Annex 5

Generic protocol for the calibration of seasonal and pandemic influenza antigen working reagents by WHO essential regulatory laboratories

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Abbreviations

CBER Center for Biologics Evaluation and Research
ERL Essential regulatory laboratory
GISRS WHO Global Influenza Surveillance and Response System
NIBSC National Institute for Biological Standards and Control
NIID National Institute for Infectious Disease
PLS Primary liquid standard
SDS–PAGE Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SRD Single radial diffusion assay
SRID Single radial immunodiffusion assay
TGA Therapeutic Goods Administration
WHO World Health Organization

1. Introduction

Vaccination is the principal measure for preventing influenza and reducing its impact.¹ Since 1973, WHO has provided formal recommendations for the composition of influenza vaccines on the basis of information provided by the WHO Global Influenza Surveillance and Response System (GISRS).

WHO technical consultations are convened each year in February and September to recommend the viruses for inclusion in influenza vaccines for the northern and southern hemispheres, respectively.² For countries in equatorial regions, epidemiological considerations influence which recommendation (northern or southern) individual national and regional authorities consider more appropriate.

High-yield candidate vaccine viruses are developed by collaboration between laboratories involved in developing reassortants and WHO collaborating centres following the strain recommendations. Once developed, these candidate reassortants are sent to WHO collaborating centres for characterization of their antigenic and genetic properties before being released to interested institutions on request. Reference reagents are subsequently developed and standardized by ERLs in collaboration with vaccine manufacturers, and are made available to manufacturers worldwide upon request.

This document provides a description of the generic protocol for the calibration of influenza antigen working reagents used by the four WHO ERLs. It represents the consensus of the ERLs on the process of assigning a potency value to a newly established influenza antigen reagent for use in potency testing of inactivated influenza vaccines. An influenza antigen working (or reference) reagent is a preparation of inactivated whole virus that has been freeze-dried and calibrated as outlined in this document.

The calibration process involves the preparation of a primary liquid standard (PLS) and a large batch of freeze-dried antigen by one of the ERLs. The PLS is distributed to all other ERLs for independent calibration by physicochemical means. Samples of the freeze-dried antigen are distributed to the ERLs at the same time and are calibrated against the PLS using the single radial immunodiffusion assay (SRID; also SRD).

2. ERLs
The ERLs are as follows:

- Australia – Therapeutic Goods Administration (TGA)
- Japan – National Institute for Infectious Disease (NIID)
- United Kingdom – National Institute for Biological Standards and Control (NIBSC)
- USA – Center for Biologics Evaluation and Research (CBER).

The participation of all ERLs is assumed, as is current practice, with a minimum of three contributing data for each calibration. The laboratories agree a timeline for completion of all calibration tests, with an expectation that most calibrations will be completed within 15 working days.

The lead ERL is the ERL that produces the freeze-dried antigen reference reagent and sends out materials to the other ERLs for use in calibration (as specified in section 4 below). The lead ERL will inform WHO in a timely manner about the availability of a new reagent and progress of the calibration.

3. Reagents supplied to collaborating ERLs
For each antigen reagent to be calibrated, the following is to be supplied:

- at least 30 ampoules of freeze-dried antigen;
- 10 vials of antiserum (if more than one lot is shipped, 10 vials of each lot);
- a batch, preferably with two aliquots, of a whole virus preparation (i.e. the “PLS”) consisting for example of an in-house live or inactivated preparation or liquid pre-freeze-dried antigen.
The PLS will be characterized by all ERLs with respect to protein content and the proportion of haemagglutinin by physicochemical means to independently determine the haemagglutinin content in micrograms. The PLS serves as the standard against which the secondary freeze-dried antigen reagent is calibrated. The PLS is supplied with a protein concentration estimated by the lead ERL.

4. Internal compliance testing and documentation

The lead ERL performs the tests on the supplied materials prior to distribution, provides test data upon request and supplies interim documentation (e.g. instructions for use). The tests performed by the lead ERL are as follows.

- A protein estimation on the PLS, preferably confirmed by using more than one method with a confirmatory value of ±20% of the estimated value.
- An estimated range of working dilutions of the specific antiserum.
- When possible, SRID assays of the PLS and the freeze-dried antigen using the specific antiserum to confirm their antigenic identity, the estimated potency value of the freeze-dried antigen and a qualitative assessment of SRID zones. If specific antiserum becomes available only after distribution of the PLS and freeze-dried antigen, SRID with a cross-reactive antiserum is performed if possible.

5. Antigen reagent supply to vaccine manufacturers

Upon request, vaccine manufacturers are to be provided with freeze-dried antigen reference reagents as soon as they are available and prior to final calibration. These may be supplied with interim estimated values for use in SRID potency assays. Manufacturers’ data (e.g. comparison of SRID values with manufacturers’ in-house methods for preliminary yield analysis of monovalent bulks) can be supplied to ERLs for review.

6. ERL calibration methodology

6.1 Procedure

- Calibration of the PLS: the haemagglutinin content of the PLS is determined by physicochemical methods. Total protein is determined by nitrogen analysis and/or Lowry assay. The percentage haemagglutinin is determined by sodium dodecyl
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sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using band densitometry, and the haemagglutinin content of the PLS is expressed in micrograms.

- **Preparation of strain-specific antiserum reagents:** the virus is digested with bromelain to remove haemagglutinin from virus particles. The haemagglutinin missing the transmembrane portion is purified and sheep are immunized multiple times to generate hyperimmune antiserum which is tested for suitability in SRID assays in terms of potency and the quality of the SRID zones produced. The serum is not calibrated but a suitable concentration range for its use is suggested. The reference antiserum is aliquoted and distributed with reference antigen.

- **Preparation of reference antigen:** a reference antigen is prepared on an industrial scale, and is aliquoted and lyophilized. The haemagglutinin content of the reference antigen is assigned by SRID against the PLS using the specific antiserum. The ERLs assign potencies to the PLS and reference antigen independently and the lead ERL collates the results and proposes a final consensus value for agreement by the ERLs. Reagents are distributed as soon as they become available. This process takes several weeks. *Note:* specific antigen standards are required for each candidate vaccine virus strain. Specific antisera are required for each recommended vaccine virus – i.e. antisera are prepared for groups of antigenically similar (“like”) viruses, and are cross-reactive between candidate vaccine viruses covered by a particular recommended virus.

6.2 **Technical details**

- **PLS protein estimation:** this should be performed by a recognized assay (e.g. Lowry or total nitrogen determination), according to the local ERL standard operating procedure or methodology. Assays should include an appropriate protein control (ideally a large batch of common protein standard shared between the ERLs).

- **PLS PAGE assay:** the PLS should be treated as appropriate prior to the PAGE analysis (e.g. reduction or deglycosylation). Assays should be performed according to the local ERL standard operating procedure or methodology, with a minimum of two independent assays, preferably performed by different analysts. Protein bands should be visualized using Coomassie blue-based staining.
■ **PAGE band analysis:** analysis of PAGE gels should be performed according to the local ERL standard operating procedure or methodology, recording any parameters that vary from their usual procedures. General guidance for confirmation of accuracy of PAGE band analysis:

- the ratio haemagglutinin 1: haemagglutinin 2 is approximately 3:2
- the haemagglutinin content should be between 20% and 50% of total protein
- the analysis is to be repeated if there is > 20% variation between replicates.

■ **SRID assay:** the assay is based on diffusion of virus antigen (e.g. detergent-disrupted virus or vaccine) through an agarose gel containing haemagglutinin-specific antiserum. The square of the diameter of the precipitin ring is proportional to the antigen concentration and a standard curve is used to quantify haemagglutinin in vaccine samples. Preferably, three to six assays should be performed involving more than one operator. Assays should be performed using the local ERL standard operating procedure or methodology. Freeze-dried antigen should be analysed using the PLS as standard antigen on plates containing the appropriate antiserum. The final potency value of the freeze-dried antigen is derived using the mean potency values of all assays.

■ **Complete the ERL data sheet:** a sample data sheet is attached to this protocol (Appendix 1). The data sheet is to be sent to the lead ERL. Supplementary data may also be included (Appendix 2).

7. **Assignment of calibrated potency value by the lead ERL**

Data generated by the ERLs (see Appendix 1 and Appendix 2) are collected by the lead ERL and compiled for the final potency value agreement and confirmation. Manufacturers’ data may also be considered, if available. Before the assigned value is made public (e.g. through the ERL web site, WHO web site or Instructions for Use), the final data sheet and proposed calibration value are sent to all participating ERLs for comment and/or approval. The lead ERL has final authority in assigning a potency value.

8. **Calibration of secondary and replacement reagents**

The antigen reagent that is developed first for a given candidate vaccine virus is calibrated according to the process outlined in sections 2–7 above. When
another antigen reagent for the same candidate vaccine virus is required, either as a replacement to replenish stocks or as an alternative reagent provided by another ERL, it is calibrated against the first antigen reagent to ensure equivalence of reagents and to ease the switch from one lot of reagent to a new one. The process for calibration (cross-calibration) of these types of secondary reagents is abbreviated: calibration uses the antigen reagent that was the first to be developed as the relevant calibrant, and not the PLS. In all cases, calibration is performed using the SRID assay. The exact process for cross-calibration varies between ERLs, and the involvement of more than one laboratory is encouraged – either within the same institution or by using external laboratories (e.g. other ERLs, or national control laboratories with proven experience in influenza vaccine potency testing). If this is not feasible, more than one operator within the calibrating ERL laboratory will need to be engaged in the calibration process.

9. Review of this document

The ERLs will review this document periodically – at least once a year – to ensure that it reflects best practice within ERLs and any updated methodology that may be implemented in the future. Review may take place through electronic means or during meetings between ERL representatives. Any updates of this document will be posted on the GISRS web site.3

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# Appendix 1

## Sample data sheet

**Date:**

**Reference:**

Primary liquid standard ([*ERL*]): [virus name] ([reassortant]) (Lot no. [yyy])

<table>
<thead>
<tr>
<th>Protein concentration (µg/ml)</th>
<th>No. of deaths/No. of animals inoculated</th>
<th>Haemagglutinin (HA) content (µgHA/ml)</th>
</tr>
</thead>
</table>

### Antiserum:

Anti-[virus name] sheep antiserum (Lot no. [zzz], [*ERL*]) x µl/ml agarose

### Sample:

Lot no. [yyy], [*ERL*]

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>Haemagglutinin (HA) content (µgHA/vial)</th>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Mean</th>
<th></th>
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</thead>
</table>

| Standard deviation of the mean (SD) | |
|-------------------------------------| |

| Coefficient of variation (CV %) | |
|---------------------------------| |
## Appendix 2

### Supplementary data sheet

Calibration of [virus name] reference antigen lot [yyy] with antiserum lot [zzz]

Collaborative study summary results

<table>
<thead>
<tr>
<th></th>
<th>TGA</th>
<th>NIID</th>
<th>CBER</th>
<th>NIBSC</th>
<th>Mean</th>
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<tbody>
<tr>
<td>Lowry total protein</td>
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<td>Haemagglutinin (µg/ml)</td>
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CBER: Center for Biologics Evaluation and Research; CV: coefficient of variation; NIBSC: National Institute for Biological Standards and Control; NIID: National Institute for Infectious Disease; SD: standard deviation; TGA: National Institute for Infectious Disease.