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**Evaluation of a candidate International Standard for Meningococcal  
Serogroup C polysaccharide**

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\*Full details of MenC IS working group in Annex 1

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## Summary

Meningococcal serogroup C (MenC) plain polysaccharide (PS) and conjugate vaccines are primarily evaluated by physicochemical methods to ensure that batches are consistently manufactured. As different assays are employed to quantify the MenC PS content of final formulations and bulk intermediaries, there is a need for an International MenC PS Standard to calibrate internal references used in the different laboratories. Twelve laboratories from nine different countries participated in a collaborative study to assess the suitability and determine the MenC PS content of a candidate International Standard MenC PS preparation (08/214). On the basis of the results from this study, it is recommended that the candidate standard 08/214 is established as an International Standard for the quantification of MenC PS content in vaccines and components. It has a content of  $1.192 \pm 0.192$  mg MenC PS/ampoule (expanded uncertainty with coverage factor of  $k=2.365$  corresponding to a 95% level of confidence), as determined by the resorcinol assays carried out by eight of the participating laboratories.

## Introduction

Physicochemical methods are the primary tests used to ensure batches of *Neisseria meningitidis* serogroup C (MenC) plain PS and conjugate vaccine are manufactured consistently to those shown to be safe and effective in clinical trials (9, 13, 15). In particular, determination of the PS content and the amount of free saccharide in the final formulations measure the potency of the vaccine.

MenC PS consists of partly *O*-acetylated or de-*O*-acetylated repeating units of sialic acids, linked with  $\alpha 2 \rightarrow 9$  glycosidic bonds. A variety of assays are used for the quantification of the MenC capsular PS in the final vaccines and bulk components. These include the resorcinol and *p*-dimethylaminobenzaldehyde (DMAB) colorimetric assays, high performance (or high pH) anion exchange chromatography-pulsed amperometric detection (HPAEC-PAD), nuclear magnetic resonance (NMR) and immunological assays to determine the *N*-acetyl neuraminic acid (NANA) or sialic acid content, or the Men PS content directly.

NIBSC proposed the production of a MenC International Standard which was endorsed at the ECBS meeting in October 2007. This reference preparation, containing a known quantity of PS can be used to cross-calibrate various methods to quantify the MenC content of the bulk saccharide, bulk conjugate and final formulations and should be made available under the auspices of WHO to facilitate calibration of assays and in-house reference materials. This is particularly required by National Control Laboratories (NCLs) and vaccine manufacturers for two main reasons. Firstly, the poor long-term stability of liquid MenC working stock solutions has led to some drift in the MenC content of in-house standards leading to potential overestimation of MenC content in vaccine samples. Secondly, the continuing and rapid development of new MenC conjugate vaccines and multivalent vaccines including MenC PS and conjugate components, the establishment of programmes to distribute MenC vaccines throughout the world, and the likelihood that more NCLs will establish methods to evaluate MenC vaccines indicate that a standard for the quantification of MenC PS will be important to establish and will be in demand.

The purpose of the Collaborative Study was to: A) assign unitage (mg MenC PS/ampoule) to the candidate standard, and, B) to evaluate the suitability of the material for determining MenC concentration/content in a bulk conjugate. The unitage of the standard is proposed in *Système international d'unités* (SI) units milligrams (mg), in accordance with the

measurement and specification of PS in  $\mu\text{g}$  units in all meningococcal vaccines, plain polysaccharide or conjugate.

### **Participants**

Twelve laboratories participated in the study including six manufacturers and six NCLs, following invitations sent to 16 laboratories. The list of participants is included in the appendix as the MenC IS Working Group. Participants were assigned a random code number, not corresponding to the order of listing.

### **Materials and Methods**

#### **The candidate standard**

NIBSC received 8.34 g of purified vaccine-grade bulk MenC PS from GlaxoSmithKline Biologicals (Rixensart, Belgium) which was stored at  $-70^{\circ}\text{C}$ . Approximately 6 g of the PS was used to make a working standard for use in ELISAs (NIBSC code 07/318). The remainder of the MenC was further processed and used to produce 08/214. The data in Table 1 was provided by the manufacturer, and is compliant with WHO Recommendations (13, 15) and the European Pharmacopoeia (2011) for purified bulk PS for use in vaccines.

MenC PS can be unstable under certain conditions; hydrolysis can occur in mild acid conditions, and experience using frozen standards of MenC PS revealed a limited shelf-life. To ensure the lyophilized MenC PS reference preparation remains stable over the long-term, a number of trial fills were performed with the PS dissolved in either water, 0.5% w/v glycine, 50 mM  $\text{CaCl}_2$ , 10 mM Tris-Cl, pH 7.5 or 10 mM sodium phosphate, pH 7.5. The inclusion of either  $\text{CaCl}_2$  or Tris caused the lyophilized cake to completely collapse; sodium phosphate caused some shrinkage of the cake. With glycine as a stabilizer, there was an acceptable cake, but a poorer recovery of the PS (70% compared with 80% with water alone) as determined using the resorcinol assay to compare the sialic acid content in the starting liquid material with the lyophilized ampoule contents.

Degradation of the MenC PS was observed in all preparations stored at relatively low temperatures ( $+20^{\circ}\text{C}$  for 3 month (mo)).  $^1\text{H-NMR}$  of a sample lyophilized in water stored at  $+56^{\circ}\text{C}$  for 3 mo suggested that the degradation was caused by a transglycolase attack rather than the expected hydrolytic pathway. Loss in molecular size of stability samples prompted exploration of improvement of the PS's stability. Due to the possibility that the high-affinity binding of divalent calcium to sialic acid (5) was a potential source of instability, further processing was performed to reduce the residual calcium and replace it with the lower-binding cation, sodium, using cation-exchange chromatography. The strong cation exchange resin, Dowex AG 50W was used.

MenC PS powder was solubilized overnight at  $+4^{\circ}\text{C}$  in 500 ml 0.56 mM NaCl to a concentration of 2.6 mg sialic acid/ml (pH 5.0-5.2). Dowex AG 50W (8% cross-linked, 200-400 mesh) biotechnology grade (Bio-Rad) resin was pre-converted to the sodium-bound form prior to addition of PS by using NaOH (to pH 13), following the manufacturer's instructions, and rinsed 5 times (to pH 7.4). MenC PS was mixed with the beads at a ratio of 10 millieq resin: 1 mmol PS (pH 5.9-6.1) for 1 hr at  $+25$ - $30^{\circ}\text{C}$  with shaking. The PS-bead slurry was filtered through 0.22  $\mu\text{m}$  filter by vacuum filtration, and washed once with a reduced volume. The filtrate, which contained MenC PS was mixed with fresh, converted resin and the process was repeated. The twice-exchanged and filtered PS (650 ml, pH 6.3, 2.4 mg/ml) was stored

frozen in glass bottles at -20°C for 3 wk prior to filling. There was an 84% recovery of sialic acid in the exchanged PS, compared with pre-processed starting material.

The WHO Recommendations were followed for the preparation, characterization and establishment of reference standards (14) of the candidate standard. The processed material was thawed and diluted in sterile distilled water to give a concentration of approximately 1.2 mg MenC PS/ml in 1000 ml. Glass ampoules were filled with 1.0 g of the stock solution at the Centre for Biological Reference Materials (CBRM), NIBSC on the 14<sup>th</sup> November 2008. The non-sterile filling was carried out at temperatures ranging from 5.6°C-6.7°C. The freeze drying process was carried out at -40°C for 20 hours, lyophilisation for 53 hours at -25°C and finally desorption for 31 hours at +30°C. The ampoules were sealed on the 18<sup>th</sup> November 2008. 1102 vials were filled and labeled **08/214** *Neisseria meningitidis* serogroup C polysaccharide'.

Ampoules were analyzed for their sodium and calcium content by atomic absorption spectrometry, and for *O*-acetyl and sialic acid content by colorimetric methods by the QC Chemistry Laboratory at Novartis Vaccines & Diagnostics, Sovicille, Italy, to determine a functional weight and sialic acid-to-MenC PS conversion factor could be supplied. Ampoules were found to contain 0.011 mg sodium/mg powder and 0.044 mg calcium/mg powder, and there were 2.4 µmol *O*-acetyl/µg sialic acid. From this it was estimated that the MenC PS was ~ 63% *O*-acetylated (mol *O*-acetyl/mol sialic acid) and that there was a near 100% co-ordination of the PS with sodium and calcium in a 50:50 molar ratio. Based on this, the functional weight was determined to be 338.27 g MenC PS/mol, and a conversion factor of 1.08 g MenC PS/g sialic acid was recommended based on the dry weight of MenC PS and its sialic acid concentration.

During the Study, laboratories 3 and 10 determined the degree of *O*-acetylation directly by NMR methods (6) using 600 MHz and 500 MHz spectrometers respectively. By performing the total integration of the free *O*Ac and the *N*-acetyl methyl resonances (NHCOCH<sub>3</sub> at ~2.0 ppm and CH<sub>3</sub>COONa at ~1.9 ppm) of sample before and after treatment with ~200 mM sodium deuteroxide for 3 hr at 37°C, values of 96% and 94% *O*-acetylation were obtained. It has been reported that the Hestrin methodology can underestimate the *O*-acetyl content. For this reason, it was decided to use the mean of the NMR-determined values of 95% *O*-acetylation. A new residue weight of 351.67 was calculated with a conversion factor of 1.137 g MenC PS/g NANA (as shown in Annex 2).

Sixty three ampoules were used to calculate the mean mass of the fill, which was 1.0050 g with a CV of 0.22%. The mean dry weight of the ampoules was calculated, using 6 ampoules, to be 1.5 mg with a CV of 22.98%. The residual moisture content is 1.87% (w/w) with a CV 23.67%, calculated using 12 ampoules. The mean oxygen head space of the standards is 0.21%, calculated using 6 ampoules with a CV of 60.22%.

Eight hundred ampoules are offered to WHO. NIBSC will act as the custodians of the material which is being stored at -20°C.

### Study materials

Participants in the collaborative study were sent two duplicate sets of 4 preparations coded A, B, C and D, where ampoules A and D were the candidate standard 08/214, and B and C were Meningococcal serogroup Y PS (NIBSC code 01/426; 1 mg/ampoule) and Meningococcal serogroup A PS (NIBSC code 98/722; 1 mg/ampoule), respectively. Upon receipt,

participants were asked to store the samples at -20°C. Prior to use in assays samples were to be reconstituted with 1ml sterile distilled water, water for injection or deuterium oxide in the case of NMR and kept at +4°C for up to 2 wk, while completing the assay(s). If longer term storage of the samples was required, samples were aliquoted and stored at -20°C until further use. Prior to use in part (B) assays, MenC-CRM<sub>197</sub> bulk conjugate was thawed and stored at +4°C for the duration of the study. Further dilutions to give test solutions in the correct concentration range for the assay were to be made in the appropriate assay solution or buffer.

### **Study design**

Participants were requested to perform the assays twice, with fresh ampoules on separate days using the laboratory's own method and standards. A value of 0.5-2 mg for the content of the preparations was provided to participants as a guideline to help in preparation of dilutions. The assay methods performed by the participants are detailed below.

### **Assays for evaluation of the candidate standard**

#### ***Resorcinol method for sialic acid***

The resorcinol method was performed by eight out of the 12 participating laboratories to measure the amount of sialic acid in the candidate standard, with 7 labs using an in-house NANA standard, and one using a MenC PS standard as detailed in Table 2. It is based on the method of Svennerholm (10), in which poly-sialic acids are hydrolysed in concentrated hydrochloric acid to release the sialic acid. The sialic acid reacts with resorcinol in the presence of copper sulphate under reducing conditions to give a blue-purple color. The absorbance by spectroscopy at 580 nm is proportional to the sialic acid concentration. To convert sialic acid concentration to MenC PS concentration, conversion factors (g MenC PS/g sialic acid), as listed in Table 2, were used to obtain mg MenC PS/ampoule based on the reported purity of the in-house NANA standards as well as the % H<sub>2</sub>O (or moisture) content, in some cases (as described in the 'Method Analysis' section. The standard curves ranged from 4-100 µg/ml. There was an assay sensitivity of about 5 µg NANA, based on the lowest level of standard used.

Following the hydrolysis and incubation step, half of the laboratories also used a butanol:butyl acetate or alcohol extraction step, which inhibits further color formation; the colored reagents separate into an organic layer, which is used for the absorbance readings. A description of the reagent preparation using this method is described in Downs & Pigman (4). The resorcinol assay is also specified for quantitation of sialic acid in the European Pharmacopoeia (3).

#### ***DMAB method for sialic acid***

An alternative colorimetric method, known as the modified Ehrlich's method (12) was used by three of the participating laboratories to measure the NANA concentration. The assay uses *p*-dimethylaminobenzaldehyde (DMAB), also called Ehrlich's reagent, which reacts with NANA under acidic conditions to form a coloured complex which absorbs at light at ~530 nm. Aluminium sulphate can be added to stabilize the color. Standard curves ranged from 40-400 µg/ml and there was a sensitivity of approximately 2 µg NANA. NANA was used as a standard by two laboratories, for which a MenC/sialic acid conversion factor was applied; the third laboratory (laboratory 5) used a MenC PS standard to directly measure MenC PS content. Table 3 contains details of the DMAB methods used by the Study participants.

***HPLC-Fluorescence***

One laboratory (laboratory 5) used a reverse-phase HPLC method to determine the sialic acid content in the supplied bulk conjugate. Following mild acid hydrolysis, the free sialic acid was derivatised with *o*-phenylenediamine and the sample was separated on a C18 column where the fluorophore-labelled sialic acid was measured with a fluorescence detector. The standard curve was generated using a MenC-TT standard.

***HPAEC-PAD***

The HPAEC-PAD assay was performed by four laboratories. For PS hydrolysis prior to chromatography, two laboratories used TFA hydrolysis and two laboratories used HCl hydrolysis. The acid hydrolysis method directly measures the content of the MenC repeating unit, NANA. If MenC PS is used as a standard (as in one laboratory), the MenC PS content can be directly measured; if sialic acid monosaccharide is used as a standard, the NANA content can be converted to MenC PS content. All laboratories used a CarboPac PA10 or PA1 column, and resolved the MenC/sialic acid peak from the internal standards using an eluent containing around ~100 mM NaOH, ~100 mM sodium acetate. Labs 3 and 4 used elution methods employing gradient elution to first elute the neutral sugars followed by a gradient to elute the anionic sugars, including NANA. Labs 5 and 10 used isocratic elution conditions. In the case of laboratory 3, who used a standard consisting of a mix of 5 monosaccharides, including NANA, as little as 0.4 nmol (~0.125 µg) NANA could be measured. Further details from laboratories performing the HPAEC-PAD assay are included in Table 4.

***<sup>1</sup>H-NMR***

Four laboratories (3, 5, 6 and 10) returned results for PS content measured by quantitative proton nuclear magnetic resonance spectroscopy (<sup>1</sup>H-NMR). The <sup>1</sup>H NMR spectrum contains signals arising from specific hydrogen atoms in the structure of the sample (Ravenscroft et al., 1999); integration of such signals, where they are well resolved, allows the estimation of the molar concentration of the sample, especially when compared with an internal standard of accurately known concentration.

Collaborators each used their own in-house method. Laboratories 3, 5 and 10 provided some NMR parameters. Some experimental details are summarised in Table 5. All three laboratories worked at or near room temperature. Signal strength increases with the spectrometer field and with the number of scans. For quantitative applications, the recycle delay between scans needs to be long enough to allow magnetization to dissipate completely. The NMR setup in Lab 3 employed a cryoprobe, rather than a conventional probe, that required fewer scans due to enhanced sensitivity. Different internal standard compounds were chosen by each participant, and the considerable variation in recycle time may be the result of optimisation by each for the compound chosen.

The best resolved MenC PS signals, from H3 eq at about 2.6 ppm and H3 ax at about 1.6 ppm, were used for concentration calculations. Calculations were performed on de-*O*-acetylated samples, to avoid line broadening due to microheterogeneity. Inspection of the <sup>1</sup>H-NMR spectrum allows ready identification of capsular polysaccharides from specific serotypes; all four laboratories returned results for sample A, three for sample D. The identities of samples B and C were reported by Lab 3.

***ELISA***

The ELISA assay was carried out by one (laboratory 11) of the 12 participating laboratories. An in-house MenC PS standard was used. The method employed was a capture or sandwich ELISA. Anti-MenC PS monoclonal antibodies (mAbs) were used to coat an ELISA plate. The test samples were added to the wells and the MenC PS captured by the bound mAbs. A secondary antibody, anti-MenC mAb conjugated to peroxidase, was added followed by its substrate, resulting in a visible colour change. The intensity of the colorimetric signal detected is proportional to the amount of polysaccharide in the sample and a value determined for the samples from a standard curve generated from samples of known MenC PS concentration in the assay.

### **Stability Studies**

To determine the stability of the candidate MenC PS standard, three studies of the candidate material were undertaken at NIBSC: a real-time stability, an accelerated degradation and a stability study of the reconstituted material. The real-time stability of samples stored at -20°C compared to a -70°C baseline sample is in progress with data available for the 1, 2, 3, 5, 6, 11 mo(s), and 1 and 2.5 year time point (Table 13). Data will be collected for further time points of, 5 and 10 yrs. The accelerated degradation study of the candidate standard (at temperatures of 4, 20, 37 and 56°C), using a -70°C baseline was carried out on samples stored for 1, 2, 3, 5, 11 and 12 mos. The samples were assessed using the colorimetric resorcinol assay as described previously.

In the final study, the candidate standard was reconstituted in 1ml of sterile distilled water and stored at -70°C, -20°C for 1, 6 and 12 mos and +4°C for 1, 2, 3 and 4 wk. The MenC PS content of the samples was determined using the resorcinol assay. In addition, a second stability study at -20°C has been set up for material reconstituted in 1 ml water or 0.09% w/v NaCl and stored at time points to 26 mo. Data is available from 1 and 2 mo time points.

### **Statistical Analysis**

All results were returned to NIBSC and analysed in a consistent manner. Conversion factors were applied to all values of sialic acid contents determined from the resorcinol to convert them into MenC PS contents, and from DMAB and HPAEC-PAD, when sialic acid was used as a standard. The moisture contents of the commercial sources of NANA (or % purity, when moisture contents were not available), as quoted by the commercial source were taken into account when determining the conversion factors,. The conversion factors used are included in the tables of methods. NMR-determined molar content of sialic acid/ampoule was converted into mg MenC PS/ampoule using the functional weights of 309.27 for free sialic acid and 351.67 for a single repeating unit of MenC PS. Further details are given in Annex 2.

## **Results**

### **Calibration of the candidate standard**

Laboratory mean estimates (mg MenC PS/ampoule) for the candidate standard 08/214 are summarised in Table 6, for all assay methods. Individual ampoule estimates are shown in Table 7 and Figure 1.

Using the results from resorcinol assays, the final estimate of ampoule content was taken as the mean of the laboratory means shown in Table 6

$$\bar{x} = 1.192$$

The uncertainty of the mean of  $n$  laboratory means was estimated as

$$u_1 = \frac{\text{standard deviation of laboratory means}}{\sqrt{n}} = \frac{0.083}{\sqrt{8}} = 0.029.$$

Uncertainty due to between-ampoule heterogeneity was estimated by laboratory 10 where six ampoules were tested in each of two independent assays (Table 8)

$$u_2 = \text{pooled standard deviation from laboratory 10 assays} = 0.032.$$

Uncertainties applicable to laboratories performing the resorcinol assay were also estimated by laboratory 10 (Table 9)

$$u_3 = 0.057 \times 1.192 = 0.068$$

An expanded uncertainty (95% confidence) for the ampoule content was estimated as

$$\bar{x} + t_{(0.975, n-1)} \sqrt{u_1^2 + u_2^2 + u_3^2} = 1.192 + 0.192$$

The between-method CV was calculated as 11.2%.

#### Determination of MenC PS in bulk conjugate sample

Laboratory mean estimates (mg MenC PS/ml) for the bulk conjugate sample are summarized in Tables 10 and 11. Individual assay estimates are shown in Table 12, Figure 2 and Figure 3. Where 08/214 has been used as the standard for determination, it has been assumed to contain 1.192 mg MenC PS/ampoule.

The between-method CV was calculated as 8.9% when using 08/214 as a common standard compared with 11.2% when using in-house standards.

#### Method Analysis

The resorcinol assay was performed with five different commercial sources of NANA as a standard, sourced from sheep, *E. coli* or synthetically made. The MenC PS used by Lab 1 was traceable to a similar commercial source. Laboratories 6 and 12 factored the purity and moisture contents of their NANA standards into its concentration. Other laboratories appeared to weigh the NANA powder directly without further drying or moisture measurements. The assay repeatability, NANA purity and weighing step were taken into consideration in the determination of the uncertainty of measurement value from Lab 10 as shown in Table 9. The pipetting/dilution and heating steps, the resorcinol reagent concentration and the absorbance readings were considered to be negligible. There is the possibility that the sialic acid standards used in the resorcinol assay had over-estimated concentrations due to unaccounted impurities. To overcome this, in part, the conversion factors used to convert NANA to MenC PS were calculated considering purities of between 98-100% and in a few cases, moisture contents between 1.2-1.9 % (w/v) water. The actual water content of the individual commercial sources may be considerably higher as reported by Laboratory 12 (8.8% water was determined by an in-laboratory Karl Fisher analysis (KF) compared with the claimed content of 0.5% by KF from the source supplier).

The mean MenC content for the candidate determined by the resorcinol assay (1.192 mg) was used for the assignment of unitage to the standard as it is a single traceable method as recommended by WHO for standards with SI values (14). The use of a single method to assign unitage has raised questions with a number of participants and NIBSC would appreciate advice from ECBS on the suitability of using multiple methods to assign a unitage to this standard. The value is 18% higher than that determined by the more 'absolute' or primary relative method of <sup>1</sup>H-NMR (1.011 mg), possibly due in part to the purity of the commercial NANA preparations, as well as to other factors discussed below. This difference should be taken into consideration by laboratories using the standard in <sup>1</sup>H-NMR in analyses.

Overall there was a 6.9% CV in the mean resorcinol estimated values, the lowest found for any of the methods (Table 6). Although, there was lab-to-lab variation in the methods used by the laboratories (reagent concentration, incubation time and temperature, extraction with organic solvent), these do not seem to account for any trend or high/low values obtained.

One laboratory returned results for PS content of the ampoule using ELISA. The mean estimated content was 1.359 mg MenC PS/ampoule (Table 6), the highest mean estimation of any of the methods. In the second part of the study the concentration of MenC PS in a bulk conjugate was estimated by this method to contain 0.842 mg MenC PS/ml, also a 'high' value.

The DMAB method was performed by three laboratories, one of which (laboratory 5) measured a MenC PS content directly using a MenC PS standard whose concentration took into account the moisture and ethanol contents. The DMAB method is a simple and sensitive assay used for determination of MenC content in final fills. Laboratory 5 miniaturized the method through the use of 96-well plates.

The HPAEC-PAD method was performed by 4 laboratories to determine the MenC PS content of the ampoule and showed the highest laboratory-to-laboratory variability (18.4% CV). Three laboratories used monosaccharide NANA and one (laboratory 10) used a MenC PS standard which was dried before weighing and gave a value in agreement with the NMR-determined values, as did the value from Lab 3, who factored in the purity of their NANA standard during the construction of the NANA calibration curve. All used an acid hydrolysis method and used an internal standard to reduce any run-to-run variability (due to injection volume, room and detector temperature, etc.). There was a wide spread of values reported from the HPAEC-PAD method in estimation of the unitage of the candidate standard, probably due to the use of different in-house standards.

The suitability of using a MenC PS-containing standard for measuring the content of a MenC oligosaccharide – CRM<sub>197</sub> bulk conjugate was demonstrated by the determination of an equivalent concentration for the bulk conjugate regardless of whether in-house standards (mainly NANA) were used or the candidate PS was used. Laboratories 1 (Resorcinol), 5 (DMAB) and 10 (HPAEC-PAD) routinely use MenC PS as a standard, which can obviate the need for a conversion factor, provided that the PS standard contains an equivalent level of O-acetylation as the test sample. The use of a standard material closely matching the vaccine sample is desirable.

#### **Traceable versus 'primary relative' methods**

The resorcinol method was used to assign unitage to the candidate standard based on its use by the majority of laboratories, and the traceability of the commercial standards used in the assay. However there are disadvantages of analytical methods, such as this and other

colorimetric methods. Assumptions are made about the working standards; these include presumptions that they are pure and do not contain water, however certificates of analysis reveal this to be untrue. Also, the sialic acid standards are assumed to consist of monosaccharides, which may behave differently than oligo- or polysaccharide in those methods which rely on destruction of the PS (resorcinol, DMAB, HPAEC-PAD and HPLC-Fluor) and its quantitation by absorption. The optimal hydrolysis conditions for a PS may partially degrade the monosaccharide standard. This was experimentally found by Lab 3 for ACW<sub>135</sub>Y serogroups across a range of acid hydrolysis conditions. In contrast, Lab 4 tested the effect of 2M TFA for 2 hr at 90°C, and found no degradation of their NANA monosaccharide (synthetic).

Quantitative NMR does not require the use of a reference material of the same kind as the unknown, and in that limited sense can be termed as a 'primary relative' method. In this it is unlike, for example, colorimetric methods where response factors can be different between the test material and that used to create a standard curve. However, high molecular weight polysaccharides give broad signals in NMR spectra, whereas small molecules (such as the internal standards) give sharp peaks. Comparison of integrals of broad and narrow peaks is always problematic, and this difficulty may possibly account for the observation that NMR determinations on the whole give lower estimates of MenC PS content than other techniques in this study. Alternatively, the problem may be that colorimetric methods are over-estimating the MenC content, due to unequal response factors between test (for example, PS) and reference (for example, monosaccharide) samples, and the poor accounting for moisture in the NANA standards. The potential superiority of NMR in the characterization of the sample is unquestionable, as seen with the *O*-acetylation determinations. However, NMR is not used for the quantitation of MenC PS in the final product, which is the purpose of this standard. ECBS have acknowledged 'the difficulties in unambiguously assigned a value in SI units, even to well-characterized [products].' (14). Guidance by ECBS on the use of 'primary relative methods' for assignment of unitage in SI-type units would be valuable for future new PS standards (other meningococcal, pneumococcal and typhoid) and replacements.

#### **Sialic acid detection in control samples containing serogroup Y and A PS**

A number of laboratories reported reactions with the control samples (Table 7). Using the resorcinol assay seven of the eight laboratories reported a reaction with ampoules B and in some cases, C. Using the DMAB assay two of the three laboratories reported a reaction with ampoules B or C. These ampoules contained serogroup Y and A polysaccharides which are composed of repeating units of *glc*-sialic acid and *N*-acetyl mannosamine-1-phosphate, respectively. The resorcinol and DMAB assays detect free sialic acids so a reaction with serogroup Y polysaccharide is not unexpected. One laboratory reported MenC polysaccharide in ampoule B using HPAEC-PAD, due to the elution of the sialic acid peak from Men A eluting very close to that of MenC PS.

#### **Stability Studies**

The amount of PS in an ampoule was compared over time using the Resorcinol assay in the three stability studies: real-time, accelerated thermal degradation and reconstituted.

The amount of polysaccharide in the ampoules remained constant (within 5% of the -70°C baseline sample) in the real-time (-20°C) up to 30 mos, reconstituted and stored at -20°C for up to 12 mos and up to +56°C up to 11 mos, with no temperature-related trend as shown in Tables 13 and 14. Due to its stability, it was not possible to predict a percentage loss of MenC content per year.

## Discussion

Meningococcal serogroup C PS and conjugate vaccines are evaluated almost completely using physiochemical methods. The relative potency of these vaccines is determined from the PS or oligosaccharide content of the vaccines. However, different methods are used across different laboratories worldwide, according to the WHO Recommendations for the production and control of meningococcal group C conjugate vaccines (15). Although country or region-specific pharmacopoeias may specify methods, these are often not well standardized. The Candidate Standard 08/214 was produced to provide a reference material for NCLs and vaccine manufacturers to standardize MenC PS quantification.

### Determination of MenC content

The establishment of a reference preparation containing a determined amount of MenC polysaccharide should facilitate calibration of in-house reference material to quantify the MenC content of the bulk saccharide, bulk conjugate and final fills of PS or PS-protein conjugate vaccines.

In line with WHO recommendations a single traceable method, the resorcinol assay, was used to derive a value with assignment of uncertainty derived from collaborative study data. The resorcinol assay is the commonest method used to determine MenC PS concentration based on the laboratories involved in this study and is commonly used in the control testing of final product world-wide. As the standard is to be given a defined amount per ampoule, which will be used to prepare secondary standards to quantitate the  $\mu\text{g}$  MenC PS content of vaccines it has been calibrated in SI units, mg MenC PS per ampoule, as was the 1<sup>st</sup> IS for Hib PS (WHO/BS/05.2018). Due to the assignment of its unitage based on a collaborative study for a biological standard by laboratories who routinely measure the MenC content of vaccines, rather than purely by analytical chemistry laboratories, it cannot be considered formally as a *Système International d' Unités*-standardized reagent, per se. This study has highlighted the need for guidance on the assignment of unitage in SI units by 'primary relative' methods.

It should also be highlighted that, when used to measure the saccharide content of a vaccine or calibrate an in-house working standard, the uncertainty on the content of the proposed standard should be incorporated into the uncertainty on the final estimate obtained and, in a small number of cases, there may be problems in meeting pharmacopoeial precision specifications when this is done. An example of such a specification is given in the EP monograph for pneumococcal conjugate vaccines (1) where the 95% confidence limits on the final estimate should be not less than 80% and not more than 120% of the estimated content. When the uncertainty on the standard cannot be considered negligible, there is a lack of pharmacopoeial guidance on how to combine this uncertainty with the uncertainty of the assay and guidance from ECBS on this would also be valuable.

### Suitability of candidate standard

Equivalent MenC contents were determined for the bulk conjugate whether using the candidate PS standard (0.765 mg/ml) or in-house standards (0.760 mg/ml), attesting to its suitability in measuring the MenC content in oligosaccharide-CRM<sub>197</sub> conjugates. The suitability of the candidate for use in different assays, such as DMAB, HPLC Fluorescence and HPAEC-PAD was also evidenced in this arm of the study. There was a reduction in the between-method variability (from 11.2% to 8.9% CV) and in the variability of all mean

results (from 13.6% to 9.5% CV) with the use of 08/214 across 5 different methods (see tables 10 and 11), although this was not statistically significant.

### **Stability and storage of the candidate standard**

Real time stability studies are on-going but data collected thus far from -20°C samples stored for up to 2.5 yr do not indicate any stability issues. Accelerated thermal degradation studies have shown the content of the ampoule to be stable at temperatures ranging from -70°C to +56°C up to 1 yr. The appearance of the material within the ampoules remained constant and no problems with reconstitution were reported. Several laboratories' methods recommended that meningococcal PS be given ample time for solubilization following reconstitution before being used in assays. A number of laboratories have fixed expiry dates for the aliquots of sialic acid or MenC PS (from 6-24 mo). A study is underway comparing whether the reconstituted material is more stable in water or dilute NaCl which could give counterion stability to the anionic MenC poly-sialic acid.

### **Proposal**

Based on the results obtained from this collaborative study, we propose that the MenC PS candidate standard 08/214 is established as the 1st International Standard for Meningococcal serogroup C Polysaccharide with a content of  $1.192 \pm 0.192$  mg MenC PS/ampoule (expanded uncertainty with coverage factor of  $k=2.365$  corresponding to a 95% level of confidence) for potential use in assays for quantification of MenC PS in bulk PS, bulk conjugate and final fills of MenC monovalent and multivalent vaccines. The standard has been assigned a unitage in SI units, because all polysaccharide and conjugate vaccines have their content specification in  $\mu\text{g}$  units. The 1<sup>st</sup> International Standard for *Haemophilus influenzae* type b capsular PS also had its unitage assigned in mg/ampoule (7). Instructions for Use are appended (Annex 3).

While the content has been assigned based on the resorcinol assays, the material is potentially suitable for use in the quantitation of MenC content by other assays. The mean content across all 5 assays is 1.165 mg which is within the uncertainty of measurement determined for the resorcinol assay. Users should determine the uncertainty of measurement in their specific assay. Although NMR, which is considered to be a 'primary relative method' determined values were 18% lower than the assigned content, NMR is not currently used to determine the MenC PS content of bulk or final vaccines. It is not recommended that this standard be used as a quantitative standard for NMR assays, as NMR quantitation relies on integrating the intensities of resolved resonances arising from an unrelated internal standard and resolved resonances from the test material, present in the same NMR tube (16). Due to the limited number of labs performing the DMAB, HPAEC-PAD, ELISA and HPLC-Fluorescence, it has not been possible to determine the reason for the differences in the individual means from these assays. All of these assays measure sialic acid, albeit through slightly different properties and in some cases, hydrolysis conditions.

This Men C PS standard is 95% O-acetylated, and is appropriate for the measurement of the MenC PS content of material that has a similar O-acetylation level. If the standard is to be used for measuring the MenC PS content of a non-O-acetylated sample, or one with lower % O-acetylation, a correction will have to be used, following the calculation of the formula weight as listed in Annex 2. For a sample with 70% O-acetylation, for example, which has a residue weight of 341.17, the MenC content measured with the IS will need to be corrected by multiplying the measured  $\mu\text{g}$  MenC PS/ml content measured by 0.97 ( $341.17 / 351.67$ ). For a

non-O-acetylated MenC PS, the measured ug MenC PS/ml content will need to be multiplied by 0.89 (311.77/351.67).

### **Participants' comments on report**

The participants were sent a draft report and asked to check their details, that their methods and results had been reported correctly, if they agreed with the recommendations and finally for any additional comments. All twelve laboratories responded; eleven agreed with the recommendations and one disagreed.

1. Laboratories 2, 3, 4, 5 and 6 queried how individual conversion factors were determined and one laboratory requested the calculation to be included in the report.

*In response this has now been added and each laboratory was provided with a full description of the calculation.*

2. Laboratories 3 and 7 identified inconsistencies with the contents of vials B and C. The vials were incorrectly stated as serogroup W-135 and Y polysaccharide in the draft report. The vials actually contained serogroup Y and A polysaccharides respectively. Laboratory 7 received samples which had been incorrectly labeled, i.e. B= serogroup A and C=serogroup Y; laboratory 3 received vials B and C both containing serogroup Y polysaccharide.

*The error in labeling was investigated and appears to have taken place with replacement samples sent to later shipments to these laboratories. We are confident that all vials labeled A and D, contained Men C polysaccharide. This is supported by the data presented in this report.*

3. Laboratory 3 agreed with the recommendations of the report but expressed concerns over the use of the resorcinol assay to assign unitage to the standard. These concerns are primarily because the resorcinol assay gives a "relative" quantitation and is prone to overestimation of MenPS content against a NANA standard. This overestimation is observed in a comparison of the resorcinol and NMR results. As the NMR method provides an "absolute" quantification this method would arguably be the preferred one to use for assigning unitage. Lab 3 felt for those who use NANA as a standard, a "resorcinol assay factor" could be applied to the absolute NMR concentration of the WHO MenC PS standard. Similarly, an "HPAEC-PAD assay factor" might also be applied. Or the standard might be accompanied by guidance that these methods overestimate MenC PS concentration and that each end user must determine the "in house" bias against the NMR-based concentration.

*The authors from NIBSC appreciate that resorcinol or other 'relative' methods relying on a monosaccharide standard may not be the most suitable for the reasons discussed. However, WHO recommendations for references calibrated in SI units specify a unitage of a standard must be derived from a single method and as we had the most results from the resorcinol assay this method was chosen amongst the others fulfilling the ECBS criteria. It was of concern that if unitage were assigned using any other method we would not have enough data to be representative. Providing an NMR derived value with conversion factors for different assays may be appropriate,*

*however we have insufficient NMR data from this study. NIBSC would appreciate further advice from ECBS regarding this point.*

4. Laboratory 4 expressed the view that this standard should be used as a reference only for the resorcinol assay as only this method was used to assign unitage.

*In response, the authors from NIBSC believe the standard can be used for other quantitative methods, as clearly demonstrated in part B of this study. The difference seen between the methods appears to be due in large part to the use of different NANA preparations, and little accounting for the moisture content, as there was a reduction in % CV when the same standard was used.*

**Acknowledgements**

The authors would like to thank Michel Duchêne and Johan Descamps, GlaxoSmithKline for the donation of the MenC PS for establishment of an international standard and for all the members of the MenC PS Working Party who participated in the Collaborative Study. Jean Petre and Neil Ravenscroft kindly offered useful suggestions for reducing the calcium ion; David Capitani, Novartis V&D generously performed chemical analysis of the ampoules; and, Brian Nunnally, Pfizer, offered helpful suggestions in the design of the study. At NIBSC, Xavier Lemercinier performed NMR analysis of trial fills and was instrumental in the further processing of the candidate standard, Paul Matejtschuk and Chinwe Duru, Standardization Science performed formulation and lyophilisation studies, Cedric Clark and Natalia Zielinska from CBRM were responsible for the ampoule filling, Dennis Crane, Fang Gao and Anne-Marie Wilkes analysed stability samples, Christopher Jones offered useful comments on the accuracy of the unitage and *O*-acetylation methods, and Ian Feavers provided guidance and support throughout the 5 yr this project was in gestation.

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**Annex 1. List of Participants involved in the collaborative study forming the *MenC IS Working Group***

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**Annex 2. Calculation of MenC PS standard (08/214) residue weight, and MenC PS/NANA conversion factor**

A formula residue weight of 351.67 g/mol for the MenC PS was calculated based on the theoretical atomic weights (as per IUPAC, 2007 values) as follows:

Free sialic acid (NANA) – FW 309.27 g/mol

For residue weight – subtract 18 (for 1 H<sub>2</sub>O) 309.27 – 18 = 291.27

To account for 95% *O*-acetylation -  
 add 0.95 \* 42 (43 for *O*-acetyl – 1 Hydrogen) 291.27 + (0.95 \* 42) = 331.17

To account for 50% of MenC PS co-ordinating with a calcium ion –  
 add 0.50 \* 19 (Half of Calcium<sup>2+</sup> (40/2)– 1 hydrogen)  
331.17+ (0.5 \* 19) = 340.67

To account for 50% of MenC PS co-ordinating with a sodium ion -  
 add 0.50 \* 22 (One Sodium<sup>1+</sup> (23) – 1 hydrogen)  
340.67+ (0.5 \* 22) = **351.67**

**MenC PS residue weight = 351.67 g/mol**

**Conversion factor of 1.137 g Men C/g sialic acid, or NANA =**

$$351.67 \text{ (MenC PS residue weight)} / 309.27 \text{ (Free NANA weight)}$$

This new conversion factor includes the additional *O*-acetylation, determined by NMR (95%), and replaced the conversion factor of 1.08 which was calculated based on 63% *O*-acetylation.

## Annex 3. Instructions for Use

«HEADER»

**International Ref. Reagent  
1st International Standard for Meningococcal Serogroup C  
polysaccharide  
NIBSC code: 08/214  
Instructions for use  
(Version 1.00, Dated )**

Not for in vitro diagnostic use

**1. INTENDED USE**

The freeze-dried preparation of *Neisseria meningitidis* serogroup C capsular polysaccharide (MenC), provided by GlaxoSmithKline Biologicals (Rixensart, Belgium) was prepared in ampoules (2008) at the Centre for Biological Reference Materials (CBRM, NIBSC) and coded 08/214. A collaborative study was carried out on this material by 12 laboratories in 2011 to determine the MenC content in SI units based on the Resorcinol assay, and to evaluate its suitability for use as a standard for MenC quantification assays (including Resorcinol, DMAB and HPAEC-PAD assays) in final fills and bulks of MenC vaccines. The content of the ampoule was determined using a resorcinol assay. The material is potentially suitable for use in the quantitation of MenC content by other assays, although users should verify its suitability and determine the uncertainty of measurement in their specific assay. NIBSC, Potters Bar, UK is the custodian and distributor of this material.

**2. CAUTION**

**This preparation is not for administration to humans.**

Not human or bovine source material

As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

**3. UNITAGE**

The First International Standard for MenC polysaccharide, ampoules coded 08/214 has a content of 1.192 ± 0.192 mg MenC PS/ampoule, as determined by the Resorcinol assay.

**4. CONTENTS**

Country of origin of biological material: United Kingdom.  
Each ampoule contains the freeze-dried powder of 1 ml of MenC PS in 0.56 mg/ml NaCl. Each ampoule contains 1.5 mg of dry material as estimated by weighing after freeze drying, with a moisture content of approximately 1.87%.

**5. STORAGE**

Ampoules should be stored at or below -20°C.

**6. DIRECTIONS FOR OPENING**

Din Ampoule

**7. USE OF MATERIAL**

**No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution**

Re-suspend the contents of the ampoule in 1ml of distilled water. To ensure complete solubilisation of the material allow to dissolve for at least 2 hours at room temperature or 12 hours at 4°C prior to use. The reconstituted material should be aliquoted and frozen at or below -20°C. The Standard can be used directly as a reference in physico-chemical assays or for the calibration of secondary standards.

This Men C PS standard is 95% O-acetylated, and is appropriate for the measurement of the MenC PS content of material that has a similar O-acetylation level. If the standard is to be used for measuring the MenC PS content of a non-O-acetylated sample, or one with lower % O-acetylation, a correction will have to be used, following the calculation of the formula weight as listed in Annex 2 of the ECBS report. For a sample with 70% O-acetylation, for example, which has a residue weight of 341.17, the MenC content measured with the IS will need to be corrected by multiplying the measured ug MenC PS/ml content measured by 0.97 (341.17 / 351.67). For a non-O-acetylated MenC PS, the measured ug MenC PS/ml content will need to be multiplied by 0.89 (311.77/351.67).

**8. STABILITY (Add or amend as necessary)**

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label. Accelerated degradation studies revealed the standard to be stable up to one year at 37°C. Real-time and extended accelerated thermal degradation studies are on-going.

NIBSC follows the policy of WHO with respect to its reference materials.

**9. REFERENCES**

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**10. ACKNOWLEDGEMENTS**

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**11. FURTHER INFORMATION**

Further information can be obtained as follows;

This material:

[enquiries@nibsc.hpa.org.uk](mailto:enquiries@nibsc.hpa.org.uk)

WHO Biological Standards:

<http://www.who.int/biologicals/en/>

JCTLM Higher order reference materials:

<http://www.bipm.org/en/committees/jc/jctlm/>

Derivation of International Units:

[http://www.who.int/biologicals/reference\\_preparations/en/](http://www.who.int/biologicals/reference_preparations/en/)

Ordering standards from NIBSC:

[http://www.nibsc.ac.uk/products/ordering\\_information/frequently\\_asked\\_questions.aspx](http://www.nibsc.ac.uk/products/ordering_information/frequently_asked_questions.aspx)

NIBSC Terms & Conditions:

[http://www.nibsc.ac.uk/terms\\_and\\_conditions.aspx](http://www.nibsc.ac.uk/terms_and_conditions.aspx)

**12. CUSTOMER FEEDBACK**

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to [enquiries@nibsc.hpa.org.uk](mailto:enquiries@nibsc.hpa.org.uk)

**13. CITATION**

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

«FOOTER2»

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«FOOTER3»

«HEADER»

**14. MATERIAL SAFETY SHEET (Add or amend as necessary)**

Physical and Chemical properties	
Physical appearance: Freeze dried white powder	Corrosive: No
Stable: Yes	Oxidising: No
Hygroscopic: No	Irritant: No
Flammable: No	Handling: See caution, Section 2
Other (specify): No special handling precautions	
Toxicological properties	
Effects of inhalation:	Not established, avoid inhalation
Effects of ingestion:	Not established, avoid ingestion
Effects of skin absorption:	Not established, avoid contact with skin
Suggested First Aid	
Inhalation:	Seek medical advice
Ingestion:	Seek medical advice
Contact with eyes:	Wash with copious amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water.
Action on Spillage and Method of Disposal	
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.	

**15. LIABILITY AND LOSS**

Information provided by the Institute is given after the exercise of all reasonable care and skill in its compilation, preparation and issue, but it is provided without liability to the Recipient in its application and use.

It is the responsibility of the Recipient to determine the appropriateness of the standards or reference materials supplied by the Institute to the Recipient ("the Goods") for the proposed application and ensure that it has the necessary technical skills to determine that they are appropriate. Results obtained from the Goods are likely to be dependant on conditions of use by the Recipient and the variability of materials beyond the control of the Institute.

All warranties are excluded to the fullest extent permitted by law, including without limitation that the Goods are free from infectious agents or that the supply of Goods will not infringe any rights of any third party.

The Institute shall not be liable to the Recipient for any economic loss whether direct or indirect, which arise in connection with this agreement.

The total liability of the Institute in connection with this agreement, whether for negligence or breach of contract or otherwise, shall in no event exceed 120% of any price paid or payable by the Recipient for the supply of the Goods.

If any of the Goods supplied by the Institute should prove not to meet their specification when stored and used correctly (and provided that the Recipient has returned the Goods to the Institute together with written notification of such alleged defect within seven days of the time when the Recipient discovers or ought to have discovered the defect), the Institute shall either replace the Goods or, at its sole option, refund the handling charge provided that performance of either one of the above options shall constitute an entire discharge of the Institute's liability under this Condition.

**16. INFORMATION FOR CUSTOMS USE ONLY**

<b>Country of origin for customs purposes*:</b> United Kingdom
* Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
<b>Net weight:</b> 5g
<b>Toxicity Statement:</b> Toxicity not assessed
<b>Veterinary certificate or other statement</b> if applicable.
<b>Attached:</b> No

«FOOTER2»

«FOOTER»

«FOOTER3»

**Table 1. Summary of results from the manufacturer's analysis of MenC purified bulk PS**

Description	White powder free from visible impurities
Identity PS C by ELISA	Positive result using C antibodies, not more than 1% w/w of group-heterologous (A, W-135 or Y) <i>N. meningitidis</i> PS
<i>O</i> -acetyl content by spectrophotometry	2.4 $\mu$ M/mg PS
Sialic acid content by HPLC	95%
Molecular size distribution by FPLC	Amount of product eluted at a $K_d \leq 0.40 = 91\%$
Protein content by Lowry	0.4% dry weight
Nucleic acid content by spectrophotometry	0.4% dry weight
Calcium content by titration	6.0%
Water content by $\mu$ Karl Fisher	5.6%
Ethanol content by spectrophotometry	3.2%
Endotoxin content by chromogenic kinetic method	<20.00 IU per mg PS

**Table 2. Details of the resorcinol assay conditions used by participants in the Collaborative Study**

Laboratory	1	2	3	6	7	8	10	12
Standard	MenC PS	Sialic acid	Sialic acid	Sialic acid	Sialic acid	Sialic acid	Sialic acid	Sialic acid
Source, cat #, % purity (% H <sub>2</sub> O)	traceable to Sigma A0812,type IV-Synthetic, min 95%	Sigma A2388, <i>E. coli</i> , min 98% (~1% H <sub>2</sub> O)	Sigma A9646, sheep, 98.7%	Sigma A2388, <i>E. coli</i> , 100%	Fluka 01398, <i>E. coli</i> , 99.9%	Aldrich 855650 Synthetic Crystalline	Sigma A2388, <i>E. coli</i> , 100% (1.2% H <sub>2</sub> O)	Nacalai (Marukin Bio) 06872-36, purity 99.6% by HPLC
Storage conditions	2.6 mg/ml, H <sub>2</sub> O, 8/10 mo. 2-8 °C	0.15 mg/ml, H <sub>2</sub> O, 1/6 mo, -20°C	1 mg/ml, H <sub>2</sub> O, 2 yr, -20°C	0.1 mg/ml, H <sub>2</sub> O, prepared fresh	0.5814 mg/ml, H <sub>2</sub> O, 1/6 mo. , 2-8 °C	1 mM, H <sub>2</sub> O, 9.5 mo, -25°C	10 mg/ml stock, 19mo/5yr; 1 mg/ml 1 wk/2 yr , H <sub>2</sub> O, -20°C	10 mg/ml, H <sub>2</sub> O, 48 hr , -20°C
Standard curve range	4-80 µg/ml	3.75-45 µg	5-25 µg/ml	5-35 µg/ml	4-64 µg/ml	5-80 nmol/ml	10-40 µg/ml	10-70 µg/ml
OD range of std. curve (rounded to 2 decimal places)	0.12-0.97	0.12-1.32	0.08-0.40	0.06-0.36	0.034 (blank), 0.09-0.90	0.08-0.65	0.2-0.64	0.09-0.93
# levels standard curve	6	5	5	5	7	4	4	7
# Repeats	2	2	4	2	2	4	3	2
Vol standard/sample	2 ml	400 µl	1 ml	1 ml	0.5 ml	400 µl	0.5 ml	1 ml
<b>Sample/reagent details</b>								
Blank/Diluent	Water	Water	Water or product-specific	Water	Water	0.9% NaCl	Water or product-specific	Water
Vol reagent	2 ml	1 ml	1 ml	1 ml	0.5 ml	400 ul	0.5 ml	1 ml
Resorcinol (final)	1.817 mM	12.7 mM	18.2 mM	9.1 mM	18.2 mM	0.1%	9.1 mM	9.1 mM
Copper sulphate (final)	0.25 mM	0.18 mM	0.125 mM	0.125 mM	0.5 mM	0.125 mM	0.125 mM	0.125 mM
HCl, % w/v (final)	29.6%	15%	15%	15%	30%	40%	15%	15%
Method Incubation	Oil bath	Boiling water bath	Heating block	Oil bath	Boiling water bath	Water bath	Heating block	Boiling water bath
Temp, time of incubation	100-105°C, 15 min	98°C, 30 min	100 ±3°C, 20±3 min	100 ±2°C, 40±1 min	95-100°C, 15 min	95±5°C, 45 min.	110±2°C, 15-18 min	100±2°C, 15 min
Volume extractant added	5 ml	1 ml	n/a	n/a	1 ml	1 mL	n/a	2 ml
Extractant added	1 pentanol	20% butanol, 80% butyl acetate (1:4)	n/a	n/a	15% n-butanol, 85% butyl acetate	isoamyl alcohol	n/a	85:15 v/v 1-butyl acetate/1-butanol
Other steps	2000-2500 rpm, 5-10 min, 2-8°C	Vortex and take organic layer	3 dilutions of sample		Vortex and take organic layer	n/a		Purity and moisture content of NANA considered
<b>Spectrophotometer</b>	580 nm	580 – 450 nm	580 nm	580 nm	585 nm	580 – 450 nm	564 nm	580 nm
<b>Purity of NANA standard</b>	94.9%	98.8%	98.7%	98.8%	100%	99.4%	98.7%	91.2%
<b>Conversion factor used (MenC/ NANA) *</b>	1.079	1.123	1.122	1.123	1.137	1.130	1.123	1.037

\*Conversion factor determined using formula (MW Men C PS/ MW NANA)\*purity (as a decimal). MenC PS and NANA MW determined as described Annex 2.

**Table 3. Details of the DMAB assay conditions used by participants in the Collaborative Study**

Laboratory code	5	8	9
<b>Standard</b>	MenC PS	NANA	NANA
Source	In-house	Aldrich 855650, Synthetic, crystalline, 99.4%	Fluka 0401, synthetic, 100.0%
Storage	1 mg/ml, 0.15 M NaCl, -70°C, 1 yr	1 mM, H <sub>2</sub> O, -25°C, 9.5 mo,	10 mM, H <sub>2</sub> O, -20°C, 6 mo,
Std curve range	50-400 µg/ml	50-200 nmol (~16-62 µg)	50-200 nmol (~16-62 µg)
OD range (rounded to 2 decimal places)	0.45-1.6	0.18-0.90	~ 0.15 – 0.75
# levels in standard curve	5	3	3
# repeats	4	4	4
<b>Sample details</b>			
Diluent	0.15 M NaCl	H <sub>2</sub> O	H <sub>2</sub> O
Volume of sample	200 µl in microplate	200 µl	200 µl
<b>Reagents</b>			
DMAB final	1.37%	1.25%	1.25%
HCl final	1.6 M HCl	1.5 M HCl	1.5 M HCl
Aluminum sulfate	n/a	15%	15%
<b>Incubation</b>			
Method	Pulsed air oven	Water bath	Water bath
Temp, time	100°C, 90 min	95°C, 30 min	95°C, 30 min
<b>Spectrophotometer</b>	502 nm	530 nm	530 nm
Other	MenC PS std corrected for moisture & ethanol content		
<b>Purity of NANA standard</b>	N/A	99.4%	100%

\*Conversion factor determined using formula (MW Men C PS/ MW NANA)\*purity (as a decimal). MenC PS and NANA MW determined as described Annex 2.

**Table 4. Details of the HPAEC-PAD assay conditions used by participants in the Collaborative Study**

Laboratory code	3	4	5	10
Standard	NANA	NANA	NANA	MenC PS
Source, cat #, % purity*	Sigma A0812, synthetic, 99.4%	Fluka 01401, synthetic 100%	Fluka 01398, <i>E. coli</i> , 100%, powder, -20°C	From vaccine manufacturer, dried prior to preparing
Storage	Individual working standards, 16, 32, 64, 96, 128, 160 nmol/ml (~5, 10, 20, 30, 40, 50 µg/ml) H <sub>2</sub> O, -22 to -24°C, 2 wk	0.1 mg/ml, H <sub>2</sub> O, -20°C, 5 mo	0.4 mg/ml, H <sub>2</sub> O, -20°C, 2.5 yr	10 mg/ml stock in H <sub>2</sub> O, -20°C 1 mg/ml, H <sub>2</sub> O, -20°C, 14/24 mo
Standard curve range, vol injected	0.4-4.0 nmol/ 25 µl injection (~0.125-1.25 µg//25 µl injection) Quadratic fit, forced origin off	0.1– 2.0 µg/ml, 50 µl injection	80-167 µg/ml, 10µl injection	0.5-27 µg/ml, 25 µl injection
# levels in standard curve, # repeats	6 levels, 4 repeats	5 levels, 2 repeats	5 levels, 2 repeats	5 levels, 2 repeats
Other standards	Fuc, ManNac, Gal, Glc	MenC PS	MenC PS, -70°C	
Internal standard	None	None	2-deoxy-glucose	glucuronic acid
Hydrolysis	0.1 M TFA	2 M TFA	1.1 N HCl	0.1 M HCl
Incubator	Heating block	Oven	Heating block	Heating block
Incubation	80°C, 2 hr	90°C, 2 hr	80°C, 2.5 hr	80°C, 2.5 hr
Make/Model Equipment	Dionex ICS 3000	Dionex DX-500	Dionex ICS3000	Dionex ICS3000
Waveform	Triple	Triple	Quadrupole	Quadrupole
Column	CarboPac PA10	CarboPac PA1	CarboPac PA10	CarboPac PA1
Guard column(s)	AminoTrap, PA10 guard	PA1 guard	BorateTrap, PA10 guard	AminoTrap, PA1 guard
Column temperature	30°C	30°C	30°C	30°C
Mobile phase	Separate: 16 mM NaOH, 30 min 6-200 mM NaOH, 0-200 mM NaAcetate, 30-45 min	100 mM NaOH; 100 mM NaOH, 100 mM NaAcetate	100 mM NaOH, 100 mM NaAcetate	100 mM NaOH, 80 mM NaAcetate
Regeneration/wash	Wash and regenerate: 200mM NaOH, 200mM NaAcetate, 45-50 min 200mM NaOH, 200-0mM NaAcetate, 50-55 min Re-equilibrate: 200 mM NaOH, 55-60 min 200-16 mM NaOH, 60-62.5 min 16 mM NaOH, 62.5-75 min	200 mM NaOH, 1 hr	n/a	n/a
Flow rate, Run Time	1 ml/min; 75 min	1 ml/min; 20 min	1 ml/min, 10 min	1 ml/min, 30 min
Elution of NANA, std	40.8 min	14 min	2-deoxyglucose, 2 min; NANA, 5 min	NANA, 6 min; GlcA, 18.3 min
Purity of NANA standard	99.4%	100%	100%	N/A
Conversion factor used (MenC/NANA) used*	1.130	1.137	1.137	Not required, MenC PS std.

\*Conversion factor determined using formula (MW Men C PS/ MW NANA)\*purity (as a decimal). MenC PS and NANA MW determined as described Annex 2..

**Table 5. Details of the NMR parameters used by participants in the Collaborative Study**

Laboratory	3	5	6	10
Field (MHz)	600	500	400	500
Number of scans	32 (cryoprobe)	96	n.a.	256
Temperature (°C)	30	25	n.a.	30
Recycle delay (s)	20	85	n.a.	26
Internal standard	Glc $\alpha$ OMe* (4 protons @ ~3.45 ppm)	sodium formate	nicotinamide	TSP**

\* Methyl- $\alpha$ -D-glucose

\*\* Trimethylsilylpropionic acid sodium salt

**Table 6. Laboratory mean estimates (mg MenC PS/ampoule) for 08/214 (ampoules coded A & D)**

Method	Lab	Mean	Method Mean	CV
Resorcinol	1	1.188	1.192	6.9%
	2	1.261		
	3	1.078		
	6	1.195		
	7	1.192		
	8	1.305		
	10	1.248		
	12	1.074		
DMAB	5	1.241	1.276	8.3%
	8	1.192		
	9	1.394		
NMR	3	1.019	1.011	9.6%
	5	0.871		
	6	1.076		
	10	1.077		
HPAEC-PAD	3	1.189	1.132	18.4%
	4	1.373		
	5	0.871		
	10	1.096		
ELISA	11	1.359	1.359	.
Mean (all results)		1.165		
CV (all results)		12.6%		
Between-method CV		11.2%		

CV = coefficient of variation

Table 7. Individual estimates (mg MenC PS/ampoule ) obtained for ampoules coded A-D

Method	Lab	Ampoule Set	Ampoule A	Ampoule B	Ampoule C	Ampoule D
Resorcinol	1	1	1.146	0.519	.	1.124
		2	1.242	.	.	1.242
Resorcinol	2	1	1.235	0.583	.	1.229
		2	1.298	0.677	0.680	1.283
Resorcinol	3	1	1.075	0.511	.	1.056
		2	1.069	.	.	1.110
Resorcinol	6	1	1.208	0.670	.	1.124
		2	1.234	0.715	0.686	1.213
Resorcinol	7	1	1.131	0.603	0.006	1.219
		2	1.197	0.606	0.017	1.220
Resorcinol	8	1	1.302	.	.	1.390
		2	1.111	.	.	1.417
Resorcinol	10	1	1.170	0.627	0.632	1.192
		2	1.334	0.688	0.624	1.295
Resorcinol	12	1	1.076	0.517	0.030	1.061
		2	1.070	0.512	0.031	1.088
DMAB	5	1	1.259	.	0.651	1.257
		2	1.213	.	0.610	1.234
DMAB	8	1	1.240	.	.	1.281
		2	1.012	.	.	1.234
DMAB	9	1	1.409	0.424	.	1.377
		2	1.389	0.439	.	1.401
NMR	3	1	1.019	.	.	.
NMR	5	1	0.902	.	.	0.872
		2	0.869	.	.	0.840
NMR	6	1	1.137	.	.	1.127
		2	0.989	.	.	1.051
NMR	10	1	0.852*	.	.	1.039
		2	1.098	.	.	1.093
HPAEC-PAD	3	1	1.179	.	.	1.180
		2	1.196	.	.	1.202
HPAEC-PAD	4	1	1.363	0.294	.	1.380
		2	1.362	0.363	.	1.388
HPAEC-PAD	5	1	0.884	.	.	0.823
		2	0.899	.	.	0.878
HPAEC-PAD	10	1	1.036	.	.	1.052
		2	1.132	.	.	1.162
ELISA	11	1	1.324	.	.	1.370
		2	1.500	.	.	1.243

\* = excluded from further calculation as recommended by lab

**Table 8. Assessment of ampoule homogeneity (mg MenC PS/ampoule) for 08/214**

Method	Lab	Assay	Ampoule	Estimate
Resorcinol	10	1	1	1.364
			2	1.290
			3	1.381
			4	1.269
			5	1.297
			6	1.323
		2	7	1.252
			8	1.255
			9	1.249
			10	1.244
			11	1.232
			12	1.230

**Table 9. Uncertainties applicable to the resorcinol assay**

Lab	Source	Value	Standard Uncertainty	Relative Standard Uncertainty
10	Repeatability <sup>1</sup>	17.0 µg/ml	0.91 µg/ml	0.054
	Purity of sialic acid standard <sup>2</sup>	99.0%	0.58%	0.006
	Weighing / mass <sup>3</sup>	20.0 mg	0.40 mg	0.020
Combined relative standard uncertainty				0.057

<sup>1</sup>determined from in-house data on single reference vaccine lot included in each assay with same NANA standard lot

<sup>2</sup>determined from purity data supplied by commercial source, assuming a rectangular distribution for calculation of standard uncertainty

<sup>3</sup>determined from manufacturer data for balance used to weigh NANA standard

**Table 10. Laboratory mean estimates (mg MenC PS/ml) for bulk conjugate sample calculated using in-house standards**

Method	Lab	Mean	Method Mean	CV
Resorcinol	1	0.697	0.710	12.1%
	2	0.671		
	3	0.658		
	6	0.686		
	7	0.726		
	8	0.893		
	10	0.636		
	12*	0.698*		
DMAB	5	0.796	0.868	7.6%
	8	0.884		
	9	0.924		
HPAEC-PAD	3	0.650	0.698	10.0%
	4	0.778		
	10	0.667		
HPLC-Fluor	5	0.885	0.885	.
ELISA	11	0.842	0.842	.
Mean (all results)		0.760		
CV (all results)		13.6%		
Between-method CV		11.2%		

CV = coefficient of variation;

\* = excluded from further calculation as outlier when using 08/214 as standard

**Table 11. Laboratory mean estimates (mg MenC PS/ml) for bulk conjugate sample calculated using 08/214 as standard**

Method	Lab	Mean	Method Mean	CV
Resorcinol	1	0.716	0.749	7.0%
	2	0.780		
	3	0.741		
	6	0.704		
	7	0.749		
	8	0.849		
	10	0.703		
	12*	1.105*		
DMAB	5	0.743	0.826	9.4%
	8	0.897		
	9	0.837		
HPAEC-PAD	3	0.666	0.694	7.1%
	4	0.752		
	10	0.665		
HPLC-Fluor	5	0.877	0.877	.
ELISA	11	0.797	0.797	.
Mean (all results)		0.765		
CV (all results)		9.5%		
Between-method CV		8.9%		

CV = coefficient of variation;

\* = excluded from further calculation as outlier when using 08/214 as standard

Table 12. Individual estimates (mg MenC PS/ml) obtained for bulk conjugate sample

Method	Lab	Assay	Calculated using 08/214 as standard	Calculated using in-house standards
Resorcinol	1	1	0.707	0.705
		2	0.726	0.689
Resorcinol	2	1	0.760	0.682
		2	0.775	0.689
		3	0.806	0.642
Resorcinol	3	1	0.744	0.681
		2	0.738	0.636
Resorcinol	6	1	0.663	0.654
		2	0.746	0.719
Resorcinol	7	1	0.730	0.717
		2	0.769	0.735
Resorcinol	8	1	0.890	0.867
		2	0.808	0.919
Resorcinol	10	1	0.703	0.632
		2	0.702	0.641
Resorcinol	12	1	1.088	0.710
		2	1.122	0.642
		3	.	0.741
DMAB	5	1	0.731	0.780
		2	0.756	0.812
DMAB	8	1	0.925	0.871
		2	0.869	0.896
DMAB	9	1	0.843	0.923
		2	0.831	0.926
HPAEC-PAD	3	1	0.668	0.652
		2	0.664	0.648
HPAEC-PAD	4	1	0.726	0.746
		2	0.777	0.810
HPAEC-PAD	10	1	0.639	0.652
		2	0.691	0.682
HPLC-Fluor	5	1	0.815	0.862
		2	0.939	0.908
ELISA	11	1	0.799	0.832
		2	0.796	0.852

**Table 13. Estimated content (expressed as % of -70°C sample) of real-time and accelerated thermal degradation samples of 08/214 determined by resorcinol assay**

Storage time (months)	Storage temperature			
	-20°C	+4°C	+37°C	+56°C
1	98.0%	99.4%	103.2%	96.7%
2	101.5%	105.5%	99.8%	100.2%
3	101.6%	99.7%	101.9%	100.8%
5	99.2%	100.2%	99.5%	97.3%
6	100.3%	99.3%	98.0%	98.9%
11	102.6%	102.5%	102.6%	101.3%
12	106.3%	103.4%	102.8%	100.6%
30	99.1%	-	-	-

**Table 14. Estimated content (mg MenC PS/ampoule) of reconstituted stability samples of 08/214 determined by resorcinol assay**

Storage time (months)	Storage temperature following reconstitution	
	-20°C	-70°C
1	1.225	1.221
6	1.176	1.150
12	1.202	1.186

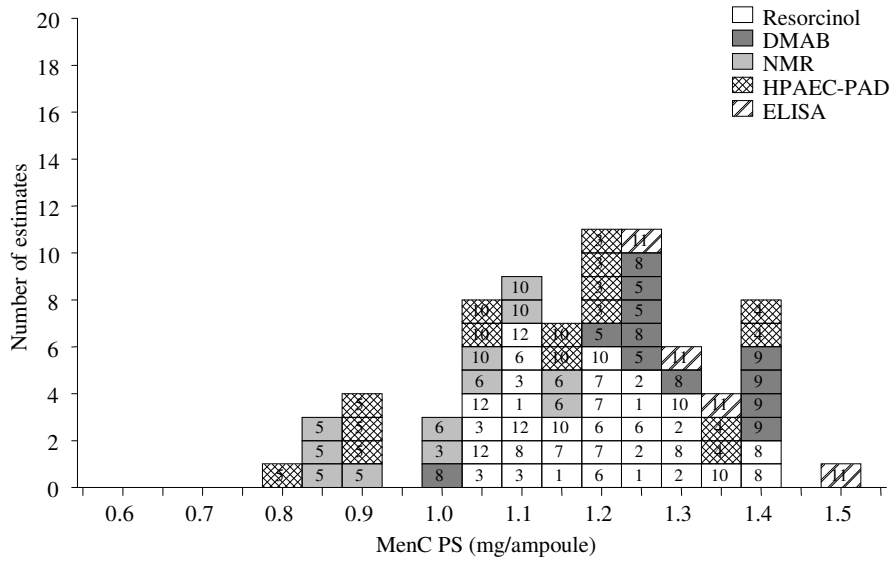
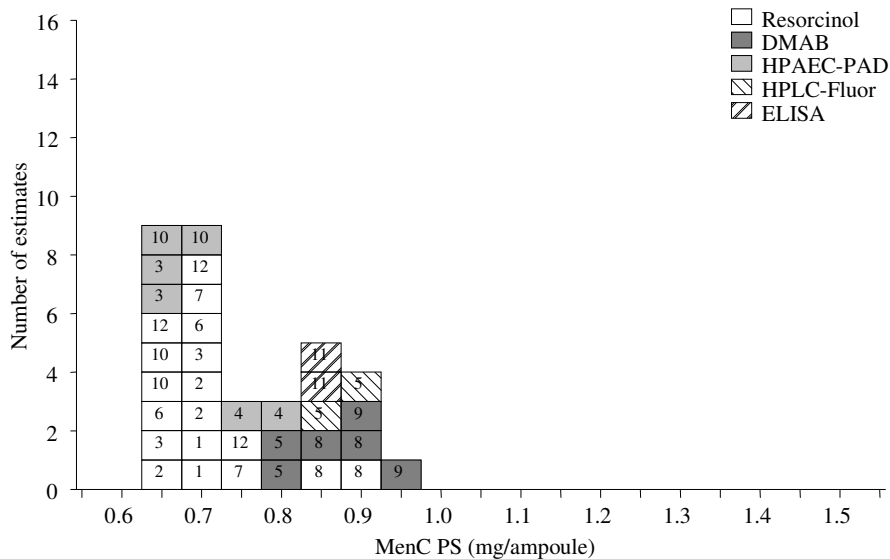
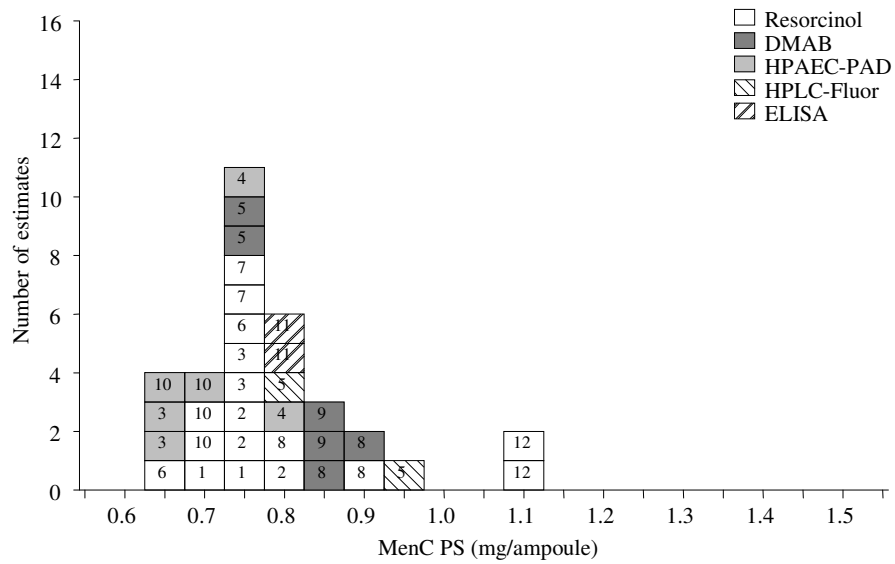


Figure 1. Distribution of individual assay estimates (mg MenC PS/ampoule) for 08/214



**Figure 2. Distribution of individual assay estimates (mg MenC PS/ml) for bulk conjugate sample calculated using in-house standards**



**Figure 3. Distribution of individual assay estimates (mg MenC PS/ml) for bulk conjugate sample calculated using 08/214 as standard**