Evaluation of Residual Protein ELISA Stability of Precoated Plates and Assay Control in a Hight Throughput Environment

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Abstract *— Evaluation of Enzyme Linked Immunoassay Sorbent Assay (ELISA) overall stability on precoated plates to appraise Residual Protein levels in a wide variety of drug substance samples and filtration process 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 and gives a swifter testing approach towards emergency runs, Process Performance Qualifications submissions, and deviations initiates 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.*

Key Terms *— ELISA, Freeze Thaw, Residual Protein Stability.*

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.

LITERATURE REVIEW

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.

The aim of this project is to see until what time point and temperature conditions the Residual Protein ELISA plates remain stable. Plate stability can be determined by comparing the signal to noise ratio, assay control titers from each week against the baseline parameters set by the control groups known as T0, calibration curve replicability across the plate.

Also, Rokni's [2] study tried a similar approach to what will be evaluated by this research project; the main difference between the method they applied and the one that is going to be considered in this project is that they tested more temperature conditions than the ones that will be considered in this research. Another key differentiator is that the antibodies they used are

different hence their stability varies and does not correlate at all to the ones that will be studied 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.

It is very important to recall that Keethkumar et al. [5] discusses some guidelines on how the freeze thaw mechanism damages monoclonal antibodies in an aqueous solution and this principle can be applied to the assay control in this study since it contains a known titter of protein that is known to be affected by the current freeze thaw method used by the analysts. The selected temperatures for storing the plates correlates to data presented by Ma et al. [3] mentioned that the most common temperatures for antibody storage are 4C, -20C and -80C. Also, Ma et al. [3] states that depending on what type of antibody analysts are dealing with, they need to take additional considerations, for instance enzyme conjugated antibodies need to be stored at 4C to avoid freeze thaw stress cycles that will translate into isotype aggregation and overall stability loss.

Additionally, it is very important to acknowledge that the studies done by Rokni et al. [2], Wailtari et al. [4], and Keethkumar et al. [5] since the studies mentioned the importance of temperature, how it can affect the conformation of the antibodies, temperature plays a key role in the studies in which it can stabilize a protein or destabilize the protein and the platform.

Curiously, Keethkumar et al. [5] makes emphasis on how important is to consider the freeze thaw cycles and how they contribute to protein aggregation within time. Therefore, within the pharmaceutical industry it is very common to freeze quick, thaw slowly and aliquot big quantities into small vials to reduce protein aggregation, reduce variability and preserve the stability of the antibody if possible. The last statement is validated by Ma et al. [3] since they show how fast thawing can denature proteins up to 11-fold more than slower thawing.

According to Farrah et al. [6] the freeze thaw cycles can impact RNA detection for SARS-CoV-2 antibodies detection in serum by ELISA immunoassays. One particular outcome was that when it came to serum testing withing an ELISA it stayed stable up to 16 cycles but when it came to test against different substances the amount of freeze cycles decreases. It is very important to test individually for every substance, antibody, or protein since all of them behave differently and they are affected by different changes in temperature and their cycles depend greatly on the size and specificity of the antigen.

Torelli et al. [7] provided an interesting point regarding influenza assays and how it detects anti-HA antibodies in serum samples to evaluate the antibody response towards influenza virus in study subjects' pre-and post-influenza vaccination. Serum samples are usually collected and stored in biobanks for long time to be processed once the sample collection is ended. To note that, since the same sample can be reanalyzed to confirm previous results or to perform further analysis, it is normal practice to prepare single usage aliquots before freezing. By preparing single use aliquots the freeze thaw cycles get reduced to just one cycle, but this technique may increase the overall cost of the assay since it requires more material usage and needs specific conditions for storage.

MATERIALS AND METHODS

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.

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 R^2 was higher than .98also, the Signal to Noise ratio, and the R^2 were taken into consideration. Figure 1 shows, how from T_0 to T_4 the mean data points started to aggregate, forming a cluster that separated them from T_5 to T_8 data points. The cluster formation can be appreciated better at -20C rather than 5C. Figure 2 shows, that most of the average data points aligned, and the OD readouts were very similar between themselves. In Figure 3, a linear behavior was identified across all dilutions during the eight-week

period this was validated by the R^2 values obtained in GraphPad statistical software at a 95% CI. In the case of Figure 4, it can be identified formations of two different clusters, one that began from timepoint 0 until timepoint 4, and the second cluster is from timepoint 5 to timepoint 8 this assay control titer behavior was like the one shown on figure 1. Figure 5 explicitly showed how at 5C it formed two clusters one from week 1 to 4 and another from week 5 to 8; in both cases the clusters impacted their S/N ratio at all concentrations but at -20C, there was no cluster formation and followed the expected pattern. Clear trends were identified in Figure 6 and 7; for instance, the optical densities for almost all weeks are like the control group, with week four as an exception, which was validated by Figure 8 close correlation above all data points. In the case of the titter behavior in Figures 6 and 7, a clear linear trend was identified, and it shows that the OD is highly correlated across the weeks mentioned above. Table 1 reflected on all the parameters that were required by the current SOP and method to assess plate viability and confirmed that plates were viable for more than a week at both temperatures.

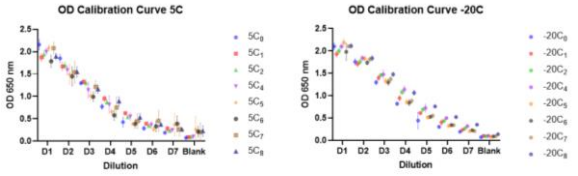


Figure 1

Mean of optical densities for calibration standard with their respective error bars at 95% CI

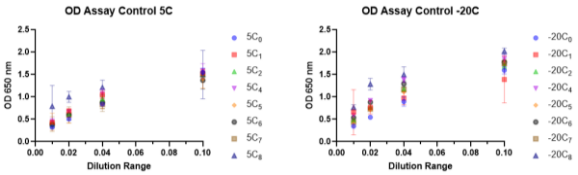


Figure 2

Mean of assay control optical densities for assay control with their respective error bars at 95% CI

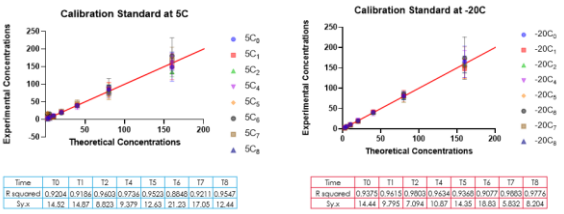


Figure 3

Linearity assessment of experimental concentrations versus theoretical concentrations with their respective standard deviations and R^2 at a 95% CI

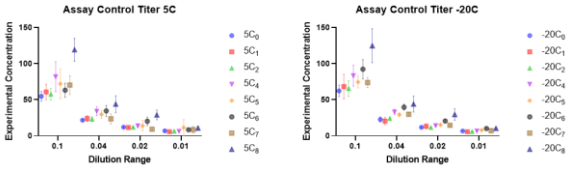


Figure 4

Mean of assay control titters at 5C and -20C with their respective error bars

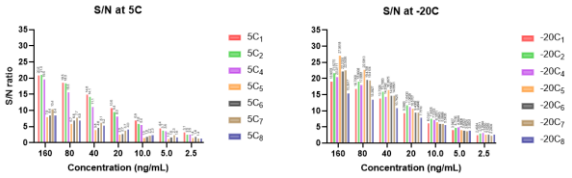


Figure 5

Signal to Noise mean ratio for 5C and -20C across all weeks tested

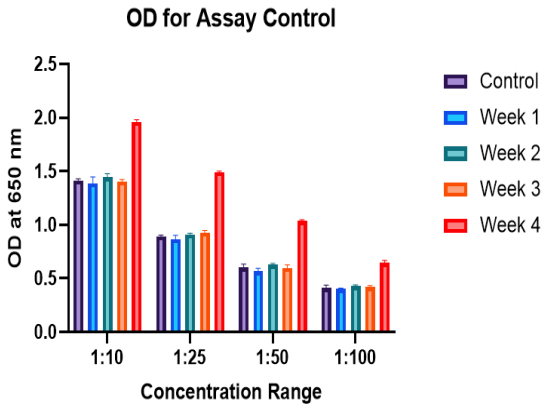


Figure 6

Comparison of optical densities across 4 weeks against their control group

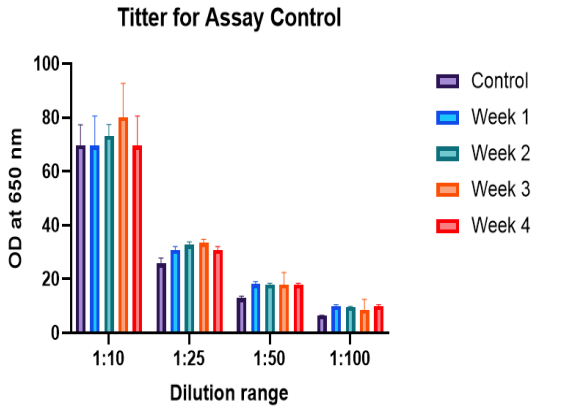
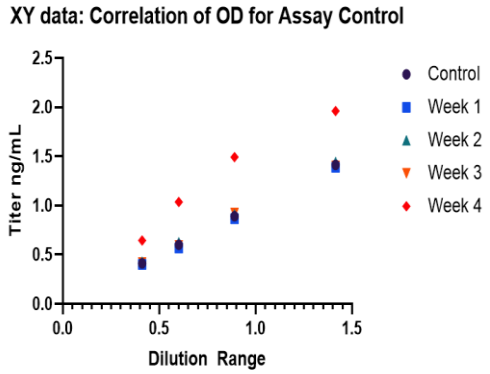


Figure 7

Comparison of titter at different dilution across 4 weeks for assay control



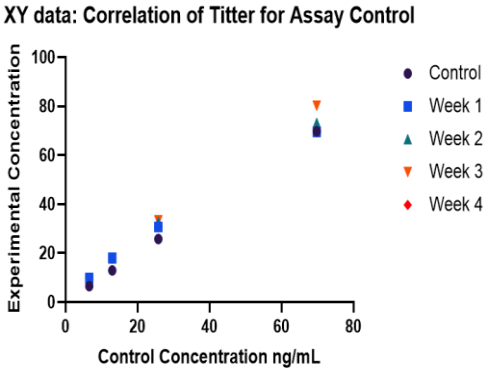


Figure 8

OD correlation of Assay Control and their respective Titter (ng)

T1

System Suitability 5C					System Suitability -20C				
Parameter	Acceptance Criteria	Value	Pass/Fail	RSD %	Parameter	Acceptance Criteria	Value	Pass/Fail	RSD %
R^2	≥ 0.98	1	Pass	N/A	R^2	≥ 0.98	0.999	Pass	N/A
B_{max}	< 3 > 2	2.136	Pass	N/A	B_{max}	< 3 > 2	2.149	Pass	N/A
LOD/LOQ = 2.5 ng/mL	S/N ≥ 3	3	Pass	N/A	LOD/LOQ = 2.5 ng/mL	S/N ≥ 3	3	Pass	N/A
Assay Control #1 Concentration (ng/mL)	< 87.0 > 40.0	60.7	Pass	17.7	Assay Control #1 Concentration (ng/mL)	< 87.0 > 40.0	68.228	Pass	25.1
Assay Control #2 Concentration (ng/mL)	< 41.0 > 15.0	23.9	Pass	16.6	Assay Control #2 Concentration (ng/mL)	< 41.0 > 15.0	20.5	Pass	24.0
Assay Control #3 Concentration (ng/mL)	< 20.0 > 8.0	11.6	Pass	9.1	Assay Control #3 Concentration (ng/mL)	< 20.0 > 8.0	11.6	Pass	25.1
Assay Control #4 Concentration (ng/mL)	< 12.0 > 3.0	5.7	Pass	6.2	Assay Control #4 Concentration (ng/mL)	< 12.0 > 3.0	5.7	Pass	8.0

T4

System Suitability 5C					System Suitability -20C				
Parameter	Acceptance Criteria	Value	Pass/Fail	RSD %	Parameter	Acceptance Criteria	Value	Pass/Fail	RSD %
R^2	≥ 0.98	1	Pass	N/A	R^2	≥ 0.98	0.999	Pass	N/A
B_{max}	< 3 > 2	3.056	Pass	N/A	B_{max}	< 3 > 2	2.468	Pass	N/A
LOD/LOQ = 2.5 ng/mL	S/N ≥ 3	3	Pass	N/A	LOD/LOQ = 2.5 ng/mL	S/N ≥ 3	3	Pass	N/A
Assay Control #1 Concentration (ng/mL)	< 87.0 > 40.0	82	Pass	25.4	Assay Control #1 Concentration (ng/mL)	< 87.0 > 40.0	83.243	Pass	17.4
Assay Control #2 Concentration (ng/mL)	< 41.0 > 15.0	34	Pass	18.3	Assay Control #2 Concentration (ng/mL)	< 41.0 > 15.0	32.877	Pass	10.9
Assay Control #3 Concentration (ng/mL)	< 20.0 > 8.0	14	Pass	15.9	Assay Control #3 Concentration (ng/mL)	< 20.0 > 8.0	14.133	Pass	6.8
Assay Control #4 Concentration (ng/mL)	< 12.0 > 3.0	6	Pass	10.5	Assay Control #4 Concentration (ng/mL)	< 12.0 > 3.0	6.7389	Pass	7.7

T5

System Suitability 5C					System Suitability -20C				
Parameter	Acceptance Criteria	Value	Pass/Fail	RSD %	Parameter	Acceptance Criteria	Value	Pass/Fail	RSD %
R^2	≥ 0.98	0.995	Pass	N/A	R^2	≥ 0.98	1	Pass	N/A
B_{max}	< 3 > 2	3.117	Pass	N/A	B_{max}	< 3 > 2	2.707	Pass	N/A
LOD/LOQ = 2.5 ng/mL	S/N ≥ 3	1	Fail	N/A	LOD/LOQ = 2.5 ng/mL	S/N ≥ 3	3	Pass	N/A
Assay Control #1 Concentration (ng/mL)	< 87.0 > 40.0	72	Pass	28.7	Assay Control #1 Concentration (ng/mL)	< 87.0 > 40.0	74.575	Pass	11
Assay Control #2 Concentration (ng/mL)	< 41.0 > 15.0	30	Pass	17.9	Assay Control #2 Concentration (ng/mL)	< 41.0 > 15.0	29.277	Pass	7.1
Assay Control #3 Concentration (ng/mL)	< 20.0 > 8.0	15	Fail	43.5	Assay Control #3 Concentration (ng/mL)	< 20.0 > 8.0	15.262	Pass	5.8
Assay Control #4 Concentration (ng/mL)	< 12.0 > 3.0	11	Fail	96.9	Assay Control #4 Concentration (ng/mL)	< 12.0 > 3.0	7.9386	Pass	6.9

T8

System Suitability 5C					System Suitability -20C				
Parameter	Acceptance Criteria	Value	Pass/Fail	RSD %	Parameter	Acceptance Criteria	Value	Pass/Fail	RSD %
R^2	≥ 0.98	0.997	Pass	N/A	R^2	≥ 0.98	1	Pass	N/A
B_{max}	< 3 > 2	2.446	Pass	N/A	B_{max}	< 3 > 2	2.597	Pass	N/A
LOD/LOQ = 2.5 ng/mL	S/N ≥ 3	1	Fail	N/A	LOD/LOQ = 2.5 ng/mL	S/N ≥ 3	3	Pass	N/A
Assay Control #1 Concentration (ng/mL)	< 87.0 > 40.0	119	Fail	13.3	Assay Control #1 Concentration (ng/mL)	< 87.0 > 40.0	124.75	Fail	18.9
Assay Control #2 Concentration (ng/mL)	< 41.0 > 15.0	44	Fail	26.3	Assay Control #2 Concentration (ng/mL)	< 41.0 > 15.0	43.91	Fail	25.1
Assay Control #3 Concentration (ng/mL)	< 20.0 > 8.0	29	Fail	23.7	Assay Control #3 Concentration (ng/mL)	< 20.0 > 8.0	29.491	Fail	26.6
Assay Control #4 Concentration (ng/mL)	< 12.0 > 3.0	11	Pass	23.4	Assay Control #4 Concentration (ng/mL)	< 12.0 > 3.0	10.263	Pass	13.4

Table 1

Summary criteria for assay acceptance

CONCLUSION

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 in Figure 5 and Table 1, 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 in Figures 1 and 4, 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 in Figure 5 and Table 1, 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 up to 3 weeks as seen on Figure 6 and 7 since this will allow to decrease the cost of the assay.

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.

During this experiment the only drawback faced was a contamination on week three plates since they were not sealed correctly. This implies that all the analysts must ensure that all plates are sealed correctly to avoid re-work and to avoid missing potential deadlines.

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