Technical Note

Identity and Purity Analysis of USP Cross-Reacting Material 197 (CRM197) Reference Standard

Polysaccharide vaccines use sugar molecules from the outer layer of bacteria to stimulate a protective immune response to infections such as pneumonia and meningitis.[1] While effective in adults, these vaccines offer limited efficacy in children under the age of two due to the immaturity of their immune systems.[2] Another limitation of this approach is that polysaccharide vaccines do not confer herd immunity.[3] To improve immunogenicity, bacterial polysaccharides can be coupled with carrier proteins to create polysaccharide protein conjugate vaccines.[4] Examples of these vaccines include Prevnar®, which protects against pneumococcal disease, and VAXEM Hib®, used for prevention of infectious diseases caused by *Haemophilus influenzae* type b.

CRM197: A Carrier Protein for Conjugate Vaccines

Tetanus toxoid, diphtheria toxoid, and Cross-Reacting Material 197 (CRM197) are three carrier proteins that have been used in a majority of licensed conjugate vaccines.[5] Recombinant CRM197 is a nontoxigenic diphtheria toxin isolated from the fermentation culture supernatant of *Corynebacterium diphtheriae* C7(β197) tox-negative and purified using chromatography and diafiltration. Unlike tetanus and diphtheria toxoids, CRM197 does not require detoxification with formaldehyde prior to polysaccharide conjugation, a process that can eliminate some of the lysine residues needed for glycan attachment; having a larger number of lysine groups in CRM197 is advantageous in vaccine manufacturing.[6]

The growing global demand for polysaccharide conjugate vaccines has necessitated the development of higher yield processes using alternative expression systems such as *Escherichia coli* and *Pseudomonas fluorescens* to replace the low yield and difficult production of CRM197 from C. *diphtheriae* C7. The carrier proteins produced by these expression systems must meet high regulatory and safety standards, particularly in terms of crucial quality traits like identity and purity.

Figure 1 shows the variability of CRM197 size, purity, and quantity of aggregates in preparations of the carrier protein from five different sources as measured by size exclusion chromatography (SEC; A) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; B).[7]

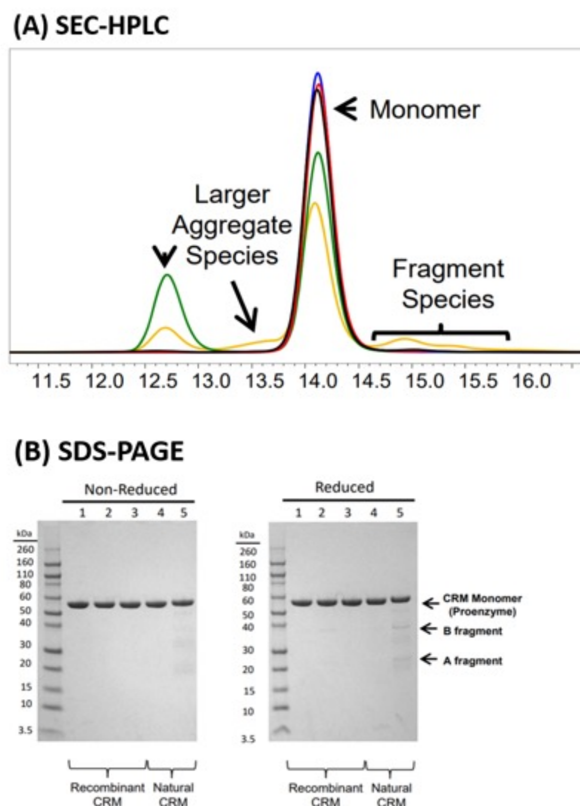


Figure 1: Size characterization of the CRM197 of different origins using SEC (A) and SDS-PAGE (B). In SEC, the yellow and green chromatograms are CRM197 from natural sources; the black, red and blue traces are recombinant CRM197 from various sources. Lanes 1, 2 and 3 in the SDS-PAGE are recombinant CRM197 from various expression systems; lanes 4 and 5 are from C. *diphtheriae*.

Hickey, et al, compared the physicochemical and immunological binding properties of CRM197 from C. *diphtheriae* and recombinant heterologous systems *(E. coli and P. fluorescens)* and found subtle physiochemical differences/impurities.[6] This finding led the authors to propose critical quality attributes (CQA) acceptance criteria to ensure the carrier protein meets the requirements for robust and sustainable manufacturing of conjugate vaccines. The authors state that purity and identity are the most important CQAs for ensuring lot-to-lot consistency, immunogenicity of the vaccine, and to mitigate manufacturing risk.

CRM197 Identity and Purity Reference Standard

To support the production of polysaccharide protein conjugate vaccines using CRM197 as a carrier protein, USP has developed a physical Cross-Reacting Material 197 Reference Standard (catalog number 1150800, CAS Number 945275-85-4).[8] The well-characterized standard can be used to support the quality assessment of CRM197 expressed by different sources and expression systems to increase the confidence in analytical results.

The USP CRM197 Reference Standard is a ~58 kDa recombinant protein expressed in *P. fluorescens* and formulated in a 10 mM sodium phosphate buffer containing 5% w/w sucrose and 0.005% polysorbate 80 at pH 7.4. The standard contains two disulfide bonds, one linking chains A and B and the second within the B chain. It is supplied in a lyophilized form for long-term stability.

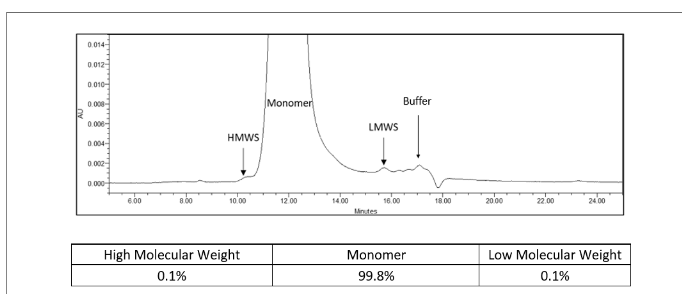
The protein's extinction coefficient and amino acid sequence are provided in the CRM197 Reference Standard certificate to support concentration and identity testing applications.

Purity Assessment by SEC

The CRM197 Reference Standard is evaluated using the size exclusion chromatography SEC method outlined in **Table 1** to determine the CRM197 monomer peak along with any high and low molecular weight species **(Figure 2)**.

Table 1. HPLC-SEC method parameters.

Concentration:	1 mg/mL
Columns:	Guard column: 6 x 40 mm, 7 µm, L59 Column: 7.8 x 300 mm, 5 µm, L59
Detector:	UV 214 nm
Mobile Phase:	10 mM Sodium Phosphate, 150 mM Sodium Chloride, pH 7.2
Method:	Injection volume: 20 µL Flow rate: 0.7 mL/min Column temperature: 30° Sampler temperature: 5°



High Molecular Weight	Monomer	Low Molecular Weight
0.1%	99.8%	0.1%

Figure 2: Separation of CRM197 Reference Standard monomer and high molecular weight species (HMWS) and low molecular weight species (LMWS) by SEC.

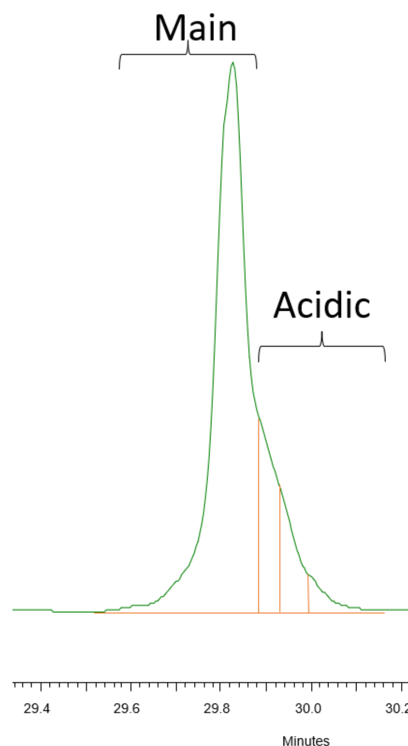
Identity and Charge Distribution Determination by Capillary Isoelectric Focusing

Capillary isoelectric focusing (cIEF), a high-resolution analytical technique that allows the separation of charge variants based on isoelectric point (pI) values, can be used to confirm the identity and charge distribution of a protein.

cIEF is performed on the CRM197 Reference Standard using the method outlined in **Table 2** to determine the isoelectric point (pI) value and charge variant profile **(Figure 3)**.

Table 2. cIEF method parameters.

Concentration	5 mg/mL
Instrument:	PA800 Plus
Capillary:	Neutral Capillary: 50 µm I.D., 30.2 cm long, 20 cm from injection site to detector with 200 µm aperture in the cartridge
Detector:	UV 280 nm
pI Standards:	pI 10.0, pI 9.5, and pI 7.0
Carrier Ampholytes	Pharmalyte 3-10
Method:	Injection pressure: 15 psi for 150 sec Separation voltage: 30KV for 35 min Capillary cartridge temperature: 20° Sampler temperature: 10°



Main Peak pI	Main	Acidic
5.8	76 %	24 %

Figure 3: Charge variant profile of CRM197 Reference Standard determined by cIEF.

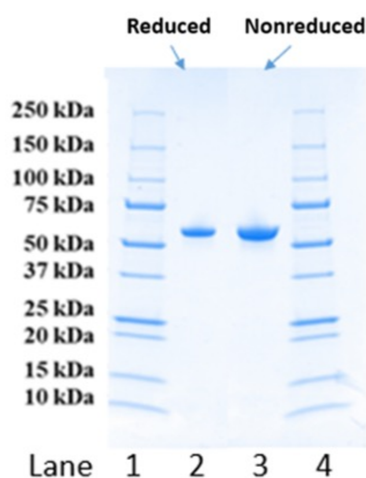
Identity Determination by SDS-PAGE

The CRM197 Reference Standard appears as a ~58 kDa band in SDS-PAGE analysis performed under both nonreducing and reducing conditions and is consistent with the reported molecular weight of 58.4 kDa. Gels were run using the method outlined in **Table 3**; results are shown in **Figure 4**.

CRM197 contains an exposed loop of three arginine residues that is clipped by proteases present in culture medium resulting in a nicked form. The CRM197 manufacturing process should produce batches with a consistently low degree of nicking. Gels run under nonreducing conditions can be used to evaluate possible covalent and noncovalent aggregation in samples and to detect impurities. Gels run in reducing conditions allow detection of the proteolytically nicked form which breaks down into two distinct polypeptides called fragments A and B that are easily detected by SDS-PAGE.

Table 3. SDS-PAGE method parameters.

Loading Mass:	2.5 µg Reduced 5.0 µg Nonreduced
Protein Marker (Unstained)	10 – 250 kDa
Power	125 V
Staining Reagent / Time	Coomassie brilliant blue R-250 / 1 hour



Lane 1, 4: Molecular Weight Markers
Lane 2: Reduced Cross-Reacting Material 197 (CRM197)
Lane 3: Nonreduced Cross-Reacting Material 197 (CRM197)

Figure 4: SDS-PAGE of reduced and nonreduced CRM197 Reference Standard displaying bands with loading mass 2.5 µg for reduced and 5.0 µg of nonreduced CRM197.

Identity Determination by Intact Mass Spectrometry

The CRM197 Reference Standard generates an average molecular weight of 58,409 Da by intact mass spectrometry which is consistent with the theoretical molecular weight of 58.4 kDa. Method parameters are shown in **Table 4** and representative spectra in **Figure 5**.

Table 4. Intact mass spectrometry method parameters.

Chromatographic Conditions			
Mobile Phase	Mobile phase A: 0.25 mL of Formic acid in 250 mL of water. Mobile phase B: 0.25 mL of Formic acid in 250 mL of Acetonitrile.		
Column	C8, 1.7µm, 2.1x100 mm		
Injection Volume (µL)	3	Column Temperature (°)	75
Flow Rate (mL/min)	0.4	Auto Sampler Temperature (°)	15
Run Time (minutes)	10	Detector (nm)	UV 214

LC Gradient			
Time (min)	**Flow Rate (mL/min)**	**Mobile Phase A %**	**Mobile Phase B %**
0	0.4	95	5
1	0.4	95	5
5	0.4	20	80
6	0.4	20	80
6.1	0.4	95	5
10	0.4	95	5

Tune Page Parameters	
Calibrant	Sodium Iodide
Ionization Mode	Positive, Resolution
Capillary (kV)	2
Source Temperature (°)	80
Sampling Cone	25
Extraction Cone	3
Source Gas Flow (mL/min)	0.0
Desolvation Temperature (°)	250
Cone Gas Flow (L/Hr)	0.0
Desolvation Gas Flow (L/Hr)	500

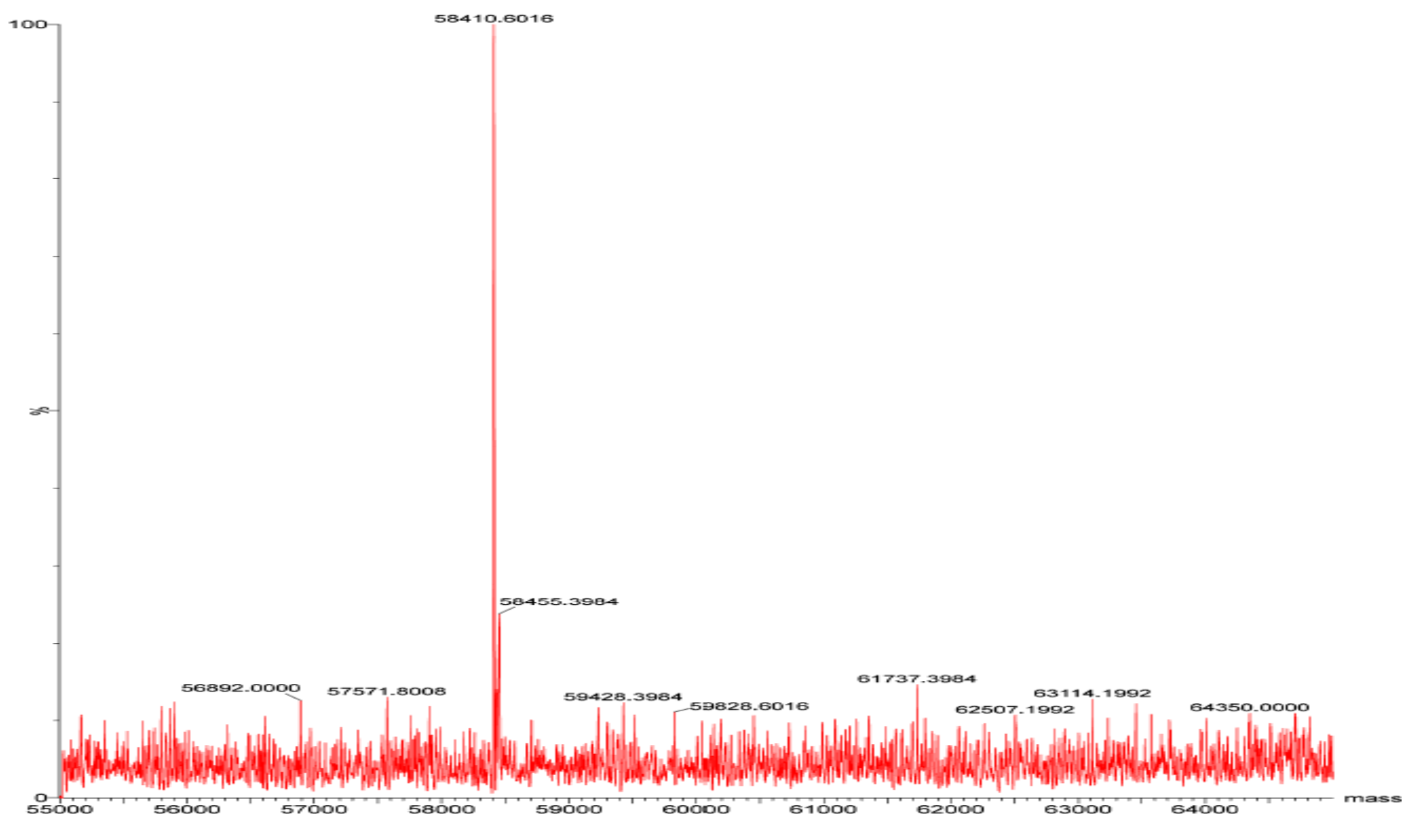


Figure 5: Representative intact mass spectrum of CRM197 Reference Standard.

Identity Determination using Peptide Mapping by Mass Spectrometry (MS/MS)

The identity of the CRM197 Reference Standard is confirmed using peptide mapping with sequence coverage. Amino acid # 52 is identified in the sequence as glutamic acid (E). The digestion is performed with trypsin, which generates 99% of peptide sequence coverage. Details of the method are summarized in **Table 5**.

Table 5. Intact mass spectrometry method parameters.

Chromatographic Conditions			
Mobile Phase	Mobile phase A: 0.25 mL of Trifluoro Acetic acid in 500 mL of water Mobile phase B: 0.25 mL of Trifluoro Acetic acid in 500 mL of Acetonitrile		
Column	C8, 1.7μm, 2.1x100 mm		
Injection Volume (μL)	15	Column Temperature (°)	75
Flow Rate (mL/min)	0.3	Auto Sampler Temperature (°)	15
Run Time (minutes)	65	Detector (nm)	UV 214

LC Gradient			
Time (min)	**Flow Rate (mL/min)**	**Mobile Phase A %**	**Mobile Phase B %**
0	0.3	95	5
1	0.3	95	5
55	0.3	50	50
60	0.3	5	95
65	0.3	95	5
10	0.4	95	5

Tune Page Parameters	
Calibrant	Positive, Resolution
Ionization Mode	3
Capillary (kV)	150
Source Temperature (°)	40
Sampling Cone	5
Extraction Cone	0.0
Source Gas Flow (mL/min)	350
Desolvation Temperature (°)	0.0
Cone Gas Flow (L/Hr)	800
Desolvation Gas Flow (L/Hr)	500

Conclusion

CRM197 is an essential component in many polysaccharide protein conjugate vaccines. The quality of this carrier protein may vary as it is available from different sources and produced from different expression systems. As physicochemical differences may alter vaccine efficacy or affect patient safety, it is essential to confirm the identity and evaluate purity as part of a quality system that ensures consistency across different lots.

The USP Cross-Reaction Material 197 (CRM197) Reference Standard supports purity and identity determinations of the protein carrier and suitability for use in conjugate vaccine production.

Resources for Glycoconjugate Vaccines: Related General Chapters

General Chapter Number	General Chapter Name
<198>	Nuclear Magnetic Resonance Spectroscopy Identity Testing of Bacterial Polysaccharides Used in Vaccine Manufacture
<1234>	Vaccines For Human Use—Polysaccharide and Glycoconjugate Vaccines
<1235>	Vaccines For Human Use—General Considerations
<1238>	Vaccines For Human Use—Bacterial Vaccines

Reference Standards *(Coming Soon)*

1.	Pullulan for Molar Mass Determination (100kDa), Item number 1581781
2.	Pullulan for Molar Mass Determination (400kDa), Item number 1581782
3.	Pullulan for Molar Mass Determination (800kDa), Item number 1581780
4.	BSA for Molar Mass Determination, Item number 1076103

[1] Grabenstein JD, Klugman KP. A century of pneumococcal vaccination research in humans. Clin Microbiol Infect. 2012:18 Suppl 5:15-24. https://doi.org/10.1111/j.1469-0691.2012.03943.x

[2] Strugnell R, Zepp F, Cunningham AL, Tantawhichien T. Vaccine antigens. In: Garçon N, Stern PL, Cunningham AL, Stanberry LR, eds. Understanding Modern Vaccines: Perspectives in Vaccinology. Amsterdam, The Netherlands: Elsevier BV; 2011:61-88.

[3] Rouphael GN, Zimmer SM, Stephens DS. Neisseria meningitidis. Published in Vaccines for Biodefense and Emerging and Neglected Diseases. 2009.

[4] Pichichero ME. Protein carriers of conjugate vaccines: characteristics, development, and clinical trials. Hum Vaccin Immunother. 2013 Dec;9(12):2505-23. https://doi.org/10.4161/hv.26109

[5] Knuf M, Kowalzik F, Kieninger D. Comparative effects of carrier proteins on vaccine-induced immune response. Vaccine. 2011;29(31):4881-4890. https://doi.org/10.1016/j.vaccine.2011.04.053

[6] Hickey JM, Toprani VM, Kaur K, Mishra RPN, Goel A, Oganesyan N, Lees A, Sitrin R, Joshi SB, Volkin DB. Analytical Comparability Assessments of 5 Recombinant CRM197 Proteins From Different Manufacturers and Expression Systems. J Pharm Sci. 2018 Jul;107(7):1806-1819. https://doi.org/10.1016/j.xphs.2018.03.002

[7] Hickey JM. Physicochemical and Immunological Comparison of CRM197 from Different Manufacturers and Expression Systems. Presented at the 8th International Symposium on Higher Order Structure of Protein Therapeutics, April 9, 2019.

[8] USP Reference Standard Certificate for Cross-Reacting Material 197 (CRM197).

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