2) remained stable within three freeze-thaw cycles<sup>20</sup>. There is no clear explanation for the discrepancies in our data and the findings of Wang and his colleagues. However, we noted the

differences in the serum storage temperature of 4°C in their case, while we stored at -80°C. Another study has reported a decrease in IL-24 following repetitive freeze/thaw cycles of the serum samples. The report is a clear contrast from our data. According to the article, variations in the laboratory results of the same analyte measured by different laboratories could be attributed to the ways samples are handling, including freeze-thaw cycles<sup>21</sup>.

Taken together, it was evident that different analytes respond differently to the impact of freeze-thaw cycles. It indicates that there could be different but specific factors responsible for these observations. Although the analytes considered in this paper are mainly proteins in nature, the instabilities of these proteins are based on biological and structural differences and could be responsible for the discrepancies reported in the effect of freeze-thaw cycles.

In practice, sample storage cannot be avoided both in routine and research laboratories, for instance, the Biobank. These stored samples can be used in the retrospective (baseline data) and prospective studies, or even to repeat the laboratory test for a patient. It is essential to control the pre-analytical process including proper storage of biological samples. It is worth pointing out that appropriate care should be taken during handling and storing samples including serum samples, as many other factors might induce protein degradation, such as storage time, microbial contamination, among others, which can affect the final test results.

This study is not devoid of limitations as the results of freeze-thaw cycles were based on short-term freeze-thaw cycles experiment. It would be ideal to evaluate the impact of freeze-thaw cycles on cytokine ELISA thoroughly, by performing the study using samples stored for extended periods. This design will allow for comparing samples with different storage timelines, and multiple freeze-thaw cycles. Sandwich ELISA data of cytokines using fresh serum samples from blood donors might reveal a clearer picture of the impact of

freeze-thaw cycles on cytokine data. The fresh serum samples will provide a baseline measurement of the cytokines in samples before and after the freeze-thaw cycles.

## **CONCLUSION:**

In conclusion, it is suggested that the use of fresh serum samples to analyse analytes in serum is preferred. However, if it is necessary to store samples for later processing, aliquots or vials of serum samples must be stored in the -80°C Freezers for single use. Also, as a quality control measure, storage of samples in facilities that are prone to an unstable power supply or constant power outages must be discouraged as fluctuations in the storage temperatures caused by these unstable supply of power may adversely affect the results and signals of serum cytokines determined by sandwich ELISA technique. Particularly, test samples must be subjected to similar treatments, for instance, analysing several samples in parallel as in Multiplex assay. These approaches will minimise variations in the values of analytes due to repetitive freeze-thaw cycles and variable sample handlings.

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