# *Evaluation of Residual Protein ELISA Stability of Precoated Plates and Assay Control in a High Throughput Environment*

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## Abstract

*The objective of this study is to assess the viability of Enzyme Linked Immunoassay Sorbent Assay (ELISA) precoated plate's overall ability to quantify Residual Protein levels in a wide variety of drug substance samples that undergo different filtration processes. The use of this methodology reduces turnaround time and give analyst more flexibility on their testing schedule. Results obtained on precoated plates at 5C and -20C showed a positive outcome when the optical densities and the actual titers were compared to the control group. Precoating plates for Residual Protein ELISA demonstrated to be cost-effective, enabling to shorten the turnaround time from one week to 72 hours. It also gives a swifter testing approach towards emergency runs, Process Performance Qualifications submissions, and deviations initiated by the manufacturing departments. Data has shown that freeze thaw mechanism at -80C extend the shelf life of the assay for up to 3 weeks.*

## Introduction

In the pharmaceutical industry, detecting and quantifying residual protein levels is very important. This specific protein should be cleared from recombinant biopharmaceuticals since they are critical to quality and present a potential immunogenicity risk for patients, product quality, and drug efficacy. In a high throughput environment, the number of samples received that need to be tested for residual protein in some instances may be overwhelming and require that analysts act with urgency and work for long shifts during extended periods. In the biopharmaceutical and immunoassay world, having a reliable antibody guarantees top- notch results and ensures staying on top of competitors. Considering that ELISA Immunoassays are the gold standard of the industry to assess residual protein levels, the current study was designed to evaluate for how long precoated plates at 5C and -20C would be valid and if freeze that mechanisms can extend the shelf life of the assay control without compromising their inherent stability and quality.

## Background

In the immunoassay world, coated antibodies and other coated proteins are the central part of a reliable immunoassay. Also, it is crucial that their stability is contingent on their reliability. For instance, a company called Candor that offers antibody stabilizers and HRP protectors ensures the overall stability of the ELISA plate. Previous research done by Richter [1] states that after successful coating, misfolding takes place in a time and temperature manner. during this project. In another study, Ma et al. [3] found that temperature affects the format or structure of antibodies. According to research done by Ma et al. [3], it was found that temperature also makes them susceptible to proteases henceforth playing a key role on the antibody stability. It is crucial keep in mind that ELISA plates are going to be stored at 5C and -20C for different periods of time and they will contain an immobilized antibody that decays over time and that is susceptible to temperature changes on their overall stability. For instance, Wailtari et al. [4] conducted stability studies with printed array plates stored at 4C and 37C and when they compared plates at these conditions, it reflected that 4C remained stable for one month while the ones stored at 37C started being unstable after a week. Also, they reflect on how storage conditions affect the overall stability of antibodies.

## Problem

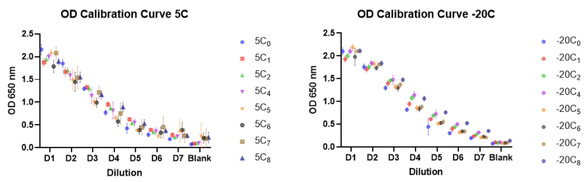
With the implementation of this methodology the turnaround time went from 7 days to 3 days and increased the testing capacity of the Analytical Technical Operations group within the ELISA workflow

## Methodology

The methodology in this study was based on taking into consideration Rokni et al. [2] two of the temperatures used in their respective study due to time and space limitations. The plates used for this experiment were Nunc-Immuno™ MicroWell™ 96 well solid plates, and these plates were coated with goat IgG polyclonal antibody. Eighteen plates were coated by preparing a 1:10 dilution of Goat anti-human antibody in PBS buffer pH 7.4 and afterward adding 100 uL to all wells of this diluted antibody and incubated for 3 hours at 300 rpm. After this step, they were washed three times using a Biotek plate washer with PBS/0.5% Tween 20. The last step of the blocking procedure is to prepare a mixture of 1:10 powdered milk in PBS and then 200 uL was added to each well to complete the overnight blocking process. The following day plates were washed again these plates were sealed with Bio-Rad PCR plate sealers and stored upside down away from light, 9 of them were stored at 5C, and the other nine were stored at -20C. Each temperature was examined sequentially from week 0 until week 8. The plates were examined using the calibration standard and assay control. The assay control starting concentration was 160 ng/mL on the top point and then it was serially diluted until it reached 2.5 ng/mL and finally the blank benchmarking the lowest point of the standard curve, for the assay control the dilution ranges were 1:10, 1:25, 1:50 and 1:100.

Once the calibrations standards and assay controls are prepared 100 uL of them were dispensed to each well. Since the assay control is known to have a great stability and higher replicability n=40, and the assay control would have n=56 for all their respective dilutions since it has a greater acceptance criterion and has more variability per lot. Once dispensed proceed to seal and incubate for 2 hours at 300 rpm. After those 2 hours wash plates and add a mixture of rabbit polyclonal IgG antibody diluted in PBS in a ratio of 1:2 and proceed to incubate for another hour at 300 rpm. Once that incubation finishes wash plates and add the anti IgG donkey polyclonal antibody, proceed to seal plates, and incubate again for 1 hour at 300 rpm and once that period expires wash plates and proceed to add horseradish peroxidase (TMB) wait for 10 minutes and then add Stop Solution.

The ELISA was read at 650 nanometers in a SpectraMax reader equipped with SoftMax Pro 7 software. The raw files generated were processed via MS Excel and GraphPad statistical software to determine averages, standard deviations, the nominal value of the OD, and the actual titers of the assay control and the respective calibration standard.

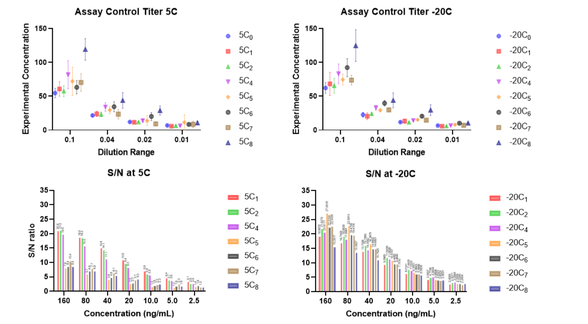


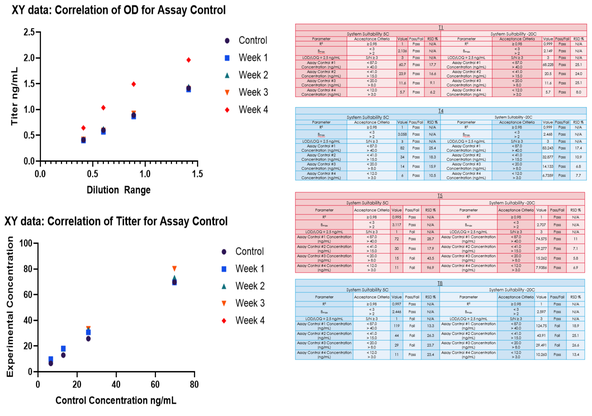

| Time | T0 | T1 | T2 | T4 | T5 | T6 | T7 | T8 |
|---|---|---|---|---|---|---|---|---|
| R squared | 0.9204 | 0.9186 | 0.9603 | 0.9736 | 0.9523 | 0.8848 | 0.9211 | 0.9547 |
| Sy.x | 14.52 | 14.87 | 8.823 | 9.379 | 12.63 | 21.23 | 17.05 | 12.44 |

| Time | T0 | T1 | T2 | T4 | T5 | T6 | T7 | T8 |
|---|---|---|---|---|---|---|---|---|
| R squared | 0.9375 | 0.9615 | 0.9803 | 0.9634 | 0.9368 | 0.9077 | 0.9883 | 0.9776 |
| Sy.x | 14.44 | 9.795 | 7.094 | 10.87 | 14.35 | 18.83 | 5.832 | 8.204 |

## Results and Discussion

SpectraMax Software, Microsoft Excel, and GraphPad were used for data generation and data deconvolution. The results showed that the precoated ELISA plates remained stable for more than a week at the two different temperatures tested. In this study, the ELISA plates corresponding to week three were discarded from the data acquisition process since these plates were not sealed correctly and were contaminated in the process; the data attained from these plates would be unusable. The raw data was obtained by reading the plates at the SpectraMax machine and based on the optical densities (OD) it detected at 650 nanometers (nm) it back calculated the calibration standards and the assay control titter. The SpectraMax software provided a graph with a R2 that evaluated the overall linearity of the optical densities and their titters for the calibration standard, for all weeks the R2 was higher than .98 also, the Signal to Noise ratio, and the R2 were taken into consideration. Small clusters were formed, from T0 to T4 and the mean data points started to aggregate, forming a second cluster from T5 to T8 data points. The cluster formation can be appreciated better at -20C rather than 5C. A linear behavior was identified across all dilutions during the eight-week period this was validated by the R2 values obtained in GraphPad statistical software at a 95% CI for the calibration standard. In the case of assay control clusters start to form in a very similar way to the ones formed on the calibration standard. One of the most important factors to determine which temperature and which range of time was appropriate for the storage of these plates was the signal to noise ratio S/N and it can be appreciated that until week 4 at 5C it has minor changes and that in -20C it appears to remain stable. Also, there is statistical and graphical evidence that shows that multiple freeze thaws start affecting the assay control optical densities and their titter after three freeze thaw cycles.




## Conclusions

The main objective of this project was to challenge convention and assess the viability of precoated plates and extend the shelf life; based on the data shown, it can be concluded that plates are stable for up to 4 weeks at 5C and up to 5 weeks at -20C. Additionally, based on the clusters formed it is safer to infer that 5C has a lesser adverse effect on optical densities and titers when plates are stored at 5C for up to 4 weeks. Dilution linearity confirmed overall stability at all temperatures for up to 4 weeks. Based on the data trends, it is proper to state that lower bound concentrations were affected more by temperature than time.

Another critical part of this study is that it enables the possibility of 72-hour turnaround time ELISA rather than the standard 1-week period. This change allows the Analytical Technical Operations team a faster response to deal with PPQ submissions and Preclinical emergency runs. Also, it allows the team a swifter testing approach towards deviations started by QC or the manufacturing department since now in 72 hours max time it can be certified if the process is within the expected parameters in respect with residual protein levels for any type of drug substance that is submitted for testing.

this experiment allowed to extend the shelf life of the assay control for three weeks

## Future Work

Future studies should focus on measuring the effects of humidity during storage. Additional research should also be done to identify the effects of temperature during each incubation step to shorten the time between each incubation step. Also, this type of study can be applied to other ELISA immunoassays to extend the plates shelf life and increase throughput.

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