

## 4. Laboratory methods

This document should be read in association with the WHO Immunization, Vaccines and Biologicals online guide, "[Manual for the Laboratory-based Surveillance of Measles, Rubella, and Congenital Rubella Syndrome, Chapter 9](#)".

### 4.1. Introduction

For a serosurvey to succeed and produce valid results, it is essential to select competent test laboratories and appropriate assays. One of the functions of the laboratories within the GMRLN is to perform serologic testing to support assessments of population immunity (1). The GMRLN has a role in the validation of measles and rubella IgG assays that are used in serosurveys and in supporting non-GMRLN laboratories when implementing serosurveys. Irrespective of whether or not testing is performed by GMRLN laboratories, the testing laboratory should have objective evidence of having a good quality management system.

All aspects of the laboratory testing and reporting should be thoroughly planned well in advance of starting the study. Many GMRLN laboratories have extensive experience with serosurveys, whereas diagnostic laboratories may primarily perform measles and/or rubella testing for confirmation of recent infection. In some regions, scaling up diagnostic laboratories to test large numbers of serologic specimens will require extensive planning and may sometimes require additional human resources and training, assay validation, data management tools and laboratory equipment. During the planning for a serosurvey, comprehensive SOPs for all laboratory activities associated with the study must be documented and approved by all collaborators. The sections that follow describe the key laboratory activities for serosurveys.

This guideline document focuses on the currently available and commonly used methods for specimen collection and laboratory testing. It is important to note, however, that ongoing research and development will likely lead to the introduction of new technologies for conducting serosurveys in the near future.

### 4.2. Select type of specimen and method of collection

A serum specimen is most commonly used for serosurveys, as it is a validated sample type for almost all commercially available measles and rubella assays. The serum is separated from the cellular components by centrifugation or by use of serum separator tubes. Vacutainers with gel separator should be considered to reduce the risk of specimen contamination. Specimens can be transported after centrifugation in vacutainers with gel separator. Ensure the quantity of whole blood collected is sufficient to perform all tests and allow for some serum to be stored for repeat testing or for future surveys. Ideally, more than 5 ml of whole blood for adults and 2.5 ml for young children is required; however, 1 mL for infants may be sufficient. Note that the amount of serum obtained will be approximately half the volume of whole blood (2).

Paediatric collection tubes may be required, especially if the target population includes infants. For very young children and infants, where venipuncture is impractical, blood can be collected by finger or heel stick using specially produced sterile lancets and collected into paediatric blood collection tubes. A minimum volume required should be established in the serosurvey's sample collection SOP, as well as instructions in the case of an insufficient specimen.

Sample types other than serum can be considered for use in the serosurvey. It should be noted that assays are usually validated for specific sample types, which are detailed in the assay's instructions for use (IFU). It is important to validate alternate sample types during the planning phase of the serosurvey, prior to testing. A range of alternative sample types have been employed in serosurveys, including dried bloodspots obtained from finger prick or venous blood, oral fluid (3), breast milk (4) and blood collected from umbilical cords (5). Generally, the concentration of antibody-containing serum in alternative sample types will be lower than serum, possibly decreasing the analytical sensitivity of the assay. A standard approach to specimen collection and preparation for sample types other than serum must be documented in the SOP.

In some cases, serosurveys will be performed using serum specimens that were collected for another purpose (e.g. antenatal screening or another study). If such specimens are to be tested, the SOP should clearly describe how the specimens will be transferred to the laboratory performing the measles and/or rubella serology. Stored specimens may need to be aliquotted, re-labelled and stored in a manner to make the subsequent testing as efficient as possible.

### **4.3. Collect, store, transport and process specimens**

An SOP describing all aspects of specimen collection, transport, labelling and storage must be developed and approved by the steering committee. Quality control mechanisms should be established to ensure adherence to the SOP.

#### **4.3.1. Collection and storage in the field**

The laboratory should select the specimen collection and storage devices, ensure that adequate supplies are available at the collection sites and collection, and train staff appropriately in their use. General guidelines and a list of basic materials for specimen collection and handling are described in **Annex 3, Collection, storage and shipment of specimens for measles and rubella serosurveys**. These supplies must include materials needed for safe collection of specimens and appropriate disposal of used consumables, e.g. sharps containers.

The laboratory should consider pre-labelling tubes or supplying pre-printed adhesive labels with a unique identifier for each specimen. Hand-written labels are strongly discouraged. The laboratory should supply a paper-based form or Excel spreadsheet with the assigned specimen numbers corresponding to pre-printed labels so that a line list can be prepared at the time of collection.

Specimens can be processed at the collection site or at a central laboratory. The laboratory SOP should provide a complete description of how the specimens will be processed. If the serum cannot be separated at the collection site, whole blood must be kept at 2–8° Celsius and shipped to the central laboratory chilled. Irrespective, serum should be separated from red blood cells by centrifugation within 48 hours of collection. Serum should be transferred to externally threaded screw top plastic, pre-labelled cryovials ensuring traceability of identifiers and the serum specimens stored at -20° Celsius. Once frozen, serum should be shipped to the testing facility on dry ice. Repeated freeze-thawing should be avoided as it can affect the antibody levels in the specimens. Ideally, the number of freeze-thaw events should be recorded for each specimen (2, 6). Samples with insufficient volume or hemolysis should be noted on the spreadsheet.

To ensure adequate cold storage is available at the central laboratory and, where necessary, at the collection site, the cold chain requirements should be assessed and additional capacity planned where required. An SOP for transporting specimens from the collection site to the central laboratory should be developed. Any additional equipment required for specimen transport and storage should be included in the budget and procured and commissioned well in advance of implementation. Appropriate training of collection staff on all aspects of the serosurvey should be performed.

Training materials such as posters, videos and web sites may be used to supplement staff training. The competency of the staff should be assessed prior to initiation of the study.

If cold storage and transport are not available, blood or serum can be collected and small volumes dried and stored on filter paper (see **Annex 3 Collection, storage and shipment of specimens for measles and rubella serosurveys**). Use of serum or blood dried on filter paper increases the amount of labour required since the serum will need to be eluted from the filter papers before being tested. Protocols for elution of serum from dried filter paper blood spot are available in the Manual for the Laboratory-based Surveillance of Measles, Rubella, and Congenital Rubella Syndrome (6) Sections 3.4.1 and 3.4.2. The advantage of dried blood and serum spots is that they are stable at room temperature when stored desiccated and are considered non-infectious, allowing easier transport. However, the disadvantages are that they are not a validated sample type in most rubella and measles assays, they lack sensitivity due to low levels of antibodies, and they cannot be used in gold standard assays such as neutralization tests.

#### 4.3.2. Shipping and receipt of specimens

The Manual for the Laboratory-based Surveillance of Measles, Rubella, and Congenital Rubella Syndrome (6) Section 3.1 details the requirements for preparation and transport of clinical specimens, which are the same for serosurveys. In brief, the following are required:

- laboratory should have all of the equipment and supplies in place and staff fully trained in required procedures before starting blood collection;
- biologic materials must be packed for air transport conforming to International Air Transport Association (IATA) regulations;
- an SOP should detail the laboratory specimen reception protocol, specimen processing procedures and the manner and location of specimen storage; and
- on receipt at the laboratory, samples should be cross-checked with documentation and verified that they are in good condition.

#### 4.3.3. Entering and storing specimen data

All specimen information should be stored in a manner that ensures that the identification of the patient is maintained throughout the entire process (traceable) and that their identity can be determined at each step from data collection and analysis to reporting. Many laboratories have electronic laboratory management systems (LMS) in place. If not, specimen management can be accomplished with spreadsheets or workbooks; however, manual logbooks and data transcription are strongly discouraged due to risk of transcription errors. The SOP should include a data management plan that includes adequate protection of confidential information and a process for data back-up. It is important that test results can be linked to the patient demographics and associated metadata as specified by the study SOP.

### 4.4. Select serologic assay(s)

The laboratory SOP should clearly describe the serologic assay(s) to be used in the serosurvey. The chosen assay(s) should have well-established performance characteristics with the selected specimen type. Irrespective of the assay chosen, the manufacturer's instructions for use (IFU) should be followed. If any deviation from the IFU is considered, the variation must be validated prior to implementation. The assays most commonly used to detect measles and rubella IgG for serosurveys are virus neutralization or commercially produced EIAs. Other technologies, including point-of-care tests and multiplex bead assays may also be considered and are discussed below. It should be noted that commercial assays are designed for testing individuals for immunity or recent infection and are often configured for high specificity. There is some conjecture about the use of 10 IU/mL as a cut-off for determining immunity to rubella (7), with lower levels having been found to be true antibody reactivity (8, 9). Although most assays are calibrated with the same WHO international standard,

there is strong evidence that the results reported by different assays are not comparable (7, 10, 11, 12). Attempts to standardize rubella assays for serosurveys have been undertaken (13), and these issues should be reviewed when considering the study design and assay selection.

Procurement of assays from international sources usually requires customs clearance. In some countries, an import permit will be required. Access to permits may take some time, so advanced planning is required. As delays in reagent acquisition or testing of the specimens may occur, it is important to request test kits with a long expiry date. Also consider obtaining the same reagent lot for the entire study to exclude a source of variation. Validation of the performance of that reagent lot is essential. For more detail regarding rubella and measles testing, refer to Manual for the Laboratory-based Surveillance of Measles, Rubella, and Congenital Rubella Syndrome (6).

#### **4.4.1. Enzyme immunoassays**

Commercial EIAs, in the form of 96 well microtitre plates, are relatively inexpensive and do not require expensive equipment or extensive training. Testing can be completed in three to four hours. Although commercial IgG EIAs generally have acceptable levels of performance, variations in relative sensitivity and specificity have been reported (10, 12, 14). Most EIAs may be automated to allow larger numbers of test runs to be performed. WHO cannot recommend a particular commercial assay, but can direct laboratories to published reports on comparisons of different test kits and provide published data on their relative sensitivities and specificities. Laboratories using EIAs for testing are strongly encouraged to use commercially-available EIAs that have an established performance record within the GMRLN.

Some laboratories are required to go through national tendering processes that may limit the choice of commercial assays. If the performance characteristics of the available test kits are not established, the test kits must be validated against established assays or plaque reduction neutralization test (PRNT), tested in a laboratory with expertise with this assay. This validation must be conducted before the serosurvey is initiated and before the laboratory SOP is finalized. Assay evaluation requires particular expertise and should include assessments of clinical sensitivity and specificity, precision and analytical sensitivity. Therefore, in addition to reviewing relevant guidelines, the survey team should conduct validation in collaboration with GMRLN laboratories or other laboratories with expertise in assay evaluations (15).

#### **4.4.2. Automated immunoassays**

In most developed countries commercial automated immunoassays are available for testing serum for viral antibodies, including anti-rubella IgG. Many of these assays are based on chemiluminescent technology and have a high throughput and excellent sensitivity, specificity and precision compared with microtitre plate EIAs. As the technology requires no manual steps, the standardization of results is high. However, the instrumentation and reagents are expensive, and the laboratory will require a level of sophistication in infrastructure. There are fewer automated immunoassays that measure measles IgG compared to rubella.

#### **4.4.3. Virus neutralization assays**

The gold standard for measuring IgG antibodies to measles are virus neutralization assays, where the number of viral plaques formed in cell culture are compared with the number of plaques formed when the virus is grown in the presence of neutralizing antibodies present in the serum (16). The PRNT detects functional neutralizing antibodies to measles and is very valuable because the results correlate with protection from infection. The serum titre used to determine measles immunity is based on the evaluation of PRN titres that appeared to provide protection from clinical disease during a measles outbreak. A study of an outbreak of measles that occurred at a Boston college included students who had participated in a blood drive prior to exposure. The PRN titre that

corresponded to the protective titre was  $\geq 120$  mIU/ml when standardized against the WHO measles antibody international standard (currently the WHO 3rd international standard; NIBSC 97/648).

There is no level of rubella neutralization that is considered protective for rubella. However, CDC has in the past defined 10 U/mL as a measure of neutralization and therefore used it as a cut-off. Others have suggested lower levels could be protective (17, 18).

At present, neutralization assays for measles and rubella are performed in a limited number of specialized laboratories. These assays require a high level of training and expertise, are labour intensive and are not amenable for testing large numbers of serum specimens (maximum 100 to 200 specimens per week). However, PRNT may be a useful tool to elucidate specimens reporting repeatedly equivocal results or as a gold standard in test kit evaluations.

For more detail, refer to Manual for the Laboratory-based Surveillance of Measles, Rubella, and Congenital Rubella Syndrome (6) Section 9.2 and 9.3.

#### **4.4.4. Multiplex (Multiimmuno) bead assays**

A technology that has more recently been employed for serosurveys is the multiplex bead assay (19, 20, 21). Viral antigens are attached to the solid phase, which are beads of a different fluorescent colour. The serum sample is incubated with the beads and subject's antibodies to each viral antigen are bound to the corresponding bead. The presence of bound antibodies is detected separately by immunofluorescence.

The advantages of this technology are that antibodies to multiple antigens can be detected and quantified simultaneously and that each test requires a small volume of serum (as low as 1  $\mu$ L). Currently, multiplex assays for measles and rubella are not commercially available and each laboratory is required to validate its own assay based on published methods and technical consultations. However, GMRLN laboratories are presently working on standardizing multiplex assays for measles and rubella IgG.

#### **4.4.5. Other assays to detect IgG**

In addition to EIA, plaque neutralization and multiplex assays, measles IgG has been detected by particle agglutination assays (22) and by haemagglutination inhibition (HAI) (10, 23, 24). Measles HAI assays are no longer used because they require a source of erythrocytes from non-human primates. Newer methods are being developed to replace measles PRNT, which leads to the possibility of shorter incubation times, automation and high throughput (25). Many of these assays were developed using recombinant measles viruses that express reporter proteins (26). Although HAI was considered the gold standard for rubella IgG detection and quantification, it is now rarely used for serosurveys as it is difficult to standardize, is labour intensive and requires considerable expertise. Point of care tests (PoCT), using finger-prick capillary blood or oral fluid, have been developed for the detection of a range of anti-viral antibodies. To date, there are no commercially available PoCTs for measles or rubella IgG detection; however, if these are developed in the future, they could simplify the serosurveys process as the testing could be performed at the time of interview.

### **4.5. Interpreting EIA results**

Most commercial EIAs and automated immunoassays for rubella and measles IgG determination have been developed for testing individuals for diagnosis and determining protective immunity, but not for population studies. The manufacturers' cut-off for "Positive" may have been optimized for increased specificity rather than sensitivity. Many EIAs for measles IgG and almost all EIAs for rubella IgG are quantitative, being calibrated against the WHO international standard and reporting results in milli-international units per milliliter (mIU/mL) or IU/mL, respectively. Manufacturers of many test kits include an equivocal range, in which the specimen result is considered neither positive nor negative (27). If equivocal results are obtained the samples should be retested. If the result is

positive or negative on repeat, this result can be used. However, some samples will be repeatedly equivocal. A process to resolve the status of these specimens is required. For detailed information regarding the interpretation of measles and rubella test results, refer to Manual for the Laboratory-based Surveillance of Measles, Rubella, and Congenital Rubella Syndrome (6) Sections 9.2 and 9.3.

#### 4.5.1. Resolving equivocal results

The serosurvey laboratory SOPs should clearly describe how specimens with repeatedly equivocal results will be resolved. In some cases, equivocal results can be considered as either positive or negative results for the purpose of analysis. The protocol must give clear guidance about how to categorize equivocal results in the prevalence calculations. The number of specimens that repeatedly test equivocal and are reclassified for prevalence calculations should be documented before proceeding with the prevalence calculation. The possible alternatives are described below.

**Counting equivocal results as seropositive:** An argument can be made to count equivocal results as positive. In general the cut-off in commercial assays is usually set at a level of IgG that is higher than the minimum level required for protection or seen in vaccinated individuals whose antibodies have waned.

**Counting equivocal results as seronegative:** Alternatively, the conservative choice would be to consider specimens with equivocal results to be seronegative, and thereby err on the side of underestimating the population seroprevalence.

**Mixture modelling:** An established method to optimize the positive/negative cut-off in populations of results. This approach avoids the need to re-categorize equivocal results and has been used extensively for seroprevalence studies; however, this approach requires adequate statistical expertise that should be sought if not available (28).

**Creating a separate category for equivocal results:** A protocol may opt to count equivocal results as a distinct category. The reader can then conduct a sensitivity analyses by combining the equivocal results with either the positive or negative results.

**Testing specimens with equivocal results in a second line or confirmatory assay:** Specimens with equivocal results could be retested with a more sensitive and biologically accurate assay such as neutralization assays. This option will usually involve collaboration with a GMRLN Regional Reference or Global Specialized Laboratory. Alternatively, specimens with repeatedly equivocal results could be tested in a second-line EIA or other technology. The testing strategy should be validated prior to use. For example, specimens with repeatedly equivocal rubella IgG may be tested using an immunoblot, which has been shown to correlate well with protective immunity (10, 8, 9).

### 4.6. Record and store specimen result data

The laboratory SOP must include procedures for collecting, storing, analysing and reporting testing data. The test results should be traceable to the patient, usually linked by patient identification, a unique patient number and/or the survey number. Data quality checks should be implemented to ensure completeness and accuracy of data stored. At a minimum the following should be considered:

- **Participant Details:** Each participant should be assigned a unique identification number that is used throughout the entire process. The unique identifiers must be transcribed onto each specimen aliquot and each laboratory worksheet if used. On receipt of the specimen at the laboratory, the information on the request form and the blood specimen should be cross checked for accuracy and information added to the data management system.
- **Laboratory Information System:** The testing information must be recorded in a data management system. At a minimum a laboratory workbook can be used, detailing each of the



identifiers for each specimen, including date of collection and date of reception at the laboratory, as well as any comments specific to the specimen.

- **Laboratory Test Results:** The results of testing must be identified with the participant's details to enable analysis. Results of initial and repeat testing should be recorded and stored for future reference in a traceable manner.
- **Specimen Storage:** The labelled specimen should be stored at -20° Celsius to enable retesting and possible future use. Storage should be in a manner that allows easy recovery of the sample, e.g. by maintaining a computer database that lists in which rack of which freezer each specimen is stored.

## 4.7. Quality of laboratory testing

The laboratory SOP must describe the quality control measure that will ensure that the laboratory testing is accurate and consistent. Quality control measures must cover all aspects of testing, including assay validation and implementation, staff training and competency, equipment maintenance, and analytical controls such as run controls and external quality assessments scheme (EQAS), also known as proficiency testing (PT).

### 4.7.1. Test kit selection and validation

The serosurvey protocol should clearly document the reasons for the selecting the test kit(s) used in the study. Where the test kit has limited documented performance characteristics, or where a deviation or a sample type other than that specified in the manufacturer's IFU is to be used, an evaluation should be undertaken to validate the suitability of the selected test kit, the variation from the IFU or the specimen type. The validation may be performed with the help of GMRLN Regional and Global Specialized Laboratories. The extent of the evaluation is dependent on the amount of existing information available. Protocols for assay evaluations are available elsewhere (15).

### 4.7.2. Instrumentation and equipment

All equipment used for the laboratory testing must be commissioned and maintained as documented in the laboratory quality management system. The SOP must include an equipment maintenance and calibration plan. A record of all calibration, maintenance and servicing should be retained. Ideally, a back-up to the electricity supply such as a generator or UPS should be available. Regular checks of all equipment, with established acceptance criteria, should be performed and documented. All refrigerators and freezers should be monitored using calibrated max-min thermometers, and the temperature should be recorded in a systematic manner. A process for dealing with instances where the acceptance criteria are not met should be established and followed. A quality officer should ensure the checks are being conducted and regularly record the results of equipment monitoring.

### 4.7.3. Internal quality control

The laboratory SOP must describe the nature and frequency of internal quality controls used for each test run. The manufacturer's controls must be tested in each EIA plate in the number and position as described in the manufacturer's IFU. If the manufacturer's validation criteria are not met, the assay is invalid and all specimens tested on that test run must be retested. If the test run repeatedly fails, a root cause investigation should be undertaken and the issue rectified before re-starting testing.

### 4.7.4. Non-manufacturer run control

The manufacturer kit controls are usually optimized for the reagent lot number. As lot numbers change, so does the reactivity of the kit controls. Therefore, it is useful to have a control, external to the manufacturer's controls, which can be tested in each test run over a long period of time. The results of this non-manufacturer control, including associated data such as reagent lot numbers, operator identification, and calibration and maintenance activities, are plotted on a Levey-Jennings

chart, and variation in the test system monitored over time (2, 29). Acceptance criteria based on upper and lower limits should be established. Results of the non-manufacturer results should not be used to validate the test run; however, if the results fall outside the acceptance limits, an investigation into the reason for the greater variation should be undertaken.

When selecting controls, ensure the level of reactivity of that control is appropriate for the assay in use before purchasing. Also make sure that the reactivity of the control does not vary from control lot to control lot. Store the control as per the manufacturer IFU. It is important that the control does not contribute to the variation being monitored.

When plotting the results of kit or non-manufacturer controls, it is important to plot the signal to cut-off or quantitative results (mIU/mL or IU/mL) rather than the optical density. The optical density will vary considerably due to incubation time, temperature and wash effectiveness. However, these variations are normalized when converted to a signal to cut-off ratio or quantitative result.

#### **4.7.5. External quality assessment scheme**

Participation in an EQAS or PT is strongly recommended (2, 29). Numerous programmes are available. EQAS providers send a panel of samples to the participants several times per year. The status of each sample is unknown by the participant. The participating laboratory tests each sample using its routine testing strategy and reports the results back to the EQAS provider. The results obtained from all participants are analysed and a report is generated. EQAS results give the participant objective evidence of a level of quality of testing. Note that EQAS providers often have remnant EQAS panels available that can be used for assay validation. It is recommended that an EQAS provider accredited to ISO 17043 is used.

#### **4.7.6. External re-testing**

Consider retesting a subset of the specimens. Retesting may be performed at the same laboratory on the same assay or at the Regional Reference or Global Specialized Laboratory; however, approval by the reference laboratory to refer specimens should be sought prior to including this activity in the study plan. The SOP should document the percentage of samples to be repeated and which laboratory will perform the re-testing. Acceptance criteria and the response to results outside those criteria must be established prior to the study starting.

### **4.8. Laboratory safety**

The laboratory protocol should describe laboratory safety for serologic testing (2). All human samples must be assumed infectious and universal precautions used. The use of appropriate personal protective equipment and biosafety cabinets as well as protocols for the safe decontamination of equipment and disposal of biologic waste must be included. Incineration or autoclaving are the preferred methods of disposal. The SOP should also note any institutional requirements for vaccination of project staff against measles, rubella and blood-borne pathogens.



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