Development and validation of a commercial ELISA for the quantification of diptheria toxin CRM197 in conjugate vaccine preparations

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ABSTRACT

Background: Carrier proteins play a pivotal role in the transformation of polysaccharides originating from T-cell independent antigens into T-cell dependent antigens through their interlinking mechanism. Additionally, they serve to enhance the immunogenicity of oligosaccharides, peptides, and proteins, thereby augmenting the body's immune response. Moreover, carrier proteins facilitate the formation of stable complexes between antigens and antibodies, ensuring efficient recognition and activation of the immune system. CRM197, a genetically detoxified diphtheria toxin, is widely used as a carrier protein in conjugate vaccines, including those against Streptococcus pneumoniae, Haemophilus influenzae b and Neisseria meningitidis. A non-toxic mutant, it lacks toxicity but retains its ability to bind to antigen-presenting cells, enhancing the immune response to attached antigens, thereby improving the vaccine's efficacy. However, accurately measuring CRM197 is essential to ensuring vaccine formulation safety, efficacy, and regulatory compliance - by maintaining quality control, optimizing antigen-carrier protein ratios, and ensuring consistent and effective immunization. Currently, techniques including both HPLC and ELISA are employed for this purpose. ELISA is preferred due to its high sensitivity, specificity, and ease of use, making it the superior method for CRM197 quantification. Currently, no other ELISA for estimation of CRM197 is commercially available, and scientists must develop, optimise and validate their own in-house assay for estimation. This allows for human error and variability in detection, and additionally creates a lack of standardisation in quantification of the CRM197 contaminant.

Objective: The objective was to develop a new, sensitive, easy to use and pre-validated sandwich assay for measurement of CRM197, using specific antibodies for detection of CRM197. This would ensure standardisation of results, and a quantitative result would allow scientists to have a better understanding of the purity of their sample, and therefore the efficiency of their vaccine formulation.

Method and Conclusion: The development of the assay involved coating micro-plates with antibodies specific to CRM197, followed by sample incubation and a subsequent enzyme-based detection system. We optimised various parameters, including antibody concentration, blocking conditions, and incubation time, to enhance assay sensitivity, dynamic range, and reproducibility. The assay offers a range of 6.25 ug/ml - 800 ug/ml. The ELISA was validated as per EMA/FDA guidelines in line with ICH M10 Code for Harmonization of Biological Assays and the Assay Guidance Manual. Validation studies using known rCRM197 standards demonstrated excellent linearity and accuracy. The simplicity, sensitivity, and specificity of this assay make it an essential tool for vaccine development, management and quality.

INTRODUCTION

Vaccines solely composed of purified polysaccharide antigens exhibit limitations due to their inherent T-cell independence, despite B-cell activation. The introduction of carrier proteins in conjugate vaccines revolutionized polysaccharide-based immunoprophylaxis. These carrier proteins, covalently linked to the polysaccharide antigen via stable chemical bonds, function as potent immunomodulators. The conjugation process transforms the polysaccharide antigen from a T-cell independent to a T-cell dependent antigen. This critical shift is mediated by the carrier protein's MHC class II presentation to CD4+ T cells, leading to robust T-cell activation and subsequent B-cell help. Conjugate vaccines therefore foster a heightened antibody response characterized by increased affinity and avidity, providing enhanced immunogenicity. The effectiveness of career protein conjugated vaccines is well established, with several approved vaccines in use currently.

One of the most widely used career proteins is Cross Reactive Material 197 (CRM197), a mutant version of the diphtheria toxin, where the single amino acid exchange of a glycine in position 52 to a glutamic acid renders the protein non-toxic. Like diphtheria toxin, CRM197 is a single polypeptide chain of 535 amino acids (58.4 kD) consisting of two subunits (linked by disulfide bridges). Conjugate vaccines such as HibTITER® (Haemophilus influenzae type b associated diseases), Prevnar™ (pneumococcal diseases) and Menveo® (meningococcal diseases) use CRM197 as carrier protein with effective results.

Conventionally, CRM197 was isolated by fermentation of Corynebacterium diphtheriae C7 (β197) cultures, which often suffered from low yield. In the last few years however, several recombinant approaches have been reported with robust processes and higher yields, which will improve the affordability of CRM197-based vaccines. One such established method is expressing the protein in e.coli, where the rCRM197 has an identical crystal structure as native CRM197, and has been used in vaccine development.

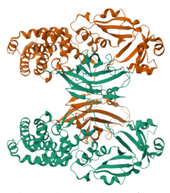


Figure 1: Crystal structure of CRM197

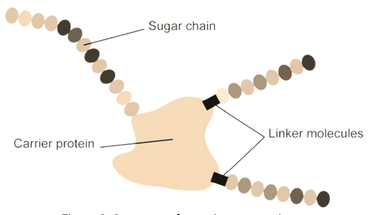


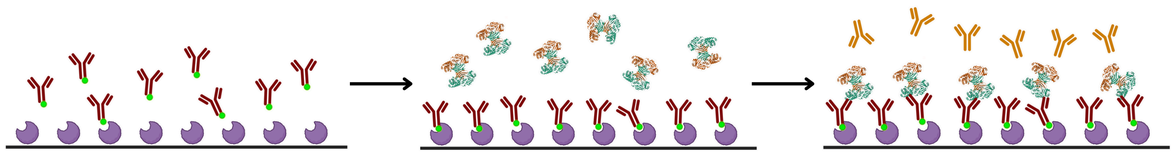
Figure 2: Structure of a conjugate vaccine

For quality control CRM197 quantities must be measured to ensure safe levels of the protein in each batch, lot consistency and for safety assessments. Currently, either HPLC or in-house ELISA are used for the detection of CRM197. However, HLPC does not offer great sensitivity and requires more expertise, and in-house assays require complete reagent preparation, plate coating and optimization on the user end. All validation of the assay must also be performed by the user to ensure accuracy and robustness in results, which may lead to inconsistency in results, slower turn-around-times and increased cost. Krishgen's objective was to develop a sensitive and optimized sandwich assay that was validated on the manufacturer end for reproducibility and accuracy. This commercially available assay could provide standardisation on the manufacturing end, ensuring unbiased quality control. Removal of manual development methods and thus, reduction in chances of error are particularly important since CRM197 must be accurately measured in each lot of vaccine released for patients.

The objective of this paper was to develop a sensitive, easy to use and pre-validated sandwich assay for measurement of CRM197, using CRM antibodies.

EXPERIMENT DESIGN

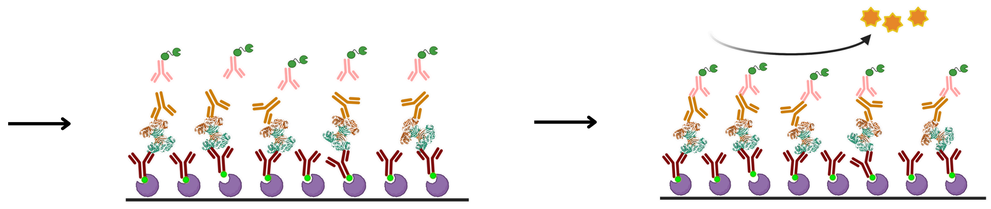
This assay was developed using two specific anti-CRM antibodies to capture samples. Streptavidin was coated overnight onto Corning CoStarTM microwell plates using a proprietary coating solution and blockers for long term immobilization and stability. The first anti-CRM antibody, a rabbit polyclonal, was biotinylated and optimized with protein stabilizers and preservatives. The second antibody used was a mouse anti-CRM antibody, treated and stabilized the same way. The Standard used was a diptheria toxin CRM197 from *E.coli* procured commercially and already used by vaccine manufacturers in successful vaccines. The standard was run at eight dilutions to form the standard curve of the kit. An anti-mouse IgG conjugated to HRP was used as the detection antibody. The assay scheme is depicted in Fig. 3.



(1) To pre-coated Streptavidin coated microtiter wells, we added biotinylated CRM antibody and incubated.

(2) After a wash, Standards and samples were added to the wells and incubated.

(3) After a wash, added the second anti-CRM antibody and incubated.



(4) After another wash, added the anti-mouse antibody conjugated to HRP and incubated.

(5) After the final wash, added TMB substrate and ended the reaction with Stop Solution.

Figure 3: CRM197 assay principle

The sandwich ELISA was optimized for CRM197 concentration and buffer composition of coating and detection antibodies, washing buffer composition, as well as incubation temperature and time of the different steps of the assay to give a commercially acceptable assay that surpasses current industry standards.

RESULTS - METHOD OPTIMIZATION

Each assay step was optimized for optimal noise-to-signal ratio and working range was determined. Streptavidin protein was used to coat the wells, which helps in orientation of the CRM protein - offering surface tethering and protein tethering to help increase sensitivity of the assay. The CRM standards were run on SDS page under both reducing and non-reducing conditions to determine the final standard for the assay (Figure 4). For the CRM antibodies, five different antibodies were studied for binding activity in ELISA (Figure 5). Two were determined to be optimal for use in the assay.

The standards were run in duplicates in various diluents, at various coating and detection conjugate levels, and other variables. Optimal concentrations of coating and detection conjugate along with appropriate diluents and incubation times were determined through a series of checkerboard titrations till optimal noise:signal ratio was observed.

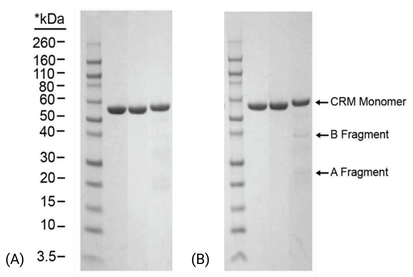


Figure 4: SDS Page of CRM197 standard and two other commercially available CRM197 molecules. (A) Non-reducing conditions (B) Reducing conditions.

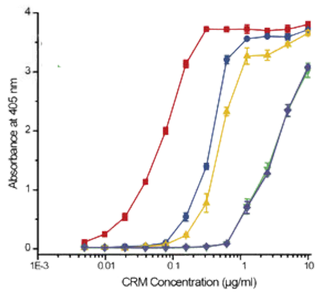


Figure 5: ELISA binding activity of CRM197 to five different anti-CRM antibodies.

The final protocol was set at 210 minutes, with multiple incubation and washing steps to ensure specific, sensitive results. Optimal noise:signal ratio and sensitivity formed the pillars of the optimization.

The ELISA was designed for an assay range of 6.25 ug/ml - 800 ug/ml, with standards 6.25, 12.5, 25, 50, 100, 200, 400 and 800 ug/ml. Upon optimization of the assay, complete validation was conducted, following internal protocols and guidelines from the ICH M10 as established by the US FDA / EMA

RESULTS - ASSAY VALIDATION

The optimized kit was then validated using the guidelines set by the ICH M10 (USA FDA / EMA). This included determining precision, sensitivity, stability and robustness. Repeatability was determined using ten replicates of the same extract in one assay. Intra-assay reproducibility was evaluated by analyzing ten extracts of the same sample in one assay. Inter-assay reproducibility was determined analyzing three extracts of the same sample in three independent assays. Additional optimization and spiking experiments were performed for minimal %CV and relative error. Assay precision was determined by both intra (n=10 assays) and inter assay (n=10 assays) reproducibility on two pools with low, medium and high concentrations, run in duplicates. Robustness was estimated by introducing deliberate changes in the established procedure in the same experiment. The Limit of Detection (LOD) was estimated as the average concentration of ten replicates of the zero standard plus three.

Finally, an accelerated stability test was conducted by keeping various temperature sensitive parameters at 37°C and calculating deterioration via %CV. Other in-house and regulatory validation processes were also compeleted. Calculations and statistical analysis were performed using the GraphPad Prism Software v5.

Limit Of Detection: It is defined as the lowest detectable concentration corresponding to a signal of Mean of '0' standard plus 2* SD. Over 10 runs in duplicates, the LOD was found to be 6 ug/ml.

***Specificity*:** The assay works on the sandwich ELISA principle and uses two CRM antibodies along with a streptavidin and biotin complex to ensure specificity of the reagents. The CRM antibodies are polyclonal (rabbit, mouse).

Standard Concentration (ug/ml)	Mean Absorbance	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.195	0.1	--
6.25	0.356	6.3	100.8
12.5	0.440	12.3	98.3
25	0.517	20.1	80.4
50	0.810	58.5	116.9
100	1.022	100.4	100.4
200	1.384	205.1	102.6
400	1.776	381.4	95.4
800	2.332	812.7	101.6

Table 1: Results obtained from CRM197 standards lyophilized, reconstituted and then run as full range standards. This table also shows recovery obtained by spiking standards, within 80-115%. Lyophilization of the standards supports easy shipping and robust results.

Precision: It is defined as the percent coefficient of variation (%CV) i.e. standard deviation divided by the mean and multiplied by 100. Assay precision was determined by both intra (n=5 assays) and inter assay (n=5 assays) reproducibility on two pools with low (6.25 ug/ml), medium (200 ug/ml) and high (800 ug/ml) concentrations. Additionally, when running the complete standard range as well in duplicates, %CV within and between plates was under 10%, ensuring robust precision and reproducibility.

High Dose Hook Effect: It is a reduction in measured signal that occurs in the presence of very high concentrations. Over several duplicate runs, the ELISA kit did not experience a high dose hook effect when it was tested up to a CRM197 concentration of 1000 ug/ml.

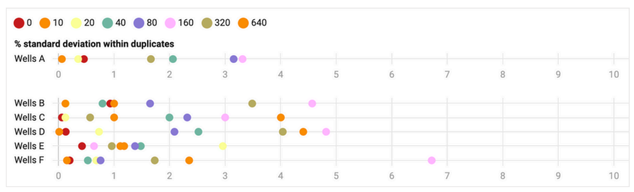


Figure 7: Six sets of complete standards were run on different days using wells from the same plate to observe the deviation in absorbance. They were run in duplicates following the protocol, and mean absorbance was noted. Standard deviation was calculated by subtracting difference between wells on each day for each standard, and the average was then noted. In all three lot runs, satisfactory recoveries were observed, and statistical results showed low standard deviation between wells, and minimal co-effecient of variation.

Standard Lyophilization: To ensure stability of the standard over the 12 month expiry, it was lyophilized using in-house proprietary solutions and methods. Validation was performed for the quality of lyophilization over various lots to ensure that it provides robust and reliable results for each run. In each complete run, acceptable recovery results were considered when between 8-12%CV only. The final concentration of the lyophilized standard was set at 1000 ug/ml, which was diluted by the user to the required standard range.

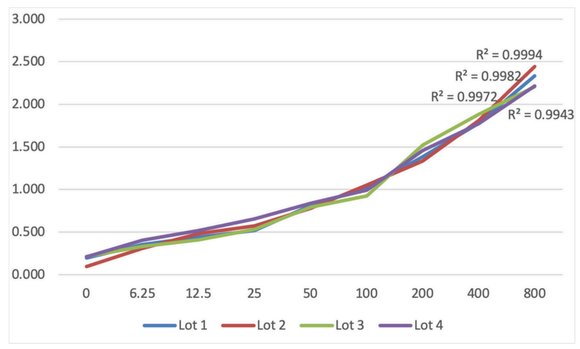


Figure 6: Four lots of lyophilised standards were reconstituted as per instructions on the manual and were run in duplicates. Absorbanced were mapped on graphpad prism and a best curve trendline of 2nd order polynomial was fit. Each standard showed acceptable recovery, with an R2 >. 0.99.

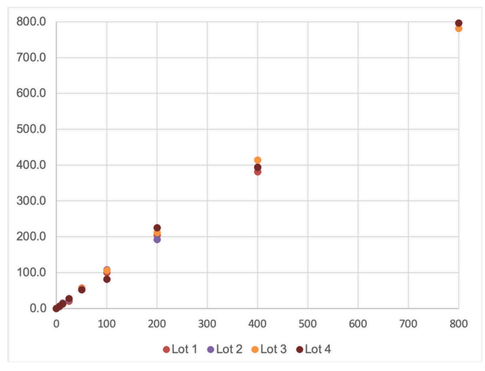


Figure 8: Four lots of complete standards were run on three different days to observe the deviation in absorbance. They were run in duplicates following the protocol, and mean absorbance was noted. Standard deviation was calculated by subtracting difference between wells on each day for each standard, and the average was then noted. In all four lot runs, actual recoveries were observed, and statistical results showed low standard deviation between wells, and minimal co-effecient of variation.

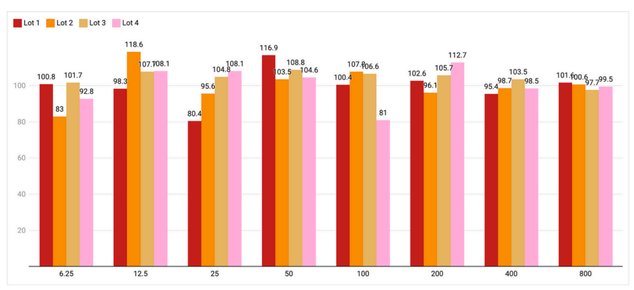


Figure 9: Represents the same lots as in figure 8 from a recovery percentage perspective. In all lots, across the standards, percentage difference between actual concentration and interpolated concentration (recovery) was between the acceptable 80 - 120%,.

***Recovery*:** It is used to determine whether analyte detection can be affected by the difference between diluent used for preparation and the experimental sample matrix. It is an important technique for analyzing the accuracy of the CRM197 ELISA. The recovery of this assay was assessed by comparing observed vs. expected values based on non-spiked and/or neat (undiluted) samples across several lots of samples. Various dilutions of spiked samples were run in duplicates and concentration was interpolated. Next, the percentage of recovery was calculated. For the three lots (n=5) run for recovery analysis, each lot provided satisfactory results. with recovery between 90-110%. Please refer to table 2 for an example of this run.

***Accelerated Stability*:** Accelerated stability testing increases the rate of degradation and physical change of components by using exaggerated storage conditions as part of the formal stability testing program. Three CRM197 kits from the same lot were subjected to a fourteen day accelerated stability study, with one critical component from each kit at stored at 37 degrees Celsius. The entire standard range was run on days 1, 2, 4, 6, 11 and 14 as per the protocol, meant to represent the stability of the kit over a period of 12 months. Inter- and intra- assay precision and recovery was analysed for each lot at each run. Satisfactory results were obtained from the accelerated stability studies under the acceptable 20% CV over all standards across all types of runs (detection conjugate, standard and plate).

CONCLUSION

Upon completion of the validation process of the assay as per both internal and regulatory standards, we report that the KRIBIOLISA CRM197 ELISA kit successfully and accurately detected CRM197 with high accuracy. The ELISA was designed for 6.25 - 800 ug/ml as assay range and achieved a sensitivity of 6 ug/ml. The sensitivity of the assay developed keeping in mind the expected levels of CRM in vaccine and biological preparations, allowing for a wide range of concentrations of CRM197 to be detected and reducing purification times. The pre-coated and validated format also allows for better precision and recovery due to the commercial standardization of the kit.

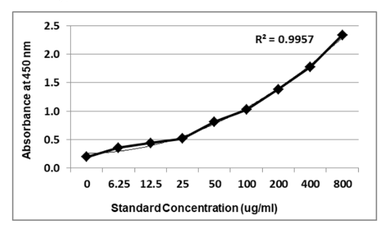
Pool	Intra Assay %CV	Inter Assay %CV
Low	<10%	<10%
Medium	<8%	<8%
High	<5%	<5%

Table 4: Inter- and Intra-assay Precision for a typical lot of CRM197 ELISA

Standard Concentration (ug/ml)	Mean Absorbance	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.195	0.1	--
6.25	0.356	6.3	100.8
12.5	0.440	12.3	98.3
25	0.517	20.1	80.4
50	0.810	58.5	116.9
100	1.022	100.4	100.4
200	1.384	205.1	102.6
400	1.776	381.4	95.4
800	2.332	812.7	101.6

Table 4: Typical results from the KRIBIOLISA CRM197 ELISA run.

- Developed as a sandwich assay format, the first of its kind, and comes with a pre-coated plate and pre-optimized reagents, with a 210 minute protocol time.
- Use of two anti-CRM197 antibodies in conjunction offers increased specificity
- It is well validated and performs within required precision parameters, as demonstrated over many lots of kits and testing.
- Final assay range was set at 6.-25 - 800 ug/ml, with a sensitivity of 6 ug/ml.
- Offers robust inter- and intra- assay precision of <12% CV each.
- Ships at room temperature or 2-8 degrees Celsius, owing to lyophilized standards.
- Provides 90% - 110% recovery and shows dilutional linearity



A typical graph that is included with each kit as part of the certificate of analysis. It represents the lot characteristics and expected graph.

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