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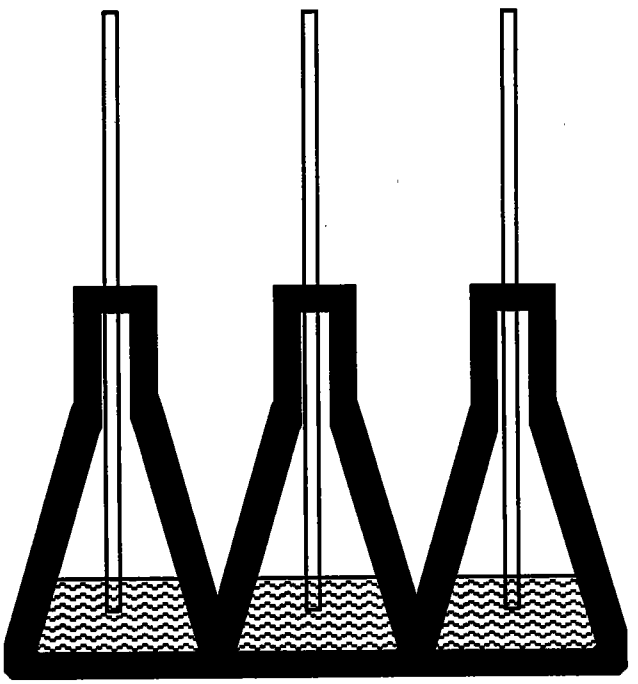
Manual of laboratory methods for potency testing of vaccines used in the WHO Expanded Programme on Immunization



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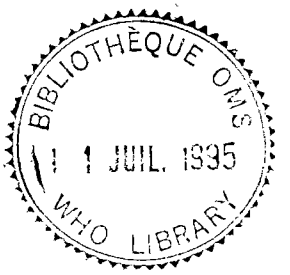

MANUAL OF LABORATORY METHODS FOR TESTING THE POTENCY OF FINAL VACCINES USED IN THE WHO EXPANDED PROGRAMME ON IMMUNIZATION

WORLD HEALTH ORGANIZATION

MANUAL OF LABORATORY METHODS FOR POTENCY TESTING OF VACCINES USED IN THE WHO EXPANDED PROGRAMME ON IMMUNIZATION

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[1] A more detailed table of contents is provided at the beginning of each of the four parts of the manual.

PART 4. CALCULATIONS AND STATISTICAL ANALYSIS OF RESULTS

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PREFACE

In 1982, with the aim of providing guidance for the standardization of tests needed to ensure the safety and efficacy of vaccines, the World Health Organization (WHO) issued a document entitled *Manual of details of tests required on final vaccines used in the WHO Expanded Programme on Immunization* (unpublished document BLG/UNDP/82.1 Rev.1). This document was updated in 1985 (unpublished document BLG/UNDP/82.1 Rev. 1 Corr. 1).

As a result of changes in some of the potency tests and the development of alternative techniques for testing the potency of bacterial vaccines, and in an attempt to present the information in a more useful way for testing laboratories, details of the potency tests for live viral vaccines and bacterial vaccines were revised and reissued provisionally in two documents, *Laboratory methods for the titration of live virus vaccines using cell culture techniques* (unpublished document BLG/EPI/89.1) and *Laboratory methods for the testing for potency of diphtheria (D), tetanus (T), pertussis (P) and combined vaccines* (unpublished document BLG/92.1).

These two documents were field tested in workshops worldwide. They have been revised in the light of comments received and combined to produce the present manual. The manual consists of four parts. Part I provides a general introduction to various aspects of vaccine potency testing; parts II and III give details of methods for testing the potency of live viral vaccines and bacterial vaccines respectively; and part IV provides information on the statistical analysis of test results. Additional details are provided in three annexes.

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Thanks are also due to the following for their contributions in the areas indicated:

R Dobbelaer, P Knight, J Lyng
- use and validation of a single vaccine dilution potency assay for diphtheria, tetanus and combined vaccines

CFM Hendriksen, JW van der Gun, FR Marsman, HJM van deDonk, JG Kreeftenberg
- toxin binding inhibition test as a secondary test for the potency estimation of vaccines containing tetanus and diphtheria toxoid

P Knight
- traditional tests for the potency of vaccines containing adsorbed diphtheria and tetanus toxoids
- pertussis potency assay of monovalent and combined whole cell pertussis vaccines
- serological tests for potency estimation of vaccines containing tetanus and diphtheria toxoids
- ELISA methods applicable to the determination of diphtheria and tetanus antibodies
- methods of measuring antitoxin in the sera of vaccinated animals

JG Kreeftenberg, JW van der Gun, CFM Hendriksen, J Lyng
- Vero cell test for potency determination of diphtheria toxoid

G Nyerges
- passive haemagglutination titration as a secondary test method for assessing the antibody response of mice in potency assays of adsorbed diphtheria, tetanus and combined vaccines

M Weiz Bentzon, F Marsman
- statistical analysis

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PART I. GENERAL

CONTENTS

1. GENERAL INTRODUCTION

Vaccines are an essential tool in the prevention of disease. They protect the vaccinated individual from developing a potentially serious disease and help protect the community by reducing the spread of infectious agents.

The success of immunization initiatives is evident from the impact they have had on the occurrence of diseases in various parts of the world. Smallpox has been eradicated worldwide, poliomyelitis is on the verge of eradication, and there have been dramatic reductions in the incidence of measles and neonatal tetanus in many areas. However, global success can be achieved only if safe and effective vaccines are readily available as and when required. It is estimated that world usage of vaccines in 1993 alone was over four billion doses, emphasizing the need for sufficient vaccines of assured quality.

The prime responsibility for the safety and efficacy of a biological product rests with the manufacturer. However, it is a country's national control authority that is responsible for establishing procedures for assuring that biological products intended for use in that country are of adequate safety and efficacy. The activities required to meet these responsibilities are incorporated in the concept of quality assurance (section 1.1).

For its part, the manufacturer must monitor and assure the quality of both its production and testing processes and the final product. Production must be undertaken in accordance with the principles of good manufacturing practices (section 1.2) and must also be subject to quality control testing (section 1.3), which should be undertaken in accordance with good laboratory practices (section 1.4).

The national control authority grants approval for the marketing of a biological product and undertakes post-marketing surveillance. The authority also has the responsibility to certify that the manufacturer's quality assurance system is working. It should therefore be capable of evaluating all aspects of the manufacturer's production and testing procedures and processes, inspecting manufacturing facilities, and performing quality control testing of all products intended for marketing by independently evaluating their safety and efficacy, where possible through a national control laboratory.

All these operations, overseen by governments, are part of the regulatory system which will permit the official certification of the quality of vaccines as they leave the production laboratory.

The guarantee that a vaccine is potent at all times is not part of the regulatory mechanism but depends on both a competent cold chain and a competent test laboratory. Countries that participate in immunization programmes should have access to laboratories that can verify, at a minimum, the potency and sterility of the vaccines used.

This activity, which is independent of production, is part of a monitoring programme, the objective of which is to guarantee the efficacy at any time, especially when the vaccine is administered to the target population. While it is true that given an effective

cold chain, a potent vaccine stored at the temperature recommended by the manufacturer may be used with confidence until the expiry date, there are cases when vaccine potency should be checked, including the following examples:

- when it is necessary to verify the conformity to the required standards of the vaccines received from distributors;
- when the vaccines are delayed in transit from the distributors and are received thawed or at ambient temperature, and it is necessary to verify the potency before approving them for use in immunization programmes;
- in case of a break in the cold chain;
- to document a request to a distributor to substitute the material sent;
- to monitor the efficiency of vaccine storage in the field;
- as part of a study evaluating immunological response, for example, a seroconversion study.

1.1 Quality assurance

Quality assurance is the term used to describe the combination of organized activities performed to demonstrate, prove and ensure that the output (product or data) of a process meets the quality criteria and specifications for its intended application.

For the vaccine manufacturer, quality assurance is the sum of all the procedures used to assure that the vaccine produced will be both safe and effective. The manufacturer is therefore responsible for ensuring that production is undertaken in accordance with the principles of good manufacturing practices and that the facilities, personnel, procedures and product comply strictly with approved quality control specifications. Total control of quality is the organized effort within an entire establishment to design, produce, maintain and assure the specific quality of each lot of vaccine distributed. It is a facility-wide activity and represents the cumulative responsibility of all components of the manufacturing company.

For the national control authority, quality assurance is the sum of all the evaluation activities that ensure the quality of vaccines. The evaluation includes control testing, approval of manufacturing processes and facilities, and monitoring of distribution systems and post-marketing performance. The authority must be certain that a vaccine manufacturer is capable of producing a safe and effective product.

1.2 Good manufacturing practices

The World Health Organization (WHO) has published guidelines for the development of

requirements for the manufacture of pharmaceutical and biological products in accordance with the basic principles of good manufacturing practices (GMP) (*2, 3*). GMP ensure the consistency of manufacture of good quality biological products that conform to established specifications. Owing to the complexity of manufacturing processes, the quality of final products may be affected by a number of elements, including personnel, premises, equipment, materials and processes.

- *Personnel.* Well trained and experienced personnel are fundamental to the successful implementation of GMP and quality assurance programmes.

- *Premises.* Buildings must be located, designed, constructed or adapted, and maintained to suit the operations carried out within them. Premises should allow optimization of the flow of raw materials, personnel and manufactured product to avoid contamination and mix-up. The working environment should be safe, comfortable and contaminant-free.

- *Equipment.* Equipment must be of high precision, with validated performance, and must be regularly maintained and cleaned.

- *Materials.* Raw materials, in-process materials and other components, such as containers, labels, and packing, must comply with specifications.

- *Process.* The manufacturing process should be considered as a sequence of well defined, inter-related activities which, if conducted under proper controls, will produce a product of desired specifications. Validation, which establishes that each step of the process achieves its intended function, must be undertaken to ensure consistency of production. It must be shown that when the materials and equipment specified are used the method will consistently yield a product of the required quality. Any significant change in production procedure must be validated to demonstrate that under these changed conditions product quality is retained. As the final quality of vaccines is also affected by post-production activities, such as storage, distribution and transportation, these must also be given careful consideration.

Documentation is an essential and often neglected part of quality assurance. Standard operating procedures and record-keeping systems for production and testing should be developed. A standard operating procedure is a certified document in which a complete minute description is given of all the materials and methods used in a certain assay or process. The materials and methods described must all have been validated and found suitable for the intended purpose. Standard operating procedures should be simple, no-nonsense, how-to-do-it documents, which lead the worker step-by-step through the operations required to attain a desired goal. These reduce the risk of error unavoidable in spoken communication. Good records permit easy access to and retrieval of information on history of production lots, their manufacture, testing and distribution. Descriptions of specifications, manufacturing formulae and procedures — almost every activity, however minor it may seem — must be available in writing. All documentation recording the manufacture of a lot must be available for scrutiny by the national control authority.

Compliance with GMP is not a static situation, but requires the manufacturer to be aware not only of what is current in the industry but also of useful innovations. Measures should be taken to update knowledge on a continuing basis. A practice is considered "good" if it contributes to ensuring greater safety, quality or purity, the benefits justify the costs, and it is feasible to implement. On-going training of personnel is essential for implementation of new technologies. Manufacturers must also establish self-inspection systems which validate procedures and implement corrective actions on a continuing basis.

1.3 Quality control

Quality control is concerned with sampling and testing, along with organization, documentation and release procedures. More specifically it assures that the necessary tests have been carried out and that the quality of a specific material has been demonstrated to be satisfactory before it is used in production or released for sale.

The results produced by a quality control laboratory will depend on several factors, including, how the data are generated, how they are handled and how auditing is undertaken to assure quality in the generation and handling of data. Quality control laboratories must therefore have appropriate facilities with adequately trained staff and must be operated in accordance with the principles of good laboratory practices. Quality control operations must also ensure that the data generated are of known accuracy to some stated, quantitative degree of probability.

1.4 Good laboratory practices

The concept of good laboratory practices (GLP) was developed as a detailed set of regulations on laboratory management to be adhered to by any laboratory providing data from non-clinical, routine toxicological studies for new drug applications or other regulatory purposes. GLP thus meant the establishment of standard procedures and conditions for the proper organization of laboratories in which studies to assess the potential health risks or safety of substances are planned, conducted, supervised, recorded and reported. They also include the organization of archives. These standards have now been generalized for any testing laboratory. A laboratory where GLP are followed possesses the capability of producing accurate test data, and can be relied upon in its day-to-day operations because it maintains high standards of performance.

The main elements of laboratory work are as follows:

- *Personnel.* The work should be undertaken by experienced laboratory personnel thoroughly trained for the task. If tests are performed in animals, personnel appropriately trained in animal care are also required.

- *Facilities.* Adequate laboratory space and services (running water, electricity, and gas) should be available. The working environment must be comfortable, safe and contaminant-free. When tests are performed in laboratory animals, a well equipped
animal house which complies with animal health and safety standards is absolutely essential.

- *Instruments and equipment* must be handled and maintained so that the accuracy required to ensure good quality results is achieved. Performance criteria for each instrument or piece of equipment must be established. Records of use and of regular maintenance must be readily available and up-to-date.

- *Test samples* must be received, recorded, handled, and stored according to standardized procedures to ensure that the quality of information gathered from them is accurate and truly representative.

- *Reagents, solutions and reference materials* must all be of high quality and used for a specific analytical method. Water is considered to be a reagent and must meet the same requirements as other reagents. Labelling and documentation of reagents is part of the monitoring and maintenance of quality assurance programmes. Reference materials are themselves validated, but storage, handling and processing conditions must be controlled.

- *Methods* must be appropriate for their intended use and compatible with the nature of the samples to be tested. They should be available in writing in a methods manual and written in the form of a standard operating procedure in a clear and unambiguous manner, such that an experienced analyst, unfamiliar with the method, is able to use the procedure and interpret the results. Standard operating procedures should follow a pre-established format which includes the following:

  - title
  - date of authorization
  - reference(s)
  - scope
  - basic principles
  - apparatus and reagents
  - procedural details
  - safety precautions
  - calculations and statistics
  - quality assurance.

1.5 Selection and implementation of methods

In choosing the analytical methods they are going to use, quality control laboratories have four major options.

- *Standard methods* are methods that have been subjected to intensive investigations by many individuals and laboratories and have come to be regarded as the best methods available, although in some cases they may be quite old. Such methods have been thoroughly tested and validated.

- *Official methods* are the methods that quality control laboratories are required to use by government or international organizations (WHO, national control authorities, pharmacopoeias). These methods have also been properly validated before being designated as official.

- *Methods from the literature*. Descriptions in specialized journals provide a good source of new techniques. However, they need to be treated with caution, and require thorough validation.

- *Methods developed in-house*. These are methods developed or modified by laboratories as a result of research to improve testing and to meet individual needs and concerns. They require stringent validation.

While standard and official methods have been thoroughly validated, a quality control laboratory wishing to use them for the first time must undertake validation tests to ensure performance is satisfactory. Any new method or major modification of an existing method considered for use in routine testing should be subjected to a rigorous selection process which includes validation. The following points should also be considered:

- Does the test meet a defined need?

- Does the test satisfy characteristics such as sensitivity, reproducibility, accuracy, precision?

- Is the test technically demanding?

- Does the laboratory have the required expertise to adapt easily and use the new procedure and, if not, is training available?

- Does the test require special equipment?

- Is the procedure costly?

- How long does the test take to perform?

1.6 Validation of methods

There are two aspects to validation. First, the suitability of the method must be established. It must give reproducible and reliable results that are appropriate for the

analysis intended. Consideration should therefore be given to the following attributes or characteristics—the significance of each will vary with the purpose of the analysis:

- accuracy (degree of correlation to the true value);
- precision (the amount of variation in the results, given usually by standard deviation or coefficient of variation);
- sensitivity (the amount of response per unit of the substance being measured);
- reproducibility (the precision of the procedure when carried out under different conditions);
- specificity (the degree of uniqueness of response to the substance being sought);
- robustness (the ability to provide results of acceptable accuracy and precision under a variety of conditions).

Secondly, a comparative study must be performed against the method usually used, in order to assess correlation. If a laboratory has not previously undertaken a test with which the new test can be compared, it should first perform a standard method. A series of samples covering the range of materials and test substances normally expected are analysed by both methods. The results obtained are compared statistically, assuming that the results of the old method represent the true values of the samples. Correlation studies are performed and a linear relationship is expected, with a slope of 1 and an intercept at (0, 0). The new method may display a proportional bias (slope different from 1) and/or a constant bias (intercept different from 0, in which case the method is invalid).

The most acceptable validation procedure for a new method is a multicentre collaborative study followed, in descending order of acceptability, by validation in two or more laboratories other than the one that developed the method, validation in a single laboratory other than the one that developed the method and, finally, validation by the laboratory that developed the method. Ideally, every method should be validated in a collaborative study. Unfortunately, this is not always possible; however, the highest level of validation should be aimed for. While validation needs to be done only once, it is restricted to the method as written. Any modification or any application not covered by the original validation, produces a new method and, as such, must be validated.

When considering alternatives for potency tests for vaccines, it is advisable to include preparations of known low potency in the validation of the tests. In this way, it is possible to establish at an early stage the ability of the method to differentiate between vaccines of acceptable potency and those of potency below acceptable limits.

1.7 Problems in the implementation of new technologies

In a vaccine quality control laboratory the validation of new technologies is of fundamental importance. However it requires considerable human, technical and financial resources, and takes time.

Lack of resources is an acute problem for quality control in some countries, particularly in relation to bioassays which require the use of a considerable number of animals in addition to specialized equipment and reagents.

Most of the difficulties encountered in raising and breeding animals are directly related to the provision of appropriate facilities in which environmental conditions, including temperature and humidity can be controlled. In warm and tropical countries, this is a major enterprise in itself; large sums of money are required for the construction of facilities and their subsequent maintenance. Other problems include difficulties in maintaining the genetic standardization of laboratory animals, limiting the incidence of intercurrent infection and in guaranteeing supplies to ensure uniform feeding regimes. All of these have a direct impact on testing and on the quality of the data generated. Countries will thus benefit significantly from simpler tests requiring fewer animals, or from *in vitro* techniques. However, the latter have their own problems, such as maintaining cell cultures free from contamination.

As mentioned earlier, newly selected techniques must undergo an evaluation process in which availability of resources, practicality of the test, ease of performance and other characteristics must be carefully considered. Several national control authorities are unable to perform animal testing on a regular basis as recommended in WHO requirements for different vaccines; others have already adopted techniques that require the use of fewer animals. The final steps of technique validation, comparison of the new test with the regular tests, can pose particular problems, especially when the laboratory has been unable to perform the regular test owing to unavailability of animals. The question for some countries, therefore, is whether it is better to undertake no test, because appropriate validation cannot be performed, or to implement a test validated elsewhere. Common sense will favour the use of some kind of testing.

Interlaboratory and intercountry collaboration and the creation of networks of quality control laboratories would address this problem. Laboratories could exchange information and organize collaborative validations, making better use of resources and expertise. This would involve the establishment of a few centres capable of performing animal testing, where vaccines can be evaluated in the "traditional" way, while other laboratories in the network would carry out the new *in vitro* tests.

As an example, a possible scheme for the evaluation and validation of an *in vitro* assay, the toxin-binding inhibition test, in a multicentre collaborative study is outlined below.

- The first step would be the introduction of the immunoassay to the different participating laboratories. A written methodology should be elaborated beforehand. The basis of the test is an immunoassay for measurement of anti-

tetanus or anti-diphtheria antibodies. For this step, the same sera (coded, but of known potency), some of which are raised against the appropriate reference preparation and others against the vaccines to be tested, should be evaluated by the different laboratories. The same reagents, standards and reference reagents should be used in all centres. This will permit the validation of the immunoassay and its performance in the different laboratories.

- The second step, to be carried out by all the laboratories, would be to perform the full test to estimate potency of a selected vaccine, which would include immunization of mice with the test and reference vaccines (mice being the animal species easiest and least expensive to handle, breed, and maintain), and evaluation *in vitro* of the sera obtained.

- The third step, probably the most ambitious, would be the evaluation of several vaccines by the different centres. One centre should perform the *in vivo* test, according to WHO requirements, on all vaccines for comparison of the results.

Once the technique had been validated, the *in vitro* assay could be implemented in the different laboratories. At regular intervals, selected vaccines could be evaluated by both procedures with the assistance of the centre responsible for animal testing.

Similar procedures could be envisaged for the validation and implementation of other alternative technologies in countries with difficulties in undertaking regular animal testing. The network could also benefit from the better use of available expertise in the development and standardization of reference reagents and standards.

1.8 References

1. *WHO Expert Committee on Biological Standardization. Twenty-fifth report,* Geneva, World Health Organization, 1973 (WHO Technical Report Series, No. 530), Annex 6. General requirements for the sterilization of biological substances.

2. *WHO Expert Committee on Biological Standardization. Forty-second report,* Geneva, World Health Organization, 1992 (WHO Technical Report Series, No. 822), Annex 1. Good manufacturing practices for biological products.

3. *WHO Expert Committee on Specifications for Pharmaceutical Preparations.* Geneva, World Health Organization, 1992 (WHO Technical Report Series, No. 823), Annex 1. Good manufacturing practices for pharmaceutical products.

2. STANDARDIZATION IN VACCINE POTENCY TESTING

2.1 Introduction

The term **standardization** as used in this manual means all the measures that should be undertaken to obtain results that are fully comparable in respect of both time and place, such as comparability of results of a certain test within one laboratory performed at different times, and by different technicians, and comparability of results of a certain test performed in different laboratories.

To achieve such comparability of results it is essential to use standardized equipment, materials and reagents, including reference materials, in accordance with a standard operating procedure.

Vaccine samples should always be tested in parallel with a reference preparation which has been calibrated against the corresponding international standard or international reference reagent. Provided that the test is carried out according to appropriate procedures, the activity of a test sample should always have the same relation to that of the standard or reference reagent, independent of the assay system.

Reference reagents are preparations used in calibrating test procedures to ensure uniformity in the designation of potency or activity of biological preparations.

International biological standards (IBSs) are preparations designated as such by the WHO Expert Committee on Biological Standardization to provide a means of ensuring uniformity throughout the world in the designation of the potency or activity of biological preparations that cannot be expressed directly in terms of chemical and physical quantities. They are defined in International Units (IU) after an international collaborative study has been completed. They are supplied in a dried and stable form in ampoules containing an assigned number of units of activity expressed in International Units.

International biological reference reagents (IBRRs) were established to provide a means of ensuring the specificity of the corresponding working reagents. The potency is assigned by the WHO Expert Committee on Biological Standardisation on the basis of an international collaborative study. International Units are not assigned to them.

National reference reagents are preparations designated as such by a national control authority for the purpose of calibrating the working reference preparations of each individual laboratory. They must be calibrated against the corresponding international biological reference reagent or international biological standard.

International biological standards and international biological reference reagents are distributed free of charge. They are available to national control authorities through WHO for the purpose of calibrating national reference reagents. A list of the preparations available is provided in *Biological substances: International Standards and*

Reference Reagents 1990, Geneva, World Health Organization, 1991.

2.2 Selection and validation of methods

As indicated in section 1, each laboratory must select test procedures based on a preliminary determination of the materials, reagents and equipment that are most suitable and available under local conditions. A step-by-step description of the methodology must be established and should be available in writing. Any method or modification of a method should be subjected to a rigorous selection process which includes validation. Such standard operating procedures will ensure reliable and reproducible results that are appropriate for the analysis intended.

In viral and bacterial vaccine potency testing, the ability of the test method to differentiate between high and low quality vaccines must be established at an early stage. It is advisable to include preparations of known low potency when validating the method, as well as vaccines from different sources and of different composition.

2.3 Selection of a working reference preparation

In order to ensure comparability of test results a suitable reference preparation as similar as possible to the samples to be tested must be selected. This working reference preparation should be tested in parallel with the samples under investigation each time the test is performed. But individual data and cumulative geometric mean values should be recorded for the continuous evaluation of reference stability and test conditions. This can be done by using a Shewhart control chart (see section 2.6).

The following characteristics should be considered in the selection of a reference preparation:

- *Composition.* A reference preparation should be of a similar composition to the biological preparation to be assayed.

- *Stability.* A reference preparation should be sufficiently stable to remain when stored under proper conditions usable for a long period.

- *Quantity.* The number of filled containers available per lot should meet the laboratory's need for several years.

2.4 Calibration of a working reference preparation

To avoid making excessive demands on supplies of national and international reference materials, working reference preparations should be developed by each laboratory.

The activity of a working reference preparation, expressed either in IU (see Example 1 below) or as a titre (see Example 2), is determined by calibration against the appropriate

national reference reagent which, in turn, has been compared with the relevant international reference. Calibration is generally achieved by parallel titration on at least six separate occasions following the established standard operating procedure.

Example 1

Following completion of an international collaborative study, the WHO Expert Committee on Biological Standardization, assigned an activity of 10 IU to the contents of one ampoule of IBS for a vaccine.

In a collaborative study, the activity of the national reference reagent was compared with that of the IBS, and it was shown that the ratio was 1:2. The activity of one ampoule of the national reference reagent is therefore,

$$1/2 \times 10 = 5.0 \text{ IU}$$

The designated potency of the national reference reagent is thus 5.0 IU.

The next step is calibration of the working reference preparation against the national reference reagent. The geometric mean titres (GMTs) of the working reference preparation and national reference reagent calculated from data obtained from six tests are 1/300 000 and 1/150 000 respectively.

The potency of the working reference preparation expressed in IU can be calculated by dividing its GMT by that of the national reference reagent multiplied by the activity of the national reference reagent in IU.

The potency of the working reference preparation = 150 000/300 000 x 5.0 = 2.5 IU.

Example 2

The established potency of an IBRR, after an international collaborative study, is 6.15 is $10^{6.15}$ per 0.1 ml.

The GMTs of the working reference preparation and IBRR calculated from data obtained from the six tests performed to establish the ratio between the two are $10^{5.40}$ per 0.1 ml and $10^{5.85}$ per 0.1 ml, respectively. The ratio is calculated by dividing the established titre of the IBRR by its GMT:

$$\text{ratio} = 10^{6.15}/10^{5.85} = 0.30.$$

The corrected titre of the working reference preparation expressed in relation to the IBRR = $10^{5.40} \times 10^{0.30} = 10^{5.70}$.

Example 3

In an alternative approach to the calibration of a bacterial reference vaccine for potency

testing could be as follows: the calibration test is repeated with the reference reagent until the cumulative 95% confidence interval of the weighted geometric mean value is one quarter of the 95% confidence interval of an individual test. The calibration of a diphtheria reference vaccine in mice (Vero cell test) is used as an example.

Based on historical data for diphtheria potency tests, the 95% confidence interval has an average lower limit of 69.9% of the estimate of potency and an average upper limit of 151.1%. Calibration tests have to be continued until the cumulative 95% confidence interval of the weighted geometric means is between the following limits:

$$\log \text{lower limit} = \log 100 - (\log 100 - \log 69.9)/4$$
$$\text{lower limit} = 91.4\%$$

$$\log \text{upper limit} = \log 100 + (\log 151.1 - \log 100)/4$$
$$\text{upper limit} = 110.9\%.$$

In practice, this criterion was reached after ten consecutive calibration tests. The more mice used per dilution, the fewer calibration tests required to reach a particular degree of accuracy.

2.5 Surveillance

Data obtained on the working reference preparation should be recorded and the geometric mean titre should be calculated each time new data are available.

The titre of the working reference preparation tested in parallel with the samples under test shall be within $10^{0.5}$ of its established titre.

Calibration of the working reference preparation against the IBRR or national reference reagent should be repeated periodically to avoid over- or under-estimation of the samples under test.

2.6 Quality control charts

In order to evaluate properly the data generated in a quality control programme, values for each of the controls in each test run should be listed on charts to permit a trend analysis. Such charts provide a visual representation of performance of the assay, enable comparisons to made, and clearly show any deviations, trends and shifts. A chart should be prepared for each control material. The most commonly used chart for internal quality control is the Shewhart control chart, also called the x chart, which is prepared in the following way.

At least 20 measurements are made on control materials, which are assumed to be stable and of uniform concentration from aliquot to aliquot. The mean and standard deviation of these measurements are then calculated and used to draw the chart. These estimates

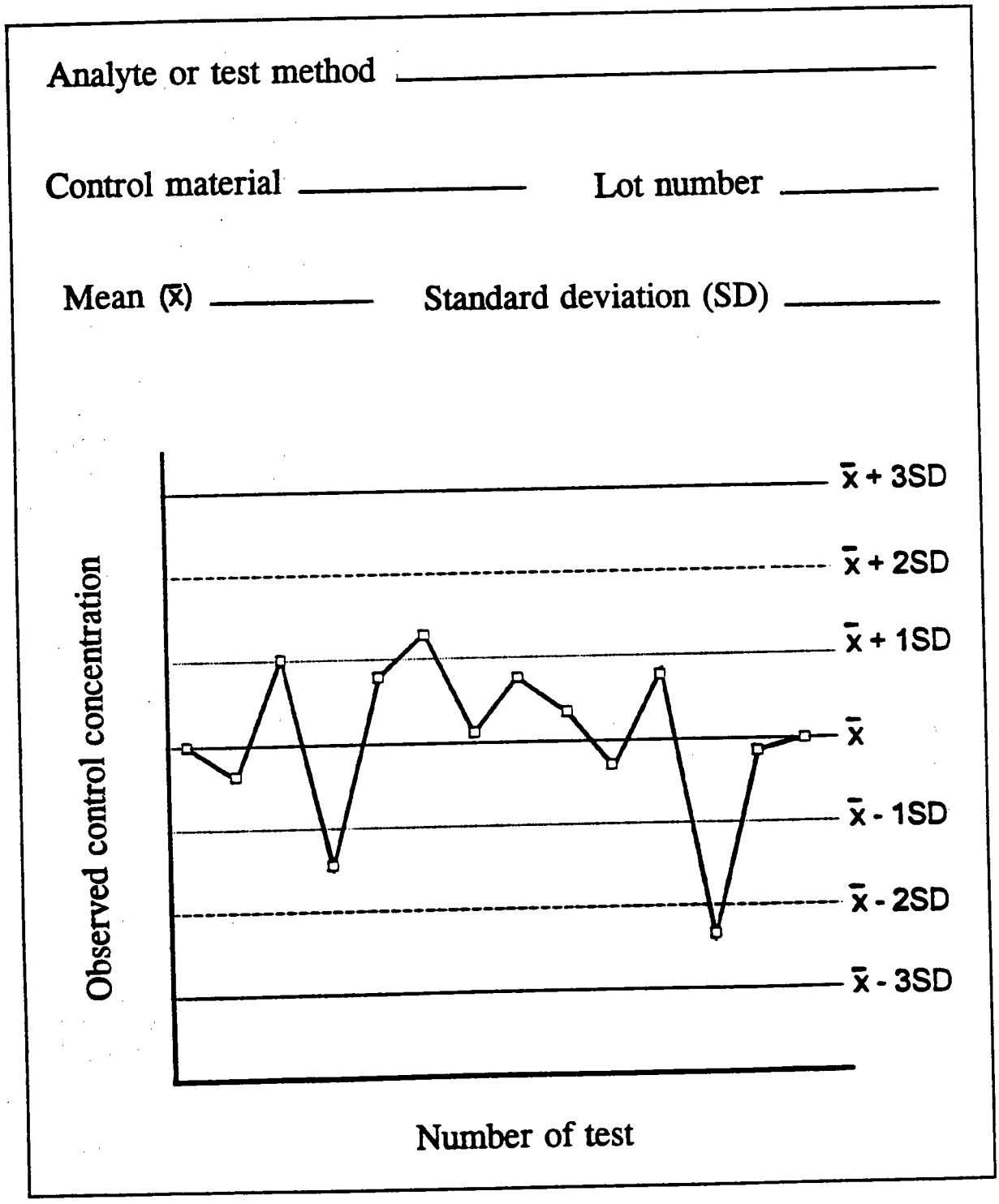
should be revised when more data become available and the cumulative values are used to prepare a new chart.

The chart displays concentration on the y-axis (vertical) versus time on the x-axis (horizontal). Horizontal lines are drawn for the mean and for the upper and lower control limits, which are calculated from the standard deviation. The two upper control limits are the mean plus three and two standard deviations (+3 SD and +2 SD) and the lower control limits are the mean minus two and three standard deviations (−2 SD and −3 SD), respectively (see Figure 1). The individual results from subsequent assays are then plotted on the chart after every run.

The graph is an extension of a Gaussian normal distribution curve (bell curve) laid on its side. Statistically, 95% of the control values are expected to fall within two standard deviations of the calculated mean value, and the data generated by the different runs should be randomly distributed around the mean. Results falling outside the range of two standard deviations (warning limit) should be cause for alert, especially if the results are from two consecutive runs. Results falling outside the range of three standard deviations (action or rejection limit) should lead to rejection of that particular run and readjustment of the assay. These are general terms — in some cases the acceptability of a test may be within ±1 SD.

Deviations are most frequently caused by random error. However, if the direction of several consecutive results points downwards or upwards from the mean line this probably indicates the presence of a systematic error or a stability problem that should be corrected. Precise test criteria should be set to increase the probability of error detection, taking care that the probability of false rejection is limited.

Figure 1. The Shewhart control chart.



3. THE USE OF LABORATORY ANIMALS IN THE QUALITY CONTROL OF VACCINES

Bioassays performed on living animals have become the method of choice for evaluating the safety and potency of bacterial vaccines. Large numbers of guinea-pigs and mice are required for these tests. In contrast to physicochemical tests, which can measure quantity, bioassays in animals can measure biological activity with very high specificity and sensitivity. Generally, responses are easily observable and quantal, meaning that the results obtained are "true or false", or "alive or dead". In addition, particular physiological responses can be measured, such as production of antibodies or body weight gain.

More than any other equipment or instrument used for testing, animals have to be handled and maintained appropriately to generate accurate, reliable, and reproducible results. It is essential to be aware of all the factors that may affect the biological functions of the test animals, and thus interfere with the outcome of a potency, safety or toxicity test.

This section contains information on the maintenance of animals, measures to be taken to ensure their quality and other factors that may influence outcome of test results. A code of practice for the use of animals in the production and quality control of vaccines, developed at a workshop organized by the European Centre for the Validation of Alternative Methods (ECVAM), is given in Annex 1.

3.1 Environment

The environment — temperature, humidity, illumination, sound — in an animal house can be a great influence on the quality of the animals. For animal houses in tropical and subtropical countries, air conditioning is recommended, since it promotes environmental stability. Changes in ambient temperature will affect the temperature regulation system of animals and thus disturb metabolism and behaviour resulting in varying experimental results. Relatively high temperatures will increase the survival of circulating microorganisms, which may become a threat to the animals. The optimum temperature for mice is 20–24 °C.

High relative humidity stimulates the production of ammonia in animal cages, facilitating respiratory diseases by depressing the defence system of the respiratory tract. A low relative humidity can lead to increased incidence of diseases such as ringtail in rats and mice. Relative humidity should be maintained at 45–75%.

Effective ventilation is necessary to promote comfort and thus reduce stress. The air should be changed 10–15 times per hour, with no recirculation of air between rooms.

To achieve a regular diurnal lighting cycle all year round, illumination with 12 hours daylight (350–400 lux) and 12 hours darkness is recommended. Cameron (*1*)

investigated the effect of illumination on the pertussis mouse weight gain test. Experiments in which mice were administered injections of saline only showed that lighting as well as temperature had an effect on weight gain; both prolonged lighting and higher temperature resulted in an increase in weight gain. Cameron suggested the use of a reference vaccine in potency testing to overcome these effects.

3.2 Nutrition

Commercially prepared laboratory animal food is preferred to freshly prepared food, since the latter carries a greater risk for introducing microbiological contamination into the animal facilities.

Van Ramshorst (*2*) investigated the influence of food on the antibody titre of guinea-pigs after immunization with a combined vaccine against diphtheria, tetanus, pertussis and poliomyelitis. During the immunization period one group received food A, the other food B, both commercially available guinea-pig foods. Food A gave a slightly higher weight gain (Table 1). The data demonstrated clearly the enhancing or suppressive effect of food on the immune response of animals emphasizing the need to use a reference vaccine in potency testing.

Table 1. Influence of diet on antibody titre and weight gain[a]

	Antibody titre	
	Food A	**Food B**
Tetanus[b]	0.02 IU/ml	0.56 IU/ml
Diphtheria[b]	0.055 IU/ml	0.44 IU/ml
Pertussis[c]	< 1/1	1/4
Weight gain (g/week)	47	39

[a] From reference (*2*).
[b] Arithmetic mean value (n = 18).
[c] Median pertussis agglutination titre (n = 6).

3.3 Genetics

At least 1 000 different strains of laboratory mice are registered. In spite of their genetic

heterogeneity, most of these strains are albinos and at first sight appear to be uniform. Most of the strains used for quality control of vaccines are outbred strains, and specific breeding methods are applied to ensure maximum genetic variation. This genetic diversity may be responsible for the variability observed in animal testing. For standardization and calibration of animals this may seem to be undesirable. Inbred strains will certainly lead to more uniform results. However, owing to their limited genetic diversity they sometimes demonstrate higher sensitivity to environmental influences. Furthermore, extrapolation to humans is easier if test results are obtained with outbred animals.

Bacterial vaccine potency control requirements are based on the lower 95% confidence limit of the estimated potency. The range of the 95% confidence interval depends to a large extent on the fluctuations in response from animal to animal. It is obvious that genetically identical animals will give a more uniform response and will facilitate comparison of data.

Hendriksen *et al.* (*3*) investigated the effect of the use of inbred strains and F_1-hybrids (progeny of the mating of two different inbred strains, which are highly uniform) on the 95% confidence interval of estimated potency. A considerable improvement was achieved when inbred strains were used. The authors concluded that, although inbred strains may be more expensive and harder to breed than random-bred stocks, the use of inbred strains seems to be favourable for potency testing of vaccines containing diphtheria and tetanus toxoid.

In a study on the possible influence of mouse strain on the assayed potency of tetanus vaccines, Hardegree *et al.* (*4*) tested a batch of tetanus vaccine in parallel with the International Standard for Tetanus Toxoid in five different mouse strains. A significant difference in relative potency between the two vaccines was found for the individual strains.

Therefore, although the use of inbred strains has been shown to have some scientific advantages, no consensus exists for the use of such strains in the quality control of vaccines.

3.4 Microbiological quality

Like people, animals can suffer a wide range of diseases. There are many well documented examples showing interference (immunosuppression and immuno-enhancement) with experimental results. In some cases infection will lead to sickness often followed by death, not only for the animals themselves but also for the animal technicians (Hantavirus, for example, can cause serious problems). Even more dangerous for animal populations as well as staff are latent infections that do not show any sign of illness.

Normally animals are free of microorganisms at birth. When they are housed under isolated conditions, introduction of pathogens can be prevented. However, for several biological functions, animals need the help of certain microorganisms. Specific

microorganisms are therefore introduced. The result is an animal from which certain potentially harmful and pathogenic microorganisms are known to be absent. This class of animals is called specific pathogen free (SPF); it is proven that these animals are free of specific pathogens. To maintain the SPF quality of an animal colony, certain hygienic and managerial measures should be taken to protect animals from contamination which may lead to unwanted infections.

Animal facilities should be suitably designed and personnel should receive appropriate training. An animal health monitoring system should be established to ensure the continuation of SPF status. It should include at least the clinical examination of the animals on a regular basis. Laboratory investigations of both sick and healthy animals are essential in the diagnosis of diseases. Postmortem examinations should be carried out on all diseased and dead animals in order to determine the responsible microorganism. Using bacteriological, virological, histopathological, mycoplasmatological and parasitological techniques, samples of healthy animals should be screened for the presence of microorganisms. Such examinations should be carried out on a routine basis to prevent diseases from spreading.

Other managerial measures include:

- quarantine of incoming animals until they are screened;
- isolation of sick animals;
- separate breeding and experimentation facilities;
- restricted entrance of staff and visitors;
- prohibition of eating, drinking and smoking;
- an "all-in, all-out" system (finish experiment, clean and disinfect animal room and equipment, and start a new experiment);
- avoidance of overlap in experiments (by using multiple small animal rooms);
- separate routing of clean (entering) and dirty (waste) materials;
- employment of well trained staff;
- limited contact of personnel with other animals;
- monitoring of health of staff.

Hygienic measures include:

- decontamination of all materials (preferably chosen for easy cleaning and decontamination) entering the animal holding rooms;

- filtration of the air;

- sterilization of food (by gamma irradiation or possibly autoclave), water, bedding (autoclave) and cages;
- personal cleanliness among animal colony personnel (change of clothing, hand-washing and showering);

- making facilities proof against insects and wild rodents.

If adequate measures are taken the SPF status of animals can be guaranteed. If no attempts are made to protect animals against pathogens misleading results could be obtained (*5*). A striking example is given by Peters (*6*) who investigated the effect of intestinal flagellates (*Hexamita muris*) on the tetanus antibody response of animals. Significant differences between flagellate positive and negative animals occurred in a potency test for tetanus vaccine. After immunization of ten flagellate positive and ten negative mice with tetanus vaccine, positive animals had a mean antibody titre of 0.8 IU/ml while negative animals had a much higher titre of 8.15 IU/ml.

Whatever the disadvantages and problems associated with the use of animals as a "measuring device" in the quality control of vaccines, no test system is known which can replace animal models completely. The use of a reference can partly overcome the effect of external factors on test results. However, attention should be paid to the care and handling of laboratory animals to minimize effects of environment and nutrition and to maximize efficacy in their use, particularly in the quality control of bacterial vaccines. Animals should be bred and maintained in such a way that the maximum possible standardization and reproducibility are obtained.

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4. GENERAL LABORATORY PROCEDURES

4.1 Guarantee of quality of reagents and equipment

Good supervision in the laboratory is essential in order to maintain appropriate operating procedures. These include a disciplined attitude, the use of correct techniques, the completion of approved protocols, and maintenance of a clean work environment.

The most important factor in guaranteeing quality is the avoidance of contamination in the operating system, which consists of the cell cultures, the reference and test vaccines, and the reagents. Contamination may come from the original material, from careless manipulation of the materials, or from the atmosphere.

The list of what to do and what not to do is too long to include here in entirety; however, it is useful to record some of the most important instructions for avoiding contamination.

- *Avoid contamination from the atmosphere.* Do not permit the formation of air currents or turbulence in the working area. If working in laminar flow cabinets, avoid barriers that can interfere with the flow of air, such as too many objects inside the hood. Always work in an area that is protected, clean, decontaminated, and free from dust and draughts.

- *Avoid contamination by personnel.* Do not permit the entry of unnecessary personnel into the working area. When in the working area use sterile gloves and cover the mouth and nose with a sterile mask. Use pipetting devices for pipettes and never pipette by mouth.

- *Avoid contamination from other laboratory materials.* Do not prepare yeast extracts in the cell culture areas. The spores can easily infect cultured cells. Sterilize all sera by filtration through a 0.22-μm filter and determine whether or not the filtrate contains mycoplasma. Filter all working solutions, including culture media, trypsin, antibiotics, and perform the necessary test to detect contamination. Store working solutions at -20 °C. Seed all cell cultures in media containing antibiotics.

- *Protect the laboratory from the consequences of contamination of cell cultures* by maintaining a bank of each cell type in liquid nitrogen or at -70 °C.

- *Perform control tests regularly and maintain a registry of results.* Table 2 gives some of the tests that should be undertaken.

Table 2. Tests that should be performed regularly in the vaccine potency testing laboratory

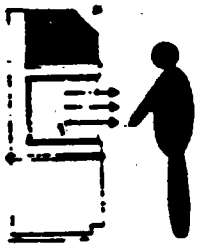
Test equipment or material	Sampling and testing frequency	What to test
Autoclave and oven	Every week	Use a biological indicator such as *Bacillus stearothermophilus*
Clean tubes and flasks (washed)	Two or more of each	Test to see if water sheets off (it will form drops on a dirty flask)
Sterile tubes and flasks	One sample from each lot sterilized	Sterility test in thioglycolate at 22 °C and 37 °C
Sterile disposable materials	One sample from each package	Sterility test in thioglycolate at 22 °C and 37 °C
Cell lines	When received in the laboratory	Tests for detection of mycoplasma, bacteria, fungi, capacity to form a monolayer
Cell cultures	One representative sample from each lot prepared	Morphology of the monolayer; viability (0.5% trypan blue absorption)
Culture media, including animal sera and diluents	One sample from each lot	Tests for detection of mycoplasma, bacteria, and fungi, test for ability to support growth and maintenance, freedom from viral inhibitors
Trypsin solution	All lots	Test for freedom from contamination; analysis of trypsin activity and effect on cell morphology
Solutions of antibiotics	All lots in concentrated form	Test for detection of contaminants; inhibiting activity using indicator microorganisms and effect on proliferation and aspect of cell monolayer
Laminar flow cabinet	Every week	Test efficacy of filters using broth and plates

4.2 Control of the atmosphere: the laminar flow cabinet

Laminar flow cabinets have a dual function. They inhibit the penetration of ambient air into the working area. This protects the quality of reagents. In diagnostic laboratories, they also protect the technician from the dangerous pathogens that can contaminate the working area.

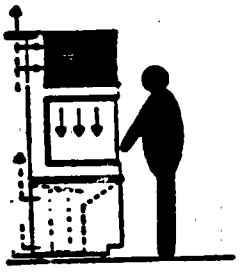
Figure 2 illustrates two types of laminar flow cabinet. A horizontal flow cabinet, which also has laminar air flow, is not a biological safety cabinet, and should not be used as such. If such cabinets are already installed in the laboratory, they should be used only with the blower turned off. If a biological safety cabinet is not available, a simple plastic shield offers better protection for the operator and the environment.

Figure 2. Types of laminar flow cabinet.



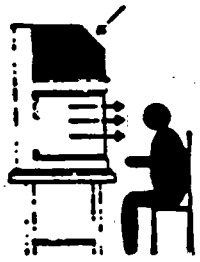
Horizontal
Flow
Cabinet

not recommended



Biological
Safety
Cabinet

recommended



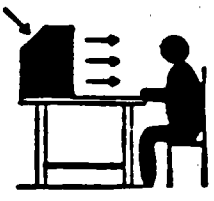
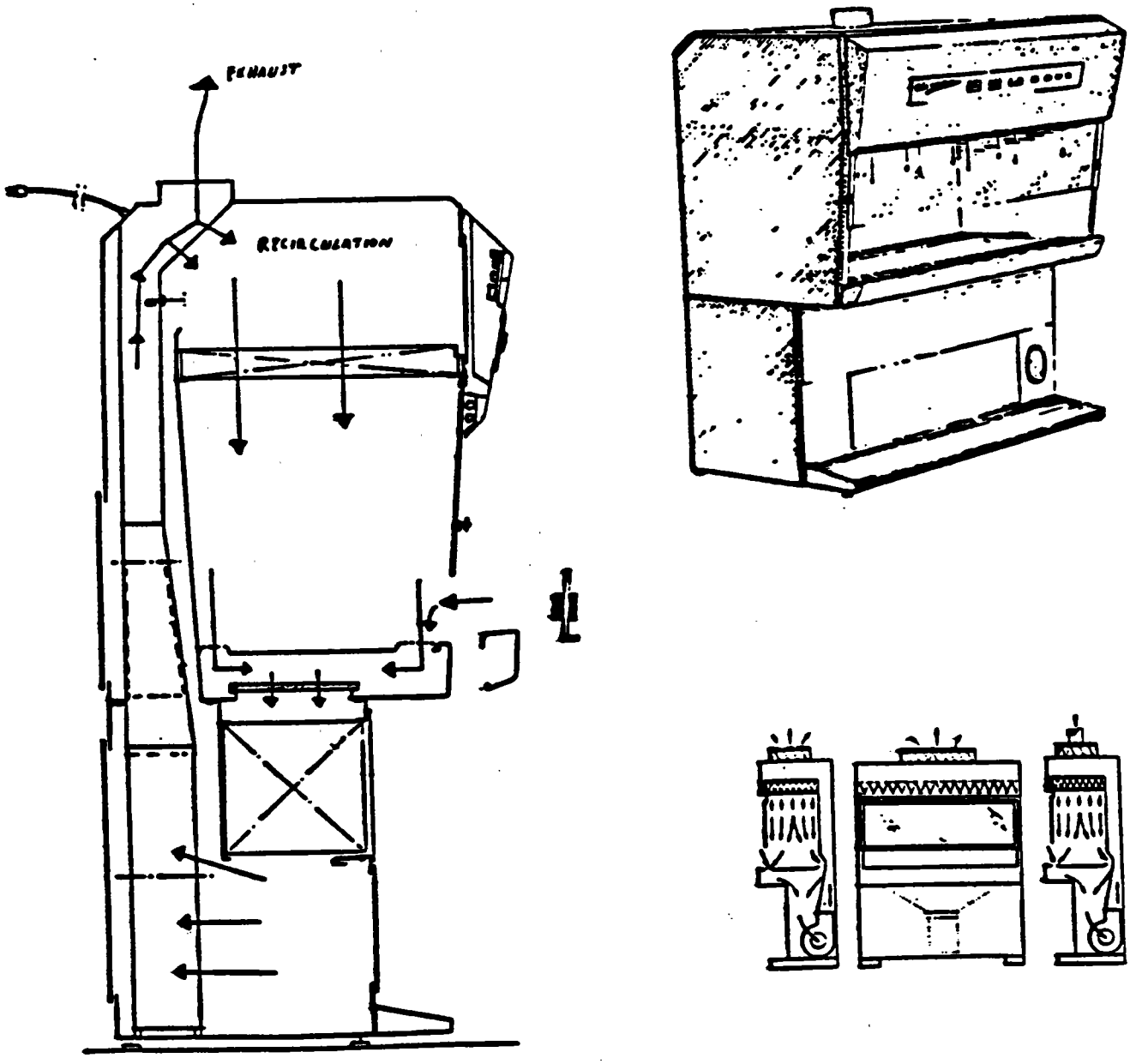


Figure 3 shows further details of a laminar flow biological safety cabinet and the dynamics of the air circulation inside it which, as the name indicates, is laminar and one-directional. The front panel separating the technician from the work area protects the technician; the barrier of air at positive pressure in the front impedes the entry of airborne contaminants, and the HEPA filters sterilize the continuously circulating air.

Figure 3. Biological safety cabinet.



A biological safety cabinet does not offer complete protection against air currents, nor is it effective against contamination produced by contact. Thus, personnel should observe strict rules of sterile technique and good working habits during manipulations.

The biological safety cabinet should be installed in a clean dust-free location as far as possible from transit areas, air conditioners, fans, doors and corridors. The cabinet and the filters should be checked for leaks at the time of installation, after each move, and at regular intervals. If trained personnel are not available to install and service the biological safety cabinet properly, it should not be purchased.

To obtain maximum efficiency from the cabinet over a long period, it is important to remember a few rules. Before beginning:

- Ensure that there are no obstructions and that the entry and exit of air are equivalent. A flow meter may be used to verify the velocity of the air current at the entrance and exit.

- Let the cabinet run for five minutes.

- Put all tubes, culture media, cell cultures, and reference materials needed for the test in the working area. These should be kept to a minimum.

- Use sterile hair and body coverings during operation.

- Avoid crossing the air barrier in front of the cabinet unless it is necessary.

- Avoid turbulence in the room or inside the cabinet, which could be caused by fans, air conditioners, heaters, centrifuges, refrigerators, rapid movements of the technician, draughts, movement of people or transport of materials.

- Avoid storing culture media in the working area as these liquids stimulate the proliferation of fungi.

After operation:

- Let the cabinet run for several minutes.

- Take all materials out of the working area.

- Clean and decontaminate the working surface.

- Maintain a registry of the use of the cabinet including date, hour, work performed, observations.

- Report any defect in functioning noted.

- Request maintenance for the cabinet according to the schedule recommended by the manufacturer.

4.3 Decontamination and sterilization

It is a good practice to separate plastic objects from those made of glass immediately after use. Disposable supplies should be collected in plastic bags, and tubes and flasks in sealed boxes. Avoid allowing culture media and cells to dry on the surface of glass objects. Tubes and flasks which are not to be washed or sterilized in an autoclave immediately after use should be kept in water.

Heat is of course the most efficient means of sterilization, when it can be used. Autoclaves that can sterilize with steam under pressure (15 lb/square inch) at a temperature of 120 °C and with dry heat at 160–180 °C are a basic requirement. Unfortunately in many cases heat cannot be used, and other methods have to be recommended.

- *Culture media and solutions*. Purify under pressure (at least 5 lb/square inch) through Seitz or membrane filters. Before filtration, wash the membrane with phosphate-buffered saline and discard the liquid of the first wash and the first 100 ml of the solution to be filtered.

- *Plastic objects (permanent use)*. Disinfectants may be used when they do not interfere with the subsequent use of these objects. Fractional sterilization is a good technique: objects are heated at 100 °C for 10 minutes, and then incubated in a protected environment overnight. Heating to 100 °C for 10 minutes is repeated the following day. This procedure is effective for the control of spore-forming bacteria.

- *Biological safety cabinet*. Regular decontamination can be done by disinfecting surfaces with liquid disinfectant. Qualified service personnel may perform a procedure which generates formaldehyde gas. The cabinet must be dismantled, and the area around it kept sealed. The procedure will take several hours, and the cabinet should then be left to ventilate until the following day.

- *Working surfaces*. Use a liquid disinfectant, for example, 5% sodium hypochlorite, or 5% phenol in water. Ultraviolet light is not recommended for sterilization.

- *Gas in cylinders*. Use a filtration unit and a sterilizing filter with μm porosity. Change the filter when necessary.

4.4 Incubators

Not all laboratories have the means to acquire a humidified incubator with carbon dioxide (CO_2) and with controls for humidity and CO_2. However, it is possible to operate without this piece of equipment. For cells of the Hep-$2_{Cincinatti}$ cell line, it is sufficient to seal the microtitre plates with plate sealers and work without the use of CO_2. Vero cells are more sensitive, however. Plates can be placed in an ordinary desiccator which is exposed to a flow of CO_2 for 30 seconds prior to closing. The desiccator is then placed in an incubator

or room at 37 °C. When a pH change is noted, shown by a change in the colour of the media, the desiccator is removed, opened, and again exposed to CO_2. Another possibility is to use a medium containing 0.025 mol/l HEPES buffer, and to incubate the plates in sealed plastic bags. This will maintain the pH and humidity sufficiently well.

4.5 Cryoconservation

Thermal analysis has demonstrated that for complete freezing of a solution, it must be brought to a temperature much lower than its freezing point. Although the freezing point of an eutectic solution of sodium chloride (the principal electrolyte in serum) is -22 °C, serum does not freeze completely unless it is brought to a temperature of -50 to -60 °C because of the other small molecules which it contains. At temperatures close to 0 °C, which is the point where ice crystals start to form, and at temperatures around -50 °C, where complete freezing occurs, the protein molecules in the serum are exposed to the damaging effects of a lowered proportion of water, and thus a higher proportion of ions, which may result in changes in pH and loss of biological activity. For maximum conservation of cell lines and sera, therefore, they should be frozen rapidly at -70 °C or lower. Ordinarily protein solutions that are used in the laboratory should be stored at temperatures below -30 °C. If this is not possible, a temperature above -10 °C is recommended. If the material is sterile, storage at 4 °C is even better.

Freezing at these low temperatures can be achieved by various means, the most common of which are liquid nitrogen, dry ice, a mixture of carbon dioxide and solvent, and a freezer with temperatures below -70 °C.

4.6 Washing cycle

To clean nondisposable glass objects, the following washing cycle is recommended. Submerge the flasks or tubes immediately after use in an aqueous solution containing a cleaning agent (experiment with various detergents, as the quality of cleaning varies, depending on the quality of water in the laboratory). Decontamination will be complete after soaking for 10 minutes. Clean manually or mechanically with a brush. Rinse with running water and twice with distilled or deionized water; for the final rinse use glass-distilled water. Manipulate clean glass objects in metal baskets. Avoid touching them with bare hands. Leave them to dry in a well ventilated, dust-free area. Determine the cleanliness of the glass and, if it is not satisfactory, repeat the washing operation. Old glassware that is crazed should not be used.

The cleaning of plastic microtitre plates for reuse is not recommended. However, some laboratories have had success using the following method. Plates are first treated for 30 minutes in a trypsin bath, followed by overnight treatment with formalin to disinfect them. After washing with chromic acid solution (10% sodium dichromate, 35% sulfuric acid), plates are subjected to the washing cycle described above, and sterilized prior to use (see section 4.3). Plates can be successfully used about five times using this method. An alternative method is overnight soaking in 5% hypochlorite solution, washing and

drying as above, soaking for 30 minutes in 70% ethanol-water solution, drying, and sterilizing.

5. LABORATORY SAFETY

It cannot be said too often that good laboratory technique is the best guarantee of laboratory safety. Moreover, staff who are adequately trained concerning the nature of their work and the possible hazards associated with it are better able to avoid unsafe practices. Laboratories should develop their own codes of safe laboratory practice, which must be uniformly enforced and regularly reviewed. Subjects to be included in such a code are immunization of laboratory workers, personal hygiene, proper attire and practices for laboratory workers, restriction of access, correct use of equipment to minimize hazards, decontamination and disposal procedures, and training. Good supervision is essential to laboratory safety.

5.1 Protection for workers

5.1.1 Physical barriers

Workers should wear laboratory coats and gowns which are not worn outside of the laboratory. Since larger particles and droplets released during microbiological manipulation settle rapidly onto bench surfaces and the hands of the operator, gloves should be worn, hands should be washed frequently, and working surfaces should be decontaminated after use.

Laboratory techniques such as pipetting, centrifugation, pouring, and mixing produce aerosols, which may put the worker at risk. To avoid aerosols from centrifugation procedures, the centrifuge should always be operated according to the manufacturer's instructions. Tubes should be thick-walled and free of defects. They should not be overfilled, and should be sealed for centrifugation of infectious materials. Tubes should be filled and opened in a biological safety cabinet, as should all containers that have been subjected to shaking and homogenization.

The biological safety cabinet is the best physical barrier to laboratory hazards if used properly (see section 4.2).

5.1.2 Immunobiological barriers

Immunization histories of personnel working in virus laboratories should be recorded and kept up to date. Staff should be immune to all viruses used in the laboratory. Consideration should be given to immunization against hepatitis B for all those laboratory workers shown not to have protective levels of antibody, with priority given to those who will be manipulating human specimens. All samples should be considered as being potentially contaminated, particularly those that contain human fluids, such as blood, and should be so handled.

5.1.3 Restriction of access

Access to the laboratory must be denied to unauthorized personnel. Waste handlers must be protected by proper disposal of infectious toxic wastes, by autoclaving or disinfecting as indicated.

Oral infection can result from the use of such hazardous practices as mouth pipetting, failure to wash hands properly, and also from eating in work areas. Separate areas for eating, drinking, and applying cosmetics should be maintained, with access limited to personnel who have been appropriately decontaminated. Food and drink should not be stored or consumed in the laboratory, and there should be no smoking, gum chewing, or application of cosmetics.

5.1.4 Training and supervision

Strict attention to detail must be maintained, and laboratory procedures must be continually reviewed to avoid danger to personnel. When staff understand the reasons for the rules and regulations of proper laboratory procedure, they are more willing to follow them scrupulously. Continuing education is important in protecting staff from laboratory hazards.

5.2 Protection of the environment

5.2.1 Proper disposal

Improper disposal of infectious waste materials or toxic chemicals endangers the environment. Such materials must therefore be autoclaved or disinfected in the case of biological hazards, and detoxified in the case of toxic chemicals. Biosafety cabinets vented to the outside must have filters that will prevent the release of hazards into the air. Radioactive wastes must be properly disposed of.

5.2.2 Containment

Laboratories that will be handling biohazards must have a negative pressure relative to the outside environment in order to avoid unintentional release of infectious materials. In those laboratories rated P3, or handling biohazards rated higher than class 2, exit of infectious wastes and of personnel must be carefully controlled. In addition, air and water effluents should be decontaminated or filtered prior to release into the environment.

5.2.3 Laboratory clothing

Protective clothing and shoes worn by personnel within the laboratory should be reserved for use in that area only. Laboratory coats should be removed when workers go to a clean area for eating, drinking or office work, and should be decontaminated prior to laundering. Shoes reserved for laboratory use are a good practice.

5.2.4 Protection of equipment and materials

Although many workers may feel that attention to details to assure laboratory safety interfere with good working procedures, this is not the case. On the contrary, strict attention to good laboratory practices, which include safety practices, will ensure a higher quality of work. For example, proper disposal of wastes will reduce the possibility of contamination of equipment and materials by microorganisms. Well thought out standard operating procedures will minimize confusion and the possibility of inadvertently mixing up or cross-contaminating samples. Physical barriers, such as gloves, will reduce the likelihood of mycoplasma contamination from the hands of the operator.

Further information is available in *Laboratory biosafety manual, second edition*, Geneva, World Health Organization, 1993.

6. PREPARATION OF HYPERIMMUNE ANIMAL SERA

6.1 Introduction

Polyclonal hyperimmune antisera of various specificities are commercially available from many sources. However, these antisera are generally rather costly, especially when needed in large quantities, such as for use in quality control of viral vaccines, or for potency or identity testing. In many circumstances, therefore, it is preferable for the individual laboratory to produce its own antisera in one of a number of species such as sheep, goats, rabbits or guinea-pigs. The following sections present protocols that have been used successfully for the preparation of specific, high-titred antisera. It is stressed, however, that such protocols cannot simply be copied to give guaranteed success under all circumstances. Apart from obvious factors such as virus strains, and the quality and type of cells used in cell culture to propagate the viruses, success depends to a great extent on the quality of animals used. Animal quality is primarily determined by breeding and housing conditions, rather than the type or strains of animals used (see section 3).

Apart from affecting the general health and well-being of the animals, microorganisms, including viruses, may well, without causing disease, interfere with the desired immunoresponse, and may give high amounts of undesired or cross-reacting antibodies. It is therefore advisable, prior to starting the immunization procedure, to test the animals for the presence of such antibodies. Further, during the course of the immunization scheme, it is advisable to monitor the desired specific immunoresponse and any undesired immunoresponses that might interfere with the test for which the antiserum is raised.

Sometimes it is better to collect hyperimmune sera early, with only moderate titres of the desired antibodies, but without undesired antibodies or cross-reactivity, rather than leaving the collection until later when titres are higher but antisera may contain large amounts of cross-reacting antibodies. Each laboratory has to determine which protocol is most suitable, depending on the specific conditions of the laboratory and the purpose for which the antiserum is needed.

6.2 Measles hyperimmune polyclonal antiserum (in rabbits)

Concentrated virus purified from cells infected with measles virus is used as an immunizing agent. A total of 0.5–1.0 mg of virus in a volume of 0.2–0.5 ml of a physiological buffer is added to an equal volume of complete Freund's adjuvant (CFA) and mixed thoroughly. The antigen-adjuvant mixture is injected intradermally or subcutaneously at multiple sites on the shaved back of a male rabbit of approximately one year of age. After two to three weeks, a booster injection of 0.1–0.5 mg of virus in Freund's incomplete adjuvant (1:1 v/v) is given subcutaneously. One week after the first booster injection, an identical second booster is given. One week later, a serum sample is taken and tested by immunofluorescence (IF), enzyme-linked immunosorbent assay (ELISA), or haemagglutination inhibition (HI) test, depending on the purpose for which the antiserum is needed.

Specific titres of >1/1 000 by IF, >1/100 000 by ELISA or >1/1024 by HI are normally reached in a total immunization time of approximately two months. If the above-mentioned titres are found in the first test bleeding sample, and the reaction to uninfected cells (nonspecific reaction) is less than or equal to 1/100 of the binding to infected cells (specific reaction), the antiserum may be accepted and the rabbit(s) are bled. It is possible that specific titres will be higher still when the animals are bled later, but it is likely that nonspecific titres will rise as well. If the nonspecific titres are too high, the antiserum may still be useful after dilution or adsorption with packed, uninfected cells (see below).

Recently, guidelines for good laboratory practices in relation to laboratory animals have indicated that the use of CFA should be avoided and is acceptable only for specific indications. Since data on the effectiveness and necessity for use of CFA in the production of specific antisera are not conclusive and often contradictory, its use for this purpose will no longer be permitted in some countries. Therefore in Tables 3 and 4 two schemes are presented, one with and one without the use of CFA. Both schemes have been claimed to be successful.

Table 3. Immunization scheme for the production of measles virus antiserum in rabbits (with CFA)

Day	Volume injected or drawn (ml)	Procedure
0	5	Bleed by ear puncture (artery) Check for cross-reactivity
0	0.5 + 0.5	MV + CFA (1:1), subcutaneous, multifocal (back)
14	0.5 + 0.5	MV + IFA (1:1), subcutaneous, multifocal
21	5	Bleed by ear puncture (artery) Check specific titre and for cross-reactivity
21	0.5 + 0.5	MV + IFA (1:1), subcutaneous, multifocal
28	5	Bleed by ear puncture (artery) Check specific titre and for cross-reactivity
35	5	Bleed by ear puncture (artery) Check specific titre and for cross-reactivity

MV, measles virus; CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant.

Table 4. Immunization scheme for the production of measles virus antiserum (without CFA)

Day	Volume injected or drawn (ml)	Procedure
0	5	Bleed by ear puncture (artery) Check for cross-reactivity
0	0.2 + 0.2[a]	MV + IFA (1:1), intradermal, multifocal
21	0.5 + 0.5[b]	MV + IFA (1:1), subcutaneous, multifocal
42	0.5 + 0.5[b]	MV + IFA (1:1), subcutaneous multifocal
56	5	Bleed by ear puncture (artery) Check specific titre and for cross-reactivity
63	5	Bleed by ear puncture (artery) Check specific titre and for cross-reactivity

MV, measles virus; CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant.
[a] Total virus content, 0.5–1 mg of MV.
[b] Total virus content, 0.1–0.2 mg of MV.

6.2.1 Propagation of measles virus

Measles virus is purified from supernatants of infected cells and from cellular debris of these cells. The virus is harvested when the cytopathic effect (CPE) has spread to from 75% to nearly 100% of the cells. Depending on the purpose for which the virus is needed — for immunization purposes to obtain hyperimmune sera, or for use as antigen in an ELISA or HI/HA — different procedures are used. A general protocol for the propagation of measles virus is given below. Section 6.2.2 gives protocols for further purification of the virus. Since measles virus is considered to be homotypic, in principle any (laboratory) strain can be used. The Edmonston B strain has been found to give the best results. High yields are produced when the virus is propagated in continuous cell lines such as Vero or HeLa, or primary cells such as chicken embryo fibroblasts (CEF), or monkey kidney cells.

Materials

- Virus: Measles, strain Edmonston B (10^{-7} $CCID_{50}$/ml)
- Cells: Vero, passage No. 167, 2.5 x 10^5/ml[1]

[1] Available on request from Chief, Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.

– Medium: M199, antibiotics, 1% glutamine and 5% fetal calf serum

Procedure

To each 850-cm^2 roller bottle (cell culture quality) add simultaneously 100 ml of the cell suspension and 1 ml of the virus suspension. Culture the cell-virus mixture at 37 °C on a roller system for approximately 4 days, until 75–95% CPE is observed.

6.2.2 Harvest and concentration of measles virus

Protocol 1 (successfully used for immunization purposes)

During the propagation procedure as described in section 6.2.1 above, most of the cells will have been infected with measles virus. Since this virus causes a lytic infection, at the end of four days of culture many of the cells will have lysed, shedding virus into the supernatant. However, there will also be large amounts of virus in the remaining intact cells and attached to the cell membranes of both lysed and intact cells. To obtain optimum yields of virus, the supernatant, cells and cell membranes are all harvested.

– Collect the supernatant and store at 4 °C.

– Collect attached cells by scraping with a rubber policeman, and add to the stored supernatant.

– Centrifuge for 10 minutes at 200 *g*, collect the supernatant, store the pellet, and centrifuge the supernatant once more for 10 minutes at 200 *g*.

– Collect the supernatant and store. Collect the pellet, add to the first pellet, and sonicate three times for 15 seconds each time in a sonicator (Branson sonifier, output 5). Pellet the sonicated material in an Eppendorf table centrifuge at maximum speed (15 000 rpm).

– Collect the supernatant and add to the previously stored supernatant.

– Ultracentrifuge the pooled supernatants at 80 000 *g* for four hours at 4 °C.

– Collect the pellet, resuspend in 10% of the original volume in physiological buffered saline, and store at -70 °C in small aliquots until use.

Protocol 2 (successfully used for the preparation of HI-antigen)

Because a large amount of haemagglutinin (H) protein is found in both the cellular membranes and in released virus, the whole culture can be harvested and used for antigen preparation.

– Freeze/thaw the roller bottles containing the cells infected with measles virus five times (be sure that the bottles can withstand this procedure!).

- Centrifuge at 10 000 *g* for 20 minutes.

- Collect the supernatant and store at -70 °C in small aliquots until use.

The haemagglutinating ability of the antigen can be increased considerably by treatment with Tween 80 or ether (*1*, *2*).

This protocol is less suitable for producing antigens for immunization purposes, since it will contain large amounts of cellular proteins and fetal serum proteins. Consequently, when used as an immunogen, undesired antibodies will be raised against these components as well.

Protocol 3 (for the preparation of crude ELISA antigens)

- Wash the measles virus-infected cell monolayer three times with cold phosphate-buffered saline (PBS).

- Disrupt the cells by hypotonic shock by adding a 1/10 dilution of PBS. Next disaggregate the cellular debris further by ultrasonification, or with the aid of a Dounce homogenizer.

- Discard the undesired debris and cell nuclei which precipitate after low speed centrifugation at 200 *g* for 15 minutes.

- Ultracentrifuge the collected supernatant for two hours at 80 000 *g* with no break.

- Collect the pellet, resuspend in PBS and store in small aliquots at -70 °C until use.

Measles virus-infected cells contain large amounts of measles nucleocapsid (NP), H and phospho-(P) proteins, and lesser amounts of matrix (M), fusion (F), and large (L) proteins.

The antigens prepared following protocol 3 are adequate for use in a simple ELISA to determine increases in anti-measles IgG antibodies in serum samples. However, if it is important to measure the levels of antibodies to all structural viral components, or if the antigen is to be used in an IgM ELISA, it will be necessary to purify the viral antigens further:

Protocol 4 (for the preparation of purified ELISA antigens)

- Collect the supernatant from the measles virus-infected cells when CPE has spread to 75–100% of the cells.

- Clarify the supernatant by centrifugation at 10 000 *g* for 20 minutes.

- Concentrate this virus suspension by overnight dialysis in a hypertonic buffer, or with the aid of a hollow fibre system.

- To the concentrated virus suspension add sodium chloride to a final concentration of 2 mol/l and ∝-D-methylmannoside to a final concentration of 4%. These compounds will reduce clumping and nonspecific binding of the virus to cellular debris.

- Hold on ice for 30 minutes.

- Carefully transfer the virus concentrate to a step gradient consisting of 10 ml of 18% and 5 ml of 36% potassium tartrate in GNTE-buffer (consisting of 0.2 mol/l glycine, 0.2 mol/l sodium chloride, 20 mmol/l tris buffer, and 2 mmol/1 EDTA, pH 7.8).

- Ultracentrifuge at 80 000 *g* for 90 minutes.

- Collect the interphase and dilute in GNTE-buffer to a tartrate concentration of less than 18%.

- Transfer the virus concentrate carefully onto a 24-ml, linear, 18–36% tartrate/GNTE-buffer gradient.

- Ultracentrifuge at 80 000 *g* for at least three hours, or overnight.

- Collect the virus band, dilute in GNTE-buffer and pellet at 80 000 *g* for 30 minutes.

- Resuspend the pellet in a buffered physiological medium such as PBS, and store at -70 °C.

(Instead of sodium tartrate, sucrose may be used to form gradients of equal densities. The concentration will have to be adapted. Both compounds are generally readily available and inexpensive.)

6.3 Poliovirus hyperimmune polyclonal antiserum (in rabbits)

For potency testing of trivalent oral poliomyelitic vaccine large amounts of antisera are required. Since these antisera are not readily available commercially in such quantities and, if available, are quite expensive, most vaccine quality control laboratories prefer to produce their own. The antisera may be produced in many different species, such as cattle, horses, goats, sheep or rabbits. Rabbits are probably the most commonly used laboratory animals for this purpose, and usually produce antisera of high quality. If large volumes of antiserum are required however, it may be preferable to use larger animals such as calves. In section 6.3.1 examples of successfully employed immunization schemes for both rabbits and calves are given.

6.3.1 General protocol for the propagation of poliovirus

Materials

Virus: poliovirus type 1 (Sabin), geometric mean titre (GMT) $10^{5.79}$; poliovirus type 2 (Sabin), GMT $10^{6.11}$; poliovirus type 3 (Sabin), GMT $10^{5.68}$ (all available from WHO[1])
Cells: Hep-$2_{Cincinnati}$ (Hep-2_c), passage no. 154, 20×10^6/ml[1]

Medium: M199 or Dulbecco's minimal essential medium (DMEM) supplemented with fetal calf serum (FCS), 1% glutamine, and antibiotics

Suspension medium for virus concentrate: M199 + antibiotics + 1% glutamine

Procedures

Two protocols may be followed: in the first, virus and cell suspension are seeded at the same time; in the second, preformed monolayers are used. The latter has the advantage that more cells are available for infection, which may result in a higher yield, and the cells may be infected in a medium containing a minimum amount of serum (desirable for immunization purposes to avoid unwanted antibody responses). The first procedure is somewhat simpler and faster, and will also render high yields, although it may be necessary to follow a more elaborate purification procedure to eliminate the contaminating serum components.

(a) Protocol 1 (infection of cell suspensions)

- To each 850-cm^2 roller bottle (cell culture quality) add 100 ml of medium in which one ampoule of the Hep-2_c cells is suspended (approximately 2 x 10^2 cells per cm^2), and one ampoule of one of the poliovirus types.

- Culture the virus-cell mixture on a roller system until 75–95% CPE is reached (approximately four days).

(b) Protocol 2 (infection of pre-formed cell monolayers)

- Seed one ampoule of Hep-2_c cells in each 850-cm^2 roller bottle in 150 ml of medium containing 5–10% FCS.

- After two days (or more, depending on the cell monolayer, which should be at least 75% confluent) discard the medium and infect the cells with one ampoule of one of the poliovirus types, prediluted 1/10 with medium.

- Place the roller drum on a roller system and incubate for one hour at 35–37 °C.

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[1]Available on request from Chief, Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.

- Refeed the cells with 100 ml of medium containing 0–2% FCS (or newborn calf serum). Normally the cells can withstand serum-free medium for a few days; use as low a concentration of serum as possible.

- Incubate further until 75–95% CPE is reached, after two to three days.

6.3.2 Harvest and concentration of polio virus

When a homogenous suspension of virus particles is ultracentrifuged over a homogenous caesium chloride (CsCl) solution, a gradient is formed in which the particles will gradually move to the place of density that equals the specific density of the particles. This process will take from 36 to more than 50 hours. To reduce this centrifugation time Brunk and Leick (*3*) developed a method in which a portion of the virus particles is suspended in a CsCl solution with low salt concentration and another portion in a solution of high salt concentration. The mean concentration of CsCl should have a density that equals the density of the virus particles (for enteroviruses about 1.34 g/cm^3). Equal volumes of the two solutions are carefully layered on top of each other in an ultracentrifuge tube. During centrifugation a steep gradient will be formed in the middle part of the tube. From the start the virus particles will move towards this middle part much faster than they would have done in the original salt solution, because the differences in density are much larger. In this way a band of RNA virus may be formed in 18 hours. The density gradient (approximately linear) will be formed within 4–12 hours, and will remain stable thereafter. The first of the protocols given below is based on this principle.

Protocol 1

- Collect the supernatant from the poliovirus-infected cells and centrifuge for 30 minutes at 1 500 *g* and 4 °C. Store the supernatant at 4–8 °C (supernatant 1).

- Freeze and thaw the roller bottles with the remaining cells twice using a freezer at -70 °C (be sure that the bottles used can withstand this procedure!) During thawing, shake the contents well while ice crystals are still present. This will improve the disruption of cells.

- Pool the contents of the roller bottles and centrifuge for 30 minutes at 1 500 *g* and 4 °C.

- Collect the supernatant and pool with the stored supernatant 1.

- Ultracentrifuge the pooled supernatants for at least four hours at 130 000 *g* and 4 °C.

- Collect the pellet and resuspend in a small volume of distilled water.

- Centrifuge in an Eppendorf centrifuge for 10 minutes at room temperature at maximum speed (about 13 000 rpm).

- Collect the supernatant and add distilled water up to 12 ml. Take 1 ml and store as a back up.

- Add 1.8 g of CsCl to 6.2 ml of the remaining supernatant and 3.6 g of CsCl to 4.4 ml of the supernatant.

- Fill an ultraclear 11-ml ultracentrifuge tube, first with the high salt concentration mixture and on top of this the low salt concentration mixture. (For ultracentrifuge tubes of other capacities use the same volume ratios and CsCl concentrations.)

- Ultracentrifuge for 18 hours at 210 000 *g* and 4 °C.

- Collect the interphase band(s) with a fine needle through the wall of the ultracentrifuge tube. Check the presence and appearance of virus particles by electron microscopy.

The virus concentrate is now suitable for immunization purposes, but may be diluted with suspension medium, or buffered saline if used immediately. The titre of the virus concentrate may be determined as the cell culture infective dose 50% ($CCID_{50}$) in an *in vitro* assay (see Part II, section 9); although the concentrated virus may have lost much of its infectivity, it is still highly immunogenic (and therefore suitable for antiserum production and ELISA).

Protocol 2

- Freeze and thaw the roller bottles containing the poliovirus-infected cells three times at -20 °C or -70 °C. Shake the bottles vigorously a few times during thawing, while icicles are still present, to disrupt the remaining cells.

- After the third freeze/thaw cycle harvest the cell/virus/medium mixture and centrifuge for 20 minutes at 200 *g*.

- Centrifuge the supernatant for 30 minutes at 4 000 *g*.

- Ultracentrifuge the resulting supernatant for three hours at 110 000 *g*.

- Resuspend the resulting pellet in 1.3% of the original volume. This suspension is ready to use for immunization purposes. If not used immediately, which is preferable, store at -70 °C.

Infectivity is preserved following this procedure, and may be checked by microtitration. The titre is generally higher by a factor of at least 10 than the starting supernatant: from 10^5–10^6 in the starting material to 10^6–10^7 in the pellet.

An immunization scheme for larger animals, such as calves, is presented in Table 5. For rabbits, schemes similar to those shown in Tables 3 and 4 for the production of measles virus antisera, are suitable.

Table 5. Immunization scheme for the production of poliovirus antiserum (with CFA) in larger animals, such as calves

Day	Volume (ml)	Procedure
0	10	Bleed Check for undesired antibodies
0	2	Poliovirus + CFA (1:1), subcutaneous, multifocal
14	5	Poliovirus, no adjuvant, intraperitoneal
28	10	Bleed Check specific and nonspecific titres
42	10	Bleed Check specific and nonspecific titres
56	10	Bleed Check specific and nonspecific titres
64	10	Bleed Check specific and nonspecific titres
70	10	Bleed Check specific and nonspecific titres

CFA, complete Freund's adjuvant

If specific antibody levels have reached acceptably high titres, with absent or low cross-reactivity and/or undesired antibodies, larger volumes of blood may be collected, up to about 0.5 l on several occasions, with intervals of at least one week. The number of times that blood may be collected, and the volumes drawn on each occasion depend on the size and condition of the animal, and on the antibody titres. Do not pool the serum samples drawn on separate occasions before the specific and nonspecific titres have been determined and found to be acceptable. Also check on the cellular toxicity of the separate serum samples (see section 7).

Blood samples are drawn from the carotid artery or vein. The rise in titre of specific antibodies and the final titre are generally monitored using a serum neutralization assay (see Part II. section 10). Appropriate checks on cross-reactivity against other poliovirus serotypes (or enteroviruses) should be carried out. Generally cross-reacting titres will rise the longer the interval after immunization and the more frequently booster doses are administered. The older the animal, the higher the chance of pre-existing undesirable antibodies (in part due to previous unapparent natural infections). Cross-reactivity is most frequently observed between poliovirus serotypes 1 and 2.

6.4 References

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6.5 Selected further reading

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7. TESTING OF ANTISERA FOR USE IN VACCINE POTENCY TESTING

Before determining the potency of one component in a composite vaccine it is necessary to neutralize the other components in the vaccine using specific neutralizing antisera. Polyclonal hyperimmune antisera or panels of (mouse) monoclonal antisera made using the hybridoma cell culture technique may be used. Whether these antisera are produced in the quality control laboratory or are obtained commercially, it is essential to test their suitability prior to use in a vaccine potency test. This section summarizes the criteria these antisera should meet and how these may be tested.

7.1 Cross-reactivity

The antisera used for neutralizing the other components of a composite vaccine should not show any reactivity against the component to be tested. In the case of trivalent oral poliomyelitis vaccine (OPV) the production of antisera that are not cross-reactive may cause some problems (see section 6.3).

Cross-reactivity can be tested in a simple neutralization assay (see Part II, section 10) in which the antiserum is challenged with a known amount of virus of each component. This is illustrated in the following example.

The aim is to produce a specific neutralizing antiserum against poliovirus type 1 for use in an OPV potency test. A rabbit is immunized with a purified amount of poliovirus type 1 (Sabin). The antiserum is tested in a microtitre serum neutralization assay with Hep-2_c cells, giving the following results:

Titre of type 1	*Titre of type 2*	*Titre of type 3*
4096	64	*16*
2^{12}	2^6	2^4

Thus in three serum neutralization tests, dilutions of the type 1 antiserum are challenged with approximately 100 $CCID_{50}$ of type 1, type 2 and type 3, respectively, resulting in a 50% cytopathic effect (CPE) at the respective dilutions of 1/4 096, 1/64 and 1/16. If this antiserum is now diluted 64 times it will no longer cross-react against type 3, but there will still be a slight cross-reactivity against type 2 (50% CPE when used at this dilution). When diluted slightly more than 64 times, say 100 times, the antiserum is monospecific, and will show neutralizing activity only against type 1.

Other methods of eliminating cross-reacting antibodies are preadsorption and affinity purification. In the former, the cross-reacting antibodies in the serum are adsorbed onto antigens towards which the antiserum shows undesired reactivity. For example, an antiserum raised against type 1 (Sabin), but showing cross-reactivity against type 2 can be purified by adsorbing the antibodies causing this cross-reactivity to type 2. Type 2

antigens are coated onto either a solid phase, such as a sepharose column, or beads which can be removed easily after adsorption either by centrifugation or magnetically. The advantage of this method is that, compared with the dilution method, a strongly reacting and specific antiserum can be obtained. Disadvantages are the costs, the need for experienced staff to carry out these procedures, and the amount of work involved.

Elimination of cross-reactivity by affinity purification involves the positive selection of the desired antibodies. In principle the method is the same as described above, except that the beads or the solid phase (column) are coated with antigens towards which the antiserum is raised. Once the desired antibodies have reacted with these antigens, the column is washed to eliminate all non-reacting antibodies, after which the bound antibodies are eluted with a solution containing a high salt concentration or a buffer with a specific pH (depending on the characteristics of the solid phase and the antibody class). The advantages and disadvantages are similar to those mentioned above, except that this method causes more loss of reactivity, owing to denaturation of antibodies or loss of low avidity antibodies during the washing procedure. Another disadvantage of this method is that antibodies that react with shared epitopes of both Sabin types are not eliminated, but are specifically selected, leaving undesired cross-reactivity. To eliminate this, the adsorption procedure described above can be followed after positive selection. A further disadvantage is the possibility that the eluted antiserum is highly diluted. It may be necessary to concentrate the antibody solution either by precipitation using ammonium chloride, by dialysis through a semipermeable membrane, or by freeze-drying.

7.2 Minimum neutralizing capacity

In principle, the minimum neutralizing capacity of the antiserum required depends on the amount of live virus particles to be neutralized. This in turn depends on the purpose for which the antiserum is needed: complete neutralization of a certain amount of virus with a high titre, or neutralization of a limited amount of virus in a known mixture of viruses, such as a composite vaccine.

Complete neutralization of a large amount of virus may be needed, for example, to investigate the presence of small amounts of contaminating viruses in a bulk vaccine during the production process. The vaccine virus of known titre has to be neutralized completely in order to find possible contaminating viruses. Strongly reacting antisera with a high specificity are required — strongly reacting, because the vaccine should not be diluted before testing; highly specific to avoid the risk that detection of a possible contaminating virus is missed because of cross-reactivity.

If, however, the antiserum is needed for potency testing of a combined vaccine, such as trivalent OPV, the antiserum need only neutralize the component(s) of the vaccine not being tested, so that the component under test is the strongest component. If the vaccine is tested in a microtitre test, the titre is determined by the CPE in the end-point dilution. This CPE will be caused by the strongest component of the vaccine. The following example should make this clear.

The aim is to determine the potency of the three components of a trivalent OPV. The (still unknown) titres of these components are type 1, $10^{6.0}$: type 2, $10^{5.0}5.0$; type 3, $10^{5.8}$; total, $10^{6.23}$. In an *in vitro* assay without using antisera, therefore, 50% CPE would be found in the dilution 10^{-6}, caused by type 1, as it is the strongest component. (Actually 50% CPE would be found in dilution $10^{-6.23}$, so the dilution 10^{-6} would produce slightly more than 50% CPE.) To titrate type 2, it is necessary to neutralize both type 1 (in this example, more than $10^{1.0}$) and type 3 (more than $10^{0.8}$) to leave type 2 as the strongest component.

Normally, prior to the test, the titres of each component are unknown, only the approximate titres and the probable difference in titres between the components are known. Since all three components are generally equally stable, all three components will be equally changed in titre if the vaccine has been damaged by improper storage or transport. In the case of trivalent OPV, the antisera needed for potency testing should be able to neutralize at least 10^2 units of each component. To be on the safe side it is advisable to use slightly stronger antisera.

7.3 Determination of the dilution in which a given antiserum should be used in a potency test

Suppose an antiserum is available which was raised against poliovirus type 1 (Sabin) with the following neutralization titres:

Titre of type 1	*Titre of type 2*	*Titre of type 3*
1/4 096	1/64	1/16
2^{12}	2^6	2^4

As explained before, this antiserum should be diluted slightly more than 64 times in order to lose all cross-reactivity. If diluted 64 times, the remaining neutralizing capacity against type 1 is 1/64 ($2^{12} \div 2^6 = 2^6$ or 1/64). This means that if the antiserum is diluted 64 times there is still a slight cross-reactivity against type 2.

If the antiserum is diluted 100 times, how much virus can it still neutralize? If diluted 4 096 times this antiserum is capable of almost neutralizing 100 $CCID_{50}$ (the challenge dose). Undiluted it is able to neutralize 4 096 x 100 $CCID_{50} = 10^{5.6}$. If the antiserum were prediluted 100 times, it would be 100 times less potent than the undiluted antiserum and able to neutralize 40.96 times more virus than a challenge dose of 100 $CCID_{50}$ or 4 096 $CCID_{50}$, which is $10^{3.6}$. This is not enough neutralizing power to neutralize the viral components not being tested for, that is, $10^{5.0}$ of type 2 and $10^{5.8}$ of type 3, so 100-fold dilution will eliminate the cross-reactivity and still serve to test the virus.

7.4 Toxicity to cells

Having dealt with cross-reactivity, and having determined the dilution at which the

antiserum will be used in a potency test using cell culture techniques, it is essential to test the antiserum in this dilution for toxicity on the cells to be used in the potency test, which could interfere with the outcome of the test. This is one of the main reasons why cell controls should be included in every potency test using cell culture techniques. Since the toxicity will generally show very early, it is essential to check the cell controls the day after adding the antiserum.

7.5 Conclusions

- Check for cross-reactivity in a serum neutralization assay. The simplest way to eliminate cross-reactivity is by diluting it out. Dilute by slightly more than the reciprocal value of the neutralization titre of the highest cross-reacting component.

- If it is necessary to neutralize all the virus of known type and of known titre the antiserum needs to be highly specific and strong; after challenge with the test virus no CPE should appear.

- If the antiserum is needed for potency testing of composite vaccines, the antiserum should be capable of neutralizing the other components to such an extent that the component to be tested is the strongest component. In general it should be slightly stronger than the expected difference between the strongest and weakest component.

  Formulae:

  (reciprocal value of neutralization titre/reciprocal value of dilution) $\times$ 100 $CCID_{50}$ = remaining neutralizing capacity in $CCID_{50}$

  (reciprocal value of neutralization titre/desired amount of virus to be neutralized) $\times$ 100 $CCID_{50}$ = number of times the antiserum has to be diluted to be able to neutralize the given desired amount of virus.

  Check test: Challenge the diluted antiserum with the amount of virus it should be able to neutralize and no CPE should appear.

- Each serum (fetal or newborn calf serum, or other sera used in cell culture, as well as antisera, or patient sera in a serum neutralization assay) used in an assay using cell culture techniques, must first be tested for toxic activity towards the cells used in the assay. Not all cells are equally sensitive to toxic substances. In general toxicity shows soon after the serum has been added. Include cell toxicity controls in each assay, and check cell growth every day.

8. PREPARATION AND PRESERVATION OF CELL CULTURES

Quality control laboratories use cell lines (heterodiploid) rather than primary or diploid cell strains as substrates for determining the identity, potency and safety of live viral and bacterial vaccines. In order to minimize the variation in sensitivity, standard cell substrates for poliovirus and other agents have been established which have been proven to be susceptible to the agent being tested.

Healthy cells in an early stage of growth are essential to maintain susceptibility. Subculturing should therefore be performed on a regular basis. Cell culture media used for growth and maintenance of the cells can also influence susceptibility. A defined medium which supports growth but has no negative influence on cell susceptibility is essential.

The passage level (number of subcultures) of the cells being used for laboratory testing of vaccines also influences their susceptibility. For this reason a cell suspension obtained after trypsinization of cell monolayers low in passage number should be preserved (see section 8.5.3) and kept at -70 °C or below to serve as a back-up (cell bank) in case of casualties, such as contamination, spontaneously occurring cellular changes or a dramatic decrease in susceptibility. Cells derived from the cell bank can be kept in culture until the maximum passage level has been reached, or any of the above mentioned casualties occur. To avoid contamination, for example by mycoplasma, it is recommended that cells are discarded after 15 passages. Contamination of the cell substrate by microorganisms, such as bacteria, fungi, mycoplasma and viruses, can cause changes in susceptibility. Cell substrates should be tested periodically for the absence of these contaminants (see section 8.4).

In handling cell cultures, laboratory personnel must be concerned not only with preventing microbial contamination of the cultures, but also with avoiding contamination of the working environment with cell culture materials. All cultures must be considered potentially hazardous, whether inoculated with an infectious agent or not. Cross-contamination between different cell types, especially continuous cell lines, is an ever-present hazard. To avoid this, *different cell types should never be processed at the same time*.

8.1 Selection of cell lines for vaccine potency testing

Cells from the Hep-$2_{Cincinnati}$ cell line (derived from a human laryngocarcinoma) are universally accepted as the best for potency testing of oral poliomyelitis vaccines.

Vero cells (from the kidney of the African green monkey) are appropriate for potency testing of measles, mumps, yellow fever and diphtheria vaccines.

RK-13 cells (from rabbit kidney) are commonly used for potency testing of rubella vaccines.

Heterodiploid cell lines have the following characteristics:

- more than 25% of the cells have two or more groups of chromosomes;
- cellular multiplication is theoretically unlimited;
- acid production is less than for diploid cells;
- they can be easily propagated.

8.2 Cell culture media

Cell culture media can be divided into two main categories, growth media and maintenance media. These contain various combinations of synthetic and natural ingredients. Growth medium have a high added serum content (5–10%). They promote rapid cell growth. Maintenance medium have a low added serum content (2–5%). They are also used to dilute antisera and virus preparations.

Fetal bovine serum (FBS) is good for promoting cell growth because of the absence of viral inhibitors. If serum from other sources is used, it must be pretested for the presence of inhibitors to the substances being studied. All sera used for cell cultures should be inactivated at 56 °C for 30 minutes.

8.3 Selection of medium for the propagation of cell lines

Medium 199 is the most suitable medium for Vero cells, although Eagle's minimal essential medium (MEM) is also appropriate.

Eagle's MEM is used as a growth and maintenance medium for Hep-2_c and RK-13 cells.

8.4 Detection of contamination in cell cultures

Contaminants come from the physical surroundings or the biological environment. The first type can be propagated by dirty objects, draughts in the working area, or sloppy technique on the part of the operator. The second type can come from inappropriate sterilization of culture media and glassware used in cell culture handling. In certain cases latent infections are present in the cells. Mycoplasma, bacteria, viruses, fungi, yeasts and other types of microorganism are examples of exogenous contamination which can be detected by appropriate culture methods, as described below.

Suspend a portion of the cells obtained by trypsinization of the cell culture in medium which does not contain antibiotics and verify the sterility as described in sections 8.4.1–8.4.4.

8.4.1 Bacterial sterility

Procedure A

- Inoculate 0.2 ml of the suspension onto the surface of two blood agar plates.
- Invert the plates and incubate them for 7 days at 37 °C and 25 °C respectively.
- Examine them daily and record the results of any growth detected.

Procedure B

- Inoculate 0.5 ml of the suspension into thioglycolate broth.
- Seal the culture hermetically and incubate for 7 days at 37 °C.
- Examine daily to determine growth, cells suspended in the upper part of the medium should not be confused with bacterial proliferation.

8.4.2 Fungal sterility

- Inoculate 0.2 ml of the suspension onto the surface of a Sabouraud agar slant.
- Incubate for two weeks at room temperature.
- Examine growth periodically.

8.4.3 Mycoplasma sterility

Mycoplasmas invalidate the final reading of the titre because of their cytopathic effects in cell cultures. The most common causes of intralaboratory infection by mycoplasmas are contaminated cell cultures, sera of animal origin, trypsin, and a break in sterile technique.

To detect human and animal mycoplasmas which can be cultivated *in vitro*, the following methods are useful:

Procedure A

- Inoculate 0.2 ml of the cell suspension or spent culture medium in a tube with broth for mycoplasma, and incubate at 37 °C.
- Given that it is difficult to detect mycoplasma proliferation in broth culture, this should be subcultured on agar after six days of incubation (see Procedure B).

Procedure B

- Inoculate 0.2 ml of the cell suspension or spent culture medium onto each of two

plates of mycoplasma agar. Tilt the plates to distribute the inoculum evenly over the surface.

- Inoculate two agar plates with 0.2 ml of sterile culture medium in the same way to serve as a control.

- Incubate one test plate and one control plate aerobically at 37 °C. These plates should be sealed with cellophane tape and inverted.

- Incubate one test plate and one control plate at 37 °C in an anaerobic atmosphere.

- Examine the plates for colonies in a microscope with a magnification of 100X. The plates must be incubated for three weeks in order to declare them negative. The microscopic examination can be facilitated by staining using the Dienes method. Mycoplasma colonies stained in this way have a dark blue centre and a light blue periphery. Bacteria and L-form colonies lose colour after 30 minutes, while mycoplasma retain colour.

8.4.4 Viral sterility

Viruses causing cytopathic effects

Incubate uninfected monolayers for seven days at 37 °C and examine with a microscope to determine cytopathic changes in the cells. These changes can be characterized by cell rounding, agglutination, total or partial destruction, separation from the surface of the culture flask, granulation, and formation of giant cells or syncytia. To increase the chance of detection of viral contaminants, several types of cells may be used.

Viruses not causing cytopathic effects

Certain viruses multiply in cell culture without causing cytopathic effects and may be detected by direct haemadsorption, the adherence of groups of erythrocytes to the monolayer following addition of a 5% suspension of erythrocytes (chicken, rabbit, guinea-pig, monkey) in a phosphate-buffered saline solution at pH 7.4 containing penicillin (1 000 IU/ml). Incubate for 30 minutes at 37 °C. Wash twice with buffer and read the results with a microscope.

Certain non-cytopathic viruses can be detected by their property of forming plaques in agar. These plaques are infected areas in the cell membrane which do not absorb neutral red, a vital stain.

Other non-cytopathic viruses can cause interference, which causes a lowering in titre of a cytopathic virus of more than 10^2. This is also an indication that the monolayer is contaminated with a non-cytopathic virus.

The fluorescent antibody technique can be used for detection of non-cytopathic viruses. Because of its specificity, this technique is most useful in diagnostic tests performed to determine the type of virus causing the contamination.

8.5 Preparation of cell cultures

8.5.1 Introduction

Cell cultures are trypsinized to obtain a cell suspension. This cell suspension can be used for the preparation of new cell cultures (subcultures) and for vaccine potency testing. The desired concentration is based either on counting the cells or on an arbitrarily chosen split ratio which depends on the type of cells being used: for Hep-2_c, one volume of cells to 12 volumes of media (1:12); Vero, 1:6; RK-13, 1:4. The ratios should be optimized for each laboratory.

8.5.2 Materials

Sterile equipment

- Disposable or reusable pipettes; 1, 5, 10 ml, and Pasteur pipettes
- Glassware: bottles and graduated cylinders of various sizes
- Plastic tissue culture flasks; 25, 75 and 150 cm^2
- Teflon-coated magnetic stirring bars
- Polypropylene-sealed ampoules

Other equipment

- Haemacytometer, Neubauer or Bürker-Türk
- Magnetic stirring base
- Binocular microscope
- Inverted microscope
- Incubator
- Water baths; 37 °C, and 56 °C
- Mechanical safety pipetting device
- Disposable glass culture tubes
- Freezers, -20 °C, -70 °C
- Refrigerator, 2–8 °C

Reagents

- Fetal bovine serum, any supplier. Store at -20 °C or colder. Before use thaw in a water-bath (5–15 °C) and store at 2–8 °C for no longer than one month.

- Penicillin and streptomycin. A stock concentrate containing benzylpenicillin 10 000 IU/ml and streptomycin 10 000 μg/ml. Store at -20 °C, before use thaw in cool tap water and store at 2–8 °C for no longer than two weeks.

- Glutamine, 200 mmol/l. Store at -20 °C. Before use thaw in cool tap water and store at 2–8 °C for no longer than one month.

- Medium: For a particular type of cell the same medium is used for maintenance and for growth in the potency tests to be described in this manual (see section 8.2). On initial day of use add: fetal bovine serum, for growth medium, 10% by volume, for maintenance medium, 5% by volume; penicillin and streptomycin, 10 ml/l; glutamine, 10 ml/l.

- Trypsin 0.25% in citrate solution. Store at -20 °C. Thaw in a water-bath at 37 °C and warm it to 37 °C for use. Trypsin may be partially thawed overnight in the refrigerator (2–8 °C) prior to being placed in the water-bath.

- Trypan blue, 0.4% in normal saline.

- Phosphate buffered saline, pH 7.2 (PBS).

- Dimethylsulfoxide (DMSO).

8.5.3 Procedures

Trypsinization of cell cultures

- Warm freshly thawed trypsin to 37 °C in a water-bath.

- Determine the number of culture flasks to be trypsinized.

- Observe the culture flasks through the inverted microscope. The following characteristics should be observed: confluent monolayer; healthy and viable cells, no or little granularity; no cytopathic effect (CPE), or other changes in cell growth present.

- Decant the spent medium from the cell culture flasks.

- Wash the cell layer gently with PBS.

- Add 0.25% trypsin solution to the monolayer: 0.5 ml to 25-cm^2 culture flasks, 1.0 ml to 75-cm^2 culture flasks, and 2.0 ml to 150-cm^2 culture flasks.

- Incubate the culture flasks in the incubator at 36 °C until the cells detach from the surface.

- Add growth medium to each cell culture flask, 10.0 ml to 25-cm^2 culture flasks, 20.0 ml to 75-cm^2 culture flasks, 30.0 ml to 150-cm^2 culture flasks. The serum in the growth medium stops the action of the trypsin still present.

- Aspirate gently to loosen the cells from the surface of the flasks and pool the cell suspension from each flask into one bottle.

- Resuspend cells in growth medium to the desired concentration, based either upon counting the cells or upon an arbitrary split ratio.

- Seed fresh culture flasks (see below)

Cell counting

Accurate numbers in a cell suspension can be calculated by counting the cells in a haemacytometer (Neubauer or Bürker-Türk). It is important to disperse the cells thoroughly.

- Prepare a 1/10 dilution of the original cell suspension (obtained by trypsinization) using 0.4% trypan blue as follows: 0.1 ml of undiluted cell suspension, 0.9 ml of 0.4% trypan blue.

- Mix well with a fine Pasteur pipette and immediately aspirate a sufficient volume to fill the haemacytometer.

- Count all viable (unstained) cells in the 8 (1.0-mm) squares constituting the four corners of each of the two haemacytometer chambers, omitting cells lying on the upper line and left line of each square. The volume of each corner square = 0.1 mm^3 (1.0 x 1.0 x 0.1).
  Note: To perform an accurate cell count, 75% of the cells in the suspension should be viable and the difference between the cell counts in the two haemacytometer chambers should be minimal.

- If cell "clumping" (aggregation) is observed, disaggregate the clumps by vigorous aspiration through a fine needle or Pasteur pipette a few times.

- Calculate the mean count of the cells per 0.1 mm^3.

- Calculate the total number of cells in the suspension using the following formula:
  $N = m \times tb \times V \times 10^4$
  where:
  N = number of cells in the cell suspension
  m = mean of cell counts per 0.1 mm^3
  tb = correction to the Trypan blue dilution (10 in the 1/10 dilution with Trypan

blue)
V = volume of the original cell suspension in ml
10^4 = conversion factor for counting chamber.

Example:

Let m = 28, tb = 10, V = 25 ml
N = 28 x 10 x 25 x 10^4 = 70 x 10^6 cells
$C1$ (cell concentration per ml) = N/V = 70 x 10^6/25 = 2.8 x 10^6 cells per ml or 8 x 10^5.

- Calculate the dilution factor (d) to obtain the working cell suspension ($C2$), the cell concentration (per ml) to be used in subsequent procedures:
  d = C1/C2
  If $C2$ = 2 x 10^5
  d = 28 x 10^5/2 x 10^5 = 14
  The cell suspension can be prepared by adding the required amount of medium.

- Dispense the cell suspension in growth medium ready for use. In this example, 14 volumes of media for each volume of cell suspension.

- Clean haemacytometer and cover slip immediately after use with 70% ethanol.

Preparation of new cell cultures

Cells are maintained in tissue culture flasks of different sizes. The volumes used in the preparation of new cultures depend on the type of cell and size of flask being used (Table 6).

Table 6. Requirements for the preparation of cell cultures

Cell type	Container (cm^2)	Volume (ml)	Cells per ml
Hep-2_c	150	80	5×10^4
	75	30	5×10^4
	25	10	5×10^4
Vero	150	80	10×10^4
	75	30	10×10^4
	25	10	10×10^4
RK-13	150	80	10×10^4
	75	30	10×10^4
	25	10	10×10^4

- Calculate the total volume (ml) of cell suspension required;
  Total volume = number of containers x volume + 50*
  *To allow for losses during seeding

Example

The total volume required to seed eight 75-cm^2 flasks = (8 x 30) + 50 = 290 ml.

- Calculate the volume of cell suspension required:
  total volume x C2/C1
  For C2 = 10 x 10^4 and the cell count in the example above
  290 ml x 19 x 10^4 cells/ml/(*C*1) 2.8 x 10^6 cells/ml = 10^4 ml of cell suspension.

- Calculate the volume of growth medium (GM) required:
  total volume − volume of cell suspension = volume of GM

Example:

290 ml − 10.4 ml = 279.6 ml of GM.

- Mix growth medium and cell suspension using a magnetic stirring bar.

- Label flasks with cell line, passage number and date of seeding.

- Dispense the appropriate amount of cell suspension into the flasks.

- Incubate the culture flasks at appropriate temperature.

- Refeed cultures with maintenance medium 3–5 days after seeding and the day before the next subculture (the optimum times should be established for each laboratory and for each type of cell being used).

- Record all information required on a cell line record sheet.

Preparation of cell suspension

Cell suspensions are prepared for live virus vaccine potency testing. The cells are suspended in growth medium at the concentration (*C*2) required.

- Calculate the volume of cell suspension required:
  Cell concentration per ml (*C*1) = 2.8×10^6 cells/ml
  Working cell suspension *C*2 = 2.0×10^5 cells/ml in this example
  C2/C1 x volume ordered = volume required

Example:

280 ml cell suspension
$(2.0 \times 10^5/2.8 \times 10^6) \times 280$ ml = 20 ml of cell suspension (*C*1) needed.

- Calculate the volume of growth medium required:
  (Total volume)−(Volume of C1) = (volume of growth medium)

Example:

280 ml − 20 ml = 260 ml of growth medium

- Mix the calculated volumes of growth medium and concentrated cell suspension into a labelled bottle of appropriate size.

- Add a magnetic stirring bar to the bottle.

- Place the bottle on a magnetic stirring base and turn it on to keep the cells in suspension.

- The suspension must be used within 6 hours of preparation.

Preservation of cell cultures

Cells, viable, in good condition and preferably low in passage number, should be preserved and kept as a back-up in case casualties occur with the cells maintained in culture. It is possible to maintain stocks of cells in a viable state for long periods at low temperature by the addition of preservatives such as dimethylsulfoxide (DMSO) to the cell growth medium. The essential features of the method are to freeze the cells slowly, keep

them at a temperature below -70 °C while frozen and to thaw them rapidly for the preparation of fresh cell culture stocks. The method is as follows.

(a) Freezing

- Use only culture flasks with cells that are in a healthy state.
- Remove cells by trypsinization (section 5.3.1).
- Resuspend cells in growth medium and perform a cell count according to the method described in section 8.5.3.
- To pellet the cells, centrifuge at 100 *g* for 10 minutes.
- Adjust the cell concentration to 5–10 x 10^6 cells per ml in growth medium containing 10% DMSO.

Example

cell count = 2 x 10^6 cells per ml
volume of cell suspension = 60 ml
total amount of cells 60 x (2 x 10^6) = 120 x 10^6 cells
cell pellet = 3 ml
(approximately 40 x 10^6 cells per ml)
dilution factor d = (40 x 10^6)/(10 x 10^6) = 4
For freezing, a cell concentration of approximately 10 x 10^6 cells per ml one volume of the cell pellet can be obtained by mixing with three volumes of growth medium containing 10% DMSO.

- Dispense in 1-ml volumes in clearly labelled polypropylene-sealed or glass-sealed ampoules. The former are suitable for storage in gaseous nitrogen, the latter for storage in liquid nitrogen. The label should indicate cell type, passage number, cell concentration and date of freezing.
- Freeze vials/ampoules slowly. Ideally the temperature should drop by 1 °C per minute. Place vials/ampoules wrapped in cotton wool in a polystyrene container with a wall thickness of 15 mm and place this in a -70 °C freezer overnight.
- Transfer the ampoules to the gaseous phase of liquid nitrogen (-150 °C to -180 °C) and when glass-sealed ampoules are used, to the liquid nitrogen (-196 °C) storage containers. For long-term storage of cells (i.e. a period of years) store cells in liquid nitrogen. Cells can be stored in a freezer at -70 °C for a few months.

(b) Thawing

- Remove the vial/ampoule from gaseous/liquid nitrogen and transfer immediately to a water-bath of 36 °C.

Note: When using gaseous/liquid nitrogen containers, visors and heavy-duty gloves must be worn to avoid injuries from nitrogen splashes or explosion of imperfectly sealed ampoules.

- When the contents are completely thawed, wipe the outside of the vial/ampoule with alcohol to reduce bacterial contamination.

- Transfer the cell suspension to a culture flask.

- Add very slowly, drop by drop, sufficient growth medium for the production of a cell monolayer.

Note: The viability of the thawed cells is severely affected if growth medium is added rapidly.

- Incubate the culture flasks overnight at 36 °C.

- Carefully decant the medium, to remove the DMSO, and add fresh growth medium with no DMSO.

(c) Interpretation

- Check inoculated culture flasks at least once a week.

- Test samples of the cells every four weeks for mycoplasma, according to the method described in section 8.4.3.

- Maintain cells until found unacceptable for use; for instance because of:
  - loss in virus susceptibility
  - change in growth characteristics
  - slow formation of a complete monolayer
  - detection of any contamination.

PART II. POTENCY CONTROL OF LIVE VIRAL VACCINES

CONTENTS

9. LIVE ORAL POLIOMYELITIS VACCINE

9.1 Introduction

The potency of trivalent live oral poliomyelitis vaccine (OPV), both total virus content and individual serotypes separately, is determined in an *in vitro* assay using cells of the Hep-$2_{Cincinnati}$ line. Titration of the individual serotypes, using mixtures of type- specific antisera, must be undertaken by the manufacturer and the national control laboratory for release of the vaccine. Total virus content (type 1 + type 2 + type 3) of trivalent OPV is determined when samples are sent from the field or to monitor the cold chain.

The preparation to be assayed and the reference preparation are diluted in an appropriate medium. It is convenient to make tenfold dilution steps of the virus suspensions initially, but the dilutions to be inoculated into cell cultures should be prepared in $10^{0.5}$ steps. The dilution range selected should encompass at least three dilutions that will infect between 0% and 100% of the cultures inoculated.

For titration of the individual serotypes, equal volumes of the selected virus dilution and the appropriate antisera are mixed in rows of 8 wells of a flat-bottomed microtitre plate. For titration of the total virus content, medium should be added instead of the antiserum mixture. After a 3-hour incubation period at 35–36 °C followed by the addition of a fixed amount of a suitable cell suspension of Hep-$2_{Cincinnati}$ cells, the plates are further incubated at 35–36 °C for 5–7 days. The potency of a reference preparation should be determined in parallel.

The cells are examined for the presence of a specific viral cytopathic effect on days 3–5, with a final reading on days 5–7, followed by fixing and staining of the viable cells. The observations are recorded and the titre in $CCID_{50}$ per human does calculated on the basis of the final observation.

9.2 Materials

9.2.1 Standard equipment

- microtitre plates (96 wells, flat-bottomed)
- multichannel pipettes, with tips to deliver 0.05-ml volumes
- micropipettes to deliver 0.05-ml and 0.1-ml volumes
- incubator
- plate sealers, if CO_2-incubator not available
- vacuum source

9.2.2 Antisera

Hyperimmune sera specific for each of the three types of poliovirus are prepared by inoculation of the appropriate antigen into a suitable animal.

To determine the titre of the individual serotypes of trivalent OPV separately, mixtures of these type-specific antisera should be prepared to contain sufficient neutralizing antibodies to inhibit at least 10_3 infectious doses of the homologous virus (see section 7).

Therefore, mixture 1 will contain antiserum against poliovirus types 2 and 3, sufficient to allow determination of the titre of poliovirus type 1; mixture 2 will contain antiserum against poliovirus types 1 and 3, sufficient to allow determination the titre of poliovirus type 2; mixture 3 will contain antiserum against poliovirus types 1 and 2, sufficient to allow determination of the titre of poliovirus type 3.

Another possibility is to use mixtures of monoclonal antibodies with the same neutralizing capacity as the polyclonal antisera.

9.2.3 Reference preparation

For each assay of trivalent OPV vaccine include a vial of live attenuated poliomyelitis vaccine, the titre of which has been well established, as a working reference preparation to control the accuracy and reproducibility of the testing system.

9.2.4 Medium and dilutions

Test medium: Eagle's minimal essential medium (MEM) supplemented with 5% fetal bovine serum.

Diluent: Eagle's MEM supplemented with 2% fetal bovine serum.

Using the refrigerated diluent, prepare tenfold dilutions. The subsequent dilutions for inoculation into the microtitre plates are prepared in $10^{0.5}$ steps. Table 7 shows a sample dilution series.

Table 7. Range of dilutions used in testing the potency of trivalent oral poliomyelitis vaccine

Volume of vaccine dilution (ml)	Volume of diluent added (ml)	Dilution
0.20 (undiluted vaccine)	1.80	$10^{-1.0}$
0.20 (from $10^{-1.0}$)	1.80	$10^{-2.0}$
1.25 ml (from $10^{-2.0}$)	2.70	$10^{-2.5}$
0.30 ml (from $10^{-2.0}$)	2.70	$10^{-3.0}$
0.30 ml (from $10^{-2.5}$)	2.70	$10^{-3.5}$
0.30 ml (from $10^{-3.0}$)	2.70	$10^{-4.0}$
0.30 ml (from $10^{-3.5}$)	2.70	$10^{-4.5}$
0.30 ml (from $10^{-4.0}$)	2.70	$10^{-5.0}$
0.30 ml (from $10^{-4.5}$)	2.70	$10^{-5.5}$
0.30 ml (from $10^{-5.0}$)	2.70	$10^{-6.0}$
0.30 ml (from $10^{-5.5}$)	2.70	$10^{-6.5}$
0.30 ml (from $10^{-6.0}$)	2.70	$10^{-7.0}$

The range of dilutions used will depend on the type of virus and the formulation of the vaccine under test. The range chosen should include the expected titre of the vaccine type being tested.

Prepare the dilutions in stoppered tubes and store them refrigerated (2–8 °C).
If mixing is done on a Vortex mixer rather than simply by pipetting, it may be preferable to omit the serum in the diluent to avoid frothing.

9.2.5 Cells

Use cells of the Hep-$2_{Cincinnati}$ line (Hep-2_c).[1] The passage level of these cells, which should be documented, should be within 15 passages of the tested stock. Watch for any change in growth characteristics such as excess acidity of medium or slowing in the time taken to achieve a complete monolayer.

The number of cells used in the assay is usually about 1–2 x 10^5 cells per ml of test medium. This concentration should provide a confluent monolayer in microtitre plate

[1] Available on request to national control laboratories and poliomyelitis diagnostic laboratories from Chief, Expanded Programme on Immunization, World Health Organization, 1211 Geneva 27, Switzerland.

medium. This concentration should provide a confluent monolayer in microtitre plate wells within two to three days. However the cell concentration should be optimized in each laboratory.

9.3 Procedure

The procedure is standard for determining the potency of trivalent OPV, both for total virus content (type 1 + type 2 + type 3) and individual serotypes separately using the microneutralization test (Figures 4 and 5).

- Add 0.05 ml of test medium to all wells of the plates for the test vaccine and reference preparation in which total virus content will be measured (plates 4 and 5).

- Add 0.05 ml of antiserum mixture 1 to all wells of the plate for titration of type 1 in trivalent OPV (plate 1).

- Add 0.05 ml of antiserum mixture 2 to all wells of the plate for titration of type 2 in trivalent OPV (plate 2).

- Add 0.05 ml of antiserum mixture 3 to all wells of the plate for titration of type 3 in trivalent OPV (plate 3).

- Add 0.05 ml of vaccine dilution to a column of 8 wells, in each of the four plates in which the trivalent vaccine is to be titrated. Start by transferring the highest dilution into column 10, and use the same dropper pipette for the whole range (plates 1, 2, 3 and 4).

- Add 0.05 ml of the dilutions made of the working reference preparation to a column of 8 wells of the plate for titration of the reference preparation. Again start by transferring the highest dilution into column 10; the same dropper pipette can be used for the whole range (plate 5).

- Add 0.05 ml of test medium, to equalize the volumes, to each of the cell control (CC, plates 4 and 5, columns 11 and 12) wells and to the wells that are to serve as serum toxicity controls (SC, plates 1–3, columns 11 and 12).

- Cover the plates with lid when using a humidified carbon dioxide incubator, or wrap them in aluminium foil or put the plates in zipper bags if a normal incubator is used.

- Incubate all plates 1–5 at 36 °C for 3 hours to allow binding of antisera to the homologous serotypes.

- During this time, wash Hep-2_c monolayer flask cultures, trypsinize, count cells and prepare a cell suspension in test medium to contain approximately 1–2 x 10^5 cells per ml (see section 8).

- Remove the plates from the incubator and add 0.1 ml of cell suspension to all wells of all plates. Avoid touching the wells with micropipette tips during this procedure.

- Cover the plates with a lid when using a humidified carbon dioxide incubator, or seal the plates with nontoxic adhesive plate sealers if a normal incubator is used.

- Incubate all plates at 36 °C for 5–7 days.

- During this time, monitor the cell control wells to ensure that the cells are forming a monolayer. Also score the positive wells starting at day 4 and keep good records of the data.

- After 5–6 days of incubation (depending on the quality of the cells), read the plates by microscope before fixing and staining with crystal violet. The optimum incubation period should be established in each laboratory.

- Air dry the plates.

- Read the plates visually and record results:
  stained cells present = no virus growth or toxicity
  stained cells absent = virus growth or toxicity.

- Calculate the titre in $CCID_{50}$ per human dose using the Kärber or Reed-Muench formula.

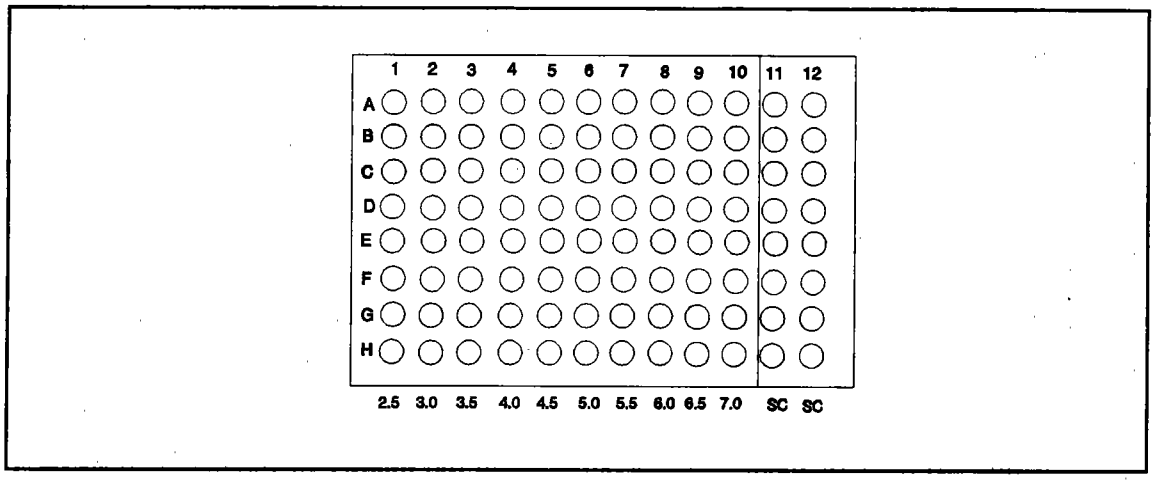

Figure 4. Arrangement of microtitre plate for OPV potency testing: plates 1—3.

Wells for serum toxicity control (SC), columns 11 and 12:

0.05 ml of serum mixture
0.05 ml of test medium
0.10 ml of cell substrate

Wells for vaccine dilution, columns 1 to 10:

0.05 ml of serum mixture
0.05 ml of vaccine dilution
0.10 ml of cell substrate

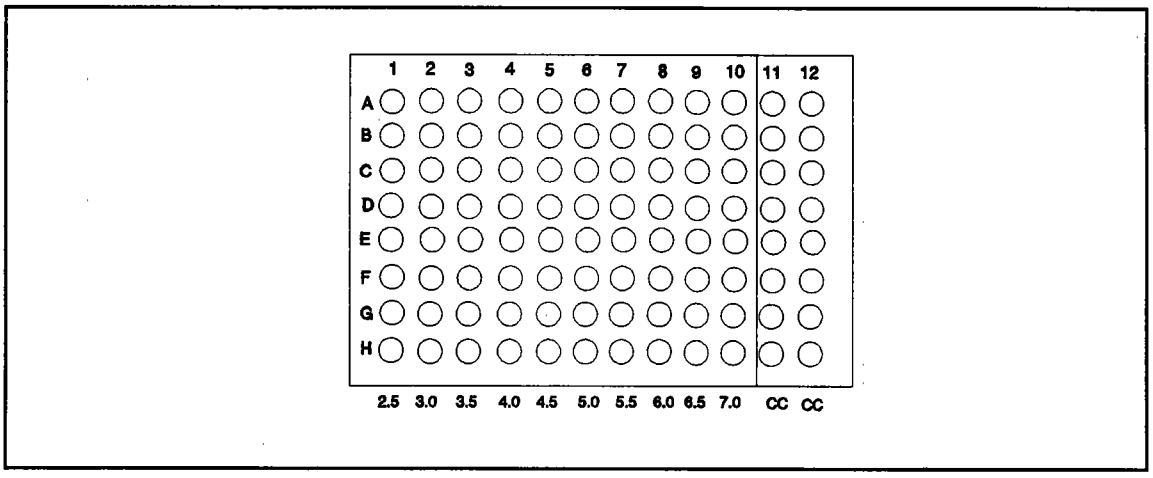

Figure 5. Arrangement of microtitre plate for OPV potency testing: plates 4 and 5.

Wells for cell control (CC), columns 11 and 12:

0.10 ml of test medium
0.10 ml of cell substrate

Wells for vaccine dilution, columns 1 to 10:

0.05 ml of test medium
0.05 ml of vaccine dilution
0.10 ml of cell substrate

9.4 Evaluation of results

For the test to be considered valid:

- The working reference preparation should be within $10^{0.5}$ of its established titre. This is based on the geometric mean titre of all valid assays for the working reference preparation performed during the previous period.

- The titre found for the working reference preparation should be within the previously established confidence limits of the test.

- The variation between the two samples of the vaccine under test should be not more than $10^{0.5}$.

- The variation of results between two samples of a vaccine should be within the previously established confidence limits of the test.

The potencies per dose ($CCID_{50}$) recommended for poliomyelitis vaccines used in the Expanded Programme on Immunization are:

- poliovirus Type 1, $10^{6.0}$

- poliovirus Type 2, $10^{5.0}$

- poliovirus Type 3, $10^{5.8}$

If the potency is unacceptable, the test can be performed again for the virus type that is low and the average of all results can be calculated. If the average of all results is within the established limits the vaccine is accepted. If the mean potency is still unacceptable after retesting, the vaccine is rejected. However, the final decision on approval of the vaccine must be made consistent with laboratory procedures and national requirements. In general, except for tests being carried out by manufacturers and national control laboratories for the release of vaccines, the potency is acceptable if it is above the allowed lower confidence limit of the test.

Poliomyelitis vaccines should also pass the following test for thermal stability:

- Two vials of vaccine which have been incubated for 48 hours at 37 °C are titrated

in parallel with vials maintained at storage temperature prior to testing. The loss in titre after the incubation period should be not more than $10^{0.5}$.

9.5 Reporting of results

Every time a titration of OPV is performed, a working reference preparation which has been calibrated against the proposed International Reference Reagent for trivalent oral poliomyelitis vaccine should be included in the same test. When the results of an assay are acceptable, the value obtained for each sample tested should be reported, together with the results found for the working reference preparation on that occasion (including previously established confidence limits for the test). If the titre of the proposed International Reference Reagent as determined in the laboratory differs from that established by collaborative study, results should be corrected for the difference. This procedure has to be repeated periodically to avoid over- or under-estimation of vaccine potencies.

10. TITRATION OF HUMAN SERA FOR NEUTRALIZING ANTIBODIES TO POLIOVIRUS

10.1 Introduction

Poliovirus neutralizing antibodies to poliovirus types 1, 2 and 3 in human serum are determined by *in vitro* microneutralization assay. Two-fold dilution ranges are made of the samples in test medium. A working reference serum of known poliovirus neutralizing capacity is tested in parallel to confirm the validity of the test. After making the dilution range of the serum samples, equal amounts of the serum dilutions and poliovirus are mixed and incubated at 36 °C.

The amount of poliovirus used as a challenge dose in the serum neutralization test should be approximately 100 $CCID_{50}$. This is calculated from previous titrations of the working poliovirus stocks, which should be kept at -70 °C in aliquots sufficient to carry out the test. An aliquot of the working poliovirus is thawed and diluted in diluent to contain ± 100 $CCID_{50}$ per 0.05 ml (see section 10.2.3). The total volume of the challenge virus preparation should be sufficient for all the serum samples.

To calculate the exact amount of challenge virus used in the serum neutralization test, a confirmatory titration of the challenge virus dose should be performed at the same time, in the same test and using the same reagents. This is done by making tenfold dilution steps of the challenge virus dose in diluent. The virus dilutions are transferred to the plates in which the confirmatory titration is performed. The titre found divided by the dilution factor of the challenge virus dose gives the exact amount of virus ($CCID_{50}$) used in the test.

After the first incubation period at 36 °C, the cell substrate, a suspension of Hep-2_c cells, is added. The cell substrate is necessary to determine the end-point dilution of the serum samples, which is considered to be the highest dilution of the serum sample in which 50% of the wells show cytopathic effects (CPE).

Since the working reference serum, titrated in parallel with the samples under test, has been calibrated against the international standard serum, results for these samples can be expressed in International Units (IU).

10.2 Materials

10.2.1 Standard equipment

- microtitre plates (96 wells, flat-bottomed)
- multichannel pipettes, with tips to deliver 0.05-ml volumes
- micropipettes to deliver 0.05-ml and 0.1-ml volumes

- incubator
- plate sealers, if CO_2-incubator not available
- vacuum source.

10.2.2 Test serum

Storage

- Prior to serum separation, store the blood specimen at 0–8 °C. Complete serum separation within 12–24 hours after collection of the blood.
- The minimum amount of serum needed for assay is 0.3 ml.
- Store the serum at -20 °C or below until ready for testing.

Testing

- Handle sera in a safety hood.
- Inactivate sera at 56 °C for 30 minutes at first thaw before testing.
- Dilute heat-inactivated sera to a starting dilution of 1/4 using diluent.
- Test each serum dilution in duplicate.

10.2.3 Reference serum

A working reference serum of known neutralizing activity must be included in each test to confirm the validity of the test. Working reference sera can usually be obtained by pooling sera from adults who have recently received a booster dose of oral poliomyelitis vaccine.

The International Standard for Anti-poliovirus Serum should be used to calibrate the laboratory working reference serum, by determining the potency of the two in parallel.

Calibration of working reference sera and expression of titres in International Units (IU) are described in sections 10.5 and 10.6.

10.2.4 Poliovirus challenge samples

- Use only Sabin strains of poliovirus types 1, 2 and 3 of known titre.

- Prepare a working virus stock from characterized Sabin virus stocks[1].
- Titrate working virus stock until reproducible results are obtained and calculate the geometric mean titre (GMT).
- Prepare aliquots of the working virus stock in volumes sufficient to carry out the serum neutralization test.
- Store aliquots preferably at -70 °C or at -20 °C. The temperatures of the storage freezer should not vary by more than ±2 °C.
- Thaw an aliquot of working virus stock and dilute in diluent to contain ±100 $CCID_{50}$ per 0.05 ml.

Example

Preparation of a challenge dose of poliovirus type 1 for the serum neutralization test

The GMT of the working stock of poliovirus type 1 is $10^{6.33}$ per dose (1 dose = 0.1 ml) Prepare a challenge virus dose of 25 ml containing ±100 $CCID_{50}$ per 0.05 ml. This is equivalent to a 10-fold dilution of the working stock:

$10^{6.33}$ => 1 $CCID_{50}$ per 0.10 ml, or
$10^{6.03}$ => 1 $CCID_{50}$ per 0.05 ml, but
$10^{4.03}$ => 100 $CCID_{50}$ per 0.05 ml

Volume of virus (ml)	*Volume of diluent (ml)*	*Dilution*
0.1	0.9	10^{-1}
0.1	0.9	10^{-2}
0.5	4.5	10^{-3}
2.5	22.5	10^{-4}

Thus, the challenge preparation is in 25 ml containing 10^{2} $CCID_{50}$ per 0.05 ml, since:

$$10^{6.03} - 10^{4.0} = 10^{2.03}$$

[1] Available to national control laboratories and poliomyelitis diagnostic laboratories from Chief, Expanded Programme on Immunization, World Health Organization, 1211 Geneva 27, Switzerland.

10.2.5 Cells

- Use cells of the Hep-$2_{Cincinnati}$ line (Hep-2_c)[1]. The passage level of these cells should be documented and within 15 passages of the tested stock. Watch for any change in growth properties.

- The number of cells used per well is usually in the range of 1–2 x 10^5 cells per ml. This concentration should provide a confluent monolayer within two to three days.

10.2.6 Cell culture media

Use Eagle's minimum essential medium (MEM) with Earle's salts and fetal bovine serum (FBS), which is free from poliovirus inhibitors.

When FBS is not available on a long-term basis, agamma newborn calf serum may be used instead. This must be tested for the absence of poliovirus inhibitors before use.

Test medium: Eagle's MEM supplemented with 5% FBS.

Diluent: Eagle's MEM supplemented with 2% FBS.

10.3 Procedure

- Inactivate the sera, prior to being tested and diluted, at 56 °C for 30 minutes.

- Prepare a 1/4 dilution of each test and working reference serum in diluent.

- Add 0.05 ml volumes of test medium to all wells of all plates (test serum, and confirmatory titration plates; Figure 6).

- Add 0.05 ml of each 1/4 diluted serum sample (test, and working reference serum) to column 1 only of the test serum plates. These wells will serve as serum toxicity controls (SC).

- Add another 0.05 ml of 1/4 diluted serum sample to column 2 only of the test serum plates. From this column start making a twofold dilution range up to column 11.

- Prepare serial two-fold dilutions of the sera under test, using a micropipette with disposable tips to mix and withdraw 0.05 ml volumes from each well in column 2. Transfer these to column 3, etc. up to column 11, to achieve serum dilutions from 1/8 to 1/4 096.

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[1] Available to national control laboratories and poliomyelitis diagnostic laboratories from Chief, Expanded Programme on Immunization, World Health Organization, 1211 Geneva 27, Switzerland.

- Repeat this procedure for the poliovirus type 2 and type 3 test serum plates.

- Prepare sufficient challenge virus suspension in diluent for the number of sera to be tested. To do this, dilute each poliovirus type to contain ± 100 $CCID_{50}$ per 0.05 ml (each plate requires approximately 5.0 ml of diluted challenge virus).

- Prepare four ten-fold dilution steps of the challenge virus preparation of each poliovirus type, for the confirmatory titration, in bottles or tubes using a separate graduated pipette at each virus dilution stage.

- Add 0.05 ml of each tenfold step dilution of the 100 $CCID_{50}$ challenge virus preparation to the appropriate wells, already containing 0.05 ml of diluent. Start with the highest dilution using the same dropper pipette.

- Add 0.05 ml of the 100 $CCID_{50}$ challenge virus preparation of the relevant poliovirus to the appropriate wells of the test serum plates, already containing 0.05 ml of serum dilution.

- Add 0.05 ml of test medium to equalize the volumes to the wells in column 12 that will serve as cell controls (CC).

- Cover the plates with lids and shake them gently to ensure adequate mixing of the well contents.

- Wrap the plates in aluminium foil or place in them zipper bags when using a normal incubator. If a humidified CO_2-incubator is used wrapping of the plates may be omitted.

- Incubate all plates at 36 °C for 3 hours.

- During this time, wash Hep-2_c monolayer flask cultures, trypsinize, centrifuge, count cells and prepare a cell suspension in test medium to contain approximately 1–2 x 10^5 cells per 1.0 ml (see section 8).

- Remove plates from incubator.

- Add 0.1 ml of the cell suspension to all wells of the microtitre plates. Avoid touching the wells with micropipette tips during this procedure.

- Wrap the plates in aluminium foil or place them in zipper bags and incubate at 36 °C in the incubator for 5 days. If a humidified CO_2-incubator is available wrapping of the plates may be omitted.

- During incubation, monitor the cell control wells to ensure that cells are in good condition and will form a monolayer. Also score the positive wells, starting at day 3, and keep good records of the results.

- After 5 days of incubation, read the plates using the microscope before fixing and staining with crystal violet.

- Read the plates visually and record results:
  stained cells present = antibodies present, no virus growth
  stained cells absent = virus growth or toxicity.

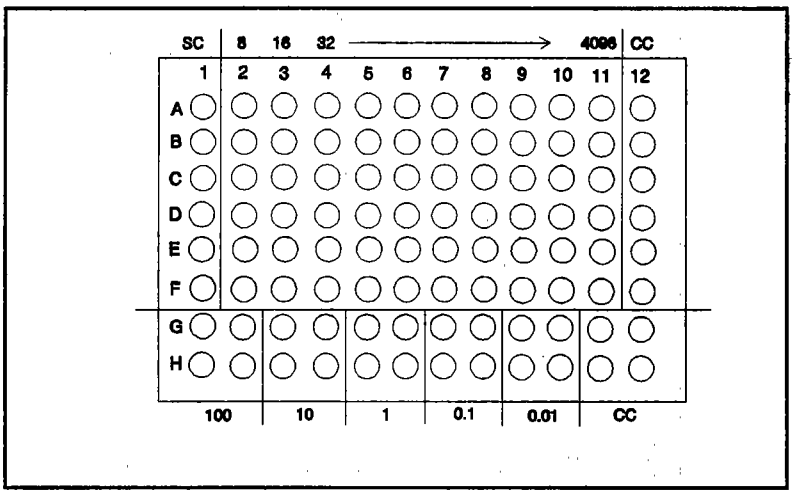
- Calculate the end-point of neutralization using the Kärber or Reed-Muench formula. The serum antibody titre is the highest dilution of serum that protects 50% of the cultures against 100 $CCID_{50}$ of challenge virus. Antibody titres are expressed as reciprocals (e.g. for 50% CPE in dilution 1/256 the titre is 256).

- Confirm the challenge virus dose preparation ± 100 $CCID_{50}$ (range 31-316 $CCID_{50}$) using the Kärber or Reed-Muench formula.

- The working reference serum antibody titre against each of the three poliovirus types should not vary beyond one two-fold dilution of the established geometric mean titre (see section 10).

- When all test sera titres have been calculated, convert into International Units.

Figure 6. Arrangement of microtitre plate for poliovirus serum neutralization test.



10.4 Evaluation of results

For the test to be considered valid:

- The cell control wells should show good viability, and cells in the serum toxicity control wells should not show any toxicity.

- The working reference serum antibody titre to each of the three poliovirus types should be within one twofold dilution step of the established geometric mean titre for the working reference serum.

  This is based on the geometric mean of all valid assays for the working reference serum performed previously in the laboratory, up to the last performed test. When the working reference serum titre is unacceptable (outside the range), the test can be performed again for the virus type that is unacceptable. If the titre of the working reference serum is still unacceptable then the titre must be reassessed.

- The challenge virus titre of the three poliovirus types should be approximately 100 $CCID_{50}$ (range 31–316 $CCID_{50}$. When the challenge virus dose is outside the range, the test should be repeated with new virus dilutions containing approximately 100 $CCID_{50}$.

10.5 Calibration of the working reference serum

The working reference serum selected for possible reference purposes and the International Standard for Anti-poliovirus Serum are titrated in parallel on at least six separate occasions using eight replicates per serum dilution.

The geometric mean titre (GMT) of the working reference serum is divided by the GMT of the International Standard. This result is multiplied by the assigned potency (the result of an international collaborative study) of the International Standard thus expressing the titre of the working reference serum in International Units (IU) (see Section 2).

Example

GMT of the working reference serum = 320
GMT of the International Biological Standard = 640
Assigned potency of International Biological Standard = 20 IU

Therefore, the potency of working reference serum is:

320/640 x 20 = 10 IU of poliovirus neutralizing antibodies.

10.6 Expression of results in International Units

The working reference serum should be tested in each assay. For the assay to be valid, the titre should be within one two-fold dilution step of the established GMT for the working reference serum.

The titre of the test serum is divided by the established GMT for the working reference serum and multiplied by the potency (IU) of the working reference serum, thus expressing the potency of the test serum in IU. Conversion tables to facilitate calculations can be constructed.

Example

The GMT of the working reference serum established from previous assays is:

Virus type	*GMT*	*Acceptable range*
1	181	90–362
2	128	64–256
3	32	16–64

The potency of the working reference serum in IU established from the previous test is:

- 10.9 IU of type 1 neutralizing antibody
- 17.3 IU of type 2 neutralizing antibody
- 2.5 IU of type 3 neutralizing antibody.

The potency of the test serum in IU, with titres of 8 to each of the poliovirus types, is:

- type 1, 8/181 x 10.9 = 0.482 IU or 482 mIU
- type 2, 8/128 x 17.3 = 1.081 IU or 1 081 mIU
- type 3, 8/32 x 2.5 = 0.625 IU or 625 mIU.

11. DETERMINATION OF MEASLES ANTIBODY BY HAEMAGGLUTINATION INHIBITION

11.1 Introduction

Haemagglutination inhibition (HI) is a useful procedure for the detection of antibodies against measles. The test is valuable for its simplicity and specificity, and is based on the fact that measles virus contain haemagglutination (H) antigens that will cause agglutination of red blood cells of various origins. The sera of individuals that have been successfully vaccinated against measles, or have suffered from the disease, will contain, *inter alia*, high titres of antibodies against theses H antigens. If present in a serum sample, these antibodies will inhibit the agglutination of red blood cells when measles virus is added in the HI test. The titre of these haemagglutination-inhibiting antibodies is determined in a serum dilution series, the end-point being the dilution in which inhibition of haemagglutination no longer occurs.

The assay is carried out as follows. Serum samples should be pretreated to remove both aspecific agglutinins and aspecific agglutination-inhibiting factors that may be present and might interfere with the test results. This is done by the kaolin/red blood cell method (see section 11.3.2). The pretreated samples are diluted and then incubated with a certain fixed amount of measles haemagglutination antigen (approximately four haemagglutination units) and appropriate red blood cells. Vervet red blood cells (VRBC) are generally used for this purpose.

When specific antibodies against measles H antigens are present, agglutination will be inhibited, resulting in a small red dot at the bottom of the well, caused by the settled red blood cells. When no specific antibodies against measles H antigens are present, agglutination will occur. The red blood cells will form complexes with the H antigens, which can be observed as a large red sediment without a clear dot at the bottom of the well. It is recommended that preliminary assays are performed not only to confirm that the suggested amount of challenge virus is appropriate but also to check whether the red blood cells being used are actually agglutinated by the antigen used. An antigen titration to determine the exact number of haemagglutination units, which is necessary to calculate the haemagglutination inhibition units of the serum samples, should be performed in parallel with the haemagglutination inhibition test itself. Serum samples of known negative and known positive haemagglutination inhibition activity should be titrated in parallel with the samples under test. The positive serum sample should be calibrated against the International Standard for Anti-Measles Serum so that the results can be expressed in International Units (IU).

11.2 Materials

11.2.1 Standard equipment

- microtitre plates (96 wells, round-bottomed)

- plate sealers
- multichannel pipettes to deliver 0.025-ml volumes
- dropper pipettes to deliver 0.025-ml volumes
- incubator
- centrifuge (Eppendorf)
- mechanical shaking apparatus
- mirror, for reading the plates.

11.2.2 Haemagglutination antigen

Freeze-dried Tween-ether inactivated measles virus of the Edmonston B strain grown in primary dog kidney cells.

11.2.3 Test serum samples

Because of the possible presence of nonspecific inhibitors or agglutinins in human serum, samples should be pretreated by adsorption (section 11.3.2).

11.2.4 Reference serum samples

Negative serum, to exclude nonspecific agglutination, and positive serum, of which the titre is well known and calibrated against the International Standard for Anti-Measles Serum.

11.2.5 Diluent

Phosphate buffered saline (PBS), pH 7.4, plus 0.1% bovine serum albumin (BSA).

11.2.6 Kaolin

Kaolin is a natural clay material consisting primarily of aluminum silicate with a varying degree of hydration. Kaolin is widely used in diagnostic virology to remove lipoproteins and glycoproteins that are non-specific serum inhibitors of viral haemagglutination and of the binding of viruses to cells.

11.2.7 Vervet red blood cells

Prepared from peripheral blood derived from the vervet monkey (*Cercopithecus*) in Alsevers medium:

- a 50% suspension of red cells is used for adsorption of the sera;

- a 0.75% suspension of red cells is used for performing the test.

11.3 Procedure

The haemagglutination inhibition assay consists of two parts: the haemagglutination test, titration of the antigen preparation to calculate how much to use to provide the appropriate challenge dose for the haemagglutination inhibition test (four haemagglutination (HA) units per 0.025 ml), and the actual haemagglutination inhibition test, in which the antibody titre against measles antigen in serum is determined.

Note: Both parts of the test should be performed at the same time using the same plate. The reciprocal of the haemagglutination (HA) titre found for the antigen gives the amount which is used in calculating the inhibitory activity of the serum, expressed in haemagglutination inhibition (HI) units.

11.3.1 Haemagglutination test

- Prepare the diluent, phosphate buffered saline (PBS) and 0.1% bovine serum albumin (BSA).
- Prepare a 0.75% red cell suspension, using vervet red cells washed three times with diluent.
- Add 0.025 ml of diluent to each of the wells of the microtitre plate in which the antigen will be titrated.
- Add 0.050 ml of diluent to the cell control wells.
- Prepare serial two-fold dilutions of the measles antigen preparation using a multichannel pipette with tips to deliver 0.025-ml volumes.
- Add another 0.025 ml of diluent to the wells in which the antigen is diluted to equalize the volumes.
- All wells will contain 0.050 ml.
- Add 0.025 ml of 0.75% red cell suspension to all wells. All wells will contain 0.075 ml.
- Mix gently.
- Incubate for 2 hours at 36 °C, shaking intermittently manually, or constantly if a mechanical shaking apparatus is used. If necessary incubate overnight at 4 °C.
- Read the plate, using a mirror, and calculate HA units.

A positive pattern (agglutination) consists of a layer of uniformly agglutinated cells covering the bottom of the well. Positive patterns are designated by a plus sign (+).

A negative pattern (no agglutination) consists of a compact, sharply demarcated button of sedimented cells in the bottom of the well. Negative patterns are designated by a minus sign (−).

An intermediate pattern (agglutination still present) may occur in the range of minimum viral activity. This pattern is designated with a plus/minus sign (±).

The end-point of HA activity of the virus is considered to be the highest dilution of the virus, before the addition of the diluent and the red cells, in which a positive pattern, a layer of uniformly agglutinated cells, is present. The virus titre, expressed as HA units contained in the undiluted sample, is the reciprocal of that end-point dilution.

Example A

Sample haemagglutination (HA) test

Well	1	2	3	4	5	6	7	8	9	10	11	12
Dilution	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096
Presence or absence of HA	+	+	+	+	+	+	-	-	-	-	-	-

The HA titre is 1/64 (2^{-6}), the end-point dilution in which a positive pattern is still present. The virus titre of the undiluted sample is therefore 64 HA units (the reciprocal of the HA titre).

11.3.2 Haemagglutination inhibition test

Adsorption of serum samples

Because of nonspecific inhibitors to agglutination found in most sera it is necessary to pretreat samples by adsorption according to the kaolin/red cell method:

- Use 0.05 ml of serum (heat inactivated).
- Add 0.05 ml of diluent.
- Add 0.10 ml of kaolin (25% w/v) suspension.
- Mix gently.

- Incubate at room temperature (20–25 °C) for 20 minutes.

- Centrifuge at 1 200 *g* for 5–10 minutes.

- Add 0.025 ml of 50% red cell suspension.

- Incubate for at least 1 hour at 4 °C, shaking intermittently. If necessary continue incubation overnight.

- Centrifuge at 250 *g* for 5–10 minutes.

- Collect the supernatant (which is not influenced by the addition of the 0.025 ml of 50% red cell suspension). The final dilution of the supernatant is approximately 1/4.

Antigen preparation

- Using the measles antigen preparation previously titrated in the HA test, dilute the antigen in diluent to contain approximately 4 HA units per 0.025 ml:

  Reciprocal of the HA titre/HA units required = dilution factor of the antigen preparation
  HA titre of the measles antigen (example A) = 1/64
  Reciprocal of the HA titre = 64
  HA units required = 4
  Dilution factor of the measles antigen, 64/4 = 16
  Add 1.0 ml of measles antigen preparation (undiluted) to 15.0 ml of diluent to give 16.0 ml of measles antigen suspension containing approximately 4 HA units per 0.025 ml, as the challenge for the HI test.

Procedure for the haemagglutination inhibition test

The assay should include a serum sample with a known haemagglutination inhibition titre as a positive control (D), and a serum sample with a known negative haemagglutination inhibition titre as a negative control (E).

- Add 0.025 ml of diluent (PBS + 0.1% BSA) to all wells of the microtitre plate (Figure 7).

- Add another 0.025 ml of diluent to the cell control wells.

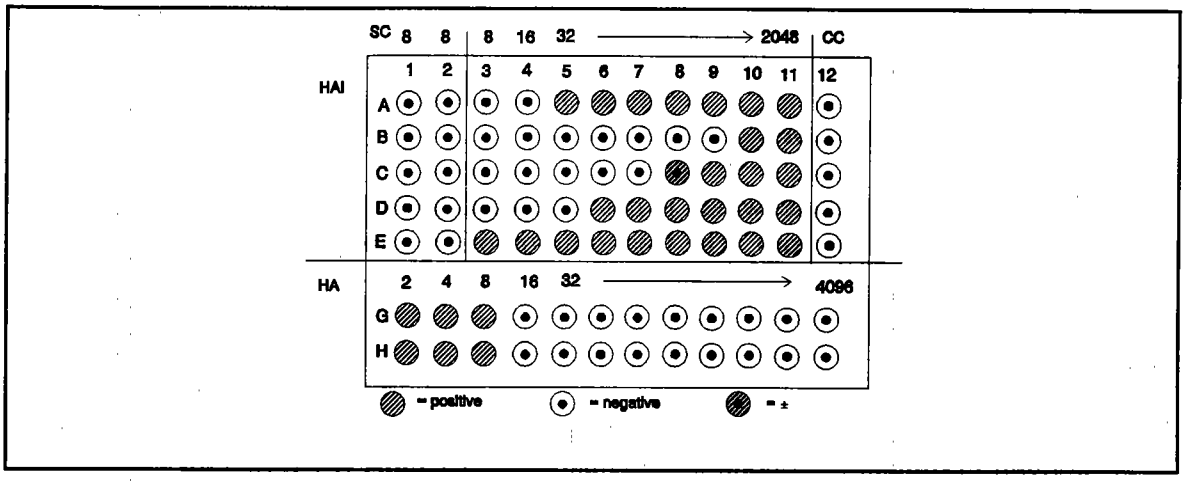
- Add 0.025 ml of adsorbed serum sample to the serum control wells.

- Prepare two-fold serial dilutions in diluent of the adsorbed serum to be tested, starting with 1/8.

- Add 0.025 ml of antigen preparation, containing 4 HA units, to the wells in which

the inhibitory activity of the adsorbed sera will be determined. No antigen is added to the cell control or serum control wells.

Figure 7. Arrangement of microtitre plate for the haemagglutination inhibition test.



- Prepare two-fold serial dilutions in diluent of the antigen preparation containing 4 HA units. This is done to determine the exact number of HA units, which is used in calculating the HI units of the serum samples under test.
- Add 0.025 ml of diluent to the wells of the antigen titration.
- All wells contain 0.050 ml.
- Mix gently, using a mechanical shaking apparatus.
- Incubate for 1 hour at room temperature (20–25 °C).
- Add 0.025 ml of 0.75% red cell suspension to all wells.
- All wells contain 0.075 ml.
- Seal the plate, using nontoxic plate sealers.
- Mix gently, using a mechanical shaking apparatus.
- Incubate 2 hours at 36 °C.
- The end-point of the inhibitory activity of the serum is the highest dilution of the serum in which haemagglutination is completely inhibited (a negative pattern: a button of sedimented cells).
- The HI titre of the serum is calculated by multiplying the reciprocal of the end-

point of the serum by the number of HA units used in the test.
Reciprocal of the HA titre of the antigen = 8 HA units
Reciprocal end-point of inhibitory activity of serum A = 16, serum B = 512, serum C = 128, serum D = 32, serum E = <8
HI titre of serum A, 16 x 8 = 128 HI units
HI titre of serum B, 512 x 8 = 4096 HI units
HI titre of serum C, 128 x 8 = 1024 HI units
HI titre of serum D, 32 x 8 = 256 HI units
HI titre of serum E = <8 HI units

11.4 Evaluation of results

For the test to be considered valid:

- The antigen should be within the range ±2 x 4 HA units.
- There should be a clear discrimination between the positive (agglutination) and negative (no agglutination) reaction.
- The titre of the positive serum sample should be within a two-fold dilution step of its established titre. This is based on the geometric mean of all valid assays for the positive serum sample.
- The negative serum sample should not show haemagglutination inhibition.
- Red cell control wells should not show haemagglutination. If they do, prepare fresh 0.75% red cell suspension and repeat the HI test.
- Serum control wells should not show haemagglutination. If they do, repeat kaolin adsorption to remove nonspecific serum inhibitors.

Note: Haemagglutination in the HI test is designated by a plus sign (+), which means that there is no haemagglutination inhibition and there are no antibodies against measles present. No haemagglutination in the HI test is designated by a minus sign (−), indicating that haemagglutination inhibition and antibodies against measles are present.

11.5 Expression of titre in International Units

For the test to be standardized, it should include a serum sample of known haemagglutination inhibition titre as a positive control. The titre of this positive control should be well established and calibrated against the International Standard for Anti-Measles Serum. This is necessary to control reproducibility of the test, and to enable the results to be expressed in International Units (IU).

The International Standard for Anti-Measles Serum, contains 10 International Units (IU) in one ampoule, established by an international collaborative study.

The example given (example C) assumes that the International Standard is reconstituted in 1 ml.

Titre determined for the International Standard = 2 560 HI units
Titre of the positive serum sample = 256 HI units = 256/2 560 x 10 IU = 1.0 IU
Geometric mean titre of the positive serum D = 256 (128—512) HI units
Potency of the positive serum D in IU, as established in previous tests = 1.0 IU
Potency of the sera under test, example B:

- serum A 128/256 x 1.0 = 0.5 IU
- serum B 4 096/256 x 1.0 = 16.0 IU
- serum C 1 024/256 x 1.0 = 4.0 IU

12. LIVE MEASLES VACCINE

12.1 Introduction

Measles, previously an almost invariable clinical experience of childhood, is in some countries a major cause of illness and of death in children. Immunization against measles has been of interest to WHO for many years, and more especially since the Expanded Programme on Immunization was launched with measles as one of the principal diseases against which it is directed.

The potency of live measles vaccine is determined by an *in vitro* microtitration assay. The vaccine is assayed in parallel with a working reference preparation calibrated against the International Reference Reagent for Measles Virus Vaccine (Live) as a means of ensuring the reproducibility of test results.

Dilutions of the vaccines under test and of the working reference preparation (WRP) are inoculated in rows of 10 wells of microtitre plates, together with a cell suspension obtained by trypsinization of a Vero cell line. The plates are incubated at 36 °C for 7–9 days. During this incubation period, starting on day 4, the cells should be checked for cytopathic changes and positive wells recorded. The final reading should preferably be done on day 9, depending on the quality of the cells; each laboratory should establish the optimum incubation period using the equipment and reagents under local conditions.

The titre in $CCID_{50}$ per human dose is calculated on the basis of the final reading using the Kärber or Reed-Muench formula.

Since measles virus is light sensitive, vaccines should be protected from direct light during storage and testing.

12.2 Materials

12.2.1 Standard equipment

- microtitre plates (96 wells, flat-bottomed)
- multichannel pipettes, with tips to deliver 0.05-ml volumes
- micropipettes to deliver 0.05-ml and 0.1-ml volumes
- incubator.

12.2.2 Test vaccine

At least two vials of freeze-dried vaccine, reconstituted with the diluent supplied with the vaccine.

12.2.3 Reference vaccine

For each assay include a vial of live measles vaccine of which the titre has been well established as the working reference preparation.

12.2.4 Medium and dilutions

Test medium: Medium 199 supplemented with 5% fetal bovine serum.
Diluent: Medium 199 supplemented with 2% fetal bovine serum.

Using the refrigerated diluent, prepare the initial dilutions in $10^{0.5}$ steps. Table 8 shows an example of a dilution range. The subsequent dilutions for inoculation into microtitre plates should range from 10^{-1} to 10^{-4}.

12.2.4 Cells

Use Vero cells[1]. The passage level of these cells should be documented and within 15 passages of the tested stock. The number of cells used in the assay is usually 2×10^5 cells per ml. This concentration should provide a confluent monolayer in microtitre plate wells within 2–3 days. However, the cell concentration should be optimized for each laboratory.

12.3 Procedure

- Wash Vero cell monolayer flask cultures, trypsinize, count cells and prepare a cell suspension in test medium to contain approximately 2×10^5 cells per ml.
- Add 0.05 ml of test medium to all wells of the plates in which the vaccines will be titrated (Figure 8).
- Add 0.05 ml of vaccine dilution to the appropriate wells. Start with the highest dilution, using one dropper pipette.
- Add 0.05 ml of test medium, to equalize the volumes, to the wells that will serve as cell controls (CC).
- Add 0.10 ml of the Vero cell suspension to all wells of all plates.
- All wells contain 0.2 ml.
- Cover the plates with lids.
- Wrap the plates in aluminium foil and place them in zipper bags. If a humidified

[1] Available on request to national control laboratories from Chief, Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.

CO_2-incubator is used wrapping of the plates may be omitted.

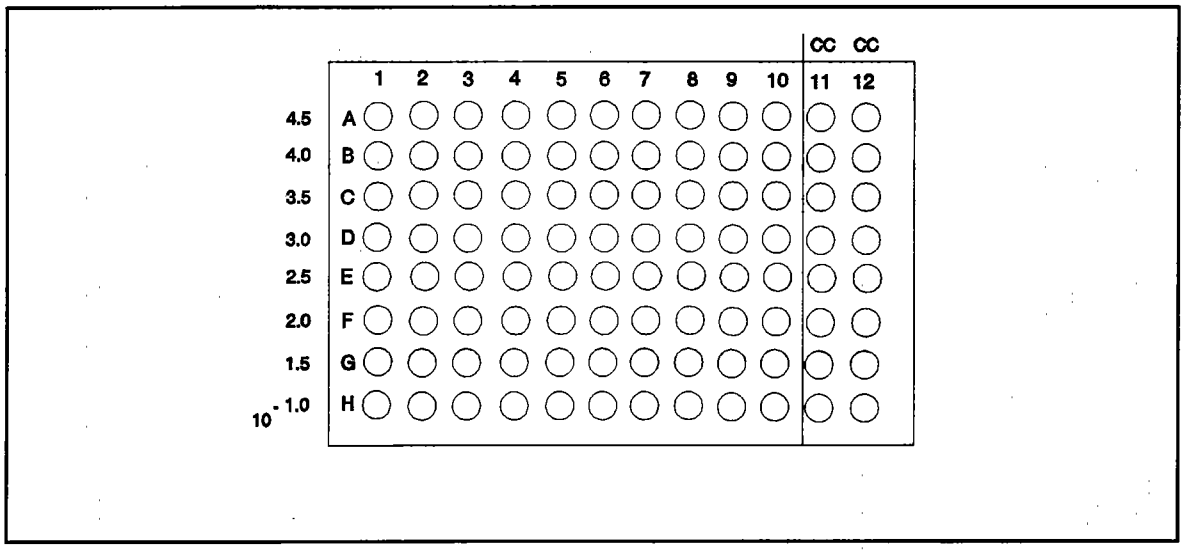
- Incubate all plates at 36 °C for 7–9 days.
- During this time, monitor the cell control wells to ensure that the cells are forming a monolayer and do not show any toxicity.
- The presence or absence of cytopathic effects is recorded starting on day 4, with the final reading performed at day 9.
- Keep good records of the data.
- Calculate the titre in $CCID_{50}$ per human dose using the Kärber or Reed-Muench formula.

Table 8. Range of dilutions for use in potency testing of live measles vaccine[a]

Volume of vaccine dilution (ml)	Volume of diluent added (ml)	Dilution
0.20 (undiluted vaccine)	1.80	$10^{-1.0}$
1.00 (from $10^{-1.0}$)	2.16	$10^{-1.5}$
1.00 (from $10^{-1.5}$)	2.16	$10^{-2.0}$
1.00 (from $10^{-2.0}$)	2.16	$10^{-2.5}$
1.00 (from $10^{-2.5}$)	2.16	$10^{-3.0}$
1.00 (from $10^{-3.0}$)	2.16	$10^{-3.5}$
1.00 (from $10^{-3.5}$)	2.16	$10^{-4.0}$
1.00 (from $10^{-4.0}$)	2.16	$10^{-4.5}$

[a] The range of used depends on the type and formulation of the vaccine. It should include the expected titre of the vaccine being tested.

Figure 8. Arrangement of microtitre plate for measles vaccine potency testing.



12.4 Evaluation of results

For the test to be considered valid:

- The titre of the working reference preparation should be within $10^{0.5}$ of the established titre, which is based on the geometric mean titre of all valid assays for the working reference preparation performed during the previous period. The titre should also be within the confidence limits of the test as established before.

- The variation between the two samples of the vaccine under test should be not more than $10^{0.5}$. It should also be within the confidence limits of the test as established before.

For the vaccine to be approved:

- The titre of the assayed vials should be at least $10^{3.0}$ $CCID_{50}$ per human dose. The variation between two tests on a vaccine should not exceed $10^{0.5}$. If the potency is unacceptable, the assay can be repeated and the mean of all assays calculated.

Live measles vaccine should also pass the following test for thermal stability:

Two vials incubated for 7 days at 37 °C are titrated in parallel with vials maintained frozen prior to testing. The loss in titre after incubation should not exceed $10^{1.0}$ and the final titre must be greater than $10^{3.0}$. Both conditions must be met.

12.5 Reporting of results

Every time a titration of measles vaccine is performed, a working reference preparation which has been calibrated against the International Reference Reagent for the Assay of Measles Vaccine (Live) should be included in the same test. If the titre of the International Reference Reagent as determined in the laboratory differs from that established by collaborative study, results should be corrected for the difference.

Example

The geometric mean titre (GMT) of the working reference preparation = $10^{3.82}$ $CCID_{50}$
The GMT of the International Reference Reagent = $10^{3.54}$ $CCID_{50}$
The established titre of the International Reference Reagent as the result of an international collaborative study = $10^{3.71}$ $CCID_{50}$
The corrected potency of the working reference preparations is, therefore:
$3.71/3.54 \times 3.82 = 10^{3.99}$ $CCID_{50}$
This procedure has to be repeated periodically to avoid over- or under-estimation of vaccine potencies.

13. LIVE MUMPS VIRUS VACCINE

13.1 Introduction

The potency of live mumps virus vaccine is determined by an *in vitro* titration in a microtitration assay. The vaccine is assayed in parallel with a reference preparation calibrated against the International Reference Reagent for Mumps Virus Vaccine (Live) as a means of ensuring reproducibility of the test results.

Dilutions of the vaccine under test and of the working reference preparation are inoculated in rows of 10 wells of microtitre plates, together with a cell substrate, obtained by trypsinization of a Vero cell line. The plates are incubated at 36 °C for 7–9 days. During this incubation period the cells should be checked for specific viral cytopathic changes and the results recorded. The final reading should preferably be done on day 9, depending on the quality of the cells; each laboratory should establish the optimum incubation period using the equipment and reagents under local conditions.

The titre in $CCID_{50}$ per human dose is calculated on the basis of the final reading using the Kärber or Reed-Muench formula.

Since the mumps virus is light sensitive, vaccines should be protected from direct light during storage and testing.

13.2 Materials

13.2.1 Standard equipment

- microtitre plates (96 wells, flat-bottomed)
- multichannel pipettes, with tips to deliver 0.05-ml volume
- micropipettes to deliver 0.05-ml and 0.1-ml volumes
- incubator.

13.2.2 Test vaccine

At least two vials of freeze-dried vaccine, reconstituted with the diluent supplied with the vaccine.

13.2.3 Reference vaccine

For each assay include a vial of live mumps virus vaccine of which the titre has been well established as the working reference preparation.

13.2.4 Medium and dilutions

Test medium: Medium 199 supplemented with 5% fetal bovine serum.
Diluent: Medium 199 supplemented with 2% fetal bovine serum.

Using the refrigerated diluent, prepare the initial dilutions in $10^{0.5}$ steps. Table 9 shows an example of a dilution range. The subsequent dilutions for inoculation into microtitre plates should range from 10^{-3} to 10^{-5}.

13.2.5 Cells

Use Vero cells[1]. The passage level of these cells should be documented and within 15 passages of the tested stock. The number of cells in the assay is usually 2×10^5 cells per ml. This concentration should provide a confluent monolayer in microtitre plate wells within 2–3 days. However, the cell concentration should be optimized for each laboratory.

13.3 Procedure

- Wash the Vero cell monolayer flask cultures, trypsinize, count cells and prepare a cell-suspension in test medium to contain approximately 2×10^5 cells per ml.
- Add 0.05 ml of test medium to all wells of the plates in which the vaccines will be titrated.
- Add 0.05 ml of vaccine dilution to the appropriate wells. Start with the highest dilution, using one dropper pipette.
- Add 0.05 ml of test medium to the wells that will serve as cell controls (CC), to equalize the volumes.
- All wells contain 0.1 ml.
- Add 0.10 ml of the Vero cell suspension to all wells of all plates.
- All wells contain 0.2 ml.
- Cover the plates with lids.
- Wrap the plates in aluminium foil and place them in zipper bags. If a humidified CO_2-incubator is used wrapping of the plates may be omitted.
- Incubate all plates at 36 °C for 7–9 days.

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[1] Available on request to national control laboratories from Chief, Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.

- During this time, monitor the cell control wells to ensure that the cells are forming a monolayer and do not show any toxicity.
- The presence or absence of cytopathic effects is recorded starting on day 4 of incubation with the final reading performed on day 9.
- Keep good records of the data.
- Calculate the titre of the vaccine in $CCID_{50}$ per human dose using the Kärber or Reed-Muench formula.

Table 9. Range of dilutions for use in the potency testing of live mumps vaccine[a]

Volume of vaccine dilution (ml)	Volume of diluent added (ml)	Dilution
0.20 (undiluted vaccine)	1.80	$10^{-1.0}$
1.00 (from $10^{-1.0}$	2.16	$10^{-1.5}$
1.00 (from $10^{-1.5}$)	2.16	$10^{-2.0}$
1.00 (from $10^{-2.0}$)	2.16	$10^{-2.5}$
1.00 (from $10^{-2.5}$)	2.16	$10^{-3.0}$
1.00 (from $10^{-3.0}$)	2.16	$10^{-3.5}$
1.00 (from $10^{-3.5}$)	2.16	$10^{-4.0}$
1.00 (from $10^{-4.0}$)	2.16	$10^{-4.5}$
1.00 (from $10^{-4.5}$)	2.16	$10^{-5.0}$

[a] The range used depends on the type and formulation of the vaccine. It should include the expected titre of the vaccine being tested.

13.4 Evaluation of results

For the test to be considered valid:

- The titre of the working reference preparation should be within $10^{0.5}$ of the established titre, which is based on the geometric mean titre of all valid assays for the working reference preparation performed during the previous period. The titre should also be within the confidence limits of the test as established before.

- The variation between the two samples of the vaccine under test should not be more than $10^{0.5}$. It should also be within the confidence limits of the test as established.

For the vaccine to be approved:

- The titre of the assayed vials should be at least $10^{3.0}$ $CCID_{50}$ per human dose. The variation between two samples of the vaccine under test should not exceed $10^{0.5}$. If the potency is unacceptable, the assay can be repeated and the mean of all assays calculated.

Live mumps vaccine should also pass the following test for thermal stability:

Two vials incubated for 7 days at 37 °C are titrated in parallel with vials maintained frozen prior to testing. The loss in titre after incubation should not exceed $10^{1.0}$ and the final titre must be greater than $10^{3.0}$. Both conditions must be met.

13.5 Reporting of results

Every time a titration of live mumps virus vaccine is performed, a working reference preparation which has been calibrated against the International Reference Reagent for Mumps Virus Vaccine (Live) should be included in the same test. When the results of an assay are acceptable, the value obtained for each sample tested should be reported, together with the results found for the working reference preparation on that occasion (including previously established confidence limits for the test). If the titre of the International Reference Reagent as determined in the laboratory differs from that established by collaborative study, results should be corrected for the difference. This procedure has to be repeated periodically to avoid over- or under-estimation of vaccine potencies.

14. LIVE RUBELLA VIRUS VACCINE

14.1 Introduction

The potency of live rubella virus vaccine is determined by an *in vitro* microtitration assay. The vaccine is assayed in parallel with a working reference preparation calibrated against the International Reference Reagent for Rubella Virus Vaccine (Live) as a means of ensuring reproducibility of the test results.

Dilutions of the vaccine under test and of the working reference preparation are inoculated in rows of 10 wells of microtitre plates, together with a cell substrate, obtained by trypsinization of a RK-13 cell line. The plates are incubated at 32 °C for 10–12 days. During this incubation period the cells should be checked for specific viral cytopathic changes and the results recorded. The final reading should preferably be done on day 12, depending on the quality of the cells; each laboratory should establish the optimum incubation period using the equipment and reagents under local conditions.

The titre in $CCID_{50}$ per human dose is calculated on the basis of the final reading using the Kärber or Reed-Muench formula.

Since the rubella virus is light sensitive, vaccines should be protected from direct light during storage and testing.

14.2 Materials

14.2.1 Standard equipment

- microtitre plates (96 wells, flat-bottomed)
- multichannel pipettes, with tips to deliver 0.05-ml volume
- micropipettes to deliver 0.05-ml and 0.1-ml volumes
- incubator.

14.2.2 Test vaccine

At least two vials of freeze-dried vaccine, reconstituted with the diluent supplied with the vaccine.

14.2.3 Reference vaccine

For each assay include a vial of live rubella virus vaccine of which the titre has been well established as the working reference preparation.

14.2.4 Medium and dilutions

Test medium: Eagle's minimum essential medium MEM supplemented with 5% fetal bovine serum.
Diluent: Eagle's MEM supplemented with 2% fetal bovine serum.

Using the refrigerated diluent, prepare initial dilutions in $10^{0.5}$ steps. Table 10 shows an example of a dilution range. The subsequent dilutions for inoculation into microtitre plates should range from 10^{-2} to 10^{-5}.

14.2.5 Cells

Use RK-13 cells. The passage level of these cells should be documented and within 15 passages of the tested stock. The number of cells in the assay is usually 1–2 x 10^5 cells per ml. This concentration should provide a confluent monolayer in microtitre plate wells within 2–3 days. However, the cell concentration should be optimized for each laboratory.

14.3 Procedure

- Wash RK-13 cell monolayer flask cultures, trypsinize, count cells and prepare a cell suspension in test medium to contain approximately 1–2 x 10^5 cells per ml.
- Add 0.05 ml of test medium to all wells of the plates in which the vaccines will be titrated.
- Add 0.05 ml of vaccine dilution to the appropriate wells. Start with the highest dilution, using one dropper pipette.
- Add 0.05 ml of test medium to the wells that will serve as cell controls (CC), to equalize the volumes.
- All wells contain 0.1 ml.
- Add 0.10 ml of the RK-13 cell suspension to all wells of all plates.
- All wells contain 0.2 ml.
- Cover the plates with lids.
- Wrap the plates in aluminium foil and place in zipper bags. If a humidified CO_2-incubator is used wrapping of the plates may be omitted.
- Incubate all plates at 32 °C for 10–12 days.
- During this time, monitor the cell control wells to ensure that the cells are forming a monolayer and do not show any toxicity.

- The presence or absence of cytopathic effects is recorded starting on day 4 of incubation with the final reading performed on day 10–12.

- Keep good records of the data.

- Calculate the titre of the vaccine in $CCID_{50}$ per human dose using the Kärber or Reed-Muench formula.

Table 10. Range of dilutions for use in the potency testing of live rubella vaccine[a]

Volume of vaccine dilution (ml)	Volume of diluent added (ml)	Dilution
0.20 (undiluted vaccine)	1.80	$10^{-1.0}$
1.00 (from $10^{-1.0}$)	2.16	$10^{-1.5}$
1.00 (from $10^{-1.5}$)	2.16	$10^{-2.0}$
1.00 (from $10^{-2.0}$)	2.16	$10^{-2.5}$
1.00 (from $10^{-2.5}$)	2.16	$10^{-3.0}$
1.00 (from $10^{-3.0}$)	2.16	$10^{-3.5}$
1.00 (from $10^{-3.5}$)	2.16	$10^{-4.0}$
1.00 (from $10^{-4.0}$)	2.16	$10^{-4.5}$
1.00 (from $10^{-4.5}$)	2.16	$10^{-5.0}$

[a] The range used depends on the type and formulation of the vaccine. It should include the expected titre of the vaccine being tested.

14.4 Evaluation of results

For the test to be considered valid:

- The titre of the working reference preparation should be within $10^{0.5}$ of the established titre, which is based on the geometric mean titre of all valid assays for the working reference period performed during the previous period. It should also be within the confidence limits of the test as established before.

- The variation between the two samples of the vaccine under test should be not more than $10^{0.5}$. It should also be within the confidence limits of the test as established before.

For the vaccine to be approved:

- The titre of the assayed vials should be at least $10^{3.0}$ $CCID_{50}$ per human dose. The variation between two samples of the vaccine under test should not exceed $10^{0.5}$. If the potency is unacceptable, the assay can be repeated and the mean of all assays calculated.

14.5 Reporting of results

Every time a titration of live rubella virus vaccine is performed, a working reference preparation which has been calibrated against the proposed International Reference Reagent for Rubella Virus Vaccine (Live) should be included in the same test. When the results of an assay are acceptable, the value obtained for each sample tested should be reported, together with the results found for the working reference preparation on that occasion (including previously established confidence limits for the test). If the titre of the International Reference Reagent as determined in the laboratory differs from that established by collaborative study, results should be corrected for the difference. This procedure has to be repeated periodically to avoid over- or under-estimation of vaccine potencies.

15. COMBINED LIVE VIRAL VACCINES AGAINST MEASLES, MUMPS AND RUBELLA

15.1 Materials

Equipment: as for the tests for the individual vaccines described in sections 12, 13 and 14.

Antisera: inactivate for 30 minutes at 56 °C. Antisera against measles, mumps, and rubella viruses are needed having a titre of 1:320 (when tested against 100 $CCID_{50}$ of the respective viruses in the tests for the individual vaccines).

Reference vaccines: obtain and reconstitute as for the tests for the individual vaccines.

Test vaccines: at least two vials of each.

Diluents and cells: as for the tests for the individual vaccines.

Since measles, mumps and rubella viruses are light sensitive, protect vaccines from direct light during storage and testing.

15.2 Methods

Before titration of specific virus components, the other virus components should be neutralized as follows.

Combined vaccines against measles and rubella:

- for titration of measles, neutralize rubella virus
- for titration of rubella, no neutralization necessary as measles virus does not grow in RK-13 cells.

Combined vaccines against rubella and mumps:

- for titration of rubella, neutralize mumps
- for titration of mumps, neutralize rubella.

Combined vaccines against measles and mumps:

- for titration of measles, neutralize mumps
- for titration of mumps, neutralize measles.

Combined vaccines against measles, mumps, and rubella:

- for titration of measles, neutralize mumps and rubella
- for titration of rubella, neutralize mumps
- for titration of mumps, neutralize measles and rubella.

15.3 Procedures

- Dilute the antisera in the appropriate manner with respect to the type of virus, the diluent, and the quantities necessary.
- Prepare sterile 13 x 100-mm tubes with diluent containing antisera and place them in a metal rack submerged in an ice-bath.
- Reconstitute the test and reference vaccine samples with sterile distilled water and prepare dilutions as required.
- Incubate all dilutions at 4 °C for one hour.
- For each virus, titrate according to the procedures described for the assays on the monovalent vaccines.

Table 11. Example of titration of a combined vaccine against measles, mumps and rubella

	Plate number		
	1	2	3
Virus to be titrated	Measles	Mumps	Rubella
Cell culture	Vero	Vero	RK-13
Medium	M199 + 2% FBS	M199 + 2% FBS	EMEM + 2% FBS
Virus diluent	47 parts medium, 2 parts anti-mumps serum (1/25), 1 part anti-rubella serum (1/50)	48 parts medium, 1 part anti-measles serum (1/50), 1 part anti-rubella serum (1/50)	24 parts medium, 1 part anti-mumps serum (1/25)
Range of dilutions	10^{-1}–10^{-4}	10^{-3}–10^{-5}	10^{-2}–10^{-4}

M199, Medium 199; FBS, fetal bovine serum: EMEM, Eagle's minimal essential medium

16. YELLOW FEVER VACCINE

16.1 Introduction

The potency of yellow fever vaccine is determined by an *in vitro* microtitration assay. The vaccine is assayed in parallel with a working reference preparation, which has been calibrated against the International Reference Reagent for Yellow Fever Vaccine, as a means of ensuring reproducibility of the test results.

An international collaborative study showed that virus plaque assays in Vero cells, using a microtitre technique, gave more reproducible results than intracerebral inoculation of dilutions made of the vaccine into mice. Since release of the vaccine by the manufacturer is based on results of the mouse LD_{50} test, each laboratory choosing to use the cell culture assay shall establish to the satisfaction of the national control authority the relationship between mouse LD_{50} and plaque-forming units (PFU).

Using the Vero cell technique, monolayers of the cell substrate are prepared in 6-well (35-mm) tissue culture plates. Serial four-fold dilutions of the reconstituted vaccine are inoculated in duplicate in the plate wells and incubated at 36 °C. After the first incubation period the inoculum is replaced by 3 ml of agarose overlay and the plates are further incubated at 36 °C. The final reading of the test is performed after 7 days of incubation. The cell cultures are stained with carbolfuchsin and washed, and the virus plaques are counted. In calculating the titre all dilutions should be considered in which the average number of plaques per well is between 1 and 30.

Since yellow fever virus is light sensitive the vaccine should be protected from direct light during storage and testing.

16.2 Materials

16.2.1 Standard equipment

- 6-well plates with wells of 35 mm
- micropipettes to deliver 0.2-ml volumes
- pipettes to deliver volumes of 1.0–5.0 ml
- incubator
- vacuum source.

16.2.2 Test vaccine

A vial of freeze-dried vaccine, reconstituted with reconstitution liquid accompanying the

vaccine, in the volume indicated. Maintain at 4 °C for 20 minutes until the vaccine has just dissolved. Use within 2 hours.

16.2.3 Reference vaccine

For each assay include a vial of yellow fever vaccine of which the titre has been well established as the working reference preparation.

16.2.4 Diluent

Diluent for vaccine dilutions: Medium 199 with 2% fetal bovine serum.
Diluent for seeding the cells: Medium 199 with 5% fetal bovine serum.

16.2.5 Cells

Use Vero cells[1]. The passage level of these cells should be documented and within 15 passages of the tested stock. The concentration of the cell suspension used for seeding the 6-well tissue culture plates depends on the time period before use:

- one day after seeding, 3.0×10^5 cells per ml
- two days after seeding, 1.5×10^5 cells per ml
- three days after seeding, 1.0×10^5 cells per ml.

16.2.6 Agarose overlay

- 50 ml of Medium 199 (2 x concentrated)
- 50 ml of agarose (1% in distilled water)
- 1 ml of antibiotics (penicillin, 1 000 IU/ml penicillin; streptomycin, 100 µg/ml; fungizone, 0.5 µg/ml)
- 1 ml of glutamine
- 5 ml of fetal bovine serum.

16.2.7 Dye

- Carbolfuchsin (may be filtered monthly and reused)
- 96% ethanol.

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[1] Available on request to national control laboratories from Chief, Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.

16.3 Test procedure

16.3.1 Seeding of Vero cell cultures

See also section 8

- Wash Vero cell monolayer flask cultures, trypsinize, centrifuge, count cells and prepare a cell suspension in Medium 199 with 5% fetal bovine serum to obtain new flasks having confluent monolayers of Vero cells.

- The cultures should be used the day on which a complete monolayer is formed, and preferably within 24 hours. The culture media should be changed one day before performing the test.
  Note: Old cell layers are not appropriate for the formation of yellow fever plaques.

16.3.2 Preparation of vaccine dilutions

Prepare a series of vaccine dilutions as shown in Table 12.

Table 12. Range of dilutions for use in the potency testing of live yellow fever vaccine

Volume of vaccine dilution (ml)	Volume of diluent added (ml)	Dilution
0.5 (undiluted vaccine)	4.5	1/10
0.5 (from 1/10)	1.5	1/40
0.5 (from 1/40)	1.5	1/160
0.5 (from 1/160)	1.5	1/640
0.5 (from 1/640)	1.5	1/2560

16.3.3 Plaque assay procedure

Remove medium from 6-well plates with complete monolayers of Vero cells.

- Inoculate two wells with 0.2 ml of each vaccine dilution, starting with the highest dilution and using one micropipette per vaccine. Inoculate two wells with 0.2 ml of diluent only, to serve as cell controls (CC).

- Repeat this procedure with reference vaccine dilutions.

- Shake the plates carefully by hand and repeat this every 15 minutes during the

incubation time, to distribute the inoculum equally over the monolayer and to prevent drying of the monolayer.

- Incubate at 36 °C for one hour.
- During incubation prepare the agarose overlay (see section 16.2.6).
- Add 3.0 ml of agarose overlay to each well of all plates.
- Let it solidify for 20 minutes at room temperature.
- Wrap the plates in aluminium foil or place them in zipper bags. If a humidified CO_2-incubator is used wrapping of the plates may be omitted.
- Incubate at 36 °C for 7 days.
- After 7 days of incubation, remove the agarose overlay by gentle shaking.
- Immerse the plates in for one minute.
- Wash the plates with fresh running tap water for one minute.
- Immerse the plates in carbolfuchsin for three minutes, with slow agitation.
- Empty the plates over the dye container and hold under fresh running tap water until the water remains clear.
- Air-dry the plates at 36 °C before reading.

Caution: Protective clothing should be worn during the staining procedure. The discarded agarose overlay should be suitably disinfected before discarding, and the ethanol used for fixation should be discarded in appropriate containers. The incubation time required to obtain clear plaques should be optimized for each laboratory.

16.4 Calculation of potency

- Count the number of plaques in all wells of all plates.
- Determine the mean number of plaques for each virus dilution.
- Determine potency using those dilutions showing 1–30 plaque-forming units (PFU) per well.

Example

Dilution of vaccine	*No. of plaques in well 1*	*No. of plaques in well 2*	*Mean no. of plaques per dilution*
1/40	TNTC	TNTC	TNTC
1/160	59	51	55
1/640	12	14	13
1/2 560	4	2	3
Diluent	0	0	0

TNTC, too numerous to count

Mean no. of plaques at dilution 1/640 = 13
Mean no. of plaques at dilution 1/2 560 = 3 multiplied by 4, resulting in equivalent count of a dilation of 1/640 = 12
Mean number of plaques at dilution 1/640 = (13 + 12)/2 = 12.5
12.5 PFU x 640 = 8 000 PFU per 0.2 ml
20 000 PFU per 0.5 ml (human dose)

Note: The titre of the vaccine should be not less than 1 000 mouse LD_{50} (the dilution at which 50% of the inoculated mice die) or its equivalent in plaque-forming units in the volume recommended by the manufacturer for use in humans.

16.5 Evaluation of results

For the test to be considered valid:

- The cell control wells should not show any plaque-forming or other cytopathic effect.

- The working reference vaccine should be within $10^{0.5}$ of its established mean titre.

Laboratories measuring the potency of yellow fever vaccine by the described procedure should establish the relation between the mouse LD_{50} and the PFU in cell culture. This relationship has been determined for some products in one laboratory as one human dose of 1 000 mouse (Swiss-Webster) LD_{50} = 3 000–30 000 PFU per 0.5 ml. Because of this variation, the range of dilutions of the vaccine used should be wide enough to ensure that the titre can be determined.

Yellow fever vaccine should also pass the following test for thermal stability:

- Three vials shall be held at 37 °C for two weeks and tested for potency, in

parallel with vials kept at storage temperature prior to testing. The mean loss in titre after heating should be not more than $10^{1.0}$.

16.6 Reporting of results

Every time the potency of yellow fever vaccine is determined, a working reference preparation which has been calibrated against the International Reference Reagent for Yellow Fever Vaccine should be included in the same test. This procedure has to be repeated periodically to avoid over- or under-estimation of vaccine potencies.