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Annex 1

Recommendations for the production and control of *Haemophilus influenzae* type b conjugate vaccines[1]

Recommendations published by WHO are intended to be scientific and advisory. Each of the following sections consitutes guidance for national control authorities and for the manufacturers of biological products. If a national control authority so desires, these Recommendations may be adopted as definitive national requirements, or modifications may be justified and made by a national control authority. It is recommended that modifications to these Recommendations be made only on condition that modifications ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the Recommendations set out below. The parts of each section printed in small type are comments for additional guidance intended for manufacturers and national control authorities which may benefit from those details.

[1] As decided by the WHO Expert Committee on Biological Standardization in October 1998, requirements for biological substances have been renamed "recommendations". These Recommendations are a revised version of the 1991 Requirements for *Haemophilus influenzae* Type b Conjugate Vaccines (Requirements for Biological Substances No. 46).

Introduction

The 1991 Requirements for *Haemophilus influenzae* type b vaccines adopted by the WHO Expert Committee on Biological Standardization in 1990 (*1*) embodied recommendations made by an informal consultation held in 1989. Much experience has been gained with the preparation and control of these vaccines since that time, including the recognition that some vaccine lots that complied with the Requirements had reduced immunogenicity in children (*2*). This emphasized a need to reassess vaccine control strategies and for continued post-marketing surveillance. A revision of the 1991 Requirements (renamed Recommendations in accordance with the decision of the Committee at its forty-ninth meeting, in October 1998) has therefore been prepared, which takes into account the above-mentioned considerations. In particular, it has been shown that the biological assay of potency recommended in 1991 does not correlate with the efficacy of the vaccine in infants and does not provide a sensitive indicator of vaccine quality. Thus, while immunogenicity testing in animals is necessary during vaccine development, the revised Recommendations state that an animal immunogenicity test need not be used for routine batch (lot) release. Instead, the testing focuses on physicochemical tests to monitor consistency of production of the polysaccharide, the protein carrier and the bulk conjugate. Where an *H. influenzae* type b conjugate is included in a combination vaccine, further consideration needs to be given to batch release testing of the final bulk and final fill because of the difficulty in performing physicochemical assays on such preparations.

General considerations

H. influenzae type b causes several diseases in humans, the most common and most serious being meningitis and pneumonia, mainly in children under 5 years of age. Other bacteraemic infections caused by this pathogen include epiglottitis, cellulitis, septic arthritis, osteomyelitis and pericarditis. There are six serotypes of encapsulated *H. influenzae*. Nearly all cases of meningitis and most cases of other bacteraemic diseases are caused by type b organisms where the capsular polysaccharide plays an important role in virulence (*3*). *H. influenzae* type b conjugate vaccines have been developed and shown to be a safe and effective means of protecting against such infections. These Recommendations deal only with *H. influenzae* type b conjugate vaccines, derived from the type b polysaccharide, a linear polymer composed of units of 3-β-D-ribofuranosyl (1→1)-D-ribitol-5-phosphate (referred to as PRP) covalently coupled to a protein carrier (*4*).

A number of epidemiological studies show the public health importance of *H. influenzae* type b disease. In the absence of the general use of the *H. influenzae* type b conjugate vaccine, the annual incidence of disease caused by this bacterium among children in the first 5 years of life is in the range 0.02–0.5% (*5–9*). The disease burden is highest in infants aged between 4 and 18 months, *H. influenzae* disease rarely occurring in infants under 3 months of age or children older than 6 years (*6*). In both developed and developing countries *H. influenzae* type b disease was the leading cause of non-epidemic bacterial meningitis in this age group and, despite prompt and adequate antibiotic treatment, frequently resulted in severe neurological sequelae, the most serious of which are hearing loss and mental retardation (*10, 11*). *H. influenzae* type b disease is contagious and can cause outbreaks of meningitis where susceptible children are crowded together, e.g. in day-care centres (*12, 13*). Transmission of *H. influenzae* occurs by means of droplets expelled by colonized individuals, and asymptomatic carriers are important disseminators of the organism. An additional concern has been the development of strains resistant to antibiotics (*14*), especially ampicillin. Vaccines are the only public health measure available to prevent the majority of disease caused by *H. influenzae* type b.

The low incidence of *H. influenzae* type b disease in older children and adults correlates with the presence of bactericidal antibodies in their serum, directed mainly to the *H. influenzae* type b capsular polysaccharide (*5*). The first vaccine against *H. influenzae* type b disease was therefore made from the type b capsular polysaccharide alone. In 1977, *H. influenzae* type b polysaccharide vaccine was shown to be protective in older children in Finland (*15*). The vaccine was, however, ineffective in inducing antibodies or providing protection in children under 18 months old (*16–19*). This lack of efficacy in the age group at greatest risk stimulated the development of improved *H. influenzae* type b vaccines.

The two immunological properties of the *H. influenzae* type b polysaccharide that limit its use in infants and young children are its age-related immunogenicity, and its failure to elicit immunological memory, and consequently a booster response on subsequent exposure to the polysaccharide (*16–18, 20*). However, immunogenicity can be enhanced by linking the polysaccharide covalently to a protein so that a T-cell-dependent antisaccharide response is elicited (*21–26*). Several different PRP conjugates stimulate T-cell-dependent antibody synthesis in infants and young children with a booster response and, in comparison with polysaccharide vaccine, an increased proportion of IgG antibody relative to IgM antibody (*24, 27–31*). Freeze-dried (lyophilized) and liquid preparations have been licensed for marketing.

Following the introduction of *H. influenzae* type b conjugate vaccines into routine childhood immunization programmes in the 1990s, disease caused by *H. influenzae* type b has largely disappeared in Australia, Canada, western Europe, New Zealand and the United States of America (*32–35*). Efficacy estimates in the range 93–100% have been reported for these vaccines (*28, 36–39*). Indeed, not only do they induce protective circulating antibodies and immunological memory in infants, but their use also results in decreased nasopharyngeal colonization with *H. influenzae* type b. Thus, a herd effect is achieved through reduced transmission of the pathogen (*40–42*).

Special considerations for the production and control of *Haemophilus influenzae* type b conjugate vaccines

The production and control of *H. influenzae* type b conjugate vaccines are more complex than those of unconjugated capsular polysaccharide vaccines, such as meningococcal polysaccharide vaccines (*43*) or pneumococcal polysaccharide vaccines. Polysaccharide vaccines consist of defined chemical entities and, when prepared to the same specifications, are expected to have comparable potencies, regardless of manufacturer. *H. influenzae* type b conjugate vaccines are less well defined chemically and different conjugation chemistries have been shown to yield effective vaccines (*27, 42*). *H. influenzae* type b conjugate vaccines all contain immunogenic determinants capable of stimulating the production of serum IgG antibodies to PRP, and are capable of inducing immunological memory in young children (*24, 26–31, 36*).

Several *H. influenzae* type b conjugate vaccines have been developed and licensed and extensive experience has been gained with the use of some of these products in Europe and the USA. The composition of some of these vaccines is described in Table A1. Immunogenicity in infants, including analysis of IgG subclasses of anti-PRP antibody, can be used to demonstrate equivalence between a new *H. influenzae* type b conjugate vaccine and existing effective vaccines. The existence of a surrogate for protection is important, because it will not be possible to carry out acceptable placebo-controlled protection-based efficacy trials of new *H. influenzae* type b conjugate vaccines or new formulations. Production of specific anti-PRP IgG has been correlated with vaccine efficacy.

Currently available effective vaccines have been found to:

- induce $\geqslant 1$ μg/ml of antibody to PRP in 70% or more of infants 1 month after completion of the primary immunization series;

- prime infants for a booster response to the native *H. influenzae* type b polysaccharide given 6–9 months after the primary immunization; and
- produce functional antibodies measured by either opsonic or bactericidal activity.

The immunogenicity in humans of the PRP and protein components of any *H. influenzae* type b conjugate vaccine should be assessed before the vaccine is licensed. Guidance on methods of evaluating immunogenicity is given in Appendix 1.

Currently, no useful biological assay is available to test potency and/or protective efficacy of individual production lots. Consequently, the strategy for control of the vaccine emphasizes the use of tests for molecular characterization and purity to ensure lot-to-lot consistency of composition with the specifications of the vaccine lot(s) employed in definitive clinical trials to prove efficacy and safety. The immunogenicity and induction of memory for antibody production by *H. influenzae* type b conjugate vaccines have been evaluated in mice and guinea-pigs (*23, 44, 45*). However, the results of such evaluations do not correlate with the immunological properties of the vaccines in human infants. Thus, whilst immunogenicity testing in animals is necessary during vaccine development to demonstrate an ability to induce a T-cell-dependent immune response, an animal potency test (immunogenicity) need not be used for routine batch (lot) release. Instead, the testing should focus on physicochemical criteria for monitoring the consistent quality of the polysaccharide, the protein carrier and the bulk conjugate.

Combination vaccines containing a *Haemophilus influenzae* type b conjugate

It is recognized that vaccine combinations may be needed for effective vaccine utilization. Each combination, produced either by preformulation or at the time of reconstitution, should be evaluated by the vaccine producer in the laboratory for possible incompatibilities between the components after mixing and for stability over time, as well as clinically. Because of the difficulty in performing physicochemical assays on *H. influenzae* type b conjugate vaccine in a combination vaccine preparation, further consideration needs to be given to batch release of final bulk and final lot of such vaccines, and the tests applied should be agreed with the national control authority. For example, an immunoassay may be considered in order to confirm that the formulated product consistently retains its immunogenic activity.

H. influenzae type b conjugate vaccines have been successfully combined by formulation with diphtheria–tetanus–pertussis vaccines containing whole-cell pertussis vaccine (DTwP), with hepatitis B vaccine, and with DTwP and inactivated poliomyelitis vaccine (IPV). Such combinations have been demonstrated to be safe and effective. Another type of combination vaccine is formed at the time of use by reconstitution of freeze-dried *H. influenzae* type b conjugate vaccine with, for example, a DTwP vaccine. Combinations with DTwP vaccine have generally shown no incompatibility. Nevertheless, the particular DTwP vaccine to be used should be evaluated clinically for possible adverse interactions with the *H. influenzae* component. A number of clinical studies have demonstrated that combinations of DTP vaccines containing acellular pertussis vaccine (DTaP) and *H. influenzae* type b conjugate vaccine may result in marked diminution of the immune response to the *H. influenzae* polysaccharide (*46*). However, despite reduced immunogenicity, such combination vaccines can prime for immunological memory (*46*).

Table A1
Formulation of some currently available *H. influenzae* type b conjugate vaccines[a,b]

H. influenzae polysaccharide material	Polysaccharide per single human dose (μg)	Nature of carrier protein	Protein per single human dose (μg)
Polysaccharide (size-reduced)	25	Diphtheria toxoid	18
Polysaccharide (low relative molecular mass)	10	Diphtheria CRM 197 protein	25
Polysaccharide (size-reduced)	7.5	Outer membrane protein complex of *Neisseria meningitidis* group B	125
Polysaccharide	10	Tetanus toxoid	20

[a] For guidance only.
[b] *H. influenzae* type b conjugate vaccine is a preparation of capsular polysaccharide from *H. influenzae* type b covalently linked to carrier protein

Part A. Manufacturing recommendations

A.1 Definitions

A.1.1 *Proper name*

The proper name of the vaccine should be "*Haemophilus influenzae* type b conjugate vaccine" translated into the language of the country of use.

The use of this name should be limited to vaccines that satisfy the recommendations formulated below.

A.1.2 *Descriptive definition*

Haemophilus influenzae type b conjugate vaccine is a preparation of capsular polysaccharide from *H. influenzae* type b covalently linked to a carrier protein.

A.1.3 *International reference materials*

No formally established international reference materials that would allow the standardization of immune responses to *H. influenzae* type b conjugated vaccines are currently available, but their development is under consideration (see also Appendix 1).

A.1.4 *Terminology*

Master seed lot. A suspension of *H. influenzae* type b organisms derived from a strain that has been processed as a single lot and is of uniform composition. It is used for the preparation of working seed lots. Master seed lots should be maintained in the freeze-dried form or frozen at or below – 45 °C.

Working seed lot. A suspension of *H. influenzae* type b organisms derived from the master seed lot by growing the organisms and maintaining them in aliquots in the freeze-dried form or frozen state at or below – 45 °C. The working seed lot is used, if applicable, after a fixed number of passages, for inoculating production medium.

Single harvest. The material obtained from one batch of cultures that have been inoculated with the working seed lot (or with the inoculum derived from it), harvested and processed together.

Purified polysaccharide. The material obtained after final purification. The lot of purified polysaccharide may be derived from a single harvest or a pool of single harvests processed together.

Processed polysaccharide. Purified polysaccharide that has been modified by chemical reaction or physical process in preparation for conjugation to the carrier.

Carrier. The protein to which processed polysaccharide is covalently linked for the purpose of eliciting a T-cell-dependent immune response to the *H. influenzae* type b polysaccharide.

Bulk conjugate. A conjugate prepared from a single lot or pool of lots of polysaccharide and a single lot of protein or a pool of lots of protein. It is the parent material from which the final bulk is prepared.

Final bulk. The homogeneous preparation present in a single container from which the final containers are filled either directly or through one or more intermediate containers.

Final lot. A number of sealed, final containers that are equivalent with respect to the risk of contamination during filling and, if appropriate, freeze-drying. A final lot should therefore have been filled from a single container and freeze-dried in one continuous working session.

A.2 General manufacturing recommendations

The general manufacturing recommendations contained in Good Manufacturing Practices for Pharmaceutical (*47*) and Biological (*48*) Products should be applied to establishments manufacturing *H. influenzae* type b conjugate vaccines with the addition of the following.

Details of standard operating procedures for the preparation and testing of *H. influenzae* type b conjugate vaccines adopted by a manufacturer, together with evidence of appropriate validation of each production step, should be submitted for approval to the national control authority. All assay procedures used for quality control of the conjugate vaccine and vaccine intermediates should also be validated (*49*). As may be required, proposals for modification of the manufacturing/control methods should also be submitted for approval to the national control authority before they are implemented.

H. influenzae type b is a class 2 pathogen and should be handled under conditions appropriate for this class of microorganism (*50*). Standard operating procedures should be developed for dealing with emergencies involved with accidental spillage, leakage or other dissemination of *H. influenzae* organisms.

Persons employed in the production and control facilities should be adequately trained. Appropriate protective measures, including vaccination against *H. influenzae* type b, should be implemented. Adherence to current good manufacturing practices is important to the integrity of the product, to protect the workers and to protect the environment.

A.3 Production control

A.3.1 *Control of the polysaccharide*

A.3.1.1 Strain of H. influenzae type b

The strain of *H. influenzae* type b to be used in preparing *H. influenzae* type b conjugate vaccine should be identified by a record of its history, including the source from which it was obtained and the tests made to determine the characteristics of the strain. The strain should have been shown to be capable of producing type b polysaccharide.

> Nuclear magnetic resonance (NMR) spectroscopy may be used to confirm the identity of the type b polysaccharide produced by the selected *H. influenzae* type b strain.

A.3.1.2 Seed lot system

The production of *H. influenzae* type b polysaccharide should be based on a working seed lot system. If materials of animal origin are used in the medium for seed production, preservation of strain viability for freeze-drying or for frozen storage, then they should comply with the guidance given in the *Report of a WHO Consultation on Medicinal and Other Products in Relation to Human and Animal Transmissible Spongiform Encephalopathies* (*51*) and should be approved by the national control authority. Cultures derived from the working seed lot should have the same characteristics as cultures of the strain from which the master seed lot was derived. Seed lots should be in conformity with the recommendations in section A.3.2.1.

> Manufacturers are encouraged to avoid, wherever possible, the use of materials of animal origin.

A.3.1.3 Culture media for production of the polysaccharide

Some media used for the growth of bacteria have been shown to contain human blood-group antigen-like material. Therefore, the *H. influenzae* type b working seed should be inoculated into a liquid medium that does not contain blood-group antigens or high-molecular-weight polysaccharides of either plant or animal origin. Assurance of the absence of human blood-group antigens should be provided by a sensitive immunoassay. This may be done on a lot-to-lot basis or by validation of the process. If materials of animal origin are used, their use should be in accordance with the guidance given in the *Report of a WHO Consultation on Medicinal and Other Products in Relation to Human and Animal Transmissible Spongiform Encephalopathies* (*51*) and should be approved by the national control authority.

> Manufacturers are encouraged to avoid, wherever possible, the use of materials of animal origin.

A.3.1.4 Single harvests

Consistency of growth of *H. influenzae* type b microorganisms should be demonstrated by monitoring the growth rate, pH and yield of polysaccharide.

A.3.1.5 Control of bacterial purity

Samples of the culture taken before killing should be tested for contamination. The purity of the culture should be verified by suitable methods that should include inoculation on to appropriate plating media. If any contaminant is found, the culture or any product derived from it should be discarded. The killing process should also be adequately validated.

A.3.1.6 Purified polysaccharide

All steps in the purification procedure should be carried out in clean containers made of material compatible with the solvents in use during the particular step of the procedure. Chemicals of an appropriate purity should be employed.

Each lot of purified polysaccharide should be tested for purity. The limits given below are expressed with reference to the purified polysaccharide, corrected for moisture. Each manufacturer should define limits for its own product, which should be agreed by the national control authority.

> Generally, purified polysaccharide is stored at or below –20 °C to ensure stability.

Identity test. A test should be performed on the purified polysaccharide to verify its identity.

> An immunological method or ^{1}H or ^{13}C NMR spectroscopy (*4*) may be used for this purpose.

Molecular size distribution. The molecular size distribution of each lot of purified polysaccharide should be estimated.

> Separation methods such as gel filtration using cross-linked agarose (e.g. gel filtration using Sepharose CL-4B or CL-2B gels) with a suitable buffer and a refractive index detector (*52*) or high-performance size-exclusion chromatography (HPSEC), either alone or in combination with light scattering and refractive index detectors (e.g. multiple angle laser light scattering; MALLS) are suitable for this purpose (*53*).

The distribution constant (K_D) should be determined by measuring the molecular size distribution of the polysaccharide at the main peak of the elution curve. The K_D value and/or the mass distribution limits should be established and shown to be consistent from lot to lot for a given product.

The K_D value should be determined by gel filtration, the mass distribution limits by MALLS; it is not necessary to do both.

Moisture content. The moisture content of the isolated purified polysaccharide should be determined by a suitable method approved by the national control authority and shown to be consistent within agreed limits.

Polysaccharide composition. The quality of the polysaccharide can be estimated by measuring the ribose content. The ribose content should be not less than 32% of the polysaccharide dry weight (i.e. corrected for moisture), as estimated by the Bial reaction for pentose, using D-ribose as a standard (*54*).

Other methods can be used to define the quantitative composition of the purified polysaccharide (e.g. high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis), but these methods and specifications should be validated for this purpose. If other methods are used, different specifications for composition may apply and should be defined.

The phosphorus content, as determined by the method of Chen et al. (*55*) or another suitable validated method, should be measured and be within defined limits.

The theoretical phosphorus content is 8.4%, and a specification of between 6.8% and 9% calculated on the dry weight has been found suitable. If other methods are used to confirm phosphorus content, direct determination of phosphorus is not required.

Protein impurity. Each lot of purified polysaccharide should contain not more than 1% of protein, calculated on the dry weight and determined by the method of Lowry et al. (*56*), using bovine serum albumin as a reference (*52*), or another suitable validated method.

Sufficient polysacccharide should be assayed to detect accurately 1% contamination.

Nucleic acid impurity. Each lot of purified polysaccharide should contain not more than 1% of nucleic acid calculated on the dry weight and determined by ultraviolet spectroscopy, on the assumption that the absorbance of a nucleic acid solution at a concentration of 10 g/l contained in a cell of 1-cm path length at 260 nm is 200 (*52*), or by another validated method.

Sufficient polysaccharide should be assayed to detect accurately 1% contamination.

Endotoxin content. The endotoxin content of the purified polysaccharide should be determined and shown to be within limits agreed by the

national control authority in order to ensure that any pyrogenic activity of the final product is acceptable.

Less than 10 IU of endotoxin per μg of polysaccharide when measured by a *Limulus* amoebocyte lysate test can be achieved. Alternatively, polysaccharide preparations should pass the rabbit pyrogenicity test when injected into rabbits in amounts of 1.0 μg of purified polysaccharide per kg.

A.3.1.7 Processed polysaccharide

Most of the processed polysaccharide preparations are partially depolymerized either before or during the chemical modification.

Chemical modification. Several methods for chemical modification (activation) have been found to be satisfactory. The methods should be approved by the national control authority.

Suitable methods include:

1. The polysaccharide is reacted with cyanogen bromide to introduce groups reactive with "spacer molecules" or with the carrier protein. Excess reactants are removed from the polysaccharide by ultrafiltration or another suitable method.
2. Following cyanogen bromide activation of the polysaccharide, adipic acid dihydrazide is covalently bound to the polysaccharide.
3. Size-reduced polysaccharides are produced by periodate oxidation of the purified polysaccharide. This generates aldehyde groups. The resulting low-molecular-weight PRP is purified.
4. Size-reduced polysaccharides are also produced by controlled acid hydrolysis, which generates reducing-end ribose. After purification, the resulting low-molecular-weight PRP is reductively aminated and converted to the adipic acid spacer *N*-hydroxy-succinimide-activated derivative.
5. The polysaccharide is reacted with carbonyldiimidazole followed by butanediamine to form a reactive intermediate with a terminal amino group. This group is further reacted to form the final derivatized polysaccharide.

The processed polysaccharide to be used in the conjugation reaction should be assessed for the number of functional groups introduced per unit of polysaccharide.

The degree of activation of the chemically modified polysaccharide should be quantitatively assessed to determine the average number of reactive sites per unit of PRP.

Molecular size distribution. The molecular size of the processed polysaccharide will depend on the manufacturing process. The size

should be specified for each type of conjugate vaccine and measured by a suitable method, as this may affect the reproducibility of the conjugation process.

The molecular size distribution may be measured by methods such as gel filtration chromatography or HPSEC using MALLS and refractive index (RI) detection (*57, 58*).

A.3.2 *Control of the carrier protein*

A.3.2.1 Microorganisms and culture media for production of the carrier protein

Microorganisms to be used for the production of the carrier protein should be grown in media free from substances likely to cause toxic or allergic reactions in humans. If any materials of animal origin are used in seed preparation or preservation, or in production, their use should be in accordance with the guidance given in the *Report of a WHO Consultation on Medicinal and Other Products in Relation to Human and Animal Transmissible Spongiform Encephalopathies* (*51*) and should be approved by the national control authority.

Production should be based on a seed lot system with the strains identified by a record of their history and of all tests made periodically to verify strain characteristics. Consistency of growth of the microorganisms used should be demonstrated by monitoring the growth rate, pH and yield of appropriate protein(s).

A.3.2.2 Characterization and purity of the carrier protein

Proteins that have been used to date as carriers in *H. influenzae* type b conjugate vaccines include diphtheria and tetanus toxoids, a non-toxic mutant of diphtheria toxin (CRM197) and the outer membrane protein complex of *Neisseria meningitidis* group B (see Table A1). Test methods used to characterize such proteins, to ensure that they are non-toxic, and to determine their purity and concentration should be approved by the national control authority. Physicochemical methods that may be used to characterize such proteins include sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), isoelectric focusing, high-performance liquid chromatography (HPLC), amino acid analysis, amino acid sequencing, circular dichroism, fluorescence spectroscopy, peptide mapping and mass spectrometry as appropriate (*59*).

Proteins and purification methods that have been used include:

1. Diphtheria and tetanus toxoids. These should be in accordance with the relevant recommendations published by WHO (*60*) and be of high purity. The purity should be, at the very least, 1500 Lf/mg (Lf = limit of flocculation) protein (nondialysable) nitrogen. Purification may

precede detoxification since this may result in a purer product, but particular care should be taken to avoid reversion to toxin when this procedure is used. Amino acids such as lysine are frequently added during detoxification and help prevent reversion. Toxoids may be rendered more suitable for conjugation by additional purification.

2. Diphtheria CRM197 protein, a non-toxic mutant of diphtheria toxin, isolated from cultures of *Corynebacterium diphtheriae* C7/β197 (*22*). Protein of purity greater than 90% as determined by HPLC is prepared by column chromatographic methods. Methods should be in place to distinguish the CRM197 protein from the active toxin.
3. Outer-membrane protein complex of *Neisseria meningitidis* group B extracted from washed bacterial cells with buffer containing detergent. The cell debris is removed and the membrane complex concentrated and washed with the buffer containing detergent to remove impurities. The composition of the purified outer-membrane complex should be determined by SDS-PAGE or a similar method and shown to be consistent from lot to lot, have not more than 8% lipopolysaccharide by weight, and pass the rabbit pyrogenicity test when injected into rabbits in amounts of 0.25 µg/kg of body mass.

A.3.2.3 Processing of carrier protein

In some conjugation procedures, the carrier protein is chemically derivatized before coupling to the polysaccharide. As a measure of consistency, the extent of derivatization of the protein needs to be monitored at this stage.

Protein activation methods that have been used include:

1. The introduction into diphtheria toxoid of a specified concentration of "spacer groups" reactive with activated polysaccharide.
2. The addition of thiol groups to the outer-membrane protein complex of *Neisseria meningitidis* group B.

A.3.3 *Control of bulk conjugate*

A number of conjugation methods are currently in use (see Table A2); all involve multi-step processes. Both the method and the control procedures used to ensure the reproducibility, stability and safety of the conjugate should be established once the immunogenicity of a particular *H. influenzae* type b conjugate vaccine has been demonstrated. The derivatization and conjugation process should be monitored by analysis for unique reaction products or by other suitable means.

Residual unreacted functional groups potentially capable of reacting in vivo may be present following the conjugation process. The manufacturing process should be validated to show that reactive functional groups do not remain at the conclusion of the manufacturing process.

Table A2
Methods currently used for conjugation of *Haemophilus influenzae* type b polysaccharide and control of conjugates[a]

Method	Procedure	Assay for conjugation
Reductive amination	Combine carrier protein and aldehyde form of polysaccharide in presence of reducing agent	Formation of unique amino acid and gel filtration
Reductive amination and attachment of spacer linked to active ester	Selective reducing end group activation and coupling to carrier protein through spacer	Gel filtration or SDS-PAGE
Carbodiimide-mediated coupling	Combine reactants in presence of carbodiimide	Gel filtration
Cyanogen-bromide activation of polysaccharide	Addition of carrier protein to cyanogen-bromide-activated polysaccharide	Gel filtration and assay for bound polysaccharide
Thioether bonding	Combine haloacyl polysaccharide with protein thiol	Formation of unique amino acid and assay for bound polysaccharide

[a] For guidance only.

After the conjugate has been purified, the tests described below should be performed in order to assess consistency of manufacture. The tests are critical for assuring lot-to-lot consistency.

A.3.3.1 Residual reagents

The conjugate purification procedures should remove residual reagents used in the conjugation process. The removal of reagents, as well as reaction by-products such as cyanide, should be confirmed by suitable tests or by validation of the purification process.

A.3.3.2 Conjugation markers

Where the chemistry of the conjugation reaction results in the creation of a unique linkage marker (e.g. a unique amino acid), each batch should be assessed to quantify the extent of covalent reaction of the PRP with the carrier protein, so that the frequency of the covalent bond is given as a function of the number of PRP repeating units or overall PRP content.

The PRP–protein ratio after purification of the conjugate is another suitable conjugation marker, although not a direct measurement.

A.3.3.3 Residual reactive functional groups

Each batch should be shown to be free of residual reactive functional groups on the chemically modified polysaccharide or the carrier protein, either by analysis of each lot or by validation of the process.

A.3.3.4 Content of PRP

The content of PRP should be chemically determined by means of an appropriate validated assay.

A.3.3.5 Conjugated and unbound (free) PRP

Only the PRP that is covalently bound to the carrier protein, i.e. conjugated PRP, is immunologically important for clinical protection.

Each batch of conjugate should be tested for free, unconjugated PRP in order to ensure that the amount present in the purified bulk is within the limits agreed by the national control authority based on lots shown to be clinically safe and efficacious. Alternatively, the total amount of PRP covalently attached to the carrier protein may be measured after separation from unconjugated PRP.

A number of methods have been used to separate unconjugated PRP from the conjugate, including precipitation, gel filtration, size-exclusion anion-exchange, and hydrophobic chromatography, ultrafiltration and ultracentrifugation (*61*). The unconjugated PRP can then be quantified by a range of techniques, including HPAEC-PAD and immunoassays with anti-PRP antibodies.

Vaccines demonstrating adequate immunogenicity in clinical studies currently contain amounts of unbound polysaccharide ranging from less than 10% to up to 40% of the total PRP, depending upon the vaccine.

A.3.3.6 Protein content

The protein content of the conjugate should be chemically determined by means of an appropriate validated assay and comply with limits set for that particular product.

If possible, unconjugated (free) protein should also be measured.

A.3.3.7 PRP-to-protein ratio

The PRP-to-protein ratio of the conjugate should be calculated. For each conjugate, the ratio should be within the range approved for that particular conjugate by the national control authority.

A.3.3.8 Molecular size distribution

The molecular size of the PRP–protein conjugate is an important parameter in establishing consistency of production and in studying

physicochemical stability during storage. The relative molecular size of the conjugate should be determined for each bulk using a validated chromatographic method appropriate to the size of the conjugate being evaluated. Suitable methods include gel filtration (for example on Sepharose CL-4B) (*52*) and size-exclusion chromatography (*53*). The method should be shown to distinguish the PRP–protein conjugate from other components that may be present, e.g. unbound protein or polysaccharide.

A.3.3.9 Sterility

The bulk purified conjugate should be tested for bacterial and mycotic sterility in accordance with the recommendations of Part A, sections 5 and 5.2 of the 1973 revised Requirements for the Sterility of Biological Substances (*62*) or by a method approved by the national control authority. If a preservative has been added to the product, appropriate measures should be taken to prevent it from interfering with the test.

A.3.3.10 Specific toxicity of carrier protein in the conjugate

The bulk conjugate should be tested for the absence of specific toxicity of the carrier protein where appropriate (for example, the use of tetanus or diphtheria toxoids).

> Absence of specific toxicity of the carrier protein may also be assessed through validation of the production process.

A.3.4 ***Final bulk***

A.3.4.1 Preparation

The final bulk is prepared by mixing the adjuvant and a preservative and/or stabilizer (as appropriate) with a suitable quantity of the bulk conjugate so as to meet the specifications of vaccine lots shown to be safe and efficacious in clinical trials.

A.3.4.2 Sterility

Each final bulk should be tested for bacterial and mycotic sterility as indicated in section A.3.3.9.

A.3.5 ***Filling and containers***

The recommendations concerning filling and containers given in Annex 1, section 4 of Good Manufacturing Practices for Biological Products (*48*) should be applied.

A.3.6 *Control tests on final product*

A.3.6.1 Identity

An identity test should be performed on at least one labelled container from each final lot.

> An immunological test, using antibodies specific for the purified polysaccharide, may be used.

A.3.6.2 Sterility

The contents of final containers should be tested for bacterial and mycotic sterility as indicated in section A.3.3.9.

A.3.6.3 PRP content

The total PRP content in the final containers should be determined; it should be shown to be within the specifications agreed by the national control authority, and within $\pm 20\%$ of the stated PRP content.

> The conjugate vaccines produced by different manufacturers differ in formulation (see Table A1). A quantitative assay for the PRP in the final container may be product-specific: colorimetric, chromatographic (including HPLC) or immunological methods may be used (*61*).

A.3.6.4 Residual moisture

If the vaccine is freeze-dried, the average moisture content should be determined by methods accepted by the national control authority. Values should be within the limits for the preparations shown to be adequately stable in the stability studies of the vaccine.

> The test should be performed on 1 vial per 1000 up to a maximum of 10 vials but on no less than 5 vials taken at random from throughout the final lot. The average residual moisture content should generally be no greater than 2.5% and no vial should be found to have a residual moisture content of 3% or greater.

A.3.6.5 Pyrogen content

The vaccine in the final container should be tested for pyrogenic activity by intravenous injection into rabbits or by a *Limulus* amoebocyte lysate test. Specifications for endotoxin content or pyrogenic activity should be established; they should be consistent with levels found to be acceptable in vaccine lots used in clinical trials. These should be approved by the national control authority.

> Existing *H. influenzae* type b conjugate vaccines pass the rabbit pyrogenicity test when injected into rabbits in amounts of PRP ranging from 0.025 to 1.0 μg per kg of body weight, depending on the protein carrier.

A.3.6.6 Adjuvant content

If an adjuvant has been added to the vaccine, its content should be determined by a method approved by the national control authority.

The amount and nature of the adjuvant should be approved by the national control authority. If aluminium or calcium compounds are used as adjuvants, the amount of aluminium should not exceed 1.25 mg per single human dose and that of calcium 1.3 mg per single human dose.

> The adsorption of the antigen to the adjuvant should be investigated. Consistency of adsorption is important, and the adsorption of production lots should be demonstrated to be within the range of values found for vaccine lots shown to be clinically effective.

A.3.6.7 Preservative content

If a preservative has been added to the vaccine, the content of preservative should be determined by a method approved by the national control authority.

The amount of preservative in the vaccine dose should be shown not to have any deleterious effect on the antigen or to impair the safety of the product in humans. The preservative, its concentration and its limits should be approved by the national control authority.

A.3.6.8 General safety test (innocuity)

Each final lot should be tested for unexpected toxicity (abnormal toxicity) using a test approved by the national control authority.

> This test may be omitted for routine lot release once consistency of production has been well established to the satisfaction of the national control authority and when good manufacturing practices are in place. Each lot, if tested, should pass a test for abnormal toxicity.

A.3.6.9 pH

If the vaccine is a liquid preparation, the pH of each final lot should be tested and shown to be within the range of values found for vaccine lots shown to be safe and effective in clinical trials and stability studies. For a lyophilized preparation, the pH should be measured after reconstitution with the appropriate diluent.

A.3.6.10 Inspection of final containers

Each container in each final lot should be inspected visually, and those showing abnormalities such as improper sealing, lack of integrity and, if applicable, clumping or the presence of particles should be discarded.

A.4 Records

The recommendations in section 8 of Good Manufacturing Practices for Biological Products (*48*) should be applied.

A.5 Retained samples

The recommendations in section 9.5 of Good Manufacturing Practices for Biological Products (*48*) should be applied.

A.6 Labelling

The recommendations in section 7 of Good Manufacturing Practices for Biological Products (*48*) should be applied with the addition of the following.

The label on the carton or the leaflet accompanying the container should indicate:

- the amounts of *H. influenzae* type b polysaccharide (PRP) and protein contained in each single human dose
- the temperature recommended during storage and transport
- if the vaccine is freeze-dried, that after its reconstitution it should be used immediately unless data have been provided to the licensing authority indicating that it may be stored for a limited time
- the volume and nature of the diluent to be added in order to reconstitute a freeze-dried vaccine, specifying that the diluent should be supplied by the manufacturer or approved by the national control authority
- the nature and amount of stabilizer contained in each single human dose (if appropriate)
- the nature and amount of preservative contained in each single human dose (if appropriate).

A.7 Distribution and transport

The recommendations in section 8 of Good Manufacturing Practices for Biological Products (*48*) should be applied.

A.8 Stability testing, storage and expiry date

A.8.1 *Stability testing*

Adequate stability studies form an essential part of vaccine development studies. The stability of the vaccine in its final form and at the recommended storage temperatures should be demonstrated to the satisfaction of the national control authority with final containers from at least three lots of final product from different independent bulk conjugates.

The polysaccharide component of conjugate vaccines is subject to gradual depolymerization at a rate that may vary with the type of conjugate, type of formulation or adjuvant, type of excipients and conditions of storage. The depolymerization can result in reduced molecular size of the PRP component, a reduction in the amount of the PRP bound to the carrier protein (i.e. an increase in free PRP) and a reduced molecular size of the conjugate (*63*). In general, PRP–protein conjugate vaccines are susceptible to gradual depolymerization and the expiry dating needs to be established accordingly.

Tests should be conducted before licensing to determine the extent to which the stability of the product has been maintained throughout the proposed validity period. The free (unconjugated) or conjugated PRP content should be determined as a percentage of the total PRP. The vaccine should meet the recommendations for final product (see Part A, sections 3.6.1 and 3.6.10) up to the expiry date.

> Molecular sizing of the final product may be carried out to ensure the integrity of the conjugate.

The desorption of antigen from aluminium-based adjuvants, if used, may take place over time and should be investigated and shown to be within the limits agreed by the national control authority.

Accelerated stability studies may provide additional supporting evidence of the stability of the product but cannot replace real-time studies.

When any changes are made in the production procedure that may affect the stability of the product, the vaccine produced by the new method should be shown to be stable.

The statements concerning storage temperature and expiry date appearing on the label should be based on experimental evidence, which should be submitted for approval to the national control authority.

A.8.2 *Storage conditions*

Storage conditions should be based on stability studies and approved by the national control authority.

> Storage of liquid vaccine at a temperature of 2–8 °C has been found to be satisfactory.
>
> Freeze-dried vaccine should be stored at 2–8 °C.

A.8.3 *Expiry date*

The expiry date should be approved by the national control authority and take into consideration the data on the stability of the bulk purified

conjugate as well as the results of the stability tests referred to in section A.8.1.

Part B. Recommendations for national control authorities

B.1 General

The general recommendations for control laboratories contained in the Guidelines for National Authorities on Quality Assurance for Biological Products (*64*) should be applied.

B.2 Official release and certification

A vaccine lot should be released only if it fulfils national requirements and/or Part A of these Recommendations.

A statement signed by the appropriate official of the national control authority should be provided at the request of the manufacturing establishments and should certify that the lot of vaccine in question satisfies all national requirements as well as Part A of these Recommendations. The certificate should state the number under which the lot was released by the national controller, and the number appearing on the labels of the containers. Importers of *H. influenzae* type b conjugate vaccines should be given a copy of the official national release document.

> The purpose of the certificates is to facilitate the exchange of vaccines between countries. An example of a suitable certificate is given in Appendix 2.

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[1] Holliday MR, Jones C. Meeting report: WHO cosponsored workshop on the use of physicochemical methods for the characterization of *Haemophilus influenzae* type b conjugate vaccines. *Biologicals*, 1999, **27**: 51–53.

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Appendix 1

Evaluation of immunogenicity of *Haemophilus influenzae* type b conjugate vaccines in humans

Different lots of *Haemophilus influenzae* type b conjugate vaccines from each manufacturer should be evaluated for immunogenicity in the target age group before licensing.

Response to polysaccharide

The results of the radioimmunoassay (RIA) of antibodies to the polysaccharide (PRP) (*1,2*) have been shown to be the best available correlate of the clinical effectiveness of *H. influenzae* type b vaccines. One method of assessing the immunogenicity is to assay the serum antibody response to the PRP using a suitable RIA or demonstrated equivalent enzyme-linked immunosorbent assay (ELISA) in which an *H. influenzae* type b reference human serum is included (*3, 4*). This applies to serum samples taken usually just before each injection of a vaccine dose and 1 month after the final dose of the primary series or after a booster dose. The percentage of vaccinees with a serum antibody concentration equal to, or greater than, 0.15 μg/ml and 1.0 μg/ml should be reported, as well as the geometric mean antibody concentration and the distribution of values. It is important to examine the duration of the protective antibody response up to 4 years of age. It is also important to show that the conjugate vaccine stimulates a statistically significant increase in anti-PRP IgG response. However, the concentration of serum antibodies sufficient to confer protection following the use of *H. influenzae* type b conjugate vaccines, or for eliminating carriage, is unclear (*5–8*). Immunological memory is thought to be a significant part of the protective response to the *H. influenzae* type b conjugate vaccine (*9*) and the demonstration of a booster response to native polysaccharide is considered an important part of the evidence of a protective response. The avidity of the induced antibodies is also another area of interest (*6–8*).

The functional activity of the conjugate-induced antibodies should be assessed by measuring the serum bactericidal activity against *H. influenzae* type b (*1, 5*). ELISAs have been used to measure total anti-PRP content, as well as isotype and subclass composition.

Response to carrier protein

Serum antibodies to the carrier protein should be measured in recipients of *H. influenzae* type b conjugate vaccine to ensure that the conjugate vaccine does not interfere with protective immunity that is relevant to

that protein. In addition, the measurement provides information about the potential of the *H. influenzae* type b conjugate to serve as a dual immunogen for both the *H. influenzae* type b polysaccharide and the carrier protein. To date, proteins such as diphtheria and tetanus toxoids and an outer membrane protein complex of *Neisseria meningitidis* group B have been used in the preparation of *H. influenzae* type b conjugates. Since some of these carriers are also components of other infant vaccines (e.g. DTP), antibody responses to those vaccines should be measured to ensure that there is no immune interference of clinical relevance. The assay for these antibodies should be a bioassay or an established equivalent.

The following reagents are available through the courtesy of manufacturers and national control authorities:

- *H. influenzae* type b reference serum USA/FDA lot 1983 with 70 μg/ml of total anti-Hib polysaccharide antibody, available from the Center for Biologics Evaluation and Research, Food and Drug Administration, HFM-428, 1401 Rockville Pike, Rockville, MD 10852-1442, USA.
- Short-chained *H. influenzae* type b polysaccharide–serum albumin conjugate (Hb0-HA antigen) prepared by Wyeth-Lederle Vaccines and available from the National Institute for Biological Standards and Control, Potters Bar, Herts. EN6 3QG, England.

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Appendix 2

Model certificate for the release of *Haemophilus influenzae* type b conjugate vaccines[1]

The following lots of *H. influenzae* type b conjugate vaccine produced by _____,[2] whose numbers appear on the labels of the final containers, meet all national requirements,[3] and Part A of the Recommendations for the Production and Control of *H. influenzae* type b Conjugate Vaccines,[4] and comply with Good Manufacturing Practices for Pharmaceutical[5] and Biological Products.[6]

Lot no.	Expiry date	Lot no.	Expiry date
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____

As a minimum, this certificate is based on an examination of the manufacturing protocol.

The number of this certificate is _____

The Director of the National Control Laboratory (or Authority as appropriate):[7]

Name (typed) _____

Signature _____

Date _____

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[1] To be completed by the national control authority of the country where the vaccine has been manufactured, and to be provided by the vaccine manufacturer to importers.

[2] Name of manufacturer.

[3] If any national requirement(s) is (are) not met, specify which one(s) and indicate why release of the lot(s) has nevertheless been authorized by the national control authority.

[4] With the exception of the provisions on shipping, which the national control authority may not be in a position to control.

[5] WHO Technical Report Series, No. 823, 1992, Annex 1.

[6] WHO Technical Report Series, No. 822, 1992, Annex 1.

[7] Or his or her representative.