

Manual for the laboratory diagnosis of measles and rubella virus infection

Second edition

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Contents

<i>Abbreviations and acronyms</i>	<i>vii</i>
<i>Acknowledgements</i>	<i>ix</i>
1. Purpose	1
2. Introduction	2
2.1 Global health burden of measles and rubella	2
2.2 Measles	4
2.3 Rubella	9
2.4 WHO global strategies for measles control and CRS prevention	15
3. Role and function of the laboratory in measles control and CRS prevention	18
3.1 Role of the laboratory in measles and rubella surveillance	18
3.2 Structure and activities of the laboratory network in measles and rubella surveillance	19
3.3 Coordination of the network	22
3.4 Molecular epidemiology and genotyping	22
4. Specimen collection, shipment, receipt and processing	30
4.1 Documenting sample collection	31
4.2 Serological samples for antibody detection	31
4.3 Samples for virus isolation	35
4.4 Samples for RT-PCR	37
4.5 Alternative sampling techniques (dried blood samples and oral fluid)	37
4.6 General safety precautions on receipt of samples	40
5. Laboratory diagnosis of measles and rubella	41
5.1 IgM assays	42
5.2 Tests for IgG antibody	46
5.3 RT-PCR	46
5.4 Virus isolation in cell culture	47
6. Data management and reporting	49
6.1 Data management goals	49
6.2 Recording receipt of specimen	52
6.3 Recording results	53
6.4 Reporting laboratory activity and results	53

7. Safe sample and isolate transport	55
7.1 Planning	55
7.2 Packaging	56
7.3 Preparation and sending	57
8. Quality assurance in Network laboratories	58
8.1 Establishing LQA systems	58
8.2 SOPs	61
8.3 Documentation	62
8.4 Equipment and instruments	62
8.5 Supplies	63
8.6 Laboratory safety	64
8.7 Annual accreditation	65
9. Annexes	68
9.1 Example measles and rubella laboratory request form	69
9.2 Extraction of measles specific IgM from dried blood specimens and detection for use in the Dade Behring indirect measles IgM antibody assay [After 39]	70
9.3 Quality control and trouble shooting of measles and rubella serological assays	73
9.4 Isolation and identification of measles and rubella virus in cell culture	77
9.5 Packaging of specimens and virus isolates for transportation	95
9.6 Composition of media and reagents	99
10. Suggested further reading	101
10.1 Global impact of measles and rubella	101
10.2 Control strategies	102
10.3 Laboratory	103
10.4 Laboratory safety and sample transport	104
11. References	105

List of Figures

Figure 1:	Reported global measles incidence.....	3
Figure 2:	Clinical features of typical measles - time course from onset of illness	5
Figure 3:	Immune responses in acute measles infection.....	6
Figure 4:	Diagram of the measles virus particle correlated with the genetic map	7
Figure 5:	Clinical features of typical rubella infection - time course from onset of illness	11
Figure 6:	Immune response in typical rubella infection	12
Figure 7:	Diagram of the rubella virus particle correlated with the genetic map. The E1 and E2 glycoproteins exist as heterodimers.....	13
Figure 8:	Countries using rubella vaccine in routine immunization schedules, 2004	17
Figure 9:	Laboratory network for measles and rubella surveillance activities at each level	21
Figure 10:	Global distribution of measles genotypes in WHO regions yet to eliminate measles 1995-2006	26
Figure 11:	Global distribution of rubella genotypes 1995-2005	29
Figure 12:	Packaging of serum samples. A - individual samples in a sealed bag or pouch. B - multiple samples in an insulated container.....	34
Figure 13:	Example format for blood spot collection card	38
Figure 14:	Capture IgM ELISA schematic	42
Figure 15:	IgM indirect ELISA schematic.....	43
Figure 16:	Measles/rubella IgM testing strategies under different disease control conditions.....	44
Figure 17:	Panel A shows cytopathic effect (CPE) caused by measles virus infection of Vero/SLAM cells. Top left panel shows uninfected Vero/SLAM cells; other panels show development of CPE (1+ to 4+ syncytium formation) after infection with wild-type measles virus	81
Figure 18:	Typical results for detection of rubella virus in cell culture using two different immunoassays: immunofluorescent assay (top row, rubella virus infected and mock-infected) and immunocolorimetric assay (bottom row, rubella virus infected and mock-infected).	82

List of Tables

Table 1:	Sequence of measles virus infection in uncomplicated primary disease	5
Table 2:	Clinical features of typical rubella infection - time course from onset of illness	10
Table 3:	Estimated measles deaths and percentage reduction, by geographical region, 1999-2004	16
Table 4:	Role of the laboratory in measles and rubella control and elimination	19
Table 5:	Reference strains to be used for genetic analysis of wild-type measles viruses	24
Table 6:	Reference strains to be used for genetic analysis of wild-type rubella viruses	28
Table 7:	Minimum requirements for measles and rubella serology and virus detection according to phase	30
Table 8:	Density of water at various temperatures and barometric pressures	76

Abbreviations and acronyms

µg	microgram
µg	microgram
µl	microlitre
B95a	Epstein-Barr virus-transformed, marmoset B lymphoblastoid cell line
BSA	bovine serum albumin
BSC	biological safety cabinet
BSL	biological safety level
CDC	Centers for Disease Control and Prevention, Atlanta USA
cDNA	complementary DNA
CO ₂	carbon dioxide
CPE	cytopathic effect
CRI	congenital rubella infection
CRS	congenital rubella syndrome
DNA	deoxyribonucleic acid
DMEM	Dulbecco's Minimal Essential Medium
DMSO	dimethyl sulfoxide
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EPI	Expanded Programme on Immunization
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
G	relative centrifugal force
g	gram
GAVI	Global Alliance for Vaccines and Immunization
GSL	Global Specialized Laboratory
HPA	Health Protection Agency, London UK
IATA	International Air Transport Association
ICAO	International Civil Aviation Organization
IgA	Immunoglobulin class A
IgG	Immunoglobulin class G
IgM	Immunoglobulin class M
IFA	immunofluorescence assay
IVB	Department of Immunization, Vaccines and Biologicals

kPa	kilopascals
LQA	laboratory quality assurance
LSM	Lymphocyte Separation Medium
MEM	minimal essential medium
mg	milligram
ml	millilitre
MM	maintenance medium
MR	measles rubella combination vaccine
MMR	measles mumps rubella combination vaccine
NL	National Laboratory
nm	nanometre
OD	optical density
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PT	proficiency test
QC	quality control
RF	rheumatoid factor
RNA	ribonucleic acid
RNase	ribonuclease
RPM	revolutions per minute
RRL	Regional Reference Laboratory
RT	reverse transcription
RT-PCR	reverse transcription - polymerase chain reaction
SIAs	supplemental immunization activities
SLAM	signaling lymphocyte-activation molecule; also known as CDw150
SNL	Sub-National Laboratory
SOP	standard operating procedure
TMB	3,3',5,5'-tetramethylbenzidine
UNICEF	United Nations Children's Fund
V&B	Department of Vaccines and Biologicals (<i>now named IVB, see above</i>)
Vero cells	continuous cell line derived from African green monkey kidney
Vero/SLAM	Vero cells transfected to express the human SLAM molecule
VTM	viral transport medium
WHA	World Health Assembly
WHO	World Health Organization

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1. Purpose

The purpose of this manual is to support measles control and prevention of congenital rubella infection activities by:

- Presenting accurate information on the pathogens, diseases, immune responses and prevention strategies;
- Describing the expected role of the laboratory in disease control and prevention;
- Outlining the requirements of effective laboratory surveillance; and
- Presenting detailed descriptions of procedures recommended for effective laboratory diagnosis of measles and rubella infection and general descriptions of procedures for the genetic characterization of wild-type strains of measles and rubella.

The manual is intended for use by virologists and technologists working in laboratories collaborating in measles and rubella control and efforts. It may also be of interest to managers of measles control programmes and prevention of congenital rubella infection programmes and field staff, who will be better able to appreciate the role of the laboratory and use it appropriately.

As of January 2005, four WHO Regions had adopted measles elimination targets (Americas, Europe, Eastern Mediterranean and Western Pacific), and two had adopted rubella elimination or major reduction of rubella targets (the Americas and Europe respectively). The remaining two WHO regions (Africa and South East Asia) had established measles mortality goals. At that time, however, approximately 60% of countries and territories reporting to WHO had introduced rubella vaccine, usually as measles-mumps-rubella (MMR) vaccine, into their routine immunization schedules. Integration of measles and rubella surveillance, particularly with regard to laboratory confirmation of infection, is a rational and cost-effective strategy in most circumstances, and this manual makes reference to integrated measles-rubella surveillance. Member States in WHO Regions that have not adopted rubella control targets, and do not use a rubella vaccine in their routine immunization programmes, should refer to the WHO Regional Office and the Regional Laboratory Coordinator for specific recommendations for surveillance and laboratory confirmation of rubella infection.

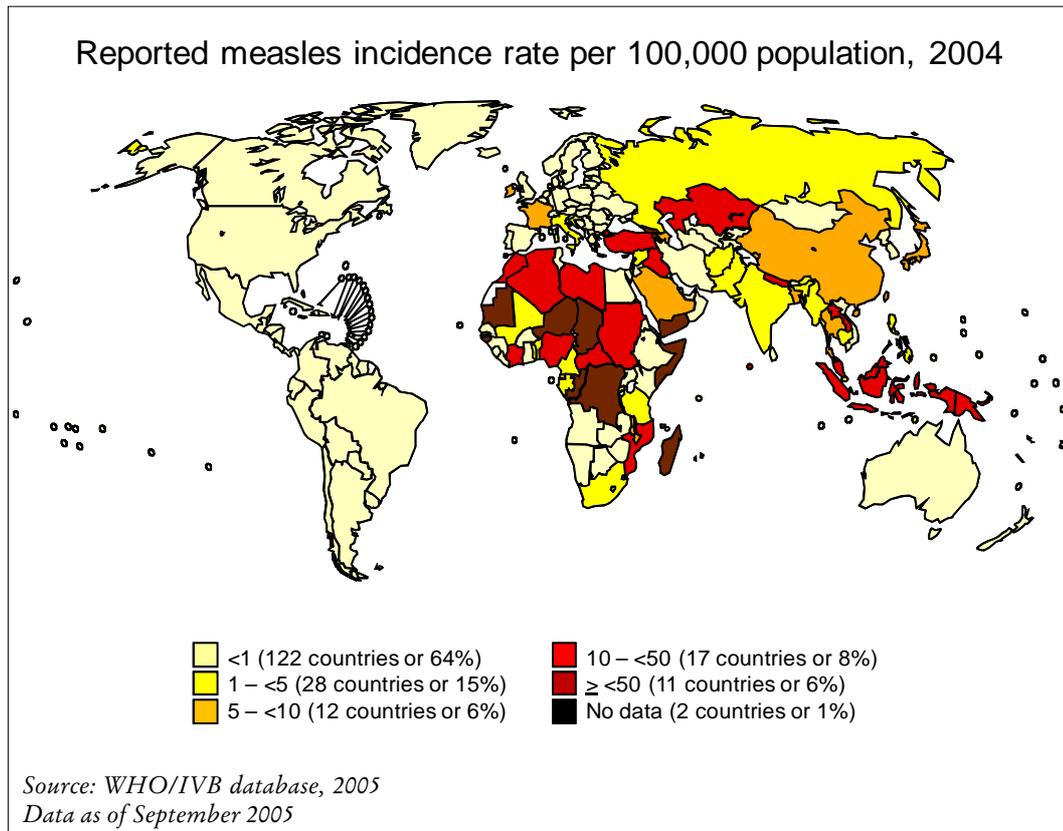
2. Introduction

2.1 Global health burden of measles and rubella

Before the introduction of measles vaccines in the 1960s, almost everyone contracted measles, usually during childhood. The result was an estimated 130 million cases and more than 2.5 million deaths due to measles (mainly children) each year [1]. Despite the availability of a safe, effective and relatively inexpensive measles vaccine for more than 40 years, measles kills more children today than any other vaccine-preventable disease, mainly in developing countries. In 2004 there were an estimated 20-30 million cases of measles worldwide and 453 000 deaths (uncertainty bounds: 329 000 deaths; 595 000 deaths) [2], one-third of all vaccine-preventable childhood deaths. Measles infection is associated with a high fever, rash, and cough, affecting mostly children, but also young adults. Children usually do not die directly of measles, but from its complications such as pneumonia and diarrhoea as a result of the immunosuppression associated with measles infection. The disease can also lead to lifelong disabilities including brain damage, blindness and deafness. Measles is one of the most contagious diseases known to man and often occurs in explosive epidemics.

In countries where measles vaccine is widely available, serious complications and measles deaths are rare. The highest mortality is found in the poorest countries. Massive efforts to improve vaccine coverage in the highest mortality countries in the world resulted in an estimated 48% decrease in measles deaths between 1999 and 2004, nevertheless more than 47% of measles deaths continue to occur in the African Region of WHO [2].

Figure 1. Reported global measles incidence



Rubella is a mild illness that presents with fever and rash. The public health importance of rubella is because infection in the early months of pregnancy usually affects foetal development [3]. Rubella infection of the foetus can result in miscarriage, foetal death or the birth of an infant with serious congenital birth defects. Congenital rubella syndrome (CRS) is an important cause of blindness, deafness, congenital heart disease, and mental retardation. Worldwide, it is estimated that more than 100 000 infants are born with CRS each year. Most of these cases occur in developing countries that have not yet introduced rubella vaccine [3].

The extent and quality of CRS surveillance remains poor, with fewer than 0.1% of estimated CRS cases reported. Many countries have not yet included CRS in their communicable disease surveillance systems, and some countries with well-established CRS surveillance fail to submit complete data to WHO/UNICEF. More countries need to establish effective CRS surveillance systems, and report their surveillance results to WHO/UNICEF, before the true burden of rubella can be measured.

2.2 Measles

2.2.1 *Epidemiology, infection and immune response*

The name measles is derived from the Latin, *misellus*, meaning miserable. The disease is also sometimes known as *rubeola* (from *rubeolus*, Latin for reddish) or *morbilli* (from *morbus*, Latin for disease). There are references to measles as far back as the 7th century. In the 10th century, Rhazes, a Persian physician, described measles as being more dreaded than smallpox. Prior to the introduction of vaccination programs, measles was almost always a disease of childhood. In densely populated areas, measles most commonly affected children aged 3-4 years old. In less crowded urban areas and in rural areas, the highest incidence was among children aged 5-10 years who contracted the disease on entering school [4].

In temperate climates, epidemics of measles tended to occur at 2-5 year intervals and lasted 3-4 months. In general, the larger the size of the community, the shorter the interval between epidemics. Measles vaccination programs have had a marked effect on the incidence of the disease and the complications associated with it. There has also been a shift in the age at which measles is most commonly contracted in these populations. After prolonged periods of high vaccine coverage in developed countries, measles transmission now occurs mainly in people that have never been vaccinated and in older children who did not seroconvert following vaccination. Measles outbreaks can still occur in countries with high immunization coverage. Such outbreaks demonstrate an immunity gap in the population involved .

Measles is one of the most easily transmitted diseases. Transmission is primarily by large droplet spread or direct contact with nasal or throat secretions from an infected person. Less commonly, it is spread by airborne aerosolised droplet nuclei or by indirect contact with freshly contaminated articles. Measles is highly communicable, with a secondary attack rate among susceptible persons of more than 90%. A number of factors tend to increase the severity of measles in developing countries. For example, overcrowding facilitates person-to-person transmission of the virus and increases the likelihood of exposure to high viral loads [5]. The measles vaccine virus is not communicable.

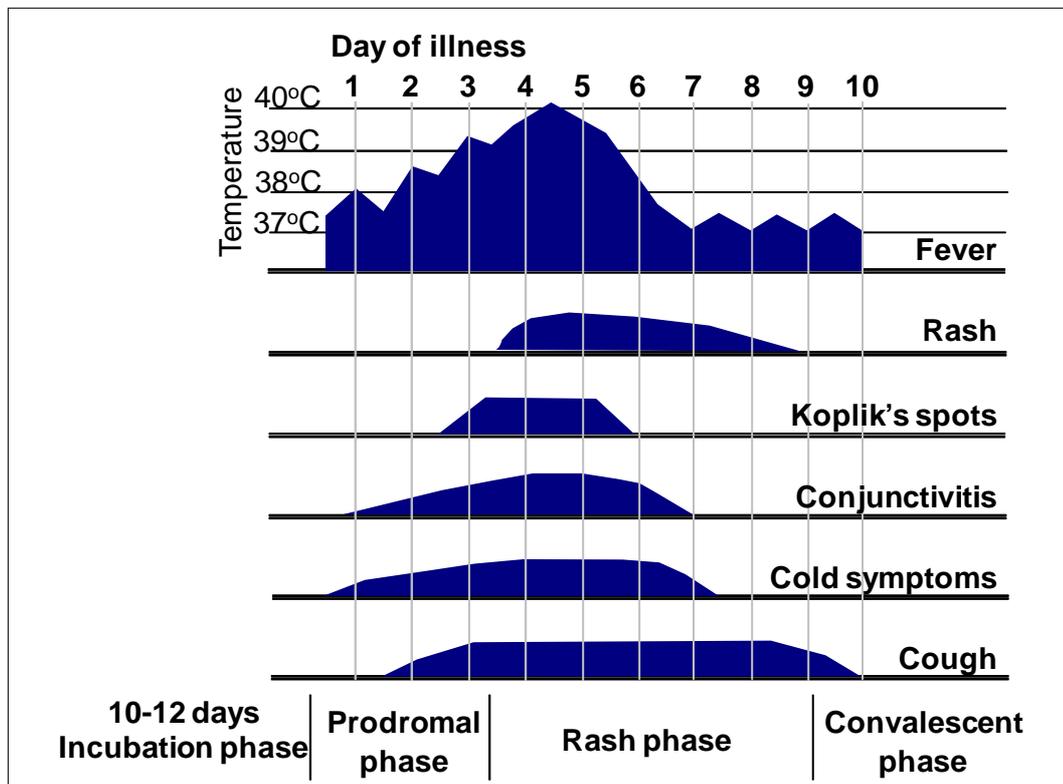
After infection, the measles virus invades the respiratory epithelium of the nasopharynx and spreads to the regional lymph nodes (Table 1). After 2-3 days of replication in these sites, a primary viraemia widens the infection to the reticuloendothelial system. Following further replication, a secondary viraemia occurs 5-7 days after infection and lasts 4-7 days. During this viraemia, there may be infection and further virus replication in the skin, conjunctivae, respiratory tract and other organs, including the spleen, thymus, lung, liver, and kidney. The viraemia peaks 11-14 days after infection, and then declines rapidly over a few days.

Table 1. Sequence of measles virus infection in uncomplicated primary disease (after [6]).

Day	Event
0	Measles virus in respiratory droplets comes into contact with the epithelial surface of the nasopharynx. Infection of epithelial cells and viral multiplication
1-2	Spread of virus to regional lymphoid tissue
2-3	Primary viraemia
3-5	Multiplication of measles virus in respiratory epithelium, regional lymph nodes and distant sites
5-7	Secondary viraemia
7-11	Establishment of infection in the skin and other sites, including the respiratory tract
11-14	Virus in the blood, respiratory tract, skin and other organs
15-17	Viraemia decreases and then ceases, viral content in organs rapidly diminishes as immunity builds

Infection of the respiratory tract gives rise to the characteristic cough and coryza (Figure 2), and the less frequent complications of croup, bronchiolitis and pneumonia. Generalised damage to the respiratory tract causes the loss of cilia and predisposes to secondary bacterial infections, such as pneumonia and otitis media. Immune reactions to the virus in the endothelial cells of dermal capillaries cause the measles rash and the measles enanthem (Koplik's spots), while interaction between virus-infected cells and local cellular immune factors is thought to be involved in measles encephalitis[4]

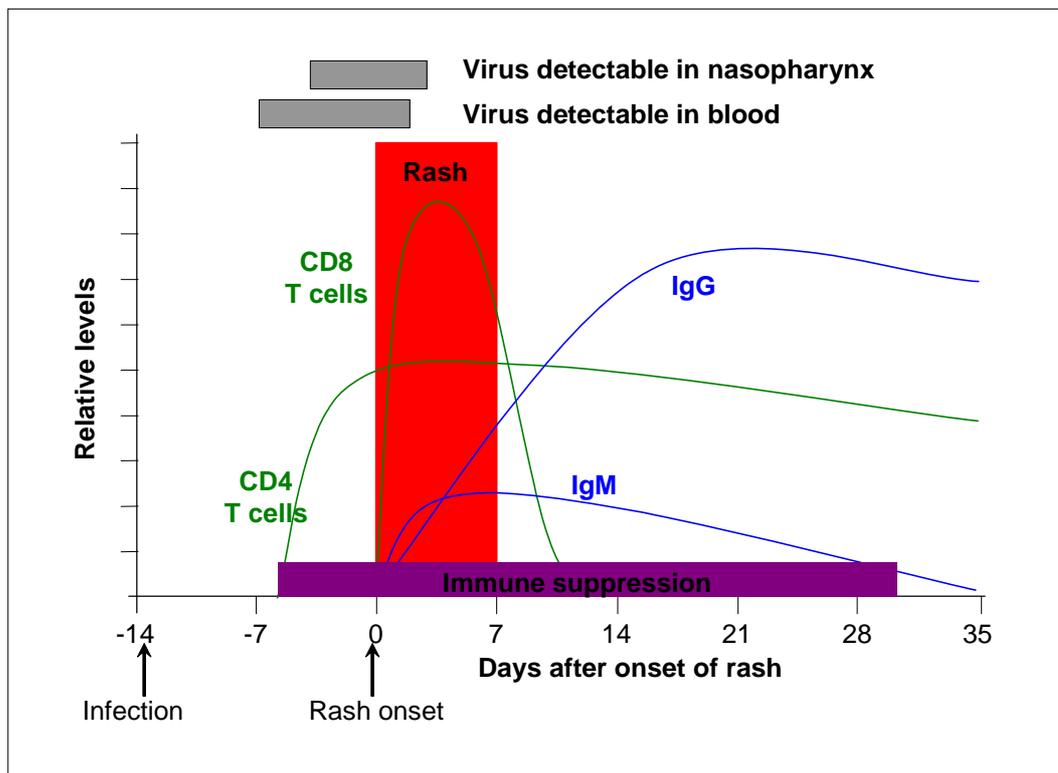
Figure 2. Clinical features of typical measles - time course from onset of illness (after [4], [6] and[7]).



Both IgM and IgG antibodies are produced during the primary immune response and can be detected in the serum within a few days of rash onset (Figure 3). Using sensitive ELISA IgM assays, 90% of measles cases are IgM positive at 3 days post rash onset [8]. IgM antibody levels peak after about 7-10 days and then decline rapidly, being rarely detectable after 6-8 weeks. IgG antibody levels peak within about 4 weeks and persist long after infection. Serum IgA and secretory IgA antibodies are also produced [9].

Re-exposure to the measles virus induces a strong anamnestic immune response with a rapid boosting of IgG antibodies, which prevents clinical disease. It appears that once the immune system has been primed through natural infection, immunity is lifelong. Cellular immunity, consisting of cytotoxic T-cells and possibly natural killer cells, plays a prominent role in immunity and recovery from acute infection. Patients with defects in cell-mediated immunity often suffer severe progressive measles infections and have a significantly increased risk of death. Measles-specific immune suppression begins with the onset of clinical disease, before the rash, and continues for several weeks after apparent recovery [7].

Figure 3. Immune responses in acute measles infection (after [7])



2.2.2 *Measles virus*

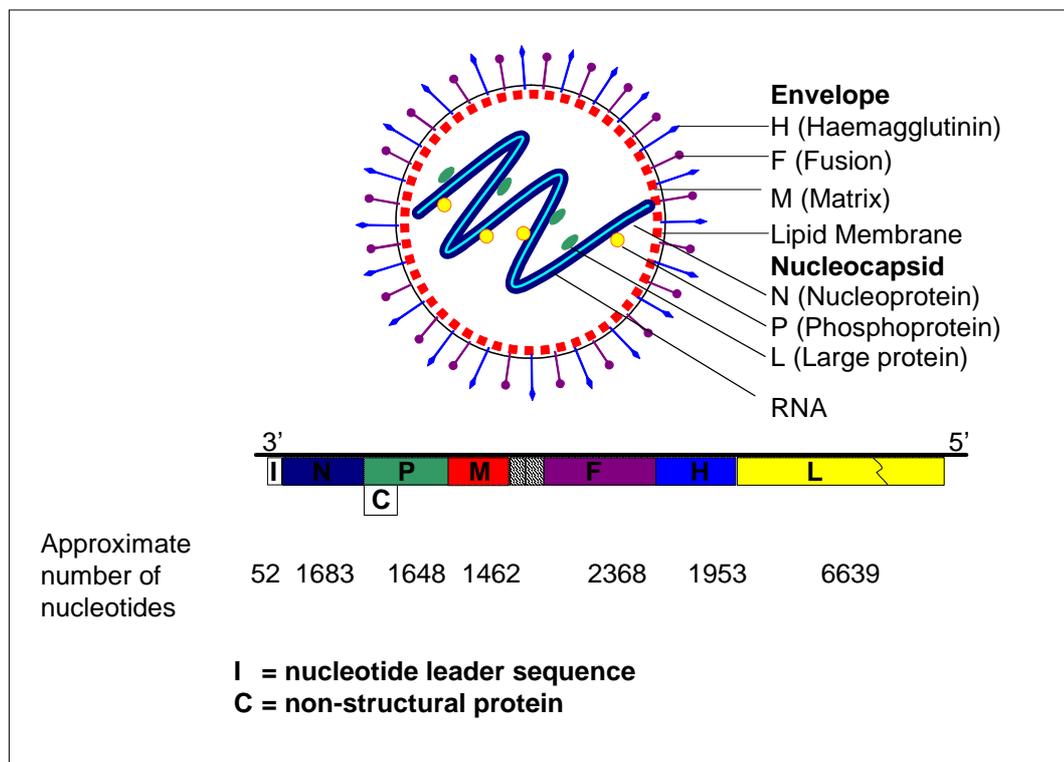
Measles virus is a paramyxovirus belonging to the genus Morbillivirus. Paramyxoviruses are so called because they have an affinity for mucous membranes (Greek: myxa = mucus). It is a pleomorphic virus ranging in diameter from 100 to 200 nm, with a mean diameter of 150 nm. Within the morbilliviruses, it is most closely related to the rinderpest virus group, and more distantly related to the canine distemper virus group. Two membrane envelope glycoproteins are important in pathogenesis. These are the F (fusion) protein, which is responsible for fusion of

virus and host cell membranes, viral penetration, and haemolysis, and the H (haemagglutinin) protein, which is responsible for binding of virus to cells (Figure 4). Although there is only one serotype of measles virus, there is genetic variability in wild-type viruses. WHO currently recognizes 23 genotypes of MV [10] with 16 genotypes identified since 1990. This genotype variation does not appear to be biologically significant, in that there is no change in vaccine efficacy.

The virus has a non-segmented, negative sense RNA genome with a linear arrangement of genes that are separated by an intergenic trinucleotide, GAA. Each gene contains a single open reading frame (except P), transcriptional start and stop signals, and a polyadenylation signal. The entire measles genome consists of 15,894 nucleotides. An example of a full length genomic sequence can be found at EMBL/GenBank under the accession numbers K01711; X16565.

Measles virus is viable for less than 2 hours at ambient temperatures on surfaces and objects, while the aerosolized virus remains infective for 30 minutes or more. It is very sensitive to heat and is inactivated after 30 minutes at 56°C. However, the virus appears to survive freeze-drying relatively well and, when freeze-dried with a protein stabilizer, can survive storage for decades at -70°C. The virus is inactivated by solvents, such as ether and chloroform, by acids (pH<5), alkalis (pH>10), and by UV and visible light. It is also susceptible to many disinfectants, including 1% sodium hypochlorite, 70% alcohol and formalin.

Figure 4. Diagram of the measles virus particle correlated with the genetic map (after [12, 13])

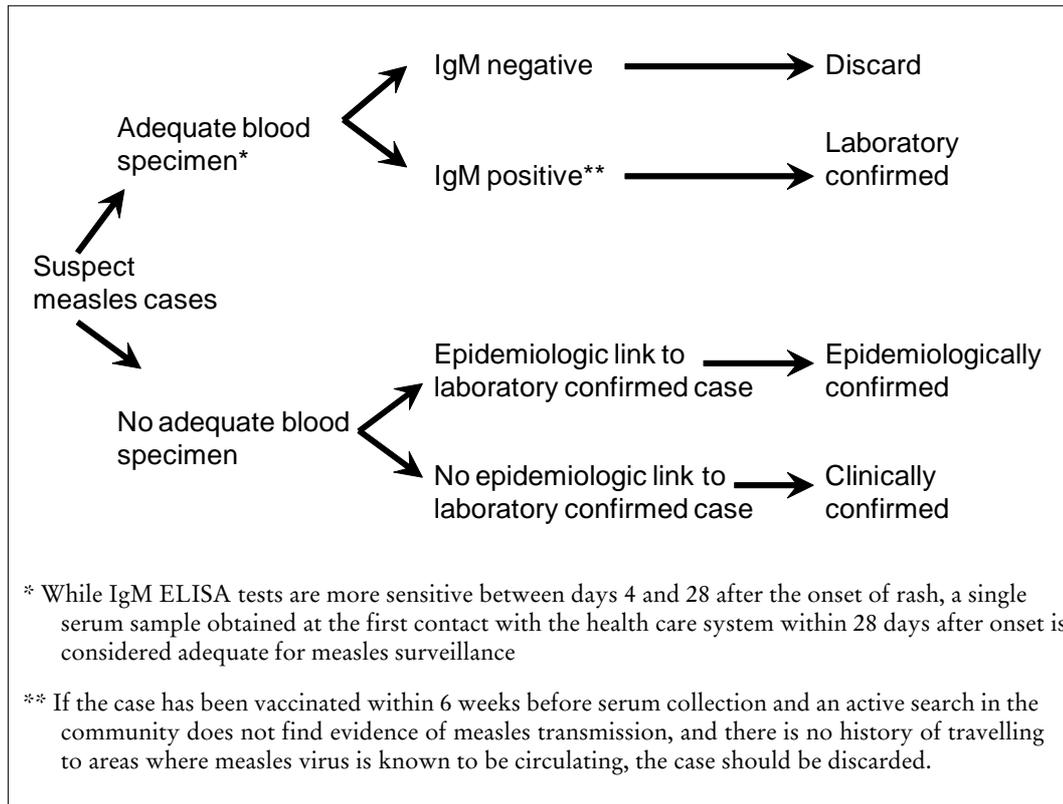


2.2.3 *Clinical and laboratory diagnosis*

Measles is an illness characterized by generalized maculopapular rash lasting 3 or more days with a temperature of 38.3°C (101°F) or higher, and cough, coryza, or conjunctivitis. Clinically, the diagnosis of measles is supported if Koplik's spots are detected and if the rash progresses from the head to the trunk and out to the extremities[7]. The non-specific nature of the prodromal signs and the existence of mild cases, however, make clinical signs unreliable as the sole diagnostic criteria of measles disease. As disease prevalence falls, many medical practitioners are inexperienced in recognizing measles, increasing the need for laboratory methods of distinguishing measles from other clinically similar diseases. Misdiagnosis of measles is, for example, more common among young infants, and outbreak-associated cases are more likely to be laboratory confirmed than sporadic cases. Measles may resemble infections with rubella, dengue fever, ECHO, coxsackie, parvovirus B19 and herpesvirus 6 viruses, as well as some bacterial and rickettsial diseases. Moreover, there are other conditions that may present in a similar form, including Kawasaki's disease, toxic shock and drug reactions.

A laboratory may use one of the following methods for confirming suspected cases:

- detection of measles specific IgM antibody in an approved or certified laboratory - EXCEPT if the case has received a measles-containing vaccine eight days to six weeks before sample collection and there has been no evidence of measles transmission in the community and no history of travel, or
- IgG seroconversion or a fourfold or greater rise in titre to measles virus (where the second serum sample is collected at least 10 days after the first, acute sample) - EXCEPT if the case has received a measles-containing vaccine eight days to six weeks before sample collection and there has been no evidence of measles transmission in the community and no history of travel. (NOTE: paired sera must be tested in parallel), or
- detection of wild-type measles virus genome in an appropriate specimen (not routinely performed for diagnosis as sensitivity is lower than serologic techniques), or
- isolation of wild-type measles virus from a clinical specimen (not routinely performed for diagnosis as sensitivity is lower than serologic techniques).
- However for countries in the outbreak control and elimination phase final classification of measles cases is usually based on the following algorithm [11]



2.3 Rubella

2.3.1 *Epidemiology, infection and immune response*

The name rubella is derived from Latin, meaning “little red.” It was initially considered to be a variant of measles or scarlet fever and was called “third disease.” It was not until 1814 that it was first described as a separate disease in the German medical literature; hence the common English name of “German measles”. In 1914, Hess postulated a viral aetiology based on his work with monkeys. In 1938 Hiro and Tosaka confirmed the viral aetiology by passing the disease to children using filtered nasal washings from acute cases. In 1941, an Australian ophthalmologist, Norman Gregg, reported the association between congenital cataracts and maternal rubella. Subsequently, the role of rubella in congenital rubella syndrome (CRS), a disease which includes cataracts, heart disease and deafness, was confirmed [14].

Except in countries where the disease has been eliminated, rubella has a worldwide distribution. It usually occurs in a seasonal pattern (i.e. in temperate zones during the late winter and spring), with epidemics every five to nine years. However, the extent and periodicity of rubella epidemics is highly variable in both developed and developing countries. The highest risk of CRS is found in countries with high susceptibility rates among women of childbearing age. Although low susceptibility rates have been reported in studies of selected populations within some countries, these may reflect local variations, and extrapolating from such studies could mask a significant national benefit from the introduction of rubella vaccination [3].

Rubella is spread through contact with nose or throat secretions of an infected person. This may result from airborne droplet spread, direct contact with an infected person or indirect contact with freshly infected articles. In closed institutions, such as in military barracks and child day-care centres, all exposed susceptible persons may become infected. Infants with CRS shed large quantities of rubella virus in their pharyngeal secretions and in urine, and can serve as a source of transmission.

Rubella is moderately contagious, mostly when the rash is erupting, but is communicable from 1 week before, to 5-7 days or more after the onset of the rash (Table 2). Infants with CRS may shed virus for up to a year after birth. There is no evidence that the vaccine virus can spread to contacts.

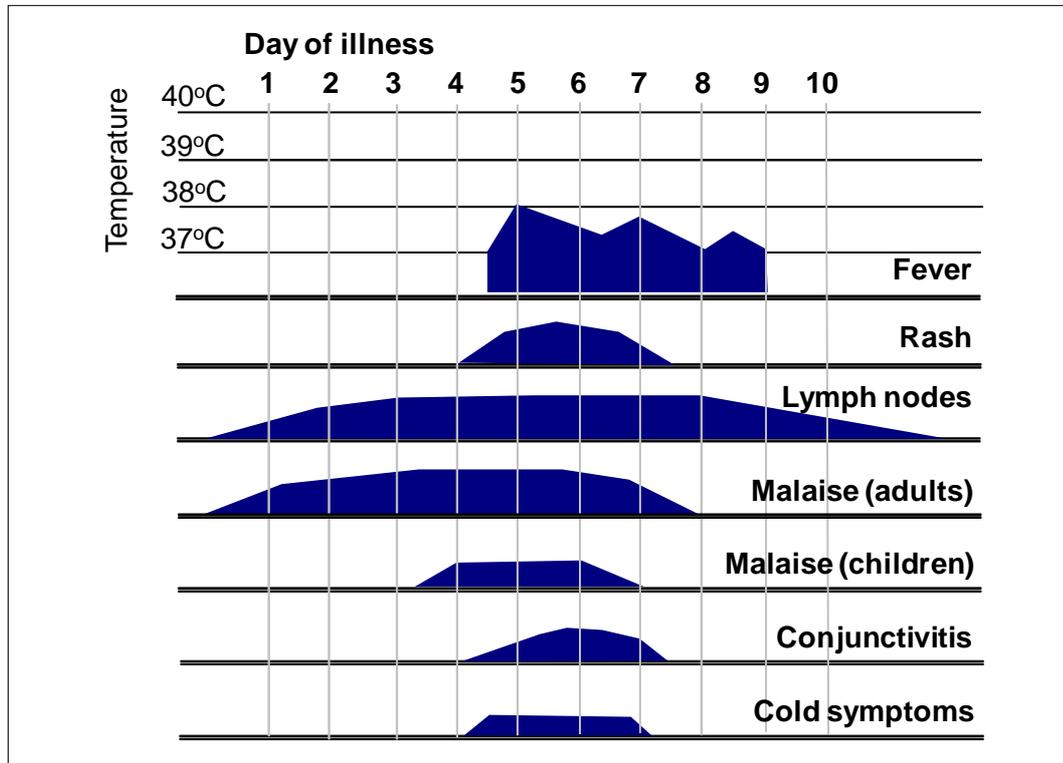
Table 2. Clinical features of typical rubella infection - time course from onset of illness (after [6, 14, 15])

Day	Event
0	Rubella virus from respiratory secretions of infected person comes into contact with the epithelial surface of the nasopharynx of a susceptible person. Localized infection in the respiratory epithelium is established and virus spreads to regional lymph nodes
1-22	Viral replication in nasopharynx and regional lymph nodes
3-8	First evidence of nasopharyngeal viral shedding
6-20	Viraemia
8-14	Infection established in skin and other sites
10-17	Maximum viraemia and viruria
10-24	Maximum nasopharyngeal viral shedding (about 3 days before to 7 days after rash onset)
17-19	Viraemia decreases and then ceases

Rubella mostly affects children, adolescents and young adults. Approximately 50% of rubella infections are subclinical and may not be detected except through laboratory confirmation. When symptoms do present, they are usually quite mild. The main symptoms include inflammation of the lymph nodes and a maculopapular rash, which may be preceded by mild catarrhal symptoms. Enlargement of the lymph nodes (lymphadenopathy) occurs from 5-7 days before the onset of the rash and up to 2 days after. Although these symptoms are not specific to rubella, lymphadenopathy may be more pronounced and last longer (several weeks) with rubella than with other exanthematic diseases, such as measles [14].

After the rubella virus infects the nasopharynx, it multiplies in the lining of the respiratory tract and in local lymph nodes before passing into the bloodstream. Viraemia begins 5-7 days after infection, spreading throughout the rest of the body, including the skin. As with measles, the rash is immunologically mediated and coincides with the development of specific anti-rubella antibodies. Virus can be isolated from the nasopharynx from up to 1 week before, to up to 2 weeks after the onset of rash.

Figure 5. Clinical features of typical rubella infection - time course from onset of illness (after [6, 13, 14])

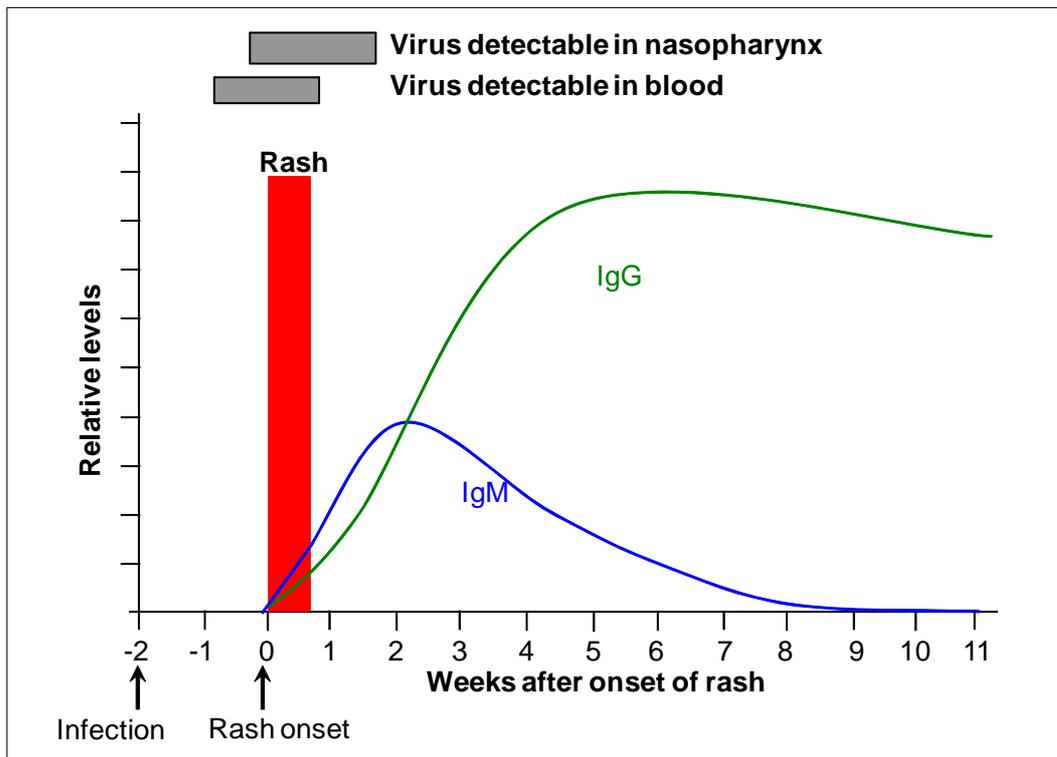


The incubation period for rubella averages 14-18 days but can range from 12-23 days. A short prodromal phase (1-5 days) occurs before the rash appears in adolescents and adults but not in children. In children, a rash is usually the first manifestation. The prodrome involves low-grade fever, headache, malaise, anorexia, mild conjunctivitis, coryza, sore throat, cough and lymphadenopathy involving the suboccipital, post-articular and cervical lymph nodes (Figure 5). Approximately 14-18 days after infection, a maculopapular rash (a pink skin rash of discrete spots) develops. The rash, which may be difficult to see, starts on the face and neck and spreads rapidly down over the trunk and extremities. The rash fades after 1-3 days, and is occasionally pruritic. Joint pain and temporary arthritis, which are uncommon in children, occur frequently in adults, especially in women.

Humoral and cell-mediated immunity develop following a rubella infection. IgG and IgM antibodies are observed about 14-18 days after rubella infection, at about the time when the rash appears (Figure 6). Rubella IgM antibodies wane quickly and are usually undetectable after 2 months, whereas rubella IgG antibodies persist. A rubella-specific cell-mediated lymphocyte response begins 1 week after the humoral response and persists for a lifetime.

Although natural rubella infection generally confers lifelong immunity, rare cases of serologically confirmed re-infections after earlier infection (or immunization) have been reported. There have also been cases of CRS following re-infection in pregnant women with natural or vaccine-induced immunity, but this is extremely rare. Maternal rubella antibodies provide protection against rubella for the first months of life and may affect the immune response if vaccination occurs at an early age.

Figure 6. Immune response in typical rubella infection (after [6, 13, 15])



2.3.2 CRS and CRI

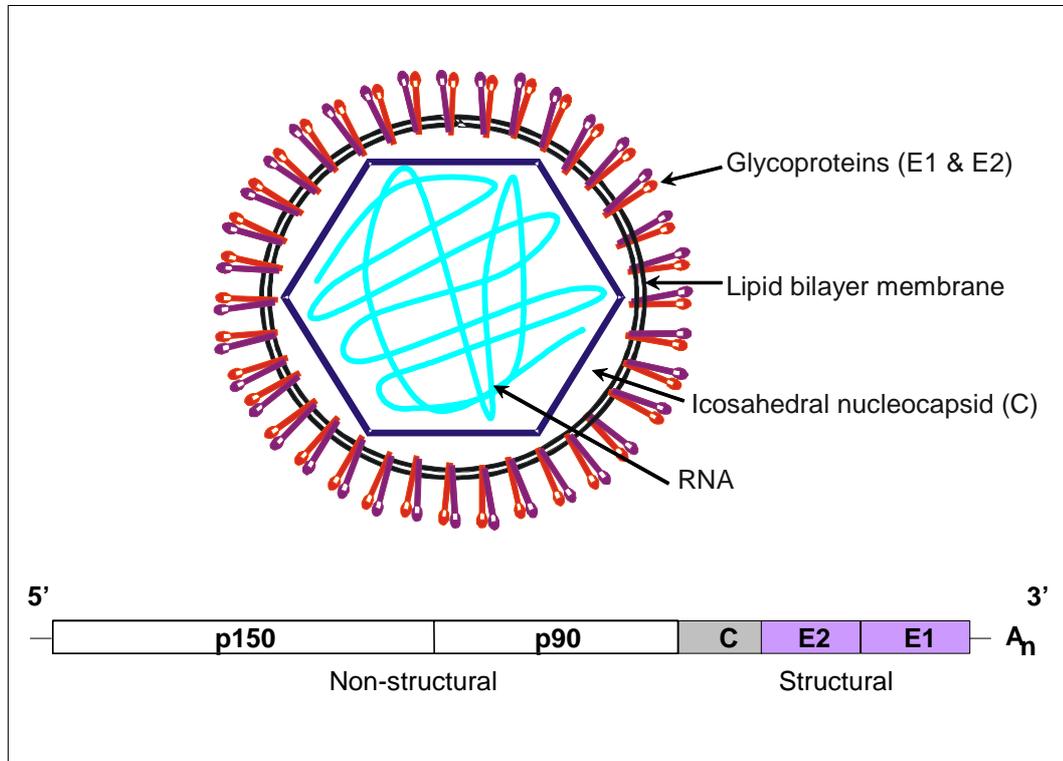
Congenital rubella syndrome (CRS) is the most serious consequence of rubella. It arises from foetal rubella infection during the first trimester of pregnancy and can cause abortion, miscarriage, stillbirth or multiple defects; virtually all organ systems can be affected. Deafness is the most common and often the sole manifestation of CRS. When foetal infection occurs without birth defects it is termed congenital rubella infection (CRI).

The risk of congenital defects following maternal rubella infection varies from 10 to 90%. The severity and nature of these defects depend on the gestational age of the foetus at the time of infection. The most dangerous time is during the first 12 weeks of gestation; defects are rare with infections after 20 weeks. The organ specificity is generally related to the stage of gestational infection; however the relationship between foetal abnormalities and the timing of infection is not always clear-cut. Once an infection has been established, it can spread to many organs and damage may accumulate. Eye and heart defects often follow infection during the first 8 weeks of pregnancy, whereas brain damage and deafness are likely to be seen when infection occurs in the first 18 weeks of pregnancy [6, 13, 14].

Although rubella vaccination is contraindicated during pregnancy, no cases of CRS were reported in more than 1,000 susceptible pregnant women who inadvertently received rubella vaccine early in pregnancy. An investigation of almost 19,000 susceptible pregnant women inadvertently administered rubella vaccine in a large mass campaign also found no evidence of CRS cases (unpublished data) thus inadvertent rubella vaccination during pregnancy is not an indication for abortion [16].

2.3.3 *Rubella virus*

Figure 7. Diagram of the rubella virus particle correlated with the genetic map (after [13, 17]) . The E1 and E2 glycoproteins exist as heterodimers.



Rubella virus is the only member of the Rubivirus genus of the Togavirus family. It is most closely related to the Alphavirus genus, such as Eastern and Western Equine Encephalitis viruses. Unlike most Togaviruses it is NOT arthropod borne, but is acquired via the respiratory route. The rubella virus is roughly spherical with a diameter of 60-70 nm. It is composed of an icosahedral nucleocapsid containing a single-stranded, positive sense RNA genome; this is surrounded by a complex lipid envelope (toga=cloak). The virus contains three structural proteins, two in the envelope (E1 and E2) and one in the core (capsid or C protein) surrounding the RNA (Figure 7). The envelope proteins, E1 and E2, are glycoproteins that exist as heterodimers that project from the virus to form 6- to 8-nm surface spikes. E1 appears to be the dominant surface molecule and is associated with neutralizing and haemagglutinating epitopes[17]. Only one serotype of the virus is known, but phylogenetic tree analysis, primarily of the coding region of E1, indicate the existence of at least 7 distinct genotypes represented in 2 clades[18]. There is no cross-reaction with other Togaviruses.

The virus contains an RNA of 9762 nucleotides. The 5' end of the genome has a cap and the 3' end has a poly (A) tract. The synthesis and processing of rubella virus proteins takes place through high-molecular weight polyprotein precursors [17]. Examples of nucleotide sequence information on rubella virus have been deposited at EMBL/GenBank under the following accession numbers M15240; M18901; and M32735.

The rubella virus is relatively temperature labile but is more heat stable than measles virus; it is inactivated after 30 minutes at 56°C, 4 minutes at 70°C, and 2 minutes at 100°C. It degrades rapidly with conventional freezing at -20° but the virus is stable at -60°C and below and when freeze-dried with stabilizers. When stabilised with protein it can be repeatedly frozen and thawed without loss of titre. Lipid solvents, weak acids and alkalis, and UV light inactivate the rubella virus. It is also susceptible to a wide range of disinfectants, and is inactivated by 1% sodium hypochlorite, 70% ethanol and formaldehyde [14, 17, 19].

2.3.4 Clinical and laboratory diagnosis

The diagnosis of rubella solely based on clinical signs and symptoms is unreliable because there are many other causes of rash that may mimic rubella infection and up to 50% of rubella infections may be subclinical [14]. A laboratory may use one or more of the following methods to provide evidence of infection:

- Detection of rubella IgM antibodies in an approved or certified laboratory - EXCEPT if the case has received a rubella-containing vaccine eight days to eight weeks before sample collection and there has been no evidence of rubella transmission in the community and no history of travel, or
- IgG seroconversion or a fourfold or greater rise in titre to rubella virus (where the second serum sample is collected at least 10 days after the first, acute sample) - EXCEPT if the case has received a rubella-containing vaccine eight days to eight weeks before sample collection and there has been no evidence of rubella transmission in the community and no history of travel. (NOTE: paired sera must be tested in parallel), or
- Detection of rubella virus genome in an appropriate specimen (not routinely performed for diagnosis as it is more difficult to perform than serologic techniques)
- A positive culture for rubella virus (not routinely performed)

Rubella virus can be isolated from nasal, blood, throat, urine and cerebrospinal fluid specimens from rubella and CRS cases. Virus may be isolated from the pharynx 1 week before and up to 2 weeks after rash onset. Although isolation of the virus is diagnostic of rubella infection, viral culture is demanding and labour intensive. Nasal, throat, blood, urine and cerebrospinal fluid specimens can be used, together with tissues from biopsy or autopsy for laboratory confirmation of CRS cases. Serology tests include the demonstration of rubella IgM antibodies or the persistence of antibodies beyond the predicted decay of passively transmitted maternal rubella IgG antibodies. In CRS cases, IgM antibodies are sometimes found for up to 1 year after birth, and persistence of IgG antibodies beyond 6 months of age has been detected in 95% of cases [14, 16].

False-positive serum rubella IgM tests have occurred in persons with parvovirus B19 infections, with a positive heterophile test for infectious mononucleosis, or with a positive rheumatoid factor [14, 20, 21].

2.4 WHO global strategies for measles control and CRS prevention

2.4.1 *Measles Mortality Reduction and Regional Elimination*

In March 2001, WHO and UNICEF jointly released their Measles Mortality Reduction and Regional Elimination: Strategic Plan 2001 - 2005 [22]. The prime objective of the plan was to reduce by half the number of global measles deaths by the end of 2005 (as compared to the number of deaths in 1999). The strategies developed to achieve this included:

- achieving and sustaining very high coverage with two doses of measles vaccine through high-quality routine immunization services;
- providing a second opportunity for measles immunization through supplementary immunization activities to populations susceptible to measles, consistent with national targets for measles control;
- enhancing measles surveillance with integration of epidemiological and laboratory information; and
- improving the clinical management of every measles case.

The release of the joint strategic plan helped to re-invigorate global, regional and national efforts to reduce measles mortality. Countries with high measles mortality were strongly urged to implement a comprehensive strategy for sustainable measles mortality reduction. This included achieving high routine measles immunization coverage (>90%) in every district and ensuring that all children were offered a “second opportunity” for measles immunization, either through routine immunization services or periodic supplemental immunization activities (SIAs). Regional measles elimination goals have now been adopted by 4 WHO Regions with the African and South East Asian Regions focussing on measles mortality reduction.

While global routine measles vaccination coverage showed moderate increase between 1999 (71%) and 2004 (76%), coverage varied significantly by geographical region [2]. There has been an increase in the proportion of countries offering children a second opportunity for measles immunization. In 2004, 168 (88%) countries offered children a second opportunity compared with 150 (78%) countries in 2001 (data is not available prior to 2001). From 2000 to 2004, more than 215 million children aged from 9 months to 14 years received measles vaccine through SIAs in 36 WHO/UNICEF priority countries. Of the 36 countries that conducted SIAs during this period, 28 (78%) were nationwide and 24 (67%) were in Sub-Saharan Africa. Substantial increases in routine coverage were evident in Sub-Saharan Africa (49% to 65%) and South Asia (54% to 61%).

These accelerated activities have resulted in a significant reduction in estimated global measles deaths (Table 3). Overall, global measles mortality decreased by 48% between 1999 and 2004. The largest gains come from the African region where measles mortality decreased by 59% and accounted for 67% of the global reduction during this period.

Table 3. Estimated measles deaths and percentage reduction, by geographical region, 1999-2004 ([2])

Geographical Region	1999 estimated measles deaths	2004 estimated measles deaths	Change (% decrease)	Contribution to Global reduction (%)
Sub-Saharan Africa	530 000	216 000	-314 000 (59%)	75%
South Asia	263 000	202 000	- 61 000 (23%)	15%
East Asia & Pacific	68 000	32 000	- 36 000 (53%)	9%
Others	10 000	4 000	- 6 000 (60%)	1%
Global totals	871 000	454 000	- 417 000 (48%)	100%

Because it is likely that the 2005 goal will be met on time, a new more ambitious goal for measles mortality reduction has been proposed in the Global Immunization Vision and Strategy (GIVS) document [23]. The new goal is a 90% reduction in measles mortality by 2010 compared with the 2000 level. The following major challenges exist to achieving this new goal. First, measles mortality reduction activities need to be implemented in several large countries with high measles burden such as Nigeria, India and Pakistan. Second, to sustain the gains in reduced measles deaths in the 45 high mortality priority countries, enhanced efforts are needed to improve immunization systems to assure that at least 90% of infants are vaccinated against measles before their first birthday. Third, the priority countries will need to continue to conduct “follow-up” SIAs every 3-4 years until their routine immunization systems are capable of providing two opportunities for measles immunization to >90% of every birth cohort. Fourth, disease surveillance systems at district, provincial and national levels need to be strengthened to enable case-based surveillance with testing of clinical specimens from suspected cases in laboratories participating in the global measles and rubella laboratory network [24]. Finally, measles case management, including appropriate vitamin A supplementation, should be strengthened.

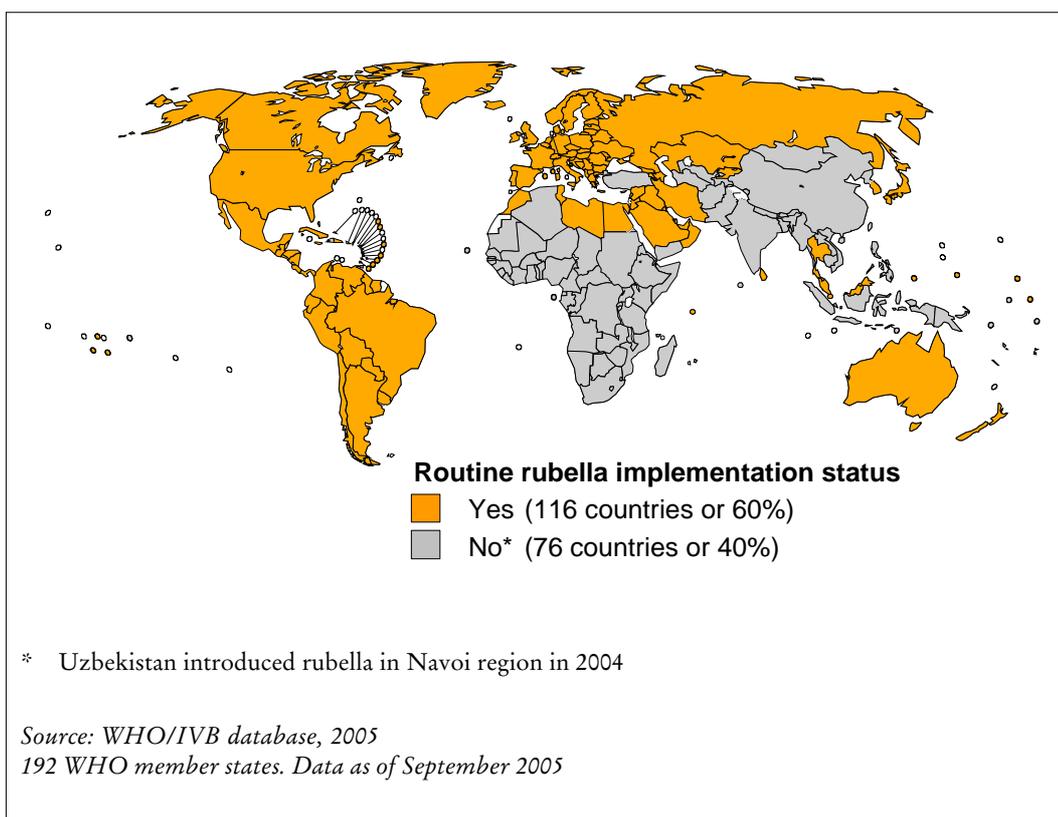
2.4.2 Prevention of CRS

The existing, internationally-licensed rubella vaccines, single or in combination with vaccines against mumps and/or measles have proved to be highly efficacious in the prevention of rubella infection and CRS in different parts of the world. WHO recommends the use of rubella vaccine in all countries with well-functioning childhood immunization programmes with sustained routine coverage of >80%, where reduction or elimination of CRS is considered a public health priority, and where resources may be mobilized to assure implementation of an appropriate strategy [15]. The global burden of CRS has now been sufficiently characterized, so that priority should now be given to advocating its control and prevention.

Countries wishing to prevent CRS are now immunizing adolescent girls and/or women of childbearing age, the precise target population addressed depending on susceptibility profile, cultural acceptability and operational feasibility. The most rapid impact can be achieved by mass campaigns for women of childbearing age; however, complete control of CRS has proved difficult by immunizing target populations as the only strategy. Vaccination through routine services alone results in delay during which CRS cases will still occur and may even increase [25] if women of child bearing age are not targeted. Routine immunization against rubella has been

increasingly adopted, with 116 out of 192 countries (60%) including rubella vaccination in routine immunization schedules in 2004 (Figure 8). All countries undertaking rubella elimination should ensure that women of childbearing age are immune and that routine coverage in children is sustained >80% [15].

Figure 8. Countries using rubella vaccine in routine immunization schedules, 2004



2.4.3 Integrated measles and rubella control

As of early 2006, four WHO Regions had adopted measles elimination goals, and two (the Americas and Europe) had adopted rubella and CRS elimination targets. WHO recommends that countries undertaking measles elimination should consider taking the opportunity to eliminate rubella at the same time, through use of MR or MMR vaccine in their childhood immunization programmes, and also in mass campaigns [15]. An integrated immunization approach to achieving measles and rubella control targets provides an opportunity for increased programme efficiency; the value of this opportunity, however, varies in different circumstances and at different times. Previous immunization activities, logistics and resource availability may make an integrated strategy unfeasible or inappropriate for some countries at present. Information and recommendations on requirements for, and appropriateness of, an integrated measles/rubella strategy can be obtained from WHO Regional Offices.

3. Role and function of the laboratory in measles control and CRS prevention

3.1 Role of the laboratory in measles and rubella surveillance

The laboratory plays an increasingly important role in measles and rubella surveillance as the level of disease control increases. In the elimination phase it is well established that surveillance based on clinical recognition of cases is inaccurate and that laboratory confirmation of suspected cases complimented by genotyping of circulating virus strains is critical for effective surveillance. The laboratory has two main functions in measles and rubella surveillance.

- Monitoring and verifying virus transmission:
 - Confirmation of outbreaks: to confirm the clinical diagnosis in the early stages of an outbreak
 - Confirmation of cases: to confirm or discard any suspected cases of measles or rubella
 - Identification of measles and rubella virus strains and genetic characterisation of viral isolates.

- Monitoring susceptibility profile of the population:
 - Determination of the age distribution of susceptibility to measles and rubella in order that the need for immunisation campaigns might be assessed
 - Evaluation of the impact of mass campaigns

For each sequential phase of the measles/rubella control programme there are specific surveillance activities (Table 4). In performing its functions, laboratories cannot act in isolation but must be organised into a supportive network, which efficiently provides accurate information to the programme.

Table 4. Role of the laboratory in measles and rubella control and elimination

Phase of measles and rubella control	Function of laboratory
Control	Confirm initial cases during outbreaks through IgM detection Analyse wild virus strains from selected cases for genetic baseline information
Outbreak prevention and elimination	Confirm the clinical diagnosis of suspected cases to help in early detection of virus circulation Analyse wild virus strains from selected cases to identify circulating genotypes, to map transmission pathways, to complete global genome mapping of measles and rubella virus and to measure impact of control strategies In special circumstances, establish seroprevalence of the population and assist in outbreak forecasting

3.2 Structure and activities of the laboratory network in measles and rubella surveillance

There are five main objectives in establishing an international network of laboratories in support of measles and rubella control activities:

- to develop protocols for the laboratory diagnosis of measles and rubella and provide the necessary support as the programme evolves;
- to establish mechanisms for reference and support for regional and national laboratories in the diagnosis of measles, rubella and other rash illness;
- to provide training resources and facilities for staff of regional and national laboratories;
- to provide a source of reference materials and expertise for the development and quality control of improved diagnostic tests;
- to serve as a strain bank of measles and rubella virus isolates for molecular epidemiology and as a resource of reference sera for quality control.

Experience has shown that the laboratory network must be organized at the same time as regional control and elimination programmes, and established with properly trained personnel, suitable equipment and reagents. The WHO Global Measles/rubella Laboratory Network is organized on four levels. (Figure 9):

- **Global Specialized Laboratories (GSL)**
These are laboratories which have set the technical standards for laboratory diagnosis. Their responsibilities extend to measles and rubella laboratories in all regions and countries.
- **Regional Reference Laboratories (RRL)**
These are centres of excellence in each region able to undertake international responsibilities. They will serve as reference laboratories for national laboratories in neighbouring countries and to serve as national laboratories in their own countries. Each WHO region may have up to 3-4 regional reference laboratories (RRLs).

- National Laboratories (NL)

These laboratories have the closest links with national programme managers. They test specimens from suspected cases by IgM enzyme-linked immunosorbent assay (ELISA) and report directly to the programme managers. The number of national laboratories will depend on the epidemiological priorities and resources available.

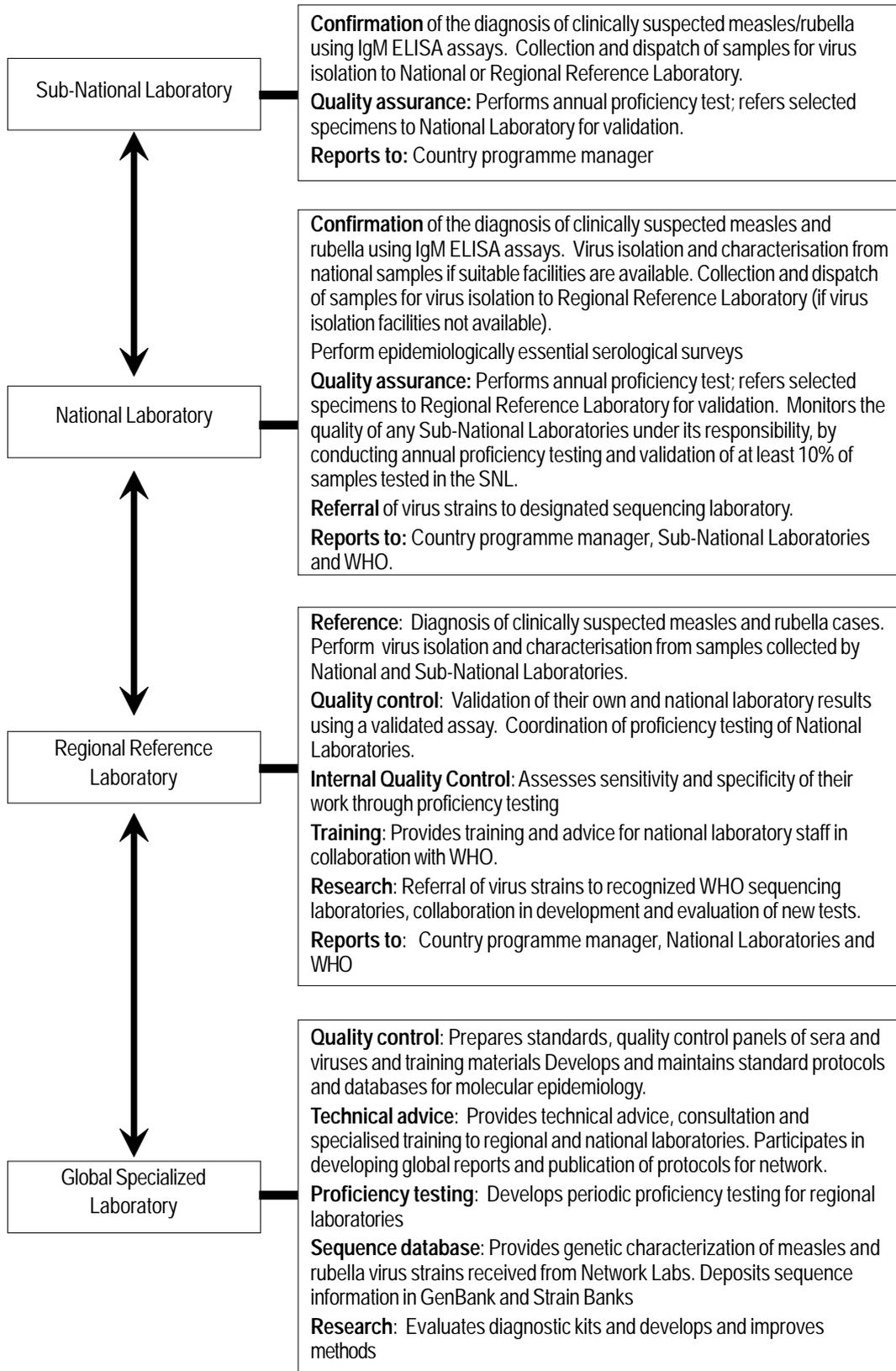
- Sub-National Laboratories (SNL)

Due to the significant population size and/or logistic challenges of some countries, testing of specimens for measles may be beyond the capacity of a single national laboratory. In these countries Sub-National laboratories (SNL) may also be established at first or second administrative levels.

To achieve the objectives outlined in Figure 9, the measles and rubella network laboratories should possess:

- known links to the immunization and surveillance units at the Ministry of Health;
- proven capability to perform accurate and timely testing;
- appropriately trained scientists and technicians;
- adequate laboratory facilities and resources to cover running costs; and
- suitable equipment to conduct routine serological assays
- capacity to communicate with the National control programme, WHO and other laboratories in the Network.

Figure 9. Laboratory network for measles and rubella surveillance activities at each level



Individual laboratories are not expected to undertake the full range of tasks listed above, but perform specific duties according to the needs of the national/regional programmes and their level within the network. Laboratories involved in the network are monitored by regular proficiency testing in selected techniques and by performance evaluation through the accreditation process.

3.3 Coordination of the network

Coordination of the Measles and Rubella Laboratory Network is carried out by WHO, based on the experience gained in establishing the Global Polio Laboratory Network. Each of the WHO regions has a Regional Laboratory Coordinator responsible for the laboratories within their region. Each of the regions works in partnership with the Global Laboratory Coordinator in WHO Headquarters, Geneva. Regular forward feeding of results, requests and queries, and feedback of analysis, comments and technical advice achieves coordination. Procurement and distribution of essential standardized laboratory equipment and reagents for selected countries is also coordinated through WHO.

An effective and efficient laboratory network is dependent on good communications, both within the network and with the disease control and immunization programmes. Standard referral and reporting forms have been developed to ensure that all essential patient information is transmitted. The format and timing of result reporting at national and Regional level will be based on global standards, but details will be agreed upon in consultation with disease control and immunization programme managers.

A system for monitoring indicators of field and laboratory performance, including annual proficiency testing and laboratory accreditation, has been established and is now being implemented. Virologists and epidemiologists at all levels must establish mechanisms to exchange information on a regular basis to monitor and evaluate performance indicators of the surveillance system. The advantages of holding regular coordination and information exchange meetings are now well established, and it is intended that representatives of the Global Specialized and Regional Reference Laboratories should meet at least once a year. Representatives from the National Laboratories should hold meetings with their control programme counterparts at least once a month and participate in regional meetings every year.

3.4 Molecular epidemiology and genotyping

3.4.1 *Measles*

Molecular characterization of measles viruses is an important component of measles surveillance as it provides a method for identifying the geographical origin and tracing the transmission pathways of a virus. It also provides a valuable tool for measuring the effectiveness of measles control and elimination programmes, and information that can be used to document the interruption of transmission of endemic measles. WHO recommends that viral surveillance be conducted during all phases of measles control and that virological surveillance activities be expanded to provide an accurate description of the global distribution of measles genotypes. The WHO Global Measles and Rubella Laboratory Network provides support for virological surveillance.

WHO has designated two measles strain banks; the measles virus section of the Centers for Disease Control and Prevention (CDC), USA, and the Health Protection Agency (HPA), UK, to acquire, analyse, store and dispense representative strains. Measles Laboratories are encouraged to submit their measles strains to one of the strain banks where the strains will be verified and submitted to GenBank, and the genotype information submitted to the WHO measles and rubella database. If Laboratories have the capacity to sequence viruses, sequence information should be also submitted to GenBank and the strain banks and genotype information shared with WHO. Sequence data for all reference strains and deposited wild-type strains will be available from the WHO strain banks or through GenBank. Clinical specimens may also be submitted to designated WHO regional virus detection laboratories for testing. If virus detection is successful, representative strains will be sequenced and entered into the strain bank's databases and GenBank. The WHO strain banks can provide advice on import permits and other requirements to facilitate the shipment of strains and clinical specimens.

In 1998, WHO published guidelines for a uniform nomenclature for designating wild type measles viruses and describing genotypes. This report also provided guidelines for the laboratory methods used for genetic characterization. The sequence of the 450 nucleotides that code for the COOH-terminal 150 amino acids of the nucleoprotein (N) is the minimum amount of data required for determining the genotype of a measles virus. Sequence data can be obtained from a viral isolate or by amplification of measles sequences directly from RNA extracted from a clinical specimen. Complete haemagglutinin (H) gene sequences (1854 nucleotides) should be obtained from representative strains from specific countries or from large outbreaks [10].

If a new genotype is suspected, a viral isolate and the complete H sequence should always be obtained in addition to the N sequence. WHO established the use of standard reference sequences for each designated genotype for analysis of sequence data obtained from viral isolates or clinical specimens. In 2001, 2003 and 2005 the WHO recommendations were updated to take into account the identification of new genotypes resulting from expanded virological surveillance[10, 26, 27, 28, 29]. Proposed genotypes should be designated by using the lower-case clade letter (e.g. d10). The terms clade and genotype are used to describe the genetic characteristics of wild-type measles viruses. For molecular epidemiological purposes, the genotype designations are the operational taxonomic unit, while the clades are used to indicate the genetic relationship between the various genotypes.

Table 5. Reference strains to be used for genetic analysis of wild-type measles viruses[10].

Genotype	Status ^a	Reference strains ^b	H gene accession	N gene accession
A	Active	Edmonston-wt.USA/54	U03669	U01987
B1	Inactive	Yaounde.CAE/12.83 "Y-14"	AF079552	U01998
B2	Active	Libreville.GAB/84 "R-96"	AF079551	U01994
B3	Active	New York.USA/94 Ibadan.NIE/97/1	L46752 AJ239133	L46753 AJ232203
C1	Active	Tokyo.JPN/84/K	AY047365	AY043459
C2	Active	Maryland.USA/77 "JM" Erlangen.DEU/90 "WTF"	M81898 Z80808	M89921 X84872
D1	Inactive	Bristol.UNK/74 (MVP)	Z80805	D01005
D2	Active	Johannesburg.SOA/88/1	AF085198	U64582
D3	Active	Illinois.USA/89/1 "Chicago-1"	M81895	U01977
D4	Active	Montreal.CAN/89	AF079554	U01976
D5	Active	Palau.BLA/93 Bangkok.THA/93/1	L46757 AF009575	L46758 AF079555
D6	Active	New Jersey.USA/94/1	L46749	L46750
D7	Active	Victoria.AUS/16.85 Illinois.USA/50.99	AF247202 AY043461	AF243450 AY037020
D8	Active	Manchester.UNK/30.94	U29285	AF280803
D9	Active	Victoria.AUS/12.99	AY127853	AF481485
D10	Active	Kampala.UGA/51.00/1	AY923213	AY923185
E	Inactive	Goettingen.DEU/71 "Braxator"	Z80797	X84879
F	Inactive	MVs/Madrid.SPA/94 SSPE	Z80830	X84865
G1	Inactive	Berkeley.USA/83	AF079553	U01974
G2	Active	Amsterdam.NET/49.97	AF171231	AF171232
G3	Active	Gresik.INO/17.02	AY184218	AY184217
H1	Active	Hunan.CHN/93/7	AF045201	AF045212
H2	Active	Beijing.CHN/94/1	AF045203	AF045217

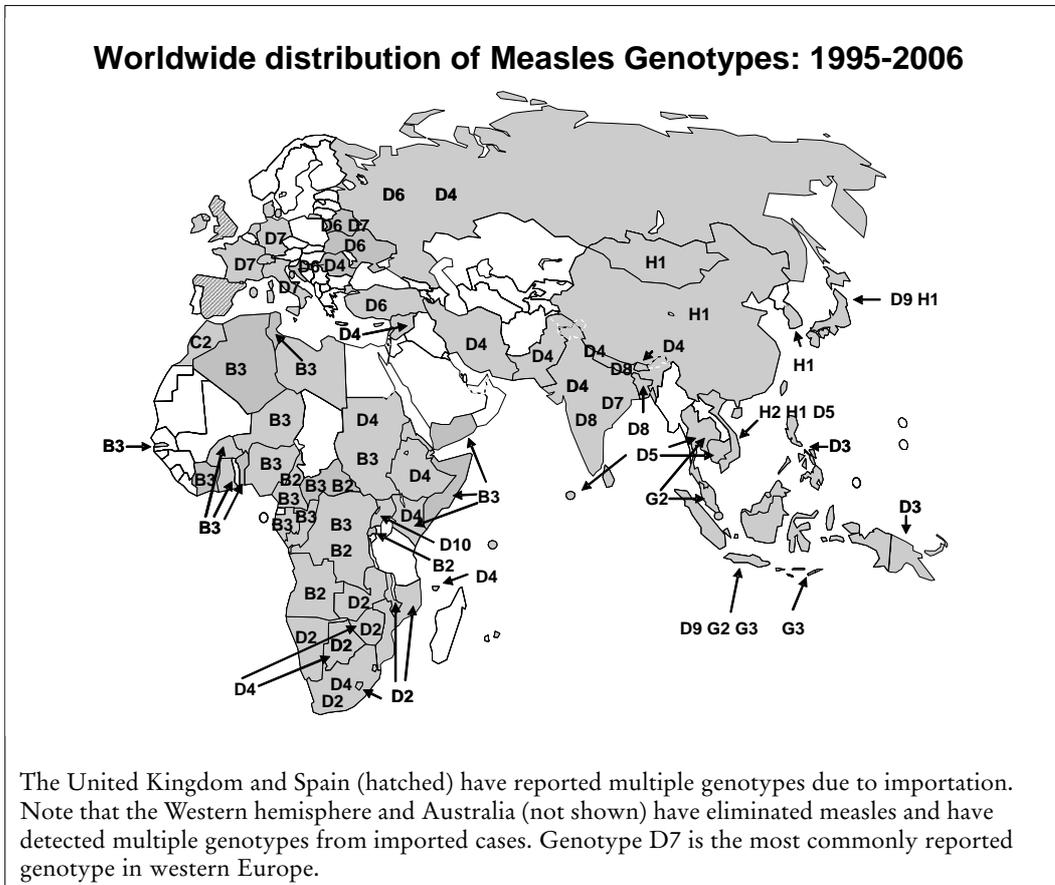
^a Active genotypes that have been isolated within the past 15 years.

^b WHO name. Other names that have been used in the literature appear in quotation marks.

Based on currently available published and unpublished information, there are eight clades, designated A-H. Within these eight clades, there are 23 recognized genotypes (Table 5). Some clades contain only one genotype, in which case the genotype designation is the same as the clade name. Other clades, such as D, contain multiple genotypes, which are designated by the clade letter (in upper case) and genotype number. Genotypes, D1, E, F, G1, will continue to be listed as inactive because no representative viruses from these genotypes have been isolated in the past 15 years. In addition, the Central African genotype B1, originally detected during the early 1980s, should also be considered as inactive with the caveat that regional measles strain surveillance is suboptimal. Genotype B2 was previously listed as inactive; however genotype B2 viruses have been isolated recently from patients with measles in Central African Republic, Angola, the Democratic Republic of the Congo and Rwanda, as well as from imported cases in South Africa and Germany. Prior to these recent detections, the last identification of genotype B2 was in the early 1980s in Gabon. Genotype B2 has now been designated as an active genotype (Table 5). Presumably, genotype B2 viruses have been continuously circulating in parts of Central Africa but had not been detected because of inadequate virological surveillance.

With the exception of genotype F, all of the genotypes have a corresponding reference strain. The reference strains were chosen to represent the earliest isolation of virus from each genotype. Designation of new genotypes must be based on sequence information from viral isolates and not on sequences obtained solely from clinical material. Several publications have referred to two clusters within genotype B3, and one representative from each cluster is included. Since designation of genotype D7 was based on the sequence of viruses that were isolated in Australia in the late 1980s, a second reference strain has been added to represent the more contemporary D7 sequences[27, 28].

Figure 10. Global distribution of measles genotypes in WHO regions yet to eliminate measles 1995-2006 (after WER Nov 05)



Convention for naming strains

The strain names provide information that is essential for interpretation of the molecular data. Since sequence data may be derived from viruses isolated in cell culture or from RNA extracted directly from clinical material, strains or sequences should be designated as either:

- MVi: measles virus isolate in cell culture; or
- MVs: measles virus sequence derived from RNA extracted from clinical material.
- Other information to be included in the strain/sequence name:
 - city of isolation, write whole name or abbreviation (required);
 - country, use ISO 3-letter designation (required);
 - date of specimen collection by epidemic week (1-52) and year (required);
 - isolate number if more than 1 per week (optional);
 - genotype (optional initially, required after sequencing of at least 450 nucleotides of the N gene is completed);
 - special designation for sequences derived from measles inclusion body encephalitis (MIBE) or subacute sclerosing panencephalitis (SSPE) cases (optional).

The following examples illustrate the nomenclature;

- MVi/NewYork.USA/03.98/2 [D2];
- MVs/London.GBR/17.97 [G3] SSPE.

3.4.2 *Rubella*

A WHO meeting in September 2004 discussed standardization of a nomenclature for describing the genetic characteristics of wild-type rubella viruses and establishment of uniform genetic analysis protocols[18]. Rubella viral surveillance is an important part of rubella surveillance during the control and elimination phases; the goal should be to obtain a virus sequence from representative samples from each outbreak during the control phase or from each chain of transmission during the elimination phase. The WHO Global Measles and Rubella Laboratory Network will support efforts to obtain isolates of currently circulating wild-type rubella viruses and will facilitate referral to designated laboratories able to perform genetic analysis. Laboratories are requested to follow the same recommendations for sharing samples and genetic information as for measles (see section 3.4.1). The two WHO strain banks designated for measles viruses are also designated for rubella viruses.

Most of the genetic studies of wild-type rubella viruses have been conducted by sequencing the full length or portions of the coding region of the E1 envelope protein. At present, different regions (windows) in the E1 coding region are used for genetic characterization, but WHO recommends a window of 739 nucleotides (nts) - nts 8731-9469 for routine molecular epidemiological analysis.

The major phylogenetic groups of rubella viruses, which differ by 8-10% in nucleotide sequence, can be separated into 2 clades. Reference viruses for 7 intraclade groups, called genotypes (1B, 1C, 1D, 1E, 1F, 2A and 2B), together with a provisional genotype (1a), are listed in Table 6. Two further genotypes (2c and 1g) were classified as provisional during the meeting [18]. A provisional genotype (lower case) will become an established genotype (upper case) when reference viruses are obtained and phylogenetic relationships between the provisional genotype and other genotypes become clear. Genotype 1a was considered provisional because the phylogeny of this group of viruses was complex and poorly understood. Proposals for new genotypes should be submitted to the WHO strain banks at the Health Protection Agency, London and Centers for Disease Control and Protection, Atlanta, for evaluation.

Table 6. Reference strains to be used for genetic analysis of wild-type rubella viruses[18].

Genotype	Reference strains ^a	Current Name ^b	Accession Number
1a	RVi/BEL/63 RVi/Con.USA/61 RVi/Toyama.JPN/67	Cendehill BEL 63 ^c HPV77 US 61 ^c TO-336 WT JP 67	AF188704 M30776 AB047330
1B	RVi/ISR/75[1B] RVi/ISR/88[1B] RVi/ISR/79[1B]	I-9 IS 75 I-34 IS 88 I-13 IS 79	AY968207 AY968209 AY968208
1C	RVi/Cal.USA/91[1C] RVi/SLV/02[1C] RVi/PAN/99[1C]	BUR US 91 QUI ELS 02 P-31 PAN 99	AY968212 AY968211 AY968217
1D	RVi/Cal.USA/97[1D]CRS RVi/Tokyo.JPN/90[1D]CRS RVi/Saitama.JPN/94[1D]	SAL-CA US 97 NC JP 90 SAI-1 JP 94	AY968206 AY968214 AY968216
1E	RVi/Shandong.CHN/02[1E] RVi/MYS/01[1E]	T14 CH 02 M-1 MAL 01	AY968210 AY968221
1F	RVi/Shandong.CHN/00[1F] RVi/Anhui.CHN/00[1F]	TS10 CH 00 TS 38 CH 00	AY968213 AY968215
2A	RVi/Beijing.CHN/79[2A] RVi/Beijing.CHN/80[2A]	BRD1 CH 79 BRD2 CH 80 ^c	AY258322 AY258323
2B	RVi/TelAviv.ISR/68[2B] RVi/Wash.USA/16.00[2B] RVi/Anhui.CHN/00/2[2B]	I-11 IS 68 TAN IND 00 TS34 CH 00	AY968219 AY968220 AY968218
Vaccine ^d	RVi/USA/64	RA27/3 US 64 ^c	L78917

^a Strains are named using the new convention recommended by WHO. Complete information is missing for some strains

^b Strains are named using old or previously published identifiers

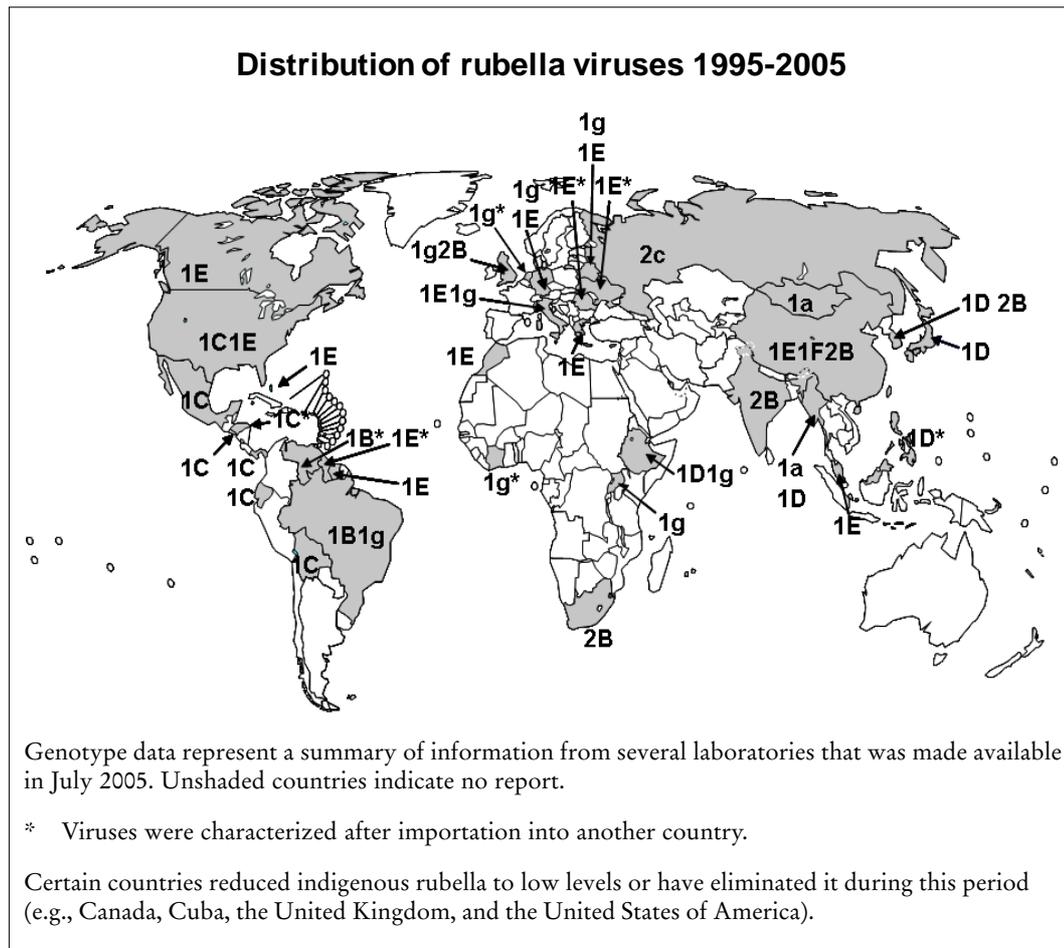
^c Attenuated vaccine virus for which original wild-type virus has been lost

^d Genotype of this virus is considered to be 1a.

Although rubella virus surveillance is recognized as being suboptimal, the information concerning the global distribution of rubella virus genotypes available in the eleven years 1995 to 2005 is shown in Figure 11. Genotype 1a was found most frequently worldwide before 1984 and has now almost disappeared, except in Mongolia and Myanmar. The last genotype 1a identified in the rest of the world was in Canada in 1985. Genotypes 1B, 1C, 1D and 1F have been found to have restricted distributions: 1B has been found in Europe and along the eastern coast of South America; 1C has been found in Central America and along the western coast of South America; 1D has been found in Asian countries, and 1 genotype 1D virus has been found in Ethiopia; 1F has been found in China. Genotype 1C was observed in a single outbreak in Japan and was thought to have been imported from a concurrent outbreak in the Americas, although there are no direct epidemiological data to support this. Genotype 1D was previously found in Canada and the United States, but was last found in these countries in 1987 in Canada and in 1988 in the United States.

Genotype 1E, first identified in 1997, now appears to be a genotype with a global distribution. Genotype 1g needs more study; nevertheless, countries where this provisional genotype has been found are shown in Figure 11. Viruses in clade 2 have been found only in Africa, Asia and Europe. Genotype 2A was isolated only in China in 1979 and 1980 and has not reappeared since then. Genotype 2B is distributed more widely than other genotypes in clade 2. Genotype 2c has been found only in the Russian Federation.

Figure 11. Global distribution of rubella genotypes 1995-2005 [after 18]



4. Specimen collection, shipment, receipt and processing

The correct timing of sampling with respect to the onset of clinical signs is important for interpreting results and arriving at an accurate conclusion. Samples for measles/rubella IgM antibody diagnosis and virus detection should be collected in accordance with the phase of measles/rubella control and elimination (Table 7). Appropriate laboratory and epidemiological staff should agree in advance on the number and type of specimens and the best locations for collection of samples for virus detection. Ideally, samples for virus detection should be collected simultaneously with samples for serological diagnosis and confirmation of measles or rubella virus as the cause of the outbreak. Since each type of sample has different requirements, however, the decision on the type of sample depends on the local resources and facilities for transportation and storage. This requires close cooperation between virologists, epidemiologists and clinicians. Detailed planning, designation of responsibilities and training are needed.

Table 7. Minimum requirements for measles and rubella serology and virus detection according to phase

Phase	Function of laboratory	Epidemiological situation	Sample for measles/rubella IgM antibody detection	Sample: specimen for virus detection
Control	To confirm initial cases during outbreaks	Isolated case	No	No
	To analyse wild virus strains from selected cases for genetic characterization of circulating measles viruses	Outbreak	Yes From 5-10 cases from early in the outbreak to confirm cause	Yes 5-10 specimens early in, and near the end of, the outbreak
Outbreak Prevention and Elimination	To confirm clinical diagnosis of all suspected cases for early detection of virus circulation	Isolated case	Yes From all suspected cases	Yes From all suspected cases
	To analyse wild virus strains and monitor their distribution and circulation for assessing the impact of immunization strategies	Cluster of febrile-rash illness	Yes From initial 5-10 cases to confirm outbreak and from newly infected districts and near the end of the outbreak	Yes 5-10 specimens; more may be collected in newly infected districts and near the end of the outbreak

Because virus is more likely to be isolated when specimens are collected within three to five days of the onset of rash, the collection of specimens for virus detection should not be delayed until laboratory confirmation of a suspected case is obtained.

4.1 Documenting sample collection

All samples collected for analysis must be documented, preferably using a standardised laboratory request form. An example of an appropriate laboratory request form is provided in Annex 9.1. The minimum information to be included on an acceptable laboratory request form includes:

- unique Identifying Number (in an agreed format);
- in-house laboratory number;
- patient name (in English);
- age (or date of birth in standard notation);
- province (or region);
- town/district;
- country code (assigned by WHO Regional Office);
- date of last measles and/or rubella vaccination;
- date of onset of rash;
- does the patient fit the case definition?
- specimen type;
- date of specimen collection;
- date specimen sent to laboratory.

4.2 Serological samples for antibody detection

The ideal test for measles and rubella diagnosis is one that:

- requires a non-invasive sample;
- requires only one sample;
- can use a sample collected at first contact with the patient;
- is highly sensitive (one which detects a high proportion of true cases) and specific (has a low level of false positivity);
- has a high positive predictive value (the proportion of cases diagnosed as measles or rubella which are truly measles or rubella); and
- is easy to perform at local level and provides quick accurate results, upon which control measures can be implemented.

The assays recommended for the WHO measles and rubella laboratory network are the ELISA tests for the detection of virus-specific IgM antibodies. These tests are commercially available and the results show very good correlation with the ability of hemagglutination inhibition and plaque neutralization tests of paired serum samples to detect a 4 fold rise in IgG. Technically, measles and rubella IgM assays resemble a number of screening assays now being performed in laboratories worldwide, and relatively little laboratory training is needed to attain a reasonable level of proficiency. Laboratories are recommended to report IgM test results from routine cases within 7 days after receipt of samples. However in outbreaks or situations where rapid turnaround of results will assist the control programme in implementing a response, a capable laboratory should be able to provide results within twenty-four hours (even within four to five hours in some laboratories) after the sample reaches the laboratory. Given the need for differential diagnosis of febrile rash illness, a battery of similar serological assays for three or more of the most frequently occurring rash diseases in a given area (for example, measles, rubella and dengue) is a practical proposition in many countries.

4.2.1 Timing of blood sampling for measles and rubella IgM

While IgM ELISA tests for measles and rubella are more sensitive between days 4 and 28 after the onset of rash, a single serum sample obtained at the first contact with the health care system at any time within 28 days after onset is considered adequate for surveillance purposes. However, in the first 72 hours after rash onset, up to 30% of tests for measles-specific IgM may give false-negative results and up to 50% of tests for rubella-specific IgM in first 72 hours may give false-negative results. [30, 31] In outbreaks where 5-10 samples have been collected, individual diagnosis is not critical, however in sporadic cases, a second serum sample may be required under the following circumstances:

- the first blood sample submitted for IgM was collected within four days of rash onset and is negative by ELISA. The laboratory may request a second sample for repeat IgM testing given the probability of false negatives on early samples;
- the IgM ELISA gives a repeatedly equivocal result;
- the clinician needs to make a definitive diagnosis on an individual patient with an initial negative result.

A second sample for IgM testing may be collected anytime between 4 and 28 days after rash onset. Collection of a second sample 10-20 days after the first will permit the laboratory not only to retest for IgM but, if a suitable quantitative method is available, test for an increase in IgG antibody level.

4.2.2 Collection and handling procedures for serum or dried blood

Blood should be collected by venipuncture in a sterile tube (5ml for older children and adults and 1ml for infants and younger children should be adequate) or if applicable, by finger or heel prick onto filter paper and labelled with the patient's identification and the collection date. Whole blood can be stored at 4–8 °C for up to 24 hours before the serum is separated, but it must not be frozen.

Whole blood should be allowed to clot and then centrifuged at 1000 × g for 10 minutes to separate the serum. If there is no centrifuge, the blood should be kept in a refrigerator until there is complete retraction of the clot from the serum (no longer than 24 hours). The serum should be carefully removed with a fine-bore pipette to avoid extracting red cells, and transferred aseptically to a sterile labelled vial with the patient's name or identifier, date of collection and specimen type.

Dried blood should be allowed to air dry and then sealed in a sealable plastic bag or envelope, with a desiccant if possible. Though dried blood samples are stable at room temperature for a limited period they should be stored at 4°C, if possible, until they can be shipped to the laboratory.

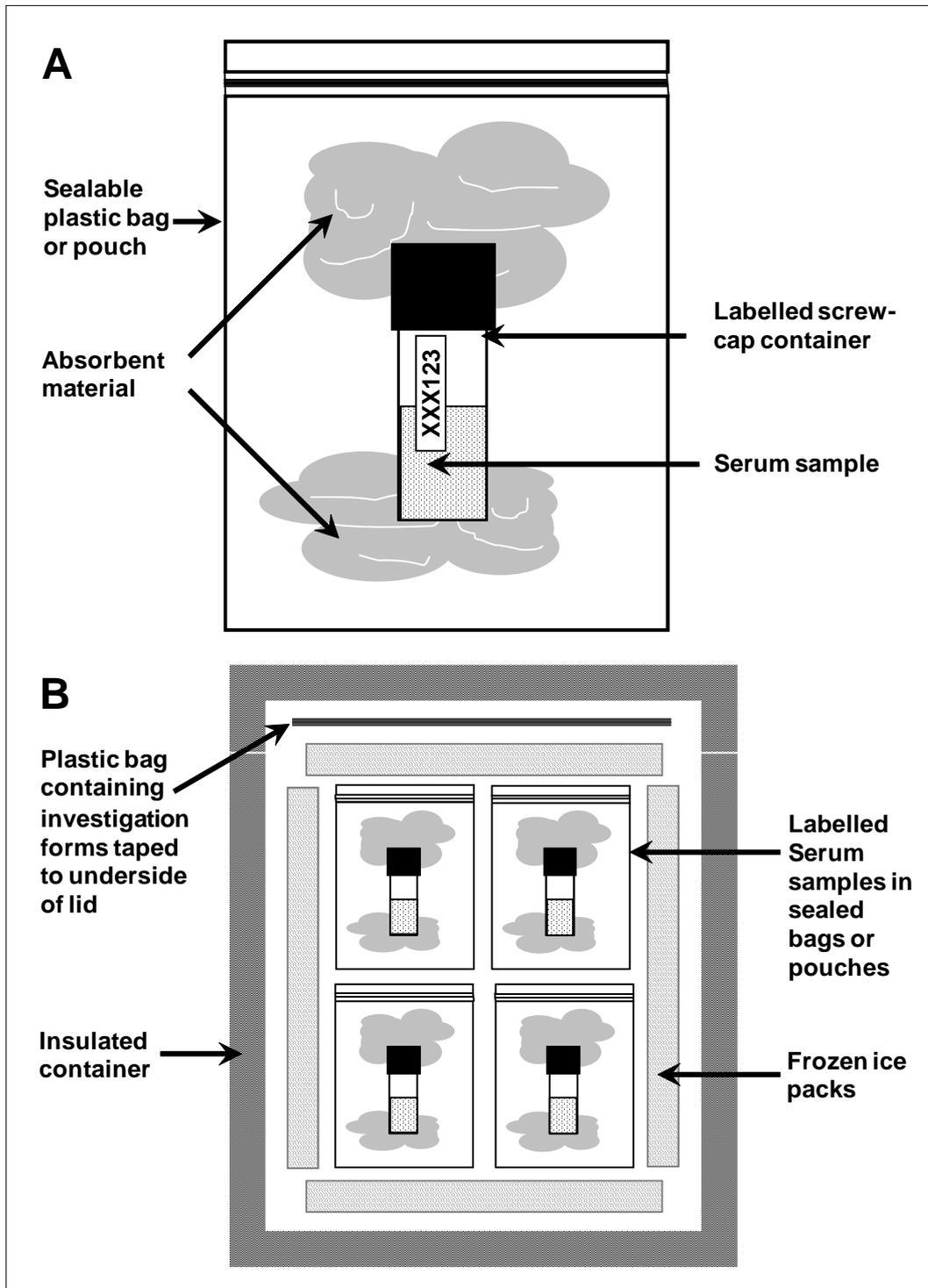
4.2.3 Storage and shipment of serum samples

Serum should be stored at 4–8°C until shipment takes place, or for a maximum of 7 days. When kept for longer periods, serum samples must be frozen at -20°C or lower and transported to the testing laboratory on frozen ice packs. Repeated freezing and thawing can have detrimental effects on the stability of IgM antibodies. As a general rule, serum specimens should be shipped to the laboratory as soon as possible and shipment should not be delayed for the collection of additional specimens.

Serum specimens, in their uniquely labelled sealed vials, should be placed in sealable plastic bags or pouches, containing absorbent materials such as cotton wool to soak up any leakage that may occur (Figure 12A). Insulated containers should be used to contain the sealed bags of specimens. The specimen form and investigation form for each specimen should be placed in a separate plastic bag and taped securely to the inner surface of the top of the insulated container (Figure 12B). If using ice packs (which should be frozen), these should be placed at the bottom and along the sides of the insulated container. The samples should then be placed in the centre and more ice packs placed on top. A shipping date should be arranged between the sample collectors and the laboratory. When arrangements have been finalized, the receiver should be informed of the time and manner of transportation. Refer to Annex 9.5 for further details.

Serum samples received for IgM analysis should be tested as soon as possible after receipt in the laboratory. Short-term storage of serum samples (1–7 days) should be at 4°C. Long-term storage of sera should be at or below -20°C.

Figure 12. Packaging of serum samples.
A - individual samples in a sealed bag or pouch.
B - multiple samples in an insulated container.



4.3 Samples for virus isolation

The laboratory should agree in advance with the epidemiologists on the type and number of samples that are most appropriate for virus isolation. Ideally, samples should be collected simultaneously with the blood samples for serological diagnosis and confirmation of measles or rubella virus as the cause of the outbreak. Since each type of sample has different requirements, the decision on the type of samples will depend on the local resources and facilities for transport and storage.

It is recommended that clinical samples (throat or nasopharyngeal swabs, nasal aspirates or 10 to 50ml of urine) for measles virus isolation be collected as soon after rash as possible. The sample should be collected at the first contact with a suspected case of measles when the serum sample for diagnosis is drawn. Measles virus isolation is most successful when samples are collected the first day of rash through the 3 days following onset of rash; however, virus may be still present at least through day 5 following rash. Rubella virus can be detected in nasopharyngeal secretions from a few days before onset of rash to several days afterwards. Both viruses are sensitive to heat, and infectivity decreases markedly when samples are not kept cold. It is important to transport samples to the laboratory with cold packs as soon as possible following sample collection.

4.3.1 *Nasopharyngeal specimens for measles and rubella virus isolation*

Nasopharyngeal specimens for virus isolation should be collected as soon as possible after the appearance of the rash, when virus is present in highest concentrations. Nasopharyngeal specimens can be taken as follows (in order of increasing yield of virus):

- *Nasal aspirates:* collected by introducing a few ml of sterile saline into the nose with a syringe fitted with fine rubber tubing and collecting the fluid in a screw capped centrifuge tube containing viral transport medium.
- *Throat washes:* obtained by asking the patient to gargle with a small volume of sterile saline and collecting the fluid in viral transport medium.
- *Nasopharyngeal/oropharyngeal swabs:* obtained by firmly rubbing the nasopharyngeal passage and back of the throat with sterile cotton swabs to dislodge epithelial cells. The swabs are placed in sterile viral transport medium in labelled screw-capped tubes.

Nasopharyngeal specimens should be refrigerated and shipped to the laboratory with ice packs (4 to 8°C) to arrive at the testing laboratory within 48 hours. If arrangements cannot be made for rapid shipment, swabs should be shaken in the medium to elute the cells and then removed. The medium or nasal aspirate should be centrifuged at 500 × g (approximately 1500 rpm) for 5 minutes, preferably at 4°C, and the resulting pellet should be resuspended in cell culture medium. The suspended pellet and the supernatant should be stored separately at -70°C and shipped to the testing laboratory on wet ice (4 to 8°C) to arrive within 48 hours or preferably, on dry ice in well-sealed screw-capped vials.

For throat or nasal washes or swabs that are in very little fluid (1 to 4ml), the entire sample can be frozen at -70°C or if low temperature freezers are not available, kept at 4°C until shipment. Repeat freeze-thaw cycles or freezing at -20°C (standard freezer temp) must be avoided because ice crystals can kill the virus. If -40°C or -70°C storage is not available, it is recommended to keep the sample in the refrigerator (4°C).

If the specimen arrives in the laboratory as frozen material in 2-3ml of cell medium or PBS, it can be stored frozen as it is. If the original swab tube is received, swab material should be collected by the addition of 2ml of DMEM, the tube contents mixed using a vortex mixer, and allowed to stand for an hour for virus elution. The swab should then be drained of as much liquid as possible by pressing against the side of the tube. If the amount of debris is heavy, the tube should be subjected to centrifugation to remove it. Sample liquid should be stored at -70°C.

4.3.2 Urine for measles virus isolation/detection

The isolation of measles virus is most successful if the specimens are collected as soon as possible after the onset of rash, and at least within 5 days after onset. It is preferable to obtain the first urine passed in the morning. About 10-50ml of urine should be collected in a sterile container and held at 4 to 8°C before centrifugation. Measles virus is present in acute cases of measles in the cells that have been sloughed off in the urinary tract. The virus is concentrated by centrifugation of the urine and the cell pellet resuspended in a suitable viral transport medium. Urine must **NOT be frozen** before the concentration procedure is carried out.

Whole urine samples may be shipped in well sealed containers at 4°C, but centrifugation within 24 hours after collection is preferable. Centrifugation should be performed at 500xg (approximately 1500 rpm) for 5 to 10 minutes, preferably at 4°C. The supernatant should be discarded and the sediment resuspended in 2 to 3ml sterile transport medium, tissue culture medium or phosphate-buffered saline. The resuspended pellet may be stored at 4°C and shipped within 48 hours to a measles reference laboratory. Alternatively, it may be frozen at -70°C in viral transport medium and shipped on dry ice in a well sealed screw-capped vial.

4.3.3 Whole blood for measles virus isolation/detection

For isolation of peripheral blood mononuclear cells (PBMC) for subsequent virus isolation, blood should be collected by venipuncture in a sterile tube supplemented with EDTA. A minimum blood volume of 5ml is recommended. The plasma fraction can be used to determine the measles-specific IgM antibodies. The tube should be labelled with the patient's identification number and the date of collection. Whole blood samples may be shipped in well-sealed tubes at 4°C. EDTA supplemented whole blood should be processed for virus isolation within 48 hours after collection and must not be frozen at any time prior to processing.

On receipt in the laboratory, PMBCs must be separated from the blood. This is most conveniently achieved using a commercial product designed to separate lymphocytes from peripheral blood through centrifugation.(e.g. Organon Teknika LSM). After dilution of the blood 1:1 with PBS it is carefully layered over 2ml of Lymphocyte Separation Medium in a test tube. The tube is subjected to centrifugation at 2000 rpm for 30 minutes at 20°C in swinging buckets and without the centrifuge brake engaged. The PMBCs should form a white/grey band above the red cell pellet. Using a pipette, the PMBC band can be carefully harvested into a clean centrifuge tube and washed with 10 to 15ml of PBS. PMBCs should be pelleted from the PBS and resuspended in a small volume (1 to 2ml) of DMEM. It is recommended that each sample be divided into at least two tubes. Resuspended PMBCs should be stored at -70°C.

4.4 Samples for RT-PCR

Measles and rubella virus can be often be detected by RT-PCR in the samples suggested above, for 3 to 4 days beyond the period after onset of rash for virus isolation. Any sample collected and transported to the laboratory for virus isolation can be used for RT-PCR analysis. However, though there are some reports of isolation of rubella virus from PMBCs they are not usually a good source of rubella virus RNA. In addition, samples collected using the alternative sampling methods (oral fluids and dried blood - see below), if collected within 7 days of onset of rash and transported to the laboratory under appropriate conditions, can be used for RT-PCR analysis. Note that oral fluid sampling devices which use a preservative for stabilising IgM (such as OraSure™) should NOT be used for collection of samples for RT-PCR.

4.5 Alternative sampling techniques (dried blood samples and oral fluid)

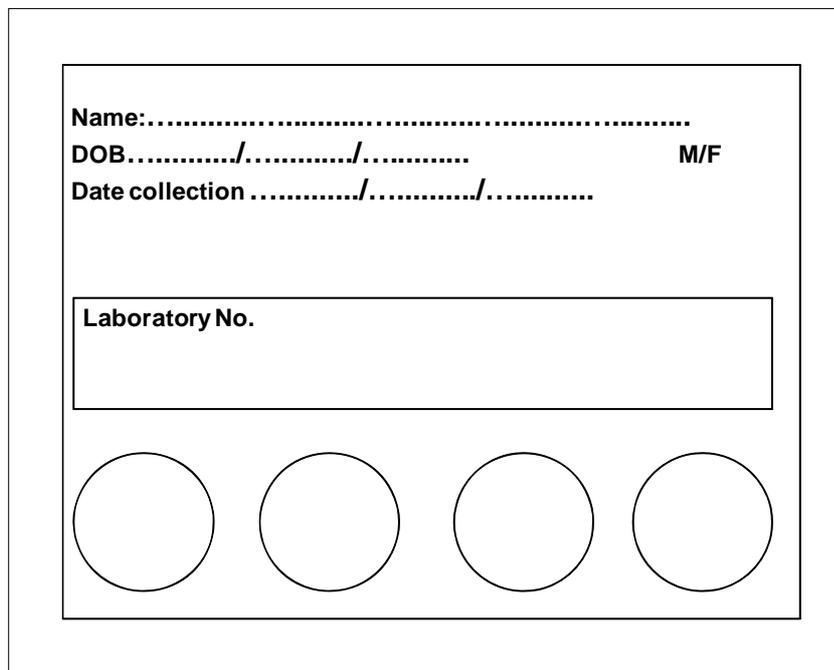
The collection of blood samples, particularly from children, is not always greeted with enthusiasm and maintaining a cold chain when transporting samples to the laboratory is not always achievable. Recently two approaches have been validated for use in the WHO Measles and Rubella Laboratory Network which have the potential to be useful tools for the measles/rubella programme; the use of dried blood spots and oral fluid samples[32-41]. Dried blood spots have been used for a range of epidemiological studies as an alternative to serum. Antibody is stable in this form and so it is particularly valuable where the lack of a cold chain is an issue. This technique has recently been applied successfully to measles cases, and there is accumulating evidence that the technique will work as well for rubella cases [41,42]. Oral fluid sampling has been used successfully for almost all measles, rubella and mumps laboratory-based surveillance in the UK since the early 1990s. Oral fluid samples are easy to collect, non invasive and more acceptable to the population. Its use enables field workers to obtain more complete sampling of suspected cases.

Oral Fluid (without serum stabilizer) and dried blood spots can also be used for viral genome detection using RT-PCR. This creates additional opportunity to test virus and antibody response in the same sample. Note; Oral fluid samples have a higher sensitivity for nucleic acid detection than dried blood samples.

4.5.1 Dried blood collection, storage and shipment procedures

The requirements for blood spot sample collection, storage, and transportation are minimal. Each participant's finger, or heel in the case of very young children, is cleaned with alcohol, then pricked with a sterile, single use microlancet. Up to four drops of whole blood are collected on standardized filter paper (for example, Whatman S&S No. 903). The filter paper should be marked, either by hand or printed with a laser printer or photocopier in a standard format that includes 14-15 mm circles within which to place the blood drops, the name, age and sex of the patient, and a space to write the laboratory or specimen number. An example of an appropriate format for a filter paper blood collection card is shown in Figure 13.

Figure 13. Example format for blood spot collection card



The figure shows a rectangular form for a blood spot collection card. It contains the following fields and elements:

- Name:** (dotted line)
- DOB:**/...../..... **M/F**
- Date collection**/...../.....
- Laboratory No.** (text box)
- Four circles arranged horizontally at the bottom, intended for blood spots.

The filter paper should be allowed to dry thoroughly (at least 60 minutes) before enclosing it in a bag for storing. Filter papers may be placed in a slide holder or similar during this process. Drying stabilizes the IgM and reduces the chance of microbiological contamination. Each filter paper should be wrapped individually, preferably in a sealable plastic bag to prevent possible cross contamination and to protect from dust and moisture. If available, a desiccant pouch should be included in the plastic bag. Thoroughly dried blood spot samples are no longer subject to IATA dangerous goods regulations and can be shipped without special documentation from the site of collection to the laboratory. Although samples do not need to be kept refrigerated or frozen during transport, it is advisable to store in a cool place and transport to the laboratory as soon as possible.

On arrival in the laboratory dried blood spots should be stored in labelled, individual sealable plastic bags or other airtight containers at 4°C until tested. Long term storage should be at -20°C, with a desiccant pouch. For safety reasons, dried blood spots should be always considered as potentially infectious and only be handled with gloves.

4.5.2 *Oral fluids collection, storage and shipment procedures*

Crevicular fluid exuded from the interface between the gums and teeth contains low levels of IgM[37]. A number of swab collection devices (such as the Oracol™ and OraSure™) have been developed specifically to collect these fluids from the mouth. The swabs are designed to be used like a toothbrush and should be rubbed along the gum until the swab is thoroughly wet. This usually takes one minute. The wet swab is placed inside the clear plastic transport tube that has an area on the outside to write the name of the patient, patient details and collection date. Some devices have virus transport medium incorporated within the plastic transport tubes, others can be transported as is. Specific instructions provided by the device manufacturer should be followed. If the daily ambient temperature is below 22°C samples should be shipped to the laboratory within 24 hours. At higher temperatures samples should be kept in a refrigerator until shipping to the laboratory on ice. The samples are usually not considered biohazardous and can be shipped without special documentation from the site of collection to the laboratory.

Oral fluids must be extracted from the sample swabs as soon as possible after receipt in the laboratory. The following process should be used for extraction and storage of oral fluid samples collected with the Oracol™ device:

- Add 1ml Virus Transport Medium (see Annex 9.6) to the tube containing the swab
- Agitate the swab manually, or use a bench vortex for 20 seconds to ensure foaming of the transport medium
- Remove swab from tube using a twisting motion, to extract as much liquid as possible from the swab
- Invert swab and replace it in the tube so that the pink foam is now at the top of the tube. Replace the cap
- Centrifuge at 2,000 rpm for 5 minutes
- Discard swab using forceps
- Extracted oral fluid can now be recovered from the tube, using a Pasteur pipette. Dispense into two tubes, one for IgM testing the other for RT-PCR.
- Store IgM sample at +4°C until tested and the RT-PCR samples at -20°C or lower until tested

4.6 General safety precautions on receipt of samples

On arrival in the laboratory, shipping cartons or carriers must be immediately unpacked in a designated area equipped with a discard container, alcohol swabs and paper towels. Safety of laboratory workers is the prime concern and, if available, a Class II Biosafety Cabinet (BSC) could be used to limit exposure of laboratory staff to potential pathogens[43]. If a BSC is not available a clean workbench can be used. This should have a surface covering that can be easily disinfected using common laboratory disinfectants (70% alcohol, sodium hypochlorite solution, 2% glutaraldehyde solution, etc.) and should be located away from areas used for other laboratory activities. Unpacking and recording of specimens should preferably be carried out by two persons: one records data while the other is gloved and is responsible for opening the package and checking for breakage and leakage of sample containers, and contamination of accompanying documents. Any contaminated paperwork should be placed temporarily in the BSC while the information is manually recorded on a clean sheet of paper. Contaminated documents should be handled in the same manner as infectious wastes. Copies of all paperwork regarding the shipment should be maintained, especially permits. It is recommended that each laboratory develop specific standard operating procedures for opening packages and logging in specimens.

5. Laboratory diagnosis of measles and rubella

Detection of measles- or rubella-specific immunoglobulin M (IgM) in serum is the standard test for the rapid laboratory diagnosis of measles and rubella. IgM testing is most commonly performed using commercial enzyme immunoassay (EIA) kits. A number of commercially available IgM assays for measles and rubella use the indirect format. This format requires the blockage of IgG antibodies and rheumatoid factor through a pre-treatment step to ensure optimal performance. IgM assays based on the capture format have been developed for both measles and rubella. These assays do not require the removal of IgG antibodies and are considered to be slightly more specific, and technically easier to perform, than the indirect EIAs for detection of IgM antibodies. Both capture and indirect assays are described in this section.

Although no longer routinely used for diagnosis of acute measles and rubella infection, detection of a rise in specific IgG in serum samples collected during the acute and convalescent phases, can be used to confirm infection. IgG assays rely on the collection of two samples about 10 to 30 days apart, and can also be used for confirmation of sporadic cases of IgM positive or equivocal results in elimination phase countries. Seroprevalence studies conducted in support of measles or rubella control activities typically use the quantitative detection by EIA of IgG in a single serum sample.

Systems for the direct detection of measles and rubella through RT-PCR are becoming more common, and although standard methods are becoming established, no single standard method has yet been developed. Well established and widely used methods for RT-PCR detection of measles, developed by the Centers for Disease Control and Prevention, Atlanta USA (CDC), and the Health Protection Agency, London UK (HPA), are available from WHO or the relevant Laboratories upon request.

Although not recommended for routine laboratory diagnosis, culture of measles and rubella virus from clinical specimens is an important component of measles and rubella control strategies. Procedures for virus isolation in cell culture, have been included in Annex 9.4.11 and 9.4.12.

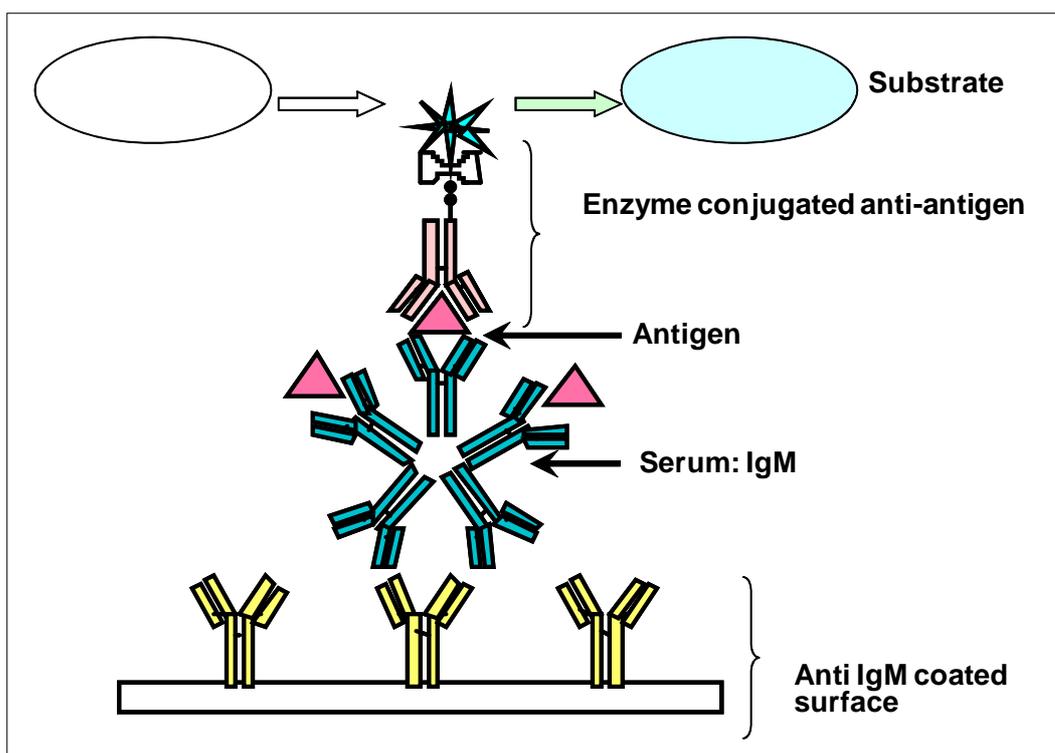
5.1 IgM assays

5.1.1 IgM capture

In the IgM-capture EIA (Figure 14), IgM antibody in the patient's serum is bound to anti-human IgM antibody adsorbed onto a solid phase. This step is non virus-specific. The plate is then washed, removing other immunoglobulins and serum proteins. Viral antigen is then added and allowed to bind to any virus-specific IgM present. After washing, bound antigen is detected using anti-virus monoclonal antibody conjugated with an enzyme, following which a detector system with chromogen substrate reveals the presence or absence of virus-specific IgM in the test sample. In some formats, the antigen-monoclonal antibody-enzyme complex is premade, eliminating one binding and washing cycle.

This test for measles is available commercially in kit form from numerous companies, however not all have been evaluated for use in the Measles and Rubella Laboratory Network. A detailed description of recommended test procedures, together with advice on interpreting results, are available on request from WHO.

Figure 14. Capture IgM ELISA schematic



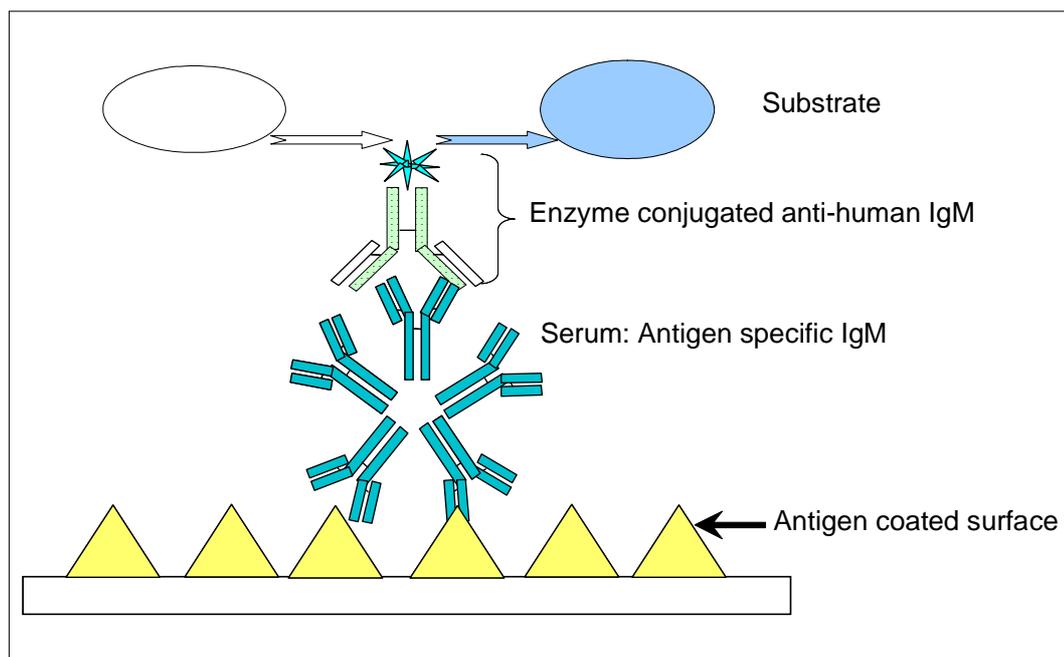
5.1.2 Indirect EIA for virus-specific IgM

In the indirect EIA for IgM (Figure 15), a rheumatoid factor absorbent is used for the complexing of IgG antibodies from test sera in a pre-treatment step. The first step of the absorption of virus antigen onto the solid phase is usually completed by the manufacturer and provided ready for use. The patient's serum is then added and any virus-specific antibody (IgM and any non-absorbed IgG) binds to the antigen.

IgM antibody is detected either directly, by means of an enzyme-labelled anti-human IgM monoclonal antibody or indirectly by means of anti-human IgM monoclonal antibody plus enzyme-labelled anti-mouse antibody. A chromogen substrate is added to reveal the presence of virus-specific IgM in the test sample.

Several commercial kits are available and kits for the detection of measles-specific IgM and rubella-specific IgM produced by Dade Behring are used in many WHO laboratories and have been validated, along with others, against a extensive panel of well validated serum samples. [8, 48]

**Figure 15. IgM indirect ELISA schematic
(Serum pre-absorbed to remove IgG and rheumatoid factor)**



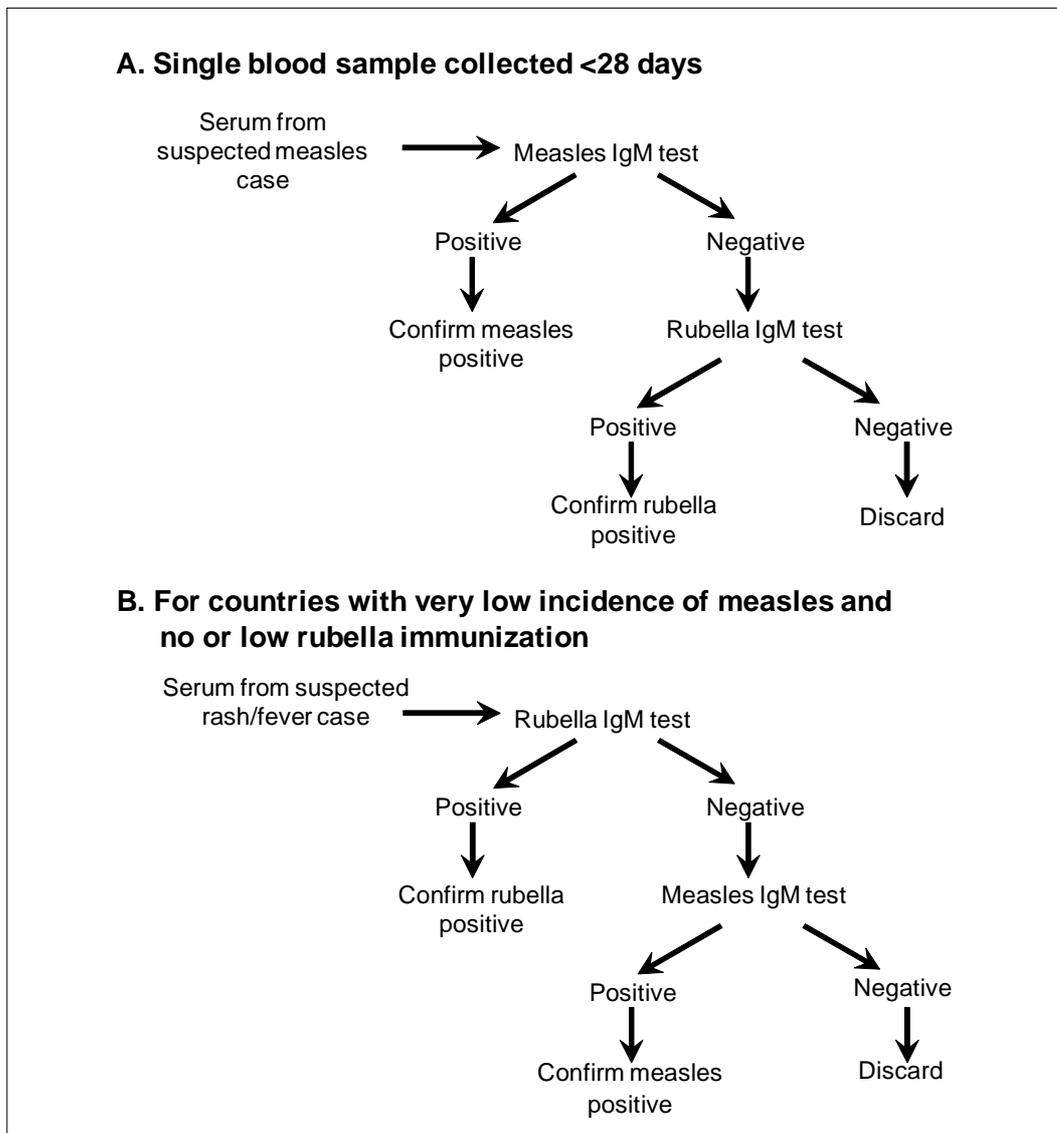
A detailed description of the test procedure, together with advice on interpreting results, are available on request from WHO.

Use of the alternative sampling methods of dried blood spot collection requires modification of the standard IgM assay procedures. Eluted fluid from dried blood spots can be tested using commercially available IgM indirect assays (such as those produced by Dade Behring), using slightly modified procedures [39]. Details of this modification are available in Annex 9.2.4. Always ensure that the EIA assay being used has been validated for the type of specimen that is being tested.

5.1.3 Differential laboratory diagnosis of measles and rubella

Laboratories in WHO Regions with both measles and rubella elimination targets will routinely test all samples from suspected cases for both measles and rubella. Laboratories in Regions without a rubella elimination target may still test samples for rubella, depending on the existing epidemiology of rubella and the status of rubella control activities, primarily because suspected measles cases are sometimes rubella cases. Since testing kits for rubella IgM detection are usually more expensive than measles, and testing all samples for both measles and rubella is often not a feasible option, a strategy of first testing all samples for measles IgM, and testing only measles IgM-negative samples for rubella IgM is an effective use of resources (Figure 16 A). Laboratories in countries with an effective measles immunization programme, and consequently a very low incidence of measles, but with no or very low rubella immunization, can make best use of resources by testing samples from rash/fever (or suspected measles or rubella) cases first for rubella IgM and then testing rubella IgM-negative samples for measles IgM (Figure 16 B).

Figure 16. Measles/rubella IgM testing strategies under different disease control conditions



5.1.4 Interpretation of IgM test results in patients with a history of recent vaccination

Since immunization with MR or MMR vaccine stimulates measles- and rubella-specific IgM responses, a history of MR or MMR vaccination within 6 weeks of rash onset makes the interpretation of serological results problematic. In a randomized double-blind trial in 581 pairs of twins, of 460 children aged 14-18 months, 32% developed moderate or severe fever after MMR vaccine (vs. 9% with placebo; $p < 0.002$), with a peak incidence at 9-10 days. Irritability (7.3% vs. 4.0% with placebo), drowsiness (3.5% vs. 1.4%) and rash (4.3% vs. 3.0%) were also more common with MMR vaccine than with placebo at around 9-10 days[44]. Serological techniques cannot distinguish between the immune response to natural infection and immunization, this can only be accomplished by viral isolation and characterization. In addition, studies conducted in Europe have shown that parvovirus B19, enterovirus, adenovirus, human herpesvirus type 6 (HHV6), and groups A and C streptococcus are the most common pathogens associated with rash-and-fever illness, accounting for between one third and one half of all cases[45, 46]. In tropical environments, approximately one third of primary dengue virus infections can present with rash and fever[47], accounting for the vast majority of rash and fever cases seen in dengue epidemic periods. Under these circumstances, recent MR or MMR vaccine recipients are likely to be represented among cases of rash-and-fever associated with other pathogens, and a vaccine-associated IgM response erroneously attributed to measles or rubella infection. It is therefore recommended that IgM-positive cases with a history of measles and/or rubella vaccination in the 1 to 6 weeks prior to rash onset can be discarded after thorough epidemiological investigation and no evidence is found of other confirmed cases in the vicinity or an epidemiological link to confirmed cases elsewhere. Countries in elimination phase should consider testing (as appropriate) for Dengue, Parvovirus B19 and HHV-6 as part of their differential diagnosis for all measles/rubella IgM positive cases.

5.1.5 Interpretation of results on samples positive for both measles and rubella IgM

A number of Network laboratories will be participating in national and international surveillance and diagnostic programmes for infectious diseases in addition to measles and rubella, and have the facilities to test for IgM specific to a range of viruses associated with rash-and-fever presentations. While commercial assays for measles and rubella IgM detection are generally $\geq 95\%$ specific, occasionally, test sera will appear to be IgM positive for a number of different agents, including measles, rubella, Epstein-Barr virus (EBV), cytomegalovirus (CMV) and human parvovirus B19 [4, 21, 48,]. The most common causes for false positive reactions include the presence of rheumatoid factor, specific IgG, and non-specific, cross-reacting IgM in the samples. In response to measles and rubella infections and vaccinations, specific IgM levels usually fall to below the level of detection within 1 to 2 months [49]. In addition, heterotypic IgM antibody responses may occur in patients infected with other viruses, particularly EBV, and sera from these patients may give false positive results for rubella and/or measles IgM [50]. This makes interpretation of the detection of measles- or rubella-specific IgM in the absence of clear clinical symptoms very difficult, particularly in low incidence countries, where use of a confirmatory assay, such as RT-PCR, positive virus isolation, 4 fold rise in IgG titer in acute and convalescent serum samples, or IgG avidity testing, can help in the interpretation.

5.2 Tests for IgG antibody

Using IgG detection to confirm a recent infection requires two serum specimens to be collected at least 10 days apart and tested in parallel. As it can often be difficult to obtain a second sample, IgM tests on a single sample are recommended. However, if the IgM test results remain inconclusive, a second (convalescent) serum specimen, collected 10-30 days after the first (acute) specimen, can be used to test for an increase in the IgG titre. However the first serum sample should be collected within the first 10 days after rash onset and IgG testing for laboratory confirmation of measles or rubella requires the demonstration of a 4-fold rise in the titre of antibody against measles or rubella. The tests for diagnostic IgG antibody should be conducted on both acute and convalescent specimens at the same time using the same type of test. The specific criteria for documenting an increase in titre depend on the test. EIA optical density (OD) values are not titres, and increases in EIA OD values do not directly correspond to titre rises.

The most widely used assays used to tests for IgG antibodies to measles and rubella are indirect EIAs. In these assays, purified virus antigen is adsorbed onto a solid phase and the patient's serum is added. Any virus-specific antibody binds to the antigen, and virus-specific IgG is detected using anti-human IgG. The binding of virus-specific IgG is measured by a direct or indirect detector system using a chromogen substrate.

5.2.1 *IgG avidity assays*

Antibody avidity refers to the strength of interaction of an antibody with a multivalent antigen. Depending upon the strength of this binding, the complex formed may or may not be easily dissociated. Antibody avidity is low after primary antigenic challenge, becomes higher with time and it usually involves IgG antibodies[51, 52]. Assays measuring the antigen-binding avidity of IgG antibodies has been developed to distinguish the low-affinity antibodies produced at an early stage of infection from those with a higher-binding affinity that reflects past immunity. IgG avidity testing can be helpful in differentiating between primary and secondary rubella infections, and in excluding the possibility of residual IgM months or years after primary infection. However, IgG avidity assays are difficult to establish, standardise, quality control and interpret, and are recommended only for laboratories experienced in using these assays. Laboratories with expertise in performing IgG avidity assays for the confirmation of rubella virus infection are available on request from WHO.

5.3 RT-PCR

The most important role of RT-PCR in measles and rubella control lies the genetic characterization of wild measles and rubella viruses and detecting genomic variation at different times and regions of the world. As RT-PCR can detect inactivated virus particles, the period of time when virus can be detected after rash onset is often several days to weeks longer than for virus isolation. However, RT-PCR presents a number of technical problems related to sensitivity and reproducibility that can invalidate the assay. In addition, cross-contamination during the RT-PCR process is a significant problem unless the strictest laboratory standards are established and maintained. For these reasons it is not recommended that laboratories set up PCR testing unless they have special designated areas for each of the PCR processes and have staff who have undergone comprehensive training.

For laboratories with appropriate facilities and trained staff, protocols for the laboratory confirmation of measles and rubella virus infection using RT-PCR are available on request from WHO.

5.4 Virus isolation in cell culture

Although rarely useful to diagnose measles, viral culture is extremely important for molecular epidemiologic surveillance of measles and rubella viruses.

5.4.1 Measles

The availability of sensitive cell lines for isolation of measles virus from clinical specimens and establishment of routine RT-PCR and sequencing techniques have allowed for rapid genetic characterization of a large number of wild-type strains of measles virus. This database of sequence information now makes it possible to use molecular epidemiological techniques to identify the source of wild-type viruses and to differentiate between wild type and vaccine strains.

Initially, an Epstein-Barr virus-transformed, marmoset B lymphoblastoid cell line, B95a, was the preferred cell line for primary isolation of measles virus. These cells are up to 10,000 times more sensitive for isolation of measles virus from clinical specimens than other commonly used cell lines. B95a cells are relatively easy to maintain in the laboratory and the cytopathic effect (CPE) from measles infection is readily observed. However, this cell line is infected with Epstein-Barr virus, and this presents a hazard to laboratory workers. These cells must be handled as infectious material at all times. For this reason B95a is no longer the preferred cell line for the isolation of measles viruses in Network laboratories.

Another cell line, Vero/SLAM, has recently been evaluated for use in the Global Laboratory Network. These are Vero cells that are transfected with a plasmid encoding the gene for human SLAM (signalling lymphocyte-activation molecule; also known as CDw150), a recently discovered membrane glycoprotein expressed on some T and B cells, which is a cellular receptor for measles virus[53]. The sensitivity of Vero/SLAM cells for isolation of measles virus is equivalent to that of B95a cells. In addition, Vero/SLAM cells are sensitive to laboratory-adapted measles strains including vaccine viruses[54]. Vero/SLAM cells have also been reported to be highly sensitive to rubella viruses, although CPE produced may be difficult to discern. The advantage of Vero/SLAM cells is that they are not persistently infected with virus, and therefore, present less of a biological hazard than B95a cells. The current disadvantage of the Vero/SLAM cells is that they require culture medium containing Geneticin to retain SLAM expression. However, once cell stocks are prepared in the presence of Geneticin, SLAM expression will be retained for at least 15 subsequent passages without Geneticin, saving on the increased cost of the tissue culture medium containing Geneticin.

Cell stocks of Vero/SLAM cells, developed by Dr Yanagi and colleagues at Kyushu University, Fukuoka, Japan [55], have been provided to the Laboratory Network by the National Institute of Infectious Diseases, Tokyo, Japan, and are now available in regional cell repositories. Vero/SLAM cells can be obtained on request through WHO.

A protocol for isolating measles or rubella virus using Vero/SLAM cells is provided in Annex 9.4.11 and 9.4.12.

5.4.2 *Rubella*

Rubella virus can be isolated from appropriately collected nasopharyngeal, blood, throat, urine, and cerebrospinal fluid specimens from rubella and CRS cases. Rubella will grow in Vero/SLAM cells, and these are currently recommended for use in the Global Laboratory Network. Vero cells can also be used for isolation of rubella virus.

A method for the detection of rubella E1 glycoprotein in infected Vero cells using monoclonal antibodies in either an immunofluorescent or an immunocolorimetric assay has been developed by CDC, Atlanta. These methods detect viral antigen in monolayers of Vero/SLAM or Vero cells that have been inoculated and incubated at 35°C for 3 or 5 days. Details of the procedure are provided in Annex 9.4.15 and 9.4.16.

6. Data management and reporting

An essential part of the work of every laboratory is to record the details of all specimens tested, to record the results of testing, and to report the results. A good laboratory will also analyse the results it obtains, interpret the results, looking for epidemiological patterns or trends, and summarize results in the form of regular reports. The term “data management” covers all of these activities, and is an essential function of any disease surveillance system. **WHO Regional offices will provide details of specific data reporting requirements appropriate to the level and activities of WHO measles and rubella laboratories within each Regional Laboratory Network.**

6.1 Data management goals

Good data management starts by understanding:

- the meaning of the information generated;
- what you need to tell to people outside of the laboratory;
- who you need to tell it to;
- how often you need to tell it.

Every laboratory needs to:

- report the results, in an organized format, to the EPI programme and back to the person who submitted the specimens;
- produce reports of its work to the director or head of the institute as annual reports or progress reports;
- produce summary reports to justify why it should continue to receive more funding.

Once all these requirements have been identified, thought can be given to what information must be recorded so that the requirements can most easily be met. As a general rule, the more information that has to be collected and recorded, the greater the chance that the information will be of lower quality, with more omissions and mistakes. It is always easier to collect and accurately record less information than more information, - but if less information is collected it is essential that it is the **required** information.

The next step in data management is to decide how the information is to be physically recorded and stored. All laboratories maintain specimen registers and laboratory results books. These are often in the form of paper records, recorded line by line, with information entered into specific columns. Such records are called line-listings, since all the information relating to that specimen or case can be found by reading along the line of information.

For laboratories with a small workload, paper records are enough to fulfil all the reporting requirements. For laboratories with larger workloads it is often more convenient to establish a computer record system. In accordance with Regional network requirements, a simple spreadsheet system (using software such as Excel), reflecting the line-listing of paper records, may be sufficient for some laboratories. Although useful for some types of analysis, computer spreadsheets are not very easy to manipulate when using large amounts of information. For large amounts of information it is better to establish a computer database.

The choice of exactly how to computerize laboratory record keeping depends on a number of factors, including:

- user preference;
- hardware availability and capacity;
- software availability and cost;
- type of programming required to use the software;
- local expertise to develop and maintain the system.

At a minimum, the system chosen should allow rapid and accurate access to chosen or selected records, be able to perform simple calculations, such as frequencies and time intervals, and be able to create tables and graphs. It is often an advantage to establish a “menu system” to help non-advanced users and to make repetitive actions, such as data entry, more efficient. The system must also be well documented for both users and programmers. The documentation should include clear descriptions of installation procedures, operation, structure, adaptation to specific needs, required maintenance activities, file management requirements, and coding lists if coded information is used.

Any computerized laboratory records system should contain the following components:

- data entry;
- data cleaning (programmes that detect errors in the information entered);
- routine backup of data;
- routine analysis and reporting (for decision-making, action, monitoring);
- feedback (information to be sent back to the case investigators);
- feedforward (information to be reported to the next level).

In designing any laboratory results recording system, it is essential to involve someone who understands the disease control objectives, strategies, surveillance needs and performance indicators of the activity. This will normally be someone from a more central level. The feedforward component in particular cannot be designed unless the next level has clearly specified its information requirements and the reporting format and structure most suited to its needs.

What information is reported, where it is reported from and where it is reported to, must be clearly agreed upon by all parties involved in the system. Feedback and feedforward reports will obviously have different formats, and different frequencies. Ideally, all information flow should be hierarchical, going from one level to the next, without missing levels. Information can also be “broadcast” (sent to several sources, at different levels, at the same time). Once a pattern of information flow is established it is very important that it be followed without exception. It is also important to review the system from time to time to make sure that it is doing the job it was designed to do, and to decide if improvements can be made. If any changes are made to the system it is essential that all parties involved are informed of the changes and agree to them.

Maintaining laboratory records and keeping them accurate and relevant involve following good management practices and a clear designation of responsibilities. The success or failure of any public health or disease control initiative depends on establishing and maintaining a good information exchange system, with accurate and timely data being provided for appropriate action. The importance of good laboratory data management cannot be overstated.

Of course, no matter what the data management system is, the raw data from testing of specimens should be retained in an easily accessible format. In addition to results from specimens, the assay worksheet should include the kit used, kit characteristics (e.g. batch number, expiration date), patient and control samples absorbance values, calculation and results of quality control samples, the name of the assay operator and the person who checked the results before they were reported.

6.2 Recording receipt of specimen

A case investigation form needs to be completed for each suspected measles case investigated. A separate laboratory request form should be completed at the time of specimen collection and should accompany all specimens sent to the laboratory. Information on specimen labels must be carefully checked to ensure that it matches information on the request forms. The following information should be included on the laboratory request form (see example Annex 9.1) accompanying the specimen:

- unique case Identifying Number (in an agreed format);
- provisional clinical diagnosis
- in-house laboratory number (optional, but often important);
- patient name (in English script);
- age (or date of birth in standard notation);
- province (or region);
- town/district;
- country code (assigned by WHO Regional Office);
- date and type (e.g. M or MR or MMR) of last vaccination;
- date of onset of rash;
- does the patient fit the case definition?
- specimen type;
- date of specimen collection; and
- date specimen sent to laboratory.

On receipt of a specimen the laboratory should record the following additional information:

- date specimen received in laboratory;
- adequacy of specimen (volume,);
- condition of specimen on receipt (for feedback to the EPI manager); and
- preparatory actions taken (elution, separation, centrifugation, storage location, etc.).

Each specimen should be allocated a specific identification number that is entered in the laboratory “day book”, on the accompanying request form and on the specimen container. This may be an abbreviated version of the case Identification Number or a sequential unique laboratory number. This number must be used on all containers, centrifuge tubes, test tubes and vials throughout subsequent laboratory procedures.

6.3 Recording results

The information to be collected and recorded on specimen processing and results should, at a minimum, include the following:

- case identification number;
- date of assay;
- type of assay (IgM, RT-PCR, virus culture);
- optical density readings;
- result of assay;
- date result reported to the EPI manager; and
- was a sample sent to the RRL (yes or no)?

If yes:

- name of regional reference laboratory;
- laboratory identification of sample sent (local laboratory specimen number);
- date of sending sample to RRL;
- date of receiving result back from RRL;
- RRL result; and
- date result reported to EPI manager.

6.4 Reporting laboratory activity and results

Laboratory results must be reported in a timely and accurate manner for several reasons. Reporting of laboratory results has a direct effect on the measles control and elimination programme through:

- feedback to national EPI teams for case follow up and planning supplementary immunization activities;
- coordination of the control and elimination programme through WHO and other international agencies and bodies; and
- monitoring of laboratory results and performance to identify possible problems and constraints.

Regular reporting of results will provide a continuous record demonstrating that recommended and acceptable procedures have been followed and laboratory accuracy has been at an acceptable level.

6.4.1 *Feedback to EPI teams*

Details of how and when laboratories report to EPI managers should be arranged locally. In general, however, all results should be reported within a week of receipt of serum sample and positive cases (in the absence of recent cases) should be reported within 24 hours. All other results should be available to the EPI managers on request. It is also helpful to the programme if a formal presentation of laboratory results is made to the EPI manager on a monthly basis.

Details of inadequate specimens and inadequate transport of specimens or missing data elements should be reported to EPI managers as soon as possible so that field staff can be informed and improvements made.

6.4.2 *Monthly reports to WHO*

All national laboratories are requested to provide a monthly report of results to WHO. This information is used to update country summaries, monitor laboratory performance and coordinate international agency activity. Data provided in the monthly reports is essential to the coordination of the programme as a whole, and it must be a priority activity of all laboratories in the network to send monthly reports in a timely and accurate manner.

Because of the amount of data involved and the time required to analyse the information it is essential that laboratories handling more than 100 specimens a year provide their monthly reports in computer database format, on computer diskettes or sent by e-mail. WHO can now provide a set of laboratory data management programs suitable for most of the measles laboratories in the global laboratory network.

7. Safe sample and isolate transport

This section is primarily concerned with the international transport of infectious and potential infectious materials from one laboratory to another within the global laboratory network.

The safe shipment of diagnostic specimens and infectious materials is the concern of all who are involved in the process. There are a number of international and national regulations covering requirements and procedures to be followed in transporting diagnostic samples and materials derived from them. Failure to meet these regulations can result in unnecessary delays, loss of viability of specimens, or an increase in the risk of accidentally exposing transport personnel, the sender, receiver, or the public, to potentially infectious materials. Hand carriage of infectious substances is strictly prohibited by international carriers, as is the use of diplomatic pouches for that purpose. Successful shipment of materials within the global laboratory network requires advanced planning, appropriate packaging, labelling, documentation and communication between all parties involved - the sender, carrier, and receiver.

7.1 Planning

It is the responsibility of the sender to ensure the correct designation, packaging, labelling and documentation of all materials sent from the laboratory. The efficient transport of infectious materials requires good coordination between the sender, the carrier, and the receiver (receiving laboratory), to ensure that the material is transported safely and arrives on time and in good condition. Such coordination depends upon well-established communication and a partner relationship among the three parties.

Once it has been decided that materials need to be shipped from the laboratory, the receiver should be contacted and informed of the nature of the materials to be sent. The sender should enquire about any import permits required by the receiving laboratory's national government. If permits are needed, the receiving laboratory will need to obtain the CURRENT permit and send it (usually a faxed copy) to the shipping laboratory so that the permit can be given to the carrier. The sender should seek information from the receiver concerning recommended carriers. The sender and receiver should then make advance arrangements for a mutually convenient time for shipment to ensure that the appropriate staff are available to receive the shipment. It is recommended that weekend arrivals be avoided.

Once the receiving laboratory knows that a shipment is necessary, the sending laboratory should contact a carrier familiar with handling infectious substances and diagnostic specimens and make arrangements to ensure that:

- the shipment will be accepted;
- the shipment is sent by the most direct routing, avoiding weekend and holiday arrival;
- documentation of the shipment progress will be kept;
- the conditions of the shipment while in transit will be monitored;
- the sender will be notified of any delays.

The sender should ask about any necessary shipping documents that the carrier may require or any specific instructions necessary to ensure safe arrival of the shipment. The carrier may also provide advice on packaging.

7.2 Packaging

Properly packaging and labelling of the material being shipped is vital to maintaining the integrity of the specimens, preventing accidents, and ensuring that there are no delays due to violations of regulations. The packaging requirements for various types of laboratory materials are subject to international and national regulations. There are a number of licensed agencies world-wide that provide training for personnel on how to package materials in compliance with international regulations.

The international regulations for the transport of infectious materials by any mode of transport are based upon the Recommendations of the United Nations Committee of Experts on the Transport of Dangerous Goods (UN). International organizations such as the Universal Postal Union (UPU), the International Civil Aviation Organization (ICAO), and the International Air Transport Association (IATA) have incorporated these recommendations into their respective regulations. The World Health Organization serves in an advisory capacity to these bodies.

Packaging which does not comply with the regulations has an increased likelihood of damage or leakage in transport. The WHO Meeting of Experts in 2001 agreed that two package performance levels would be adequate to contain the hazards posed by the full range of known pathogens. Based on the risk assessment, infectious substances containing pathogens for which particular precautions are warranted were placed in category A. These must be consigned following Packaging Instruction 620 (PI 620). Category B agents (including measles and rubella virus) are considered to be of less risk because they are not easily transmissible and basic precautions and hygienic practices will serve to prevent exposure and infection in the event of an incident. Category B agents should be consigned following Packaging Instruction 650. See section 9.5 for full details.

7.3 Preparation and sending

The documentation required to be completed for shipping materials is determined by the nature of the materials being sent. In general, each shipment should be accompanied with the following documents:

- a packing list/proforma invoice/customs declaration/commercial invoice which includes the receiver's address, the number of packages, detail of contents, weight, value (required for international shipping only);
- airway bill if shipped by air;
- export/import documentation, if required;
- the airway bill marked with:
 - the name, address, and telephone/fax number of receiver
 - number of specimens
 - “highly perishable”
 - “telephone receiver upon arrival” (repeat telephone number)
- handling information:
 - “URGENT: DO NOT DELAY: Biological specimens - highly perishable
- store at 4°C to 8°C”

Once the package has been sent, the receiver should be immediately notified of the following:

- number of specimens;
- estimated number of cartons and weight;
- flight and arrival date/time;
- airway bill number.

In addition, the receiver should be informed that a copy of the airway bill has been mailed to the receiving laboratory and be requested to inform the sender if the package is not received.

Once the package has been received, the receiver should immediately notify the sender of the receipt and condition of the shipment and any problems encountered. This can be facilitated by the sender including a “fax back” form in the shipment that the receiver can use for that purpose.

8. Quality assurance in Network laboratories

Laboratory Quality Assurance (LQA) is concerned with the organizational processes and the conditions under which laboratory activities are planned, performed, monitored, recorded and reported. Adherence by laboratories to the principles of LQA ensures the proper planning of activities and the provision of adequate means to carry them out. It promotes full and accurate reporting, and provides a means whereby the integrity of the activities can be verified.

8.1 Establishing LQA systems

Setting up a LQA system in a laboratory means defining the organizational structure, responsibilities, procedures, processes and resources necessary to achieve the following objectives:

- to prevent risks;
- to detect deviations;
- to correct errors;
- to improve efficiency;
- to ensure data quality and integrity.

It is the responsibility of the Director or Chief of the Laboratory to establish, implement and ensure compliance with LQA. However, LQA is the responsibility of all laboratory personnel. There are a number of elements that make up the LQA process, which are detailed below:

8.1.1 *Staff*

The measles/rubella laboratory should have the necessary staff with suitable qualifications and experience to carry out safely and accurately all the functions and responsibilities required of the measles/rubella laboratory. The laboratory should prepare an organigram of the measles/rubella laboratory that reflects the hierarchy and lines of authority, and include the functions and responsibilities of each person.

Staff should include:

- director or chief of the laboratory;
- head of each section or unit if appropriate e.g. serology, cell culture or molecular biology' laboratories etc.;
- scientific, technical and auxiliary staff;
- administrative support, maintenance, cleaning and service staff;

Each post should have a job description including: functions and responsibilities, academic training required and experience necessary.

8.1.2 Staffing levels

Staffing levels should be adequate to enable all the functions expected of the measles/rubella laboratory to be carried out without compromising safety or the integrity of the processes performed in the laboratory. There are specialized activities within the laboratory that require staff with considerable experience, such as EIA testing, cell culture production, reading of cytopathic effect in virus cultures, performing virus detection procedures, RT-PCR and sequencing techniques.

There should be at least one person with at least 12 months relevant experience to carry out these activities. It is advisable for at least one other person to work together with the experienced person to gain understanding of the activity and build capacity within the laboratory and allow for backup in the event of staff absence.

8.1.3 Human resources

The fundamental objective of the human resources policy is to have reliable staff with scientific and/or technological training to apply appropriate laboratory procedures correctly, and remunerated according to the labour market. The laboratory must regularly arrange and coordinate training courses to extend and update the skills of both technical and scientific staff according to needs identified and as proposed by the heads of department. This training is offered as a means of contributing to the success of the LQA process. A continuing education programme must be developed which includes on-site as well as external training. Documentation should be kept describing the staff training programme.

The human resources programme should include the technical evaluation of staff and follow-up of the performance of each staff member based on the job description. This system allows the correction of errors or weaknesses, and can also be used as a tool for promotion, where merited.

8.1.4 Space allocation

The measles/rubella laboratory should have adequate space to safely perform all activities, store all necessary equipment and allow for easy cleaning and maintenance. There should be enough rooms to enable separation of infectious from non-infectious activities. Cell culture and media-making facilities should be separated as much as possible from all other activities and preferably be in a room(s) completely separated from the laboratory where viral or other microbiological activities are being carried out. There should be a clear delineation of different working areas to minimize the chances of contamination of clean areas. If possible, there should be a logical arrangement of activities in a laboratory or laboratories to minimize the distance infectious materials must be carried and to ensure that infectious materials are not being transported through clean areas. If space allows, specific areas and preferably specific rooms should be allocated for:

-
- reagents and consumables storage;
 - instruments and equipment;
 - washing, preparation and sterilization (clean and dirty);
 - serology
 - cell culture;
 - specimen receipt and recording;
 - specimen processing;
 - inoculation, harvesting and typing;
 - specialized activities;
 - documentation, archiving and control;
 - the administrative area;
 - disposal of contaminated and medical wastes.

The following are the general characteristics with which the laboratory areas should comply:

- Lighting and ventilation should correspond to the needs of each working area, according to the specific requirements of the activity carried out. The surfaces of the workbenches should be smooth, easy to clean and made of material resistant to chemicals.
- Safety systems should cover fire, electrical emergencies, emergency shower and eyewash facilities.
- Hot and cold water, treated water, vacuum, gas, steam and electricity installations should be arranged so that they guarantee adequate use during the work and also facilitate maintenance and repair operations. Electrical installations should be arranged so that they do not pose any risk to workers, and electrical wires should not cross walkways. A standby generator is desirable for the support of essential equipment such as incubators, biological safety cabinets (BSCs), freezers etc, especially if power supply is erratic.
- Storage space must be adequate to hold supplies for immediate use and thus prevent clutter on bench tops and in aisles. Additional long-term storage space conveniently located outside the working areas should also be provided.
- Hand wash basins, with running water if possible, should be provided in each laboratory room, preferably near the door.
- An autoclave should be available in the same building as the laboratory.
- Facilities for storing outer garments and personal items, and for eating and drinking, should be provided outside the working areas.

Installation of equipment and organization of the laboratories should take biosafety and other safety standards into account.

8.2 SOPs

Standard operating procedures (SOPs) describe in detail the activities performed in the laboratory so as to:

- provide uniformity, consistency and reliability in each of the activities performed in the laboratory;
- reduce systematic errors;
- provide training and guidance for new staff.

Standard operating procedures should be drawn up by specialized technical staff in the laboratory, revised by their immediate supervisor and approved by the Director of the laboratory. Standard operating procedures should be prepared for all general procedures and should closely follow the WHO recommended procedures. Ideally SOPs should be drawn up as follows:

Title: Descriptive

Code: This code will identify:

- the laboratory;
- the number relating to each procedure;
- the number that identifies the revisions, with 00 being used for the original document.

Objective: The aim of the procedure being described should be expressed clearly and concisely.

Scope: the operating unit that will apply the procedure, and the field of application of the procedure.

Definitions: The meaning of the principal terms used in the procedure should be stated.

General description: Each SOP should be drawn up clearly, without ambiguity, so that it can be understood by staff with and without experience. Each step for performing the activity that is regulated by the procedure should be described in detail. Flow diagrams may be used to complement the description.

Safety conditions: These should reflect the safety measures and conditions to be kept in mind for the correct execution of the SOP. Material Safety Data Sheets should be included for hazardous chemicals used.

Documentation: the form or protocol in which the data and measurements involved in the procedures should be recorded.

References and documents: the references used to draw up the SOP.

8.3 Documentation

Documentation is the set of quality manuals, standard operating procedures, instructions, forms, reports, analytical protocols and record of data that serve as evidence of the LQA and permit the traceability of data. Responsibility for the preparation and revision of documents should rest with the LQA or Quality Assurance department, or with the person appointed, depending on the complexity of the laboratory.

8.4 Equipment and instruments

The laboratory should have the necessary equipment and instruments for the accurate performance of all tests performed. New instruments and equipment should be installed and calibrated if possible by the distributor or a suitably qualified person. All manuals and operating instructions should be stored in an area accessible to all users and a regular maintenance and calibration schedule established. All users should be completely familiar with the operating, maintenance and validation procedures to ensure correct functioning. Documentation of all malfunctions, maintenance and validation activities should be recorded in a central register.

The laboratory should have a list of equipment and instruments that include:

- the name;
- brand;
- donor or supplier;
- maintenance company;
- maintenance schedule;
- inventory number;
- serial number;
- model and year;
- location;
- date of purchase;
- date of first use;
- copy of manufacturer's handbook.

8.5 Supplies

8.5.1 Reference materials

These comprise material used to calibrate the test procedures and to guarantee uniformity in determining activity such as: reference positive and negative control sera, or RT-PCR controls. A central registry or logbook should be kept containing the following:

- name of the reference material;
- supplier;
- origin;
- lot number;
- date of analysis to determine whether it complies with the stipulated requirements;
- place and conditions of storage;
- expiry date, where applicable;
- storage in an appropriate form (corresponding SOP).

This registry should contain all the information relating to the properties of the reference material. The quality of the reference material should be verified when the conditions have been altered and routinely once a year.

8.5.2 Reagents (including diagnostic kits)

Reagents can be defined as materials of chemical or biological origin used in laboratory assays. At least a six months' reserve stock of reagents should be held in the laboratory at all times. Given the long delivery times and difficulty of transport to some regions, reagents should be ordered six to 12 months ahead of need. For those laboratories that routinely undertake virus isolation, cell culture medium should be considered a reagent.

A central registry or logbook should be kept containing the following:

- name of the reference material;
- supplier;
- origin;
- lot number;
- date of analysis to determine whether it complies with the stipulated requirements;
- place and conditions of storage;
- expiry date, where applicable;
- storage in an appropriate form (corresponding SOP).

This registry should contain all the information relating to the properties of the reference material. The quality of the reference material should be verified when the conditions have been altered and routinely once a year.

Characteristics of reagents:

- They should be of appropriate quality.
- They should be obtained from recommended suppliers in their original packaging.
- A record should be kept of purchasing, reception and distribution to guarantee continuity, particularly with substances that need to be acquired in advance.
- They should be inspected to ensure that the seals are intact when received in the stockroom or when distributed to the laboratory. These inspections should be recorded with the initials of the person responsible for the inspection and the date on the label.
- There should be a specific SOP for the transport, storage and handling of reagents and disposal.

Any changes to the composition of reagents or media or to the lot numbers of biological products (antisera, conjugates etc) should be fully documented in the central registry or logbook. Water should be considered a reagent and should comply with purity specifications or other technical requirements for use in the laboratory.

Reagents prepared in the laboratory should be prepared in conformity with written procedures and, where applicable, according to WHO standard recommendations, validated and labelled appropriately, stating the following:

- identification of the reagent;
- concentration;
- preparation and expiry date;
- storage conditions;
- initials of the technician responsible.

8.6 Laboratory safety

Each laboratory should have available the *WHO Laboratory Biosafety Manual* (WHO/CDS/CSR/LYO/2004.11) available at: <http://www.who.int/csr/resources/publications/biosafety/en/Biosafety7.pdf>.

This describes the essential biosafety, chemical, fire and electrical safety requirements to protect staff, the community and the environment. All staff should be familiar with the contents of this manual and should proceed accordingly. All new staff should be made aware of the risks involved in working in a measles/rubella laboratory before starting work in the laboratory and should be required to have read the Biosafety Manual. The director is responsible for implementation of and compliance with the provisions of the manual.

The major risk to staff in the measles and rubella laboratory is in handling serum samples. Serum should always be considered as a potentially infectious material and personnel should wear gloves when opening packages containing serum, aliquoting or transferring samples and when performing assays. Personnel who receive and unpack specimens should be aware of the potential health hazards involved, and should be trained to adopt standard precautions, particularly when dealing with broken or leaking containers. Primary specimen containers should be opened in a biological safety cabinet where possible. Disinfectants should be available in case of spills.

All laboratory staff should be vaccinated against Hepatitis B. Measles and rubella vaccination should be considered, especially in laboratories which are attempting to culture measles or rubella virus from clinical samples. Women of child bearing age working in the measles and rubella laboratory should have demonstrable immunity to rubella.

8.7 Annual accreditation

Accreditation provides documentation that the laboratory has the capability and the capacity to detect, identify, and promptly report measles and/or rubella positive samples. The accreditation process further provides a learning opportunity, a mechanism for identifying resource and training needs, a measure of progress, and a link to the Global WHO Laboratory Network.

Accreditation of National Measles and Rubella Laboratories is reviewed annually by the WHO Regional Office and is based on laboratory performance during the immediately preceding 12 months with complete data, usually from 13 months to 1 month prior to evaluation. Accreditation is given for the upcoming calendar year.

There are six criteria for accreditation for National Laboratories:

- Test results are reported by the laboratory on at least 80% of measles IgM samples within 7 days of receipt.
 - To allow an appropriate response to measles cases, all test results should be reported to the EPI programme in a timely manner.
- Serological tests are performed on at least 50 serological specimens annually.
 - To maintain skills in performing serological assays, Virus laboratories should maintain appropriate reagents and assay kits to have capacity to test continually through the year. To maintain expertise it is envisioned that laboratories will test a minimum of 50 serological specimens annually spread across the year.
- The accuracy of measles and rubella IgM detection is at least 90%.
 - Accuracy is determined by the agreement in test results on sera submitted by the National Laboratory to the Regional Reference Laboratory (RRL) during the 12-month review period. The percentage of samples sent for validation is dependant on the quality of the laboratory and could range from 10-100% with the lower range for a fully accredited laboratory and 100% for a laboratory which has failed accreditation. Samples for validation should be representative of all results (positive, negative and equivocal) and outbreaks, and should be sent to the Regional Reference Laboratory at regular intervals.

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- Internal quality control (QC) procedures for IgM assays are implemented.
 - Appropriate QC procedures are in place and followed, including; appropriate serological controls, micro-pipettor calibration and temperature recording of incubators and refrigerators/freezers. QC data sheets and summaries of corrective action are retained and available for review.
 - The score on the most recent WHO approved proficiency test is at least 90%.
 - Proficiency test (PT) results to be reported within 10 days of panel receipt to receive full credit.
 - The score from the annual on-site review of laboratory operating procedures and practices is at least 80%.
 - For National Laboratories with consistently high performance indicators an on-site review may be waived upon satisfactory completion of the annual check-list by the laboratory.

Regional Reference Laboratories undergo a similar annual accreditation review and are assessed under the following seven criteria:

- Confirmation results on at least 80% of National Laboratories' referred samples are reported within 14 days.
 - To ensure National laboratories receive a timely response to all validation test results.
- Serological tests are performed on at least 50 specimens annually.
 - To maintain skills in performing serological assays, Virus laboratories should maintain appropriate reagents and assay kits and test a minimum of 50 specimens annually, spread over the year.
- The score on the most recent WHO approved proficiency test is 90%.
 - Proficiency test (PT) results to be reported within 10 days of panel receipt to receive full credit.
- Internal quality control (QC) procedures are in place.
 - Appropriate QC procedures are in place and followed, including; appropriate serological controls, micro-pipettor calibration and temperature recording of incubators and refrigerators/freezers . QC data sheets and summaries of corrective action are retained and available for review.
- For the Regional Reference laboratories that also serve as National Laboratories:
 Test results are reported by the laboratory on at least 80% of measles IgM samples within 7 days of receipt.
 - To allow an appropriate response to measles cases, test results should be reported to the EPI programme in a timely manner

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- For laboratories performing measles and/or rubella genotyping:
 - Genotyping is completed within 2 months of receipt of virus sample and genotype data reported to the appropriate WHO regional office monthly (including zero reporting) for at least 80% of virus samples received.
 - Genotype information can assist national control programmes in determining transmission pathways and needs to be provided in a timely manner. Laboratories are also encouraged to submit sequence data to GenBank once sequencing is completed.
 - The score from the annual on-site review of laboratory operating procedures and practices is at least 90%.
 - For Regional Reference Laboratories with consistently high performance indicators, the Global Laboratory Coordinator may waive the on-site review upon satisfactory completion of the annual check-list by the laboratory (see below).

A Laboratory that achieves less than the passing score on any one of the applicable criteria will work with the Laboratory Coordinator to:

- Identify areas where improvement is needed.
- Develop and implement a work plan.
- Monitor laboratory progress.
- Provide for re-testing where required.
- Continue steps to achieve full accreditation.

A Laboratory that fails to achieve a passing proficiency test score within 6 months after annual review is deemed non-accredited and arrangements must be made for an accredited Laboratory to perform duplicate tests on all specimens.

All laboratories should be assessed annually but for laboratories with a consistently high accreditation score the Measles Laboratory Coordinator may waive annual onsite reviews and determine accreditation status after review of the other performance indicators. In this situation, onsite reviews of well performing laboratories may be carried out every 2-4 accreditation cycles.

9. Annexes

9.1 Example measles and rubella laboratory request form

Measles and rubella laboratory request form							
Country ¹ :		Date ² :		Patient ID ¹ :			
Patient Name:		M	F	Date of birth:			
Age in months:		Name of parent or guardian:					
Address:							
Number of doses of measles vaccine:			Date last dose received:				
Number of doses of rubella vaccine:			Date last dose received:				
Date of onset of fever:			Date of onset of rash:				
Provisional clinical diagnosis:							
Sample ID ¹		Sample type ³		Collection date		Shipment date	
1)							
2)							
3)							
Additional comments on patient or samples ⁴ :							
Name of person to whom laboratory results should be sent:							
Address:							
Telephone number:				Fax number:			
For use by the receiving laboratory							
Name of person receiving the sample:							
Sample ID as written on sample ⁵		Sample type		Date of receipt		Condition on receipt ⁶	
1)							
2)							
3)							
Additional comments:							
Sample ID		Sample type		Action taken on receipt in laboratory ⁷			
1)							
2)							
3)							

Notes:

- ¹ Formats for Country names, Patient IDs and Sample IDs should be agreed in advance with the WHO Regional Office and used consistently.
- ² The same date format should be used consistently – preferable the same format (e.g. dd-mm-yy) will be used throughout the Region. The preferred format will be advised by the WHO Regional Office.
- ³ Sample type may include: serum, whole blood (EDTA, heparinized), dried blood spot, swab (oral fluids, throat, nasal), aspirate (nasopharyngeal, respiratory), urine (whole sample, pelleted), and others.
- ⁴ Any additional comments on the patient or the samples collected that may be of importance to the epidemiological investigation or the laboratory, such as: patient died; patient relationship to another case under investigation; second set of samples collected from the same patient; samples exposed to sub-optimal conditions prior to shipment; etc.
- ⁵ Note the Sample ID exactly as written on the sample container – to confirm this matches the Sample ID assigned by the case investigator.
- ⁶ Either confirm that the sample was received in good condition, or note the inappropriate or sub-optimal condition of receipt. This information should be transmitted to the case investigator or individual responsible for sending the sample.
- ⁷ This information is particularly important for samples received for virus isolation, but applies to all samples received. Example entries may include: stored in sample refrigerator; stored in sample freezer; processed to separate blood, pellet urine, etc.

9.2 Extraction of measles specific IgM from dried blood specimens and detection for use in the Dade Behring indirect measles IgM antibody assay [After 39]

9.2.1 Reagent Preparation:

Extraction buffer containing skim milk should be prepared in advance. To make 100ml of extraction buffer use:

PBS	100ml
0.5% Tween 20	500µl
Dried skim milk powder (blotting grade, non fat dry milk)	5 g

- Dissolve the milk powder whilst stirring on a magnetic heated stirrer (approximately 50°C) for about 15-30 minutes, (do not allow to boil), allow to cool to room temperature before use.
- Store the extraction buffer at 4°C for maximum of 1 week or dispense in 10ml or 50ml aliquots and freeze at -20°C.

(Thaw frozen aliquots at approximately 45 - 50°C to ensure the milk totally dissolves and that there are no particulates in the diluent.)

9.2.2 Elution of blood spot

- Punch out three (3) dried blood spot discs from one blood spot with using a hole punch (3 x 6mm discs).
- Place the three discs into one well of a clean dry flat bottomed microtitre plate or round-bottom Eppendorf tube (or similar).
- Add 330µl extraction buffer.
- (This is equivalent to a 1:23 dilution assuming each 6 mm disc has approximately 5µl serum. i.e. 15µl equivalent serum in 330µl of diluent).
- Cover plate and shake using plate shaker or rotator for 15 minutes at room temperature. (If using Eppendorf tubes, vortex for 30 seconds, incubate 15 minutes at room temperature and vortex again before incubating overnight at 4°C)
- Seal plate, place in sealed moistened box (plastic box with tight lid) and incubate overnight at 4°C.

9.2.3 Absorption of Eluate

- Shake plate for 15 minutes at room temperature (vortex Eppendorf tube for 30 seconds).
- Centrifuge plate or tubes approx 3800 rpm 15 minutes at room temperature (this spins down any particulates in the microtitre well including the discs to facilitate good access to the eluate).
- Remove 170µl of eluate and mix with 170µl RF absorbent, supplied with the Dade Behring kit and prepared as directed in kit instructions.

Final dilution of specimen should be 1:46

9.2.4 *Immunoassay method (for dried blood assay only)*

- For each test run, prepare 1 tube of negative assay control (P/N) control and 2 tubes of positive assay control (P/P).
- Pipette 150µl of P/N and P/P into Measles Antigen and Control Antigen wells from initial dilution tubes (1:21) into first and second positions. Do not add P/P to last position wells until end of eluate sampling.
- Transfer 150µl of the eluate/RF mixture into both the control and test wells (see Figure 14).
- Incubate at 37°C for 1.5 hours (NB. this is 30 minutes longer than standard Dade Behring assay for serum)
- Wash x 5 with 1-2 minute soak time with Behring wash buffer (supplied with the supplementary kit) (NB: this is once more than in the standard assay)
- Make up kit anti-measles IgM conjugate according to kit instructions and dispense 100µl to both test and control wells
- Incubate conjugate for 1.5 hours at 37°C. (NB. this is 30 minutes longer than standard Behring assay for serum)
- Wash x5 as above and complete assay with substrate and stop solutions as per kit instructions
- Read at 450nm (using reference wavelength 650nm)

9.2.5 *Determining results*

Refer to the manual and kit insert for validation criteria. Record validation results with your results.

For each test sample and also for the reference samples, calculate the difference (ΔA) between the measured absorbance of the measles antigen well and the control antigen well:

$$\Delta A = A_{\text{measles antigen}} - A_{\text{control antigen}}$$

For a run to be considered valid, the following conditions for the Dade Behring assay must be met:

- the ΔA for the negative control (P/N) must be <0.10
- the ΔA for each of the positive controls (P/P1 and P/P2) must be >0.2
- the ΔA for each of the positive controls (P/P1 and P/P2) must be within the upper and lower specified margins (see kit insert values)
- the ΔA of each of the P/Ps must not deviate from the mean of both P/Ps by more than 20%
- Dried blood samples in which the control well has an OD > 0.15 should be repeated

9.2.6 Interpretation of results

The following criteria are required for a specimen to be identified as measles specific IgM positive, negative or equivocal:

Measles specific IgM positive:	$\Delta A > 0.20$
Measles specific IgM negative:	$\Delta A < 0.10$
Measles specific IgM equivocal:	$\Delta A \geq 0.10$ and ≤ 0.20

- Samples with $\Delta A \geq 0.1$ and ≤ 0.2 should be retested after re-eluting from the dried blood spot.

9.2.7 Comment on rubella specific IgM from dried blood specimens.

- There is accumulating evidence that protocols similar to the one described above will be useful for diagnosis of rubella cases [41, 42], but the optimal protocols are not yet available.

9.3 Quality control and trouble shooting of measles and rubella serological assays

9.3.1 Quality Control

Each assay has a specific set of quality control criteria which must be met before the assay can be considered valid. It is essential that the quality control guidelines from the instructions provided by the manufacturer of the assay are followed for each assay run. It is also helpful to have a set of in-house positive and negative control sera which should be run in assays at regular intervals. These in-house samples can be helpful for comparing variation between batches of assays, helping trace assay problems and in trouble-shooting. To ensure that in-house control sera remain stable, they should be aliquoted in single use volumes and stored at -20°C or lower. Ideally, in-house sera should be selected which are of sufficient volume to allow aliquots to be run at least every 2 to 4 weeks over a 12 month period. Presenting the absorbance values of kit and in-house controls in a graphic form can allow easy monitoring of the consistency of ELISA assays being run in each laboratory. These can be used for the early detection of assay problems and may be used to even monitor individual technician's performance.

For the purposes of illustration, the quality control measures required for the Dade Behring measles and rubella IgM assays are shown below:

For each test sample and also for the reference samples, calculate the difference (DA) between the measured absorbance of the measles antigen well and the control antigen well:

$$\Delta A = A_{\text{measles antigen}} - A_{\text{control antigen}}$$

For a run to be considered valid, the following conditions for the Dade Behring assay must be met:

- Kit must not have expired
- ΔA for the negative control (P/N) must be <0.10
- ΔA for each of the positive controls (P/P₁ and P/P₂) must be >0.2
- ΔA for each of the positive controls (P/P₁ and P/P₂) must be within the upper and lower specified margins (check kit insert values)
- ΔA of each of the P/Ps must not deviate from the mean of the positive controls (P/P₁ and P/P₂) by more than 20%. For example:
- If ΔA P/P₁ = 0.488 and ΔA P/P₂ = 0.452
- Mean of ΔA P/P₁₊₂ = (ΔA P/P₁ + ΔA P/P₂) / 2 = 0.470 (for example above)
- 20% of mean = Mean ΔA P/P₁₊₂ x 0.2 = 0.470 x 0.2 = 0.094 (for above)
- Each ΔA P/P should be within the values; $\pm 20\%$ of the mean of ΔA P/P₁₊₂
- Or between 0.376 and 0.564 (for example above)

If a test is invalid it is necessary to check all reagents and procedural steps in order to eliminate the problem. If the problem cannot be identified with any certainty then it is essential that the assay should be investigated step by step, examining or resolving each variable one by one. The in-house control samples are invaluable for this purpose. Below are some general guidelines for avoiding and resolving problems with ELISA assays.

9.3.2 Trouble shooting problems in ELISA assays

Reagent problems

- Ensure that all reagents and samples have been stored correctly, that they have not been contaminated, and that they have not passed their expiry date.
- Always label all reagents and include the date of preparation/ reconstitution.
- During the assay keep the timing of reagent addition consistent, and process the plates in a consistent order during all steps of the procedure
- Regularly use an internal quality control sample with known absorbance value
- Avoid repeated freeze thawing cycles of sera, especially those used for internal quality controls. If internal control samples need to be frozen, make multiple aliquots of single use volumes.

Operator, mechanical or procedural errors

- Ensure that the assay protocols are followed exactly, especially incubation temperatures and incubation times. Be aware that assay protocols may change. The manufacturer's instructions should be re-read with every new batch of assays and SOPs adjusted as appropriate.
- Monitor and record temperature of refrigerators, freezers and incubators daily.
- Ensure the correct washing procedures are followed. Under-washing can cause high background and over-washing can result in low absorbance. Follow exactly the recommended number of wash cycles and wash buffer dwell-time (time the wash buffer is left in the wells for each wash cycle).
- Check all channels on the ELISA washer are working correctly and not blocked. Use distilled water to rinse washer after every use to avoid salt crystallisation in the delivery head. High background absorbances in every well of a row or column may be caused by a blocked channel.
- Check that the correct filter in the ELISA reader is used for the appropriate substrate (for example; 405nm for ABTS, 450nm for TMB, 492nm for OPD). Read plate with another ELISA reader or try other filters if visual colour changes do not match the ELISA reader absorbance readings. The use of a dual wavelength ELISA reader reduces the chance of absorbance readings being influenced by non-substrate components. For a TMB substrate (450nm optimal filter wavelength) an appropriate reference filter wavelength would be 630nm.
- If no colour changes occur in the assay the problem may be in either the conjugate, the substrate or both. One quick test to identify this problem can be to mix approximately 10µl of the correctly diluted conjugate with 100µl of the diluted substrate and observe whether colour develops. It is recommended to use low protein binding plastic containers for the dilution of both conjugate and substrate as some glass containers can be inhibitory to the enzymatic reaction as can residual chemicals from cleaning fluids.

-
- Confirm that the pH of the dilution or wash buffer is optimal. Always use freshly distilled water for preparation of reagents and wash buffers and check that the pH conforms with the protocol.
 - Allowing a plate to dry out during the washing process can result in non-specific binding with a resultant high background problem.
 - Ensure that the reagent dilutions have been correctly calculated by recalculating all of them and getting someone else to check them.
 - Record assay performance data in a laboratory logbook, including:
 - kit batch number(s) and expiry date(s)
 - date of the assay
 - name of person performing the assay
 - name of person checking the results (usually the laboratory supervisor)
 - assay data calculations; dilutions; time of the start and end of each incubation
 - any other observations, errors, inadvertent changes
 - positive and negative control and QA data
 - raw absorbance data

Micropipettor inaccuracies

- Regularly (at least every 3 months) clean and check the accuracy of the pipettors that are being used for the assay. Most inaccuracies related to micro-pipetting can be minimized by correctly using the micropipettor (see micropipettor instruction leaflet) and ensuring the barrel of the pipettor is kept clean, especially the area where the tip is fitted, and preventing any sample entering the pipette barrel. The outside of the barrel should be cleaned after each use with a soft cloth wetted with 70% ethanol. The barrel and piston can be cleaned the same way every month, after being dismantled by an experienced technician.
- The volume delivery of a micropipettor can be assessed by determining the weight of set volumes of distilled water with an analytical balance.
- Distilled water has a density of 1µl /1mg at 4°C and a barometric pressure of 1 atmosphere (or 1013.25 hectopascals (hPa)). The density of water at other temperatures and barometric pressures can be found using table 8.
- An accuracy of >98% in each micropipettor's delivery should be attainable, but check with the manufacturers package insert. Before proceeding it is essential that the balance has been calibrated and is accurate and preferably able to measure to 3 or 4 decimal places (i.e. 0.001g or 0.0001 g).
- Use appropriate volume pipettors for all measurements:
 - for 1-10 µl, use a Gilson P10 or equivalent;
 - for 5-20 µl, use a Gilson P20 or equivalent;
 - for 20-200 µl, use a Gilson P200 or equivalent;
 - for 200-1000 µl, use a Gilson P1000 or equivalent.

Table 8. Density of water at various temperatures and barometric pressures

Temperature °C	Air Pressure hPa					
	800	853	907	960	1013	1067
15	1.0018	1.0018	1.0019	1.0019	1.002	1.002
15.5	1.0018	1.0019	1.0019	1.002	1.002	1.0021
16	1.0019	1.002	1.002	1.0021	1.0021	1.0022
16.5	1.002	1.002	1.0021	1.0022	1.0022	1.0023
17	1.0021	1.0021	1.0022	1.0022	1.0023	1.0023
17.5	1.0022	1.0022	1.0023	1.0023	1.0024	1.0024
18	1.0022	1.0023	1.0024	1.0024	1.0025	1.0025
18.5	1.0023	1.0024	1.0025	1.0025	1.0026	1.0026
19	1.0024	1.0025	1.0025	1.0026	1.0027	1.0027
19.5	1.0025	1.0026	1.0026	1.0027	1.0028	1.0028
20	1.0026	1.0027	1.0027	1.0028	1.0029	1.0029
20.5	1.0027	1.0028	1.0028	1.0029	1.003	1.003
21	1.0028	1.0029	1.003	1.003	1.0031	1.0031
21.5	1.003	1.003	1.0031	1.0031	1.0032	1.0032
22	1.0031	1.0031	1.0032	1.0032	1.0033	1.0033
22.5	1.0032	1.0032	1.0033	1.0033	1.0034	1.0035
23	1.0033	1.0033	1.0034	1.0035	1.0035	1.0036
23.5	1.0034	1.0035	1.0035	1.0036	1.0036	1.0037
24	1.0035	1.0036	1.0036	1.0037	1.0038	1.0038
24.5	1.0037	1.0037	1.0038	1.0038	1.0039	1.0039
25	1.0038	1.0038	1.0039	1.0039	1.004	1.0041
25.5	1.0039	1.004	1.004	1.0041	1.0041	1.0042
26	1.004	1.0041	1.0042	1.0042	1.0043	1.0043
26.5	1.0042	1.0042	1.0043	1.0043	1.0044	1.0045
27	1.0043	1.0044	1.0044	1.0045	1.0045	1.0046
27.5	1.0044	1.0045	1.0046	1.0046	1.0047	1.0047
28	1.0046	1.0046	1.0047	1.0048	1.0048	1.0049
28.5	1.0047	1.0048	1.0048	1.0049	1.005	1.005
29	1.0049	1.0049	1.005	1.005	1.0051	1.0052
29.5	1.005	1.0051	1.0051	1.0052	1.0052	1.0053
30	1.0052	1.0052	1.0053	1.0053	1.0054	1.0055

For example: 20µl distilled water should correspond to 20.058mg at 20° C and 1013 hPa. (20 x 1.0029)

9.4 Isolation and identification of measles and rubella virus in cell culture

9.4.1 *Collection and shipping of clinical specimens*

The type of sample to collect for measles virus isolation depends on the situation in hand. Generally, the most convenient samples to collect are throat or nasal swabs (for measles and rubella) and/or urine samples (for measles only) and it is acceptable to collect both a respiratory sample and a urine sample. Nasopharyngeal aspirates and heparinized blood samples are also useful sources of virus, but these require more equipment, specialized personnel and laboratory support. **Specimens for virus isolation should be obtained as soon as possible after the onset of rash.** Samples for virus isolation should be collected in addition to a serum sample, but should never be substituted for serum samples. Protocols are described below.

9.4.2 *Respiratory Specimens*

Materials required:

- sterile swabs (or proprietary swab-based viral culture collection kits, with VTM included)
- sterile saline
- 3ml aliquots of viral transport medium (VTM: sterile PBS or suitable isotonic solution such as Hank's BSS, etc. containing antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin) and either 2% foetal bovine serum in centrifuge tubes
- 5ml plastic syringes
- plastic aspirators or 30ml syringe
- Cryovials
- Insulated shipping containers

Sample collection:

- Attempt to obtain the sample as soon as possible after onset of rash. Many samples from cases are positive if collected by 5 days after rash onset.
- Nose and throat swabs: Sterile swabs can be used to wipe the nasopharynx and oropharynx. A wooden spatula tongue depressor can be used if necessary. Place both swabs in a tube containing 2-3ml of VTM. Measles virus is extremely cell-associated, so attempt to swab the back of the throat and nasal passages to collect epithelial cells. The swab can be placed in the tube of VTM. Break off the handle of the swab and seal the tube.
- The nasal wash (nasopharyngeal aspirate) respiratory specimen is a difficult sample to collect but can be done by using a syringe attached to a small piece of plastic tubing. After placing about 3-5ml of saline in the nose, aspirate as much of the material as possible and add to the centrifuge tube containing the VTM. (In a clinic or hospital setting, if available, a vacuum may increase the recovery of fluid.) Rinse the syringe and collection tubing into the VTM.
- Keep all specimens on wet ice or at 4°C and ship to an appropriate laboratory as soon as possible on wet ice, preferably within 24-48 hours.

9.4.3 *Urine specimens*

Materials required:

- Urine collection cups, preferably with leakproof lid.
- 50ml polystyrene centrifuge tubes.
- PBS or DMEM
- Cryovials
- Shipping containers

Sample collection:

Urine should be collected within 5 days of rash onset (1-3 days best!). First morning voided specimens are ideal, but any urine collection is adequate. Collect up to 50ml of urine in a urine specimen container. Urine should be transferred to a 15 or 50ml centrifuge tube before shipping.

It is best to centrifuge the urine specimen as soon after collection as possible. After collection, keep the specimen cool (refrigerator or wet ice). For processing, transfer 50ml of the specimen to 15 or 50ml plastic centrifuge tube(s) and centrifuge at 1500 rpm for 5-10 minutes at 4°C to pellet the sediment. Discard the supernatant and suspend the sediment in 1-2ml of VTM (above) or any cell culture medium (DMEM, EMEM, RPMI plus antibiotics) and ship. Preferably, specimens that have been centrifuged and resuspended should be frozen at -70°C and shipped on dry ice. If dry ice is not available, however, they can be stored at 4°C and shipped on wet ice.

If centrifugation is not available, **do not freeze the urine sample**. The entire urine specimen should be stored at 4°C, and shipped to the lab on wet ice. It is best to deliver the specimen within 24 hours so that it can be processed and frozen at -70°C for optimal virus recovery and less opportunity for contamination. Seal the specimen container tightly to prevent leakage.

9.4.4 *Blood samples*

Virus may also be isolated from lymphocytes. This technique is not widely used because it requires an additional blood sample (with anticoagulant) and lymphocytes are technically challenging to recover. However, if it is possible to collect several ml of heparinized blood, the lymphocytes can be a good source of virus. The whole blood should be stored at 4°C and transported to the laboratory with 24-48 hours of collection. Lymphocytes can be purified from the whole blood using Ficoll-Hypaque or Lymphocyte Separation Medium (LSM). See protocol in section 9.4.6.

9.4.5 Shipping of clinical specimens and viral isolates

For shipping of viral isolates in cell culture, it is best to send a plastic 25cm² tissue culture vessel. Cells should be infected just prior to shipping. Once cells have been infected (including the 1 hour incubation), fill the vessel almost to the top with DMEM (plus antibiotics and 2% FBS) ensuring that an air bubble (~0.5ml) is left to allow for liquid expansion during shipping. Screw the top on tightly and seal with plastic film or tape. Wrap the flask in sufficient absorbent material to contain the entire liquid content of the flask, if spilled. Place the vessel and absorbent material in a leak-proof container such as a zip-lock plastic bag and then a leak-proof outer container meeting PI 620 packaging and documentation requirements, and ship at room temperature.

If preferred, infected cells (showing >50% syncytia) can be pelleted, resuspended in a small volume (1-2ml) of DMEM and frozen at -70°C before shipping on dry ice. (See Section 9.3.11 below for instructions on preparing viral stocks)

Remember to use proper shipping containers, obtain necessary permits and to notify the recipient of shipment. See Appendix 9.5.

9.4.6 Processing of specimens

As each specimen is logged into the laboratory, identification number and information on the patient and specimen are recorded in a logbook or spreadsheet. This information may be helpful in identifying problems that may contribute to loss of virus and inability to make isolations.

Patient Information	Specimen Information
Age	Type (throat swab/nasal washing/urine/blood)
Date of Birth	Date of collection of sample
Rash onset date	Volume (urine)
Blood draw date	Condition (temp. upon arrival)
IgM result	Actions taken (centrifugation, storage location)
Measles/rubella vaccination date	

Throat, nasal or nasopharyngeal swabs or aspirates: If the specimen arrives as frozen material in 2-3ml of cell medium or PBS, it can be stored at -70°C or below as is. If the original swab tube is sent, add 2ml of DMEM and vortex to collect swab material, allow an hour for virus elution and drain swab as much as possible against the side of the tube. Store at -70°C.

Urine: If received in bulk quantity, transfer to tubes to pellet sediment (1500 rpm, 10 minutes, 4°C). Resuspend all sediment in 1-2ml DMEM. Store at -70°C.

Heparinized Blood: Use a proprietary lymphocyte separation medium to separate lymphocytes from peripheral blood during centrifugation.

Example of lymphocyte separation protocol (Organon Teknika LSM): Dilute blood 1:1 with PBS. Add 2 volume of LSM solution to a plastic centrifuge tube. Carefully layer diluted blood over the LSM. Centrifuge at 2000 rpm for 30 minutes at 20°C (use swinging buckets, with brake off). The lymphocytes should form a white/grey band above the red cell pellet. Using a pipette, carefully harvest the lymphocyte band into a clean centrifuge tube and wash the lymphocytes with 10-15ml of PBS. Lymphocytes should be pelleted from the PBS and resuspended in a small volume (1-2ml) of DMEM. Store at -70°C.

Store all samples at -70°C and ship on dry ice. It is recommended that each sample be divided into at least two tubes.

Do not routinely filter clinical specimens before inoculation of cell culture. However, if a culture is contaminated after the first isolation attempt, the remaining specimen can be filtered. To filter specimen, bring the volume to 1-2ml with DMEM, and then filter contents through a nitrocellulose filter (0.45 µm) fitted to a 5ml syringe.

9.4.7 Introduction to cell culture

The Vero/SLAM cell line is now recommended for routine isolation of measles or rubella viruses in the WHO laboratory network. These cells are Vero cells which have been transfected with a plasmid encoding the gene for the human SLAM (signalling lymphocyte-activation molecule) protein [55]. SLAM has been shown to be a receptor for both wild-type and laboratory-adapted strains of measles. Vero/SLAM cells were developed by Dr Yusuke Yanagi, Kyushu University, Kuyokyo, Japan. He has kindly agreed to allow the WHO Measles and Rubella Laboratory Network to use these cells in Network Laboratories under the following conditions:

- The cell line Vero/SLAM is used only for Laboratory diagnosis of measles and rubella viral infection by virus isolation and/or investigation of measles or rubella strains for molecular epidemiological purposes.
- The cell line is not used for commercial purposes.
- The cell line is not distributed to laboratories outside the WHO Laboratory Network without Dr Yanagi's and WHO's permission.

Any publication of work using the Vero/SLAM cell line acknowledges the original publication (Ono et al. J. Virol. 2001, 75:4399-4401).

The sensitivity of Vero/SLAM cells for isolation of measles virus is equivalent to that of B95a cells and measles infection of Vero/SLAM results in the characteristic CPE, syncytium formation (Figure 17). The advantage of the Vero/SLAM cells compared to B95a cells is that they are not persistently infected with Epstein-Barr virus, and therefore, are not considered as hazardous material. This provides a significant safety advantage for laboratory workers and greatly facilitates international shipments. The disadvantage of the Vero/SLAM cells is that they must be cultured in medium containing Geneticin to retain SLAM expression. This increases the cost of the tissue culture medium. Figure 17 shows CPE from wild-type measles virus in Vero/SLAM cells and results from an immunofluorescence test to confirm isolation of measles virus.

Vero/SLAM cells can also be used to isolate rubella viruses from clinical samples with a sensitivity that is similar to that of standard Vero cells. Unlike measles virus, rubella virus from clinical specimens does not produce CPE in the vast majority of cases, even after several passages. However, the presence of rubella virus in the culture can be detected by other methods, most commonly an immunofluorescence assay or an immunocolorimetric assay (Figure 18). Both protocols are included in this manual (See section 9.4.15 and 9.4.16).

Figure 17. Panel A shows cytopathic effect (CPE) caused by measles virus infection of Vero/SLAM cells. Top left panel shows uninfected Vero/SLAM cells; other panels show development of CPE (1+ to 4+ syncytium formation) after infection with wild-type measles virus

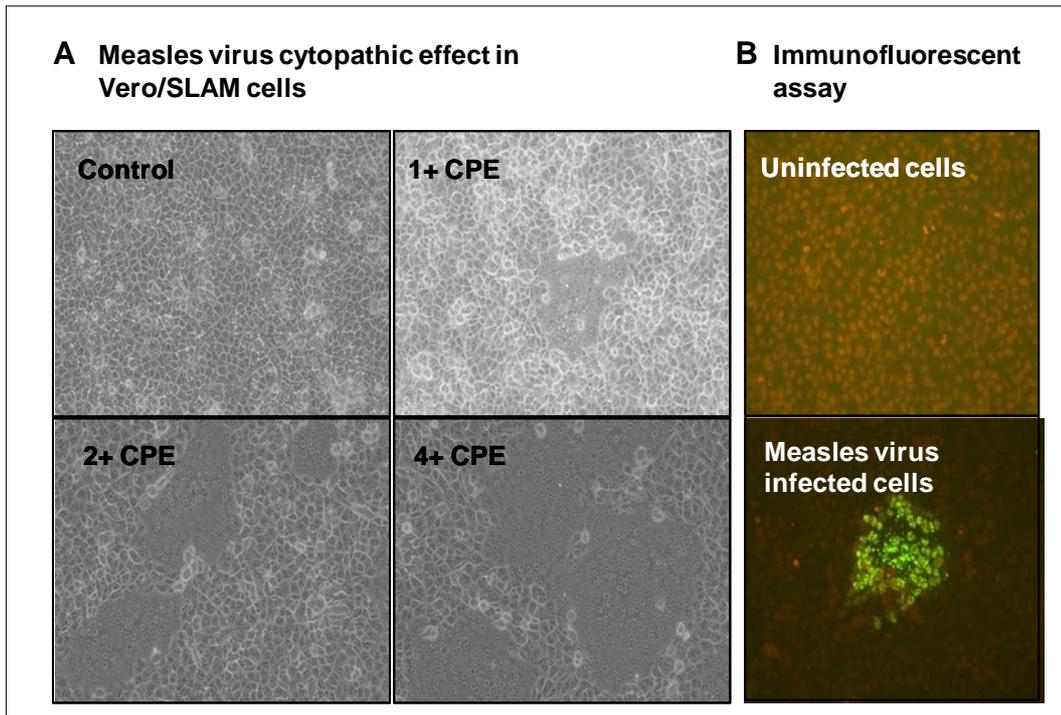
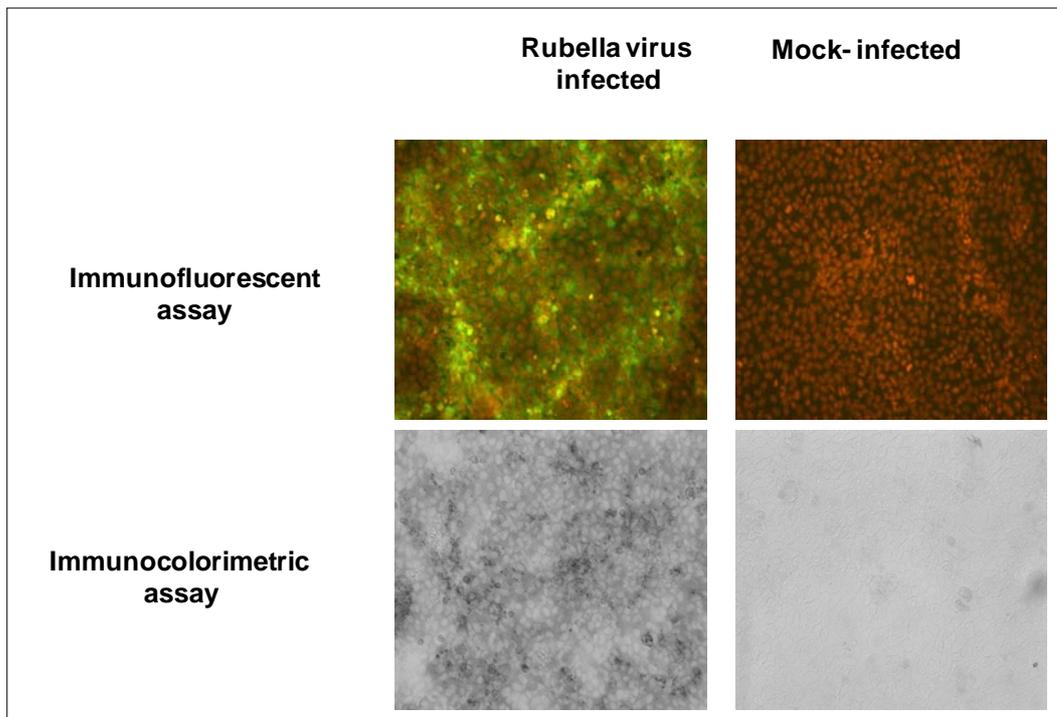


Figure 18. Typical results for detection of rubella virus in cell culture using two different immunoassays: immunofluorescent assay (top row, rubella virus infected and mock-infected) and immunocolorimetric assay (bottom row, rubella virus infected and mock-infected).



9.4.8 Geneticin Requirements for Vero/SLAM Cells

Because of the cost of Geneticin, several laboratories have investigated the Geneticin requirements for the maintenance of SLAM expression in Vero/SLAM cells. These studies indicated that SLAM expression is stable for at least 15 passages in cell culture medium without Geneticin. The cells are also fully susceptible to infection by wild-type measles viruses following up to 15 passages without Geneticin in the medium. Fifteen passages is the maximum number of passages recommended for any cell line used for virus isolation. Based on this information, the WHO recommends the following procedures for use of Vero/SLAM cells in network laboratories:

Preparation of cell stocks for liquid nitrogen storage: Network laboratories should only accept Vero/SLAM cells from a WHO-approved source (RRL or GSL). Upon receipt, the cells should be passaged in medium containing 400µg/ml Geneticin as described below. Laboratories should passage the cells 2 to 4 times in the presence of Geneticin to prepare a sufficient number of cell culture vessels to prepare 20-50 vials for liquid nitrogen storage. A cryopreservation procedure is included below.

Passage of Vero/SLAM cells for routine virus isolation: To prepare cells for virus isolation procedures, Vero/SLAM cells should be recovered from liquid nitrogen and passaged up to 15 times in medium without Geneticin. These cells should be used for virus isolation attempts only and should be discarded after 15 passages. Cells that have been passaged without Geneticin in the medium should never be used to prepare cell stocks for liquid nitrogen storage or shipped to another network laboratory for use in virus isolation.

9.4.9 *Materials Needed for Maintenance of Vero/SLAM cells*

- 1) Dulbecco's Modified Eagle Medium (DMEM):
with 4,500mg/L D-glucose (high glucose)
with L-glutamine
without sodium pyruvate
Alternate: EMEM
- 2) Antibiotics (100X)
10,000 units/ml penicillin G and 10,000 µg/ml streptomycin sulfate in 0.85% saline
- 3) Trypsin-EDTA
0.05% Trypsin (porcine pancreas), in 0.53 mM EDTA in HBSS without Ca⁺⁺ and Mg⁺⁺
- 4) Fetal Bovine Serum (Defined)
- 5) Geneticin (G418), 50mg/ml, pre-prepared liquid is available or alternative.

9.4.10 *Maintenance of Vero/SLAM Cells*

Vero/SLAM cells being passaged for storage or for preparing flasks or tubes for virus isolation should be manipulated in a “clean” Biological Safety Cabinet (BSC) completely separate from where any infectious material is being handled and ideally, in a specifically designed cell culture laboratory.

- 1) Prepare DMEM by adding the 5ml of penicillin/streptomycin solution to 500ml DMEM (DMEM-PS). If preparing cells stocks for liquid nitrogen storage, and Geneticin to a final concentration of 400µg/ml (4ml of 50mg/ml stock to 500ml DMEM) of DMEM (DMEM-PSG).
- 2) When confluent, Vero/SLAM cells can be passaged by trypsinization as with any other adherent cell line. Cells are usually maintained in 25cm² or 75cm² flasks, but the volumes given below can be adjusted for larger or smaller vessels.
- 3) For 25cm² or 75cm² tissue culture flasks, wash cell monolayer 1X with 5ml pre-warmed trypsin solution (or warm PBS) for about 30 sec to 1 minute. Discard wash medium into hypochlorite solution. Add 5ml pre-warmed trypsin solution and allow flask to incubate on the bench top for 4-5 minutes. Remove most of the trypsin leaving just enough fluid in the flask to keep the monolayer wet (approximately 1ml). Place flask at 37° C for about 3-4 minutes. Observe flask every few minutes and to check if cells are detaching. When cells are detaching, hit flask quickly with palm of hand to dislodge remaining cells. Pipette up and down several times with a 1 or 2ml pipette to break up any cell clumps.
- 4) Resuspend cells in 5ml DMEM-PS or DMEM-PSG plus 10% FBS and pipette up and down to break up clumps. Seed cells into flasks containing DMEM-PS or DMEM-PSG plus 10% FBS. Split ratios of up to 1:5 are acceptable. A 1:2 or 1:3 split usually will produce monolayers of sufficient density for virus isolation after 24 hours incubation (Total volume of medium required: 25cm² flask is 10ml/flask; 75cm² flask is 30ml/flask; 150cm² is 50ml/flask).
- 5) Cells should be passaged at least one time each week. Cells can be maintained for several days by switching to medium containing 2% FBS to prevent overgrowth.
- 6) Cell lines should be passaged only 15 times after recovery from liquid nitrogen.

9.4.11 *Inoculation of Vero/SLAM for Isolation of Measles Virus*

Preparation of samples for virus isolation and the process of virus inoculation and subsequent passaging of virus in Vero/SLAM cells should be carried out only in a BSC specifically used for handling infectious material. Potentially infectious material should be kept completely separate from where “clean” cell stocks are being handled.

For inoculations, cells should be seeded into 25cm² tissue culture flasks (T-25). Cells should be at approximately 85-90% of confluency and at least one day after seeding. If cells are overgrown, virus isolation will be unsuccessful. Virus inoculation and subsequent incubation should be in DMEM-PS plus 2% FBS.

- 1) For inoculation of a T-25 flask, (equals virus passage number1), decant growth medium, add 5ml of DMEM-PS plus 2% FBS and 0.5- 1ml of specimen. Incubate at 37° C for 1 hour and observe the cells under the microscope to ascertain if the sample was toxic to the cells (rounding of cells, cells floating).
- 2) Inoculated cells should be observed by light microscopy for CPE on a daily basis. Passage the infected Vero/SLAM cells by trypsinization after 4-5 days (in a Biological Safety Cabinet used specifically for handling potentially infectious material), at a 1:3 split ratio (passage number 2).
- 3) Check the flasks daily. If no CPE is observed for 4-5 days after passage number 2, then discard. Record as negative result.
- 4) When CPE is visible, continue to feed the cells (replace the medium with fresh DMEM-PS with 2% FBS, if necessary) until the CPE becomes extensive. It may be necessary to passage the cells one more time to allow the infection to spread before cells become overgrown. When CPE is visible over at least 50-75% of the cell layer, cells can be harvested for preparation of a viral stock (step 5 below).
- 5) To prepare a viral stock, scrape the cells into the medium with a cell scraper or 1ml pipette. Transfer medium and cells to a sterile, plastic centrifuge tube and centrifuge the cells at approximately 1000 x g for 10 minutes. Discard the supernatant into hyperchlorite solution and resuspend the cell pellet in 1.0ml of DMEM-PS. Transfer 0.5ml to each of 2 cryovials and store at -70° C. Alternatively, discard all but about 1ml of the supernatant medium into hypochlorite solution. Scrape the cells into the remaining medium and pipette 0.5ml each into 2 cryovials and store at -70° C.
- 6) If successful virus isolation has been achieved, using an immunological assay such as immunofluorescence can be used to confirm the presence of measles. Refer section 9.3.14. Always prepare a viral stock for long- term storage at -70° C.
- 7) Do not attempt to passage the virus in cell lines other than Vero/SLAM cells. Many clinical isolates do not readily adapt to growth in standard Vero cells. Viral stocks may be lost upon passage on the wrong cell line. For molecular epidemiological studies, RNA should be prepared from infected Vero/SLAM cells.
- 8) Cell lines should be passaged only 15 times after recovery from cryopreservation.

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- 9) Always include a negative control of uninfected Vero/SLAM during virus isolation attempts. A positive control may be included, but the operator should be aware of the possibility for cross contamination. As the operator becomes more familiar with appearance of measles CPE in Vero/SLAM cells, the positive control will no longer be necessary. Positive controls, if used, should be wild-type viruses with known genetic characterization.

Note: Susceptibility testing of Vero/SLAM cells is not currently recommended by WHO. However, if this test is performed, a low passage, wild-type virus should be used. This virus should produce CPE on Vero/SLAM but not Vero. This result would verify the appearance of CPE is linked to SLAM expression.

9.4.12 Inoculation of Vero/SLAM for Isolation of Rubella Virus

Growth of rubella virus is preferred to be at 35°C because some rubella virus strains grow best at this temperature. If a 35°C incubator is not available, 37°C is an acceptable alternative temperature.

If low amounts of virus are suspected in a specimen, passages can be done with a freeze-thaw lysate of the infected cell culture (i.e. cell associated virus and virus in medium) instead of medium from the infected cell culture, which is described below.

- 1) For inoculations, a cell monolayer(s) at approximately 85-90% confluency in a 25cm² tissue culture flask(s) should be ready. For inoculation of a 25cm² flask, (= virus passage number 1), decant growth medium, add 2ml of PBS plus 1% FBS and 0.5- 1ml of specimen. Incubate at 35° C for 1 hour. After 1 hour remove the inoculum and add 5mls DMEM-PS containing 5% FBS.

Note: If contamination is a problem, the clinical sample can be passed through a low retention 0.2 µm syringe filter to remove contaminating fungi and bacteria. Alternately, add 0.1ml of a solution containing 1000 µg/ml of gentamicin, 0.1ml of a solution containing 100 µg/ml of fungizone, and 5µl of a 1000x solution of Penn/Strep to 1ml of clinical sample and incubate at 4° C for 30 minutes before inoculation.

- 2) Allow the cells to incubate for 7 days at 35°C. Check the cells on a daily basis and discard any that show signs of contamination (cloudy, yellow media, many floating cells, or fungus). Rubella CPE (rounding and detachment of some cells) will most likely NOT be visible.
- 3) On the 7th day post-inoculation, remove 0.5ml of media. (Note: unlike measles, rubella virus virions are released into the media and, therefore, media or cells from infected cultures can be used to transfer the virus to the next passage.) Transfer the 0.5ml of media to a new 25cm² flask containing Vero/SLAM cells as was done in the inoculation step (This is viral passage number 2). In addition, remove 1ml of media from passage number 1 and store in a cryovial at -70°C.
- 4) After 7 days of culture for viral passage number 2, remove 100µl of media and use to infect a chamber slide containing Vero/SLAM cells for an IFA or immunocolorimetric assay or a well of a 48-well plate for an immunocolorimetric assay. Store 1ml of media from passage 2 in a cryovial at -70°C.

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- 5) If the IFA/colorimetric assay is positive, use the media stored in the freezer from passage 2 to prepare a viral stock. Always prepare a viral stock if isolation is successful. Thaw the stored aliquot of media and use 0.5ml to infect a T-75 tissue culture flask as above (refreeze the other 0.5ml in the freezer as a back-up. Media saved from passage 1 is also a back-up). Incubate the 75cm² flask for 1 week at 35° C. After 1 week, remove 5-10ml of media, aliquot and store at -70° C. For molecular epidemiological studies, RNA should be prepared from the infected Vero/SLAM cells in the 75cm² flask. If the IFA or colorimetric assay is negative, record as a negative rubella specimen.

If low amounts of virus are suspected in a specimen, passages can be done with a freeze-thaw lysate of the infected cell culture (i.e. cell associated virus and virus in medium) instead of medium from the infected cell culture, which is described below.

9.4.13 Preparation of Stock (frozen) Vero/SLAM Cells

It is extremely important to prepare multiple frozen vials of Vero/SLAM cells as soon as they are available in the laboratory. It is recommended that Vero/SLAM be passaged no more than 15 times. Cells can be frozen using any standard cryopreservation technique. Commercial freezing media is available or the reagents and procedure described below should be adequate.

- 1) Vero/SLAM cells should be passaged in DMEM-PSG (with Geneticin) for preparation of cell stocks. A monolayer of Vero/SLAM cells in a 150cm² flask should be available; however, 75cm² flasks can be used and the volumes adjusted accordingly.
- 2) Before starting the protocol, label a sufficient number of screw-top cryovials with cell identity, passage number and date.
- 3) Cells should be removed from the flask by trypsinization as described above (take care not to over-trypsinize). All of the cells from a 150cm² flask should be placed in 10ml DMEM-PS plus 10% FBS and pelleted by centrifugation at 1500 rpm for 10 min at 4°C. Discard supernatant. To the cell pellet add 5ml DMEM-PS (with antibiotics) containing 30% FBS and resuspend the cells by vortexing. Add an equal volume (5ml) of DMEM-PS (with antibiotics) and 15% DMSO (reagent grade). Pipette gently up and down briefly to mix and dispense 1ml into each of 10 plastic cryovials. It is important to work very quickly here because prolonged exposure to DMSO at room temperature will be toxic to the cells. The vials should be cooled slowly using a programmed cell freezer or a commercial product designed for gradual temperature reduction (optimally -1°C /minute between 20°C and -70°C). Store vials in liquid nitrogen.
- 4) Perform a test recovery from the frozen stocks before discontinuing routine culture of the seed stocks Vero/SLAM cells (with Geneticin in the medium). Recover cells from 1 vial using the procedures described below in #5. Observe the recovered cells; adherent cells should be observable on day one after recovery. It may take several days for the monolayer to become confluent. This is normal. If the cells do not recover properly (ie. no or very few adherent cells and/or visible contamination), it will be necessary to prepare another stock of frozen cells from the cultures.

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- 5) To recover cells from liquid nitrogen storage, remove the vial from the freezer and transfer to the laboratory on dry ice. Thaw the cells quickly in a 37° C water bath and immediately place in a T-25 flask containing 10ml DMEM-PS plus 10% FBS. Allow the cells attach to the flask for approximately 4 hours. After cells have attached, decant the medium and replace with 10ml of DMEM-PS plus 10% FBS. Continue to observe cells and passage by trypsinization after the cells become confluent.

Note: If recovering cells for preparation of additional frozen cell stocks, Geneticin should be added to the medium.

9.4.14 Immunofluorescence for Confirmation of Measles Virus Isolation

This immunofluorescence assay (IFA) uses a monoclonal antibody to detect the nucleoprotein of measles virus in infected cells. The infected cells are fixed onto a microscope slide. Binding of the measles-specific antibody is detected using a goat anti-mouse antibody that is conjugated to fluorescein isothiocyanate (FITC). Binding of the detector antibody is visualized by fluorescence microscopy.

Both direct and indirect IFA kits are commercially available from a number of sources. This discussion will describe the Light Diagnostics Measles Indirect Immunofluorescence Assay from Chemicon, Inc (catalogue number 3187).

It is also possible to configure an indirect IFA test without using a commercial kit. Most monoclonal antibodies to the nucleoprotein (e.g. 80-2 KK2, available from Chemicon) will perform well in the IFA procedure described below. Monoclonal antibodies directed against other viral proteins such as the hemagglutinin and fusion proteins may recognize conformational epitopes that are not stable after acetone fixation. When configuring an in-house IFA assay the appropriate working dilutions of monoclonal antibody and FITC-labelled conjugate will have to be determined by experimental titration.

Chemicon IFA Procedure (modified):

- 1) Dislodge cells from surface of the flask with a cell scraper. Place 1ml of dislodged cells (ie 1/5th volume of dislodged cells from a T-25 flask) into a small centrifuge tube and pellet the cells by centrifugation at 1500 rpm for 10 min at 4°C. Decant the supernatant medium and resuspend the cells in 0.25ml of ice cold 50% ethanol in PBS and vortex to resuspend. Spread about 15µl into one chamber of a microscope slide using a micropipette or Pasteur pipette and allow the cells to air dry on the slide. Remember to include uninfected cells as a negative control.

Note: An alternative fixation procedure is to resuspend the cell pellet (from above) in 0.1ml PBS and add 10-20 ul/ per chamber (spot) on the slide. Allow to air dry completely. Dip slide into ice cold 80% acetone for 1 minute, carefully blot excess with filter paper and allow to air dry.

- 2) Prepare the PBS-Tween buffer supplied with the kit (PBS, 0.1% Tween 20).
- 3) Overlay the cell spots on the slides with one drop (or 25µl) of the measles monoclonal antibody.
- 4) Incubate the slide at 37°C for 30 minutes-1 hour in a humid chamber. Petri plates containing a wet paper towel will work very well.

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- 5) Wash the slides for 15-20 seconds in the PBS-Tween buffer and shake off excess buffer. Blot dry with paper towel being careful not to touch cells.
 - 6) Add one drop (or 25µl) of anti-mouse IgG/FITC conjugate to the cell spot.
 - 7) Incubate the slide at 37°C for 30 minutes in a humid chamber.
 - 8) Wash the slides for 15-20 seconds in the PBS-Tween buffer and shake off excess buffer. Blot dry and prepare slides for viewing with mounting fluid and a cover slip.
 - 9) Observe for fluorescence using a fluorescence microscope. FITC absorbs at 495 nm with peak emission at 525 nm. Under these conditions, positively staining cells will show a granular, green fluorescence in the cytoplasm. The Evan's blue counter stain will appear red.

9.4.15 Indirect Immunofluorescence Assay (IFA) for Detection of Rubella Virus in Cell Culture

This is an indirect immunofluorescence assay developed by the Rubella Laboratory, CDC, Atlanta, using a CDC-developed monoclonal antibody specific to the rubella virus E1 glycoprotein. Reagents are very important since rubella virus does not produce large amounts of antigen. The protocol for measles IFA using acetone fixation does not work well for rubella virus because background using acetone fixed cells exceeds the signal from rubella virus proteins. The protocol presented here using paraformaldehyde fixation and highly cross-adsorbed fluorescent antibody does work well.

Equipment and reagents required:

- CO2 Incubator
- 8 chambered slides
- Sterile pipettes
- Vero or Vero/SLAM cells
- Fluorescent microscope, with appropriate filters
- 2% Paraformaldehyde*
- Foetal bovine serum
- Methanol
- DMEM, PBS
- Antibiotics
- Propidium Iodide** counterstain (stored at 4°C)
- Bovine Serum Albumin (BSA)
- Tween 20
- Fluorescent mounting medium*** (stored at 4°C)
- Cover slips

* Paraformaldehyde can be purchased as a 16% stock solution (e.g. from Electron Microscopy Sciences, #15710). Dilute 1:8 with cold PBS immediately before use.

** e.g. Molecular Probes, #P-3566. Dilute 1:2000 in blocking buffer.

*** e.g. Dako Fluorescent Mounting Medium, #S3023.

Antibodies: Goat anti-mouse IgG conjugate (Alexa Fluor® 488 from Molecular Probes, Eugene, OR, USA, Web: <http://www.probes.com>) (store at 4°C)

Monoclonal antibody (anti-rubella E1 glycoprotein) from CDC rubella lab (stored at 20°C)

Growth of Vero or Vero/SLAM cells and virus infection:

Growth of rubella virus in the following protocols is at 35°C because some rubella virus strains grow best at this temperature. If a 35°C incubator is not available, 37°C is an acceptable alternative temperature.

- 1) Grow Vero or Vero/SLAM cells to about 50% confluence in an 8-chambered slide, e.g. Lab-Tek Chamber Slide (catalogue number 177445). Do not allow cells to overgrow. Typically this is done using DMEM with 5% FBS and Pen/Strep in 35°C, CO₂ incubator.

Note: If a CO₂ incubator is not available, the slide will have to be sealed to allow cell growth and virus growth after inoculation (see #2 below). The seal needs to be gas impermeable, for example coating the edges of the chamber slide lid with petroleum jelly.

- 2) Remove medium and add 100µl of specimen (media from passage 2 of the culture of a clinical specimen inoculated on Vero or Vero/SLAM cells) to one chamber.
- 3) A known positive specimen (such as a well characterized wild type strain) should be added to one of the corner chambers. Three negative control chambers (add media only) should surround the positive control to reduce the chance of the positive control contaminating a specimen chamber. If a high titre virus is used as a control, it should be diluted before adding to chamber. Incubate 1 hour in 35°C, CO₂ incubator, in order to allow virus attachment.
- 4) Add 200µl DMEM containing 5 % FCS. Incubate for 3 days in 35°C, CO₂ incubator (or 37°C). At the end of this 3 day incubation, IFA results will be optimal if cell monolayer is about 80% confluent, but a completely confluent monolayer is acceptable.
- 5) If the tissue culture medium in the virus-containing wells becomes acidic (yellow), this may indicate that virus is present.

Fixing cells:

The cells are fixed using 2% paraformaldehyde in PBS that has been chilled at 4°C. Cells are then permeabilized with 20°C methanol.

- 1) Remove chambered slide from incubator and place on ice for 10 minutes; support the slide above the ice by laying the slide on a metal or foil strip in contact with ice.
- 2) In a Biological Safety Cabinet, remove the tissue culture medium and wash 1X with cold PBS. For media removal, insert pipette tip into one corner of chamber and continue to use this one corner throughout the procedure to minimize cell loss. Add reagents down the side of the chamber for the same reason.

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- 3) Add 200µl of 2% paraformaldehyde for 30 minutes on ice.
 - 4) Remove paraformaldehyde and wash 1X with cold PBS. The rest of procedure can be done on the bench top as virus has been inactivated by the paraformaldehyde.
 - 5) Add 200µl of 20°C methanol and incubate for 10 minutes at 20°C. This is easily done by placing the slide in a 20°C freezer. If a freezer is not available, placing the slide on a frozen (20°C) freezer pack (e.g. Frigid Brick manufactured by Touchpad Solutions) on the lab bench is acceptable.
 - 6) Remove the methanol and wash 1X with room temperature PBS.
 - 7) Fixed cells may be stored at this point, covered with blocking buffer (1% BSA, 0.5% FBS, 0.1% Tween 20 in PBS). Fill the chambers/wells with blocking buffer; fixed cells covered with blocking buffer can be stored in a humidified chamber at 4°C for at least 1 month. For long term storage, add 1X Pen/Strep to blocking buffer.

Note: A humidified chamber can be as simple as placing the slide on damp paper towels and storing in a plastic box or wrapped with plastic wrap.

IFA Procedure:

Blocking, dilutions of antibodies and propidium iodide, and washes are all performed in blocking buffer at room temperature. Final washes after propidium iodide are with PBS only.

- 1) Block non-specific antibody reactions by adding 200µl of blocking buffer per chamber for 1 hour at room temperature (if the slide has been stored in blocking buffer, this step can be omitted.)
- 2) Remove blocking buffer and add diluted monoclonal antibody (100µl per well) in blocking buffer. Incubate 1 hour at room temperature. Dilute monoclonal stock 1:1000 to use in assay (for lot # 03-031). Note that the dilution of the monoclonal may change depending on the lot used.
- 3) Wash 2 times with blocking buffer, and then add second fluorescent antibody in blocking buffer (100µl per well). Typically this is Alexa Fluor 488 goat anti-mouse IgG (H+L) “highly cross-adsorbed” at a 1:500 dilution. Incubate 30 minutes at room temperature; cover slide with foil to keep it in the dark.
- 4) Wash 2 times with blocking buffer, and then add propidium iodide at 0.5 µg/ml in blocking buffer. Incubate 5-15 minutes at room temperature covered with foil.
- 5) Wash 2 times with PBS. Remove chambers and gasket from slide.
Wick off excess PBS with absorbent tissue or filter paper. Allow slide to dry completely, and then add 2-3 drops of mounting media (e.g. Dako). Carefully add the cover slip and press slightly to remove air bubbles. Wipe off excess mounting media from edges with absorbent paper.
- 6) Observe results with fluorescent microscope using blue light (e.g. Zeiss Axiovert BlueH 485 filter).

Note: Rubella virus infected cells will be green; propidium iodide-stained nuclei will be red. There should be no green background antibody staining in uninfected cells (negative control chambers), although there is sometimes some staining near edges of chambers, presumably due to the gasket. If there is background antibody staining, most likely it is too much fluorescent second antibody. The utility of IFA test is dependent on low background antibody staining in uninfected cells. Depending on how much virus was contained in the inoculum, all cells may not exhibit green fluorescence (in fact, with clinical samples only a very small number of cells may exhibit fluorescence, e.g. 2 foci of 10 cells each on the entire lawn of cells).

9.4.16 Immunocolorimetric Assay for Detection of Rubella Virus in Cell Culture

This is an indirect colorimetric assay using monoclonal antibody developed by the Rubella Laboratory, CDC, Atlanta which is specific to the rubella E1 glycoprotein. The procedure is the nearly the same as the rubella IFA up to the step where the secondary antibody is added, which in this case is HRP conjugated rather than fluorescent. Twice the amount of monoclonal antibody is used in the immunocolorimetric assay. The immunocolorimetric assay is described for both chamber slides and 48 well plates. If using 48 well plates instead of chamber slides, there is a difference in the way the positive controls are processed.

Equipment and reagents required:

- CO2 Incubator
- 8 chambered slides or 48 well tissue culture plates
- Sterile pipettes
- Vero or Vero/SLAM cells
- 2% Paraformaldehyde*
- Foetal bovine serum
- Methanol
- DMEM, PBS
- Antibiotics
- Bovine Serum Albumin (BSA)
- Tween 20
- BM Blue POD substrate, precipitating: Roche, cat# 1442066, 100ml \$112.00

* Paraformaldehyde can be purchased as a 16% stock solution (e.g. from Electron Microscopy Sciences, #15710). Dilute 1:8 with cold PBS immediately before use.

Antibodies: Goat anti-mouse, HRP conjugated antibody: Molecular Probes: cat# G21040, \$122.00.

Monoclonal antibody (anti-rubella E1 glycoprotein) from CDC rubella lab (stored at 20°C)

Growth of Vero or Vero/SLAM cells and virus infection:

Growth of rubella virus in the following protocols is at 35°C because some rubella virus strains grow best at this temperature. If a 35°C incubator is not available, 37°C is an acceptable alternative temperature.

- 1) Grow Vero or Vero/SLAM cells to about 75% confluence in an 8-chambered slide, e.g. Lab-Tek Chamber Slide (catalogue number 177445) or wells of a 48 well tissue culture plate. Do not allow cells to overgrow. Typically this is done using DMEM with 5% foetal bovine serum (FBS) and Pen/Strep in 35°C, CO₂ incubator.

Note: If a CO₂ incubator is not available, the slide or plate will have to be sealed to allow cell growth and virus growth after inoculation (see #2 below). The seal needs to be gas impermeable, for example coating the edges of the chamber slide lid or edges of 48 well plate lid with petroleum jelly.

- 2) Remove medium and add 100µl of specimen (media from passage 2 of the culture of a clinical specimen inoculated on Vero or Vero/SLAM cells) to one chamber/well.
- 3) A known positive specimen (such as a well characterized wild type strain) should be added to one of the corner chambers. Three negative control chambers (add media only) should surround the positive control to reduce the chance of the positive control contaminating a specimen chamber. If a high titre virus is used as a control, it should be diluted before adding to chamber. Incubate 1 hour in 35°C, CO₂ incubator, in order to allow virus attachment.
- 4) Add 200µl DMEM containing 5 % FCS. Incubate for 5 days in 35°C, CO₂ incubator (or 37°C). At the end of this 5 day incubation, immunocolorimetric results will be optimal if cell monolayer is about 80% confluent, but a completely confluent monolayer is acceptable.
- 5) If the tissue culture medium in the virus-containing wells becomes acidic (yellow), this may indicate that virus is present.

Note: Positive controls for 48 well plates should be prepared in advance and stored for use in specimen testing as described below. The separate preparation of positive controls greatly reduces possible contamination of specimens by positive controls. A known positive specimen (such as vaccine virus or lab strain) should be prepared on a separate 48 well plate. If a high titer lab strain virus is used, dilute 1:10 to 1:1000 with PBS, 1% FBS before adding to cells. Several wells of the 48 well plate can be infected with the positive control virus. After the cell fixation step (see below) plates can be stored at 4°C for at least 1 month, provided wells are covered with blocking solution. To use as a positive control, remove the blocking buffer from 1 fixed well and complete the colorimetric assay, starting with the monoclonal antibody addition step. The plate can then be refrigerated until the next assay.

Fixing cells:

The cells are fixed using 2% paraformaldehyde in PBS that has been chilled at 4°C. Cells are then permeabilized with 20°C methanol.

- 1) Remove chambered slide or tissue culture plate from incubator and place on ice for 10 minutes; support above the ice by laying the slide on a metal or foil strip in contact with ice.
- 2) In a Biological Safety Cabinet, remove the tissue culture medium and wash 1X with cold PBS. For media removal, insert pipette tip into one corner of chamber (or a position on the well) and continue to use this one corner/position throughout the procedure to minimize cell loss. Add reagents down the side of the chamber/well for the same reason.
- 3) Add 200µl of 2% paraformaldehyde for 30 minutes on ice.
- 4) Remove paraformaldehyde and wash 1X with cold PBS. The rest of procedure can be done on the bench top as virus has been inactivated by the paraformaldehyde.
- 5) Add 200µl of 20°C methanol incubate for 10 minutes at 20°C. This is easily done by placing the slide/plate in a 20°C freezer. If a freezer is not available, placing the slide/plate on a frozen (20°C) freezer pack on the lab bench is acceptable.
- 6) Remove the methanol and wash 1X with room temperature PBS.
- 7) Fixed cells may be stored at this point, covered with blocking buffer (1% BSA, 0.5% FBS, 0.1% Tween 20 in PBS). Fill the chambers/wells with blocking buffer; fixed cells covered with blocking buffer can be stored in a humidified chamber at 4°C for at least 1 month. For long term storage, add 1X Pen/Strep to blocking buffer.

Note: A humidified chamber can be as simple as placing the slide/plate on damp paper towels and storing in a plastic box or wrapped with plastic wrap.

Colorimetric Procedure: Blocking and antibody dilutions are done in blocking buffer (1% BSA, 0.5% FBS, 0.1% Tween 20 in PBS) at room temperature.

- 1) Block nonspecific antibody reactions by adding 200µl of blocking buffer per chamber/well for 30 minutes at room temperature. This step can be omitted if slides or plates have been stored in blocking buffer.
- 2) Dilute the rubella monoclonal antibody 1:500 (for lot# 03-031) in blocking buffer (This is twice the amount of monoclonal antibody used in IFA protocol). Remove blocking buffer from the chambers/wells and add 100µl of the diluted monoclonal per chamber/well. Incubate 30 minutes at room temperature.
- 3) Wash the chambers/wells with blocking buffer 2 times (about 0.5ml each time).
- 4) Dilute goat anti-mouse, HRP conjugated antibody 1:1000 in blocking buffer. Add 100µl per chamber/well at room temperature for 30 minutes.
- 5) Wash the chambers with PBS 2 times (about 0.5ml each time).

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- 6) To each chamber add 50µl BM Blue POD substrate, for each well in 48 well plate add 100µl. The colour should appear in 2 minutes. The colour will get darker if incubated longer and the colour in the mock-infected chamber/well should be significantly less than positive control. If the infection is widespread, the result should be visible to the naked eye. If only a few cells are infected, the result may not be visible so chambers that appear negative should be examined using a regular light microscope. The staining will appear as dots. Note: if using chamber slides, the chambers do not have to be removed from the slide. A cover slip is not needed. To store cells, remove substrate and add 200µl color stabilization buffer (50mM Tris, pH6.8, 100mM NaCl, 1mM EDTA) at room temperature for 5 minutes. Remove stabilization buffer. Stained cells can now be stored in the dark without loss of color. Depending on how much virus was contained in the inoculum, all cells may not exhibit staining. In fact, with clinical samples only a very small number of cells may exhibit staining.

Note: Monoclonal antibodies should be stored at 20°C.

HRP conjugate diluted as described by manufacturer is stored at -20°C and BM Blue can be stored at 4°C.

9.5 Packaging of specimens and virus isolates for transportation

The IATA Dangerous Goods Regulations governing the transportation of biological specimens were updated and are applicable from 1 January 2007. The following is a summary of the changes and the current regulations. More details can be found in the Guidance on regulations for The Transport of Infectious Substances, 2007-2008. http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_EPR_2007_2/en/index.html

9.5.1 Classification of specimens

There are 3 categories of specimens under the new regulations

1) Infectious Substance, Category A

Substances that contain **highly pathogenic agents**, also known as “Category A” infectious substances. Examples of Category A pathogens are listed in Annex 2 of Guidance on regulations for The Transport of Infectious Substances, 2007-2008.

Dangerous goods are assigned UN numbers and proper shipping names. For category A substances these are: **UN 2814, INFECTIOUS SUBSTANCE, AFFECTING HUMANS, or UN 2900, INFECTIOUS SUBSTANCE, AFFECTING ANIMALS**

The following conditions are required to ship Category A Infectious Substances

- Shippers' Declaration for Dangerous Goods
- Full training and certification
- UN Specification Packaging must follow **Packing Instruction 620, (PI 602 for IATA)**

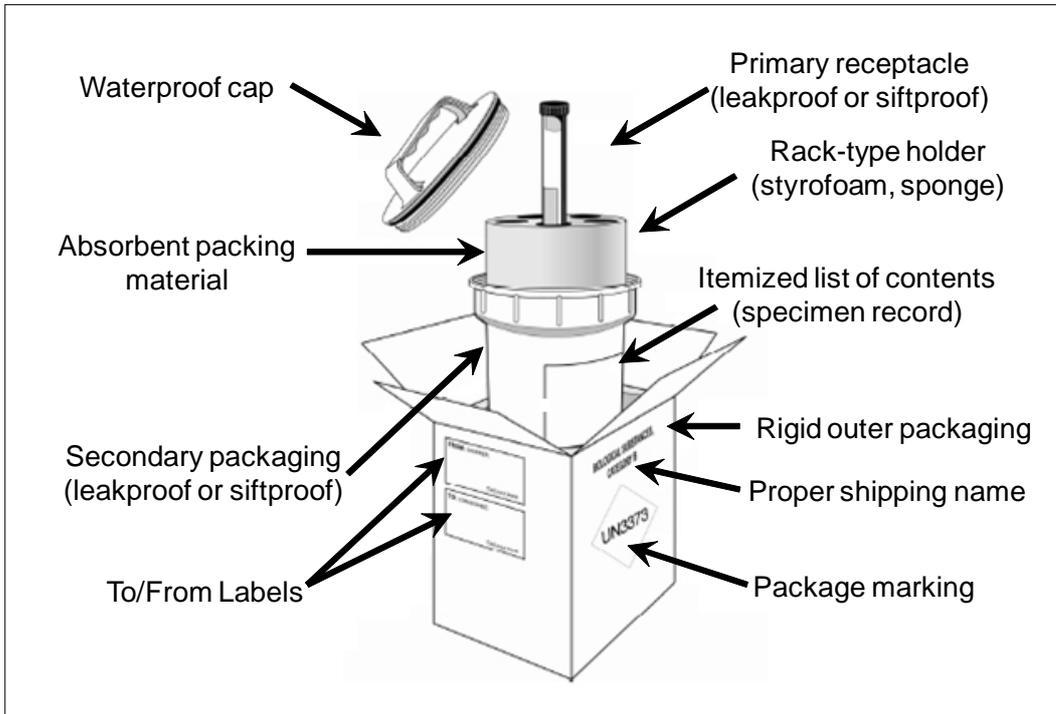
2) Biological Substance, Category B

Materials containing or suspected to contain infectious substances that do not meet Category A criteria. **Live measles and rubella virus cultures are included in this category.**

UN number and proper shipping name for category B substances are: **UN 3373, BIOLOGICAL SUBSTANCE; CATEGORY B**. The conditions required to transport category B substances include:

- The triple component Packing Instruction 650 (see example below) to be followed
- Package labelled with “Biological Substance, Category B” in letters at least 6mm high
- Does not require Shipper's Declaration for Dangerous Goods
- Label UN3373 in diamond-shaped mark used

Example of shipping container meeting Packing Instruction 650.



Marking

Each package shall display on the external surface of the outer packaging

- the shipper's (sender's, consignor's) name, address and telephone number
- the telephone number of a responsible person knowledgeable about the shipment
- the receiver's (consignee's) name, address and telephone number
- the proper shipping name (“BIOLOGICAL SUBSTANCE; CATEGORY B”)
- temperature storage requirements (optional)
- marking UN 3373 as below



3) Exempt:

Human (or animal) specimens for which there is minimal likelihood that pathogens are present are not subject to dangerous goods requirements and regulations if they are transported in packaging which will prevent any leakage and are correctly labelled.

Patient diagnostic specimens are in this category if there is minimal likelihood that pathogens are present and if they are packaged and labelled appropriately.

- The exterior packaging must be marked as “**Exempt human specimen**”
- The packaging must consist of three components (triple packing):
 - leak-proof primary receptacle
 - leak-proof secondary packaging
 - outer packaging of adequate strength for its capacity, mass and intended use
- at least one side of the packaging must have minimum dimensions of 100 x 100 mm
- For liquids: Absorbent material in sufficient quantity to absorb the entire contents (placed between the primary and secondary packaging to prevent leakage to the outer packaging, see figure 12)
- When multiple “fragile” primary receptacles are used in a single package, they must be wrapped or separated so that contact between them is prevented (see figure 12)

NOTE: Packing Instruction 650 (as for UN 3373/Category B shipments) can be used, except labelled with “Exempt human specimen” in place of “UN3373”.

Exceptions

Because of the low hazard they present, the following substances are exempted from dangerous goods requirements and regulations.

- Substances that do not contain infectious substances or will not cause disease in humans or animals
- Substances containing microorganisms that are not pathogenic to humans or animals
- Substances in a form in which any pathogens present have been neutralized or inactivated such that they no longer pose a health risk
- Environmental samples that are not considered to pose a significant risk of infection
- **Dried blood spots** and faecal occult blood screening tests
- Decontaminated medical or clinical wastes

Dried blood samples **do not** need to meet the triple packaging requirement, but should be packaged in airtight containers to ensure no contact with personnel may occur during the shipping process.

Refrigerants

Refrigerants may be used to stabilize infectious substances in Categories A and B during transit and should be used when shipping samples for attempted virus isolation or when virus cultures are being shipped for further investigation.

Ice or ice packs pre-frozen at -20°C are suitable for maintaining temperatures $4-8^{\circ}\text{C}$ in a suitably well insulated shipping container for up to 3 days. For maintaining cold-chain conditions for longer than 3 days, dry ice may be considered, if supplies are readily available.

Ice or dry ice shall be placed **outside** the secondary receptacle. Wet ice shall be placed in a leak-proof container; the outer packaging or overpack shall also be leak-proof. **Dry ice must not be placed inside the primary or secondary receptacle because of the risk of explosions.** A specially designed insulated packaging may be used to contain dry ice. The packaging must permit the release of carbon dioxide gas if dry ice is used. ICAO/IATA Packing Instruction 904 shall be observed.

The secondary receptacle shall be secured within the outer package to maintain the original orientation of the inner packages after the refrigerant has melted or dissipated.

If dry ice is used to ship infectious substances in Category A, the details shall appear on the shipper's Declaration for Dangerous Goods. In addition, the outermost packaging shall carry the hazard label for dry ice and the appropriate marking. If dry ice is used to ship infectious substances in Category B, the package shall be marked "Carbon dioxide, solid" or "Dry ice".

If liquid nitrogen is used as a refrigerant, special arrangements shall be made in advance with the carrier. Primary receptacles shall be capable of withstanding extremely low temperatures, and packaging and documentation requirements for liquid nitrogen shall be observed. In particular, the outermost packaging shall carry the hazard label for liquid nitrogen. For air transport, the handling label for cryogenic liquids shall also be affixed.

9.6 Composition of media and reagents

Phosphate buffered saline, pH 7.2 (PBS)

NaCl	8.00g
KCl	0.20g
NaHPO ₄	1.15g
KHPO ₄	0.20g

- Dissolve reagents in distilled water. Make up to 800ml.
- Adjust to pH 7.2 with HCl.
- Autoclave at 10 PSI for 15 minutes. This gives a working solution of PBS without calcium or magnesium ions.
- (PBS is also commercially available in powder, tablet or liquid form)

PBS-Tween wash solution

PBS	100ml
Tween 20 (Commercially available)	0.05ml

- Add 0.05ml Tween 20 per 100 ml PBS. Prepare sufficient volume for one test.

Virus transport medium (VTM)

Hanks x10 Basal Salt Solution (Gibco/Invitron)	100 ml
Sterile deionised water	900 ml
Sterile 20% Bovine albumin	10 ml
Sterile Penicillin Streptomycin solution (PSS)	10 ml
Sterile 4.4% sodium bicarbonate to give pH 7.4	

- Aseptically add Hanks x10 Basal Salt Solution; 20% Bovine albumin and PSS to the deionised water and mix thoroughly
- Aseptically adjust to exactly pH 7.4 with sodium bicarbonate solution, mixing thoroughly after each addition
- Aseptically dispense 2ml aliquots into sterile 25ml screw cap bottles/flasks. Ensure the caps are very tight.
- Store at 4°C. Shelf life at 4°C is expected to be 6 months.

Penicillin Streptomycin solution (PSS)

- Dissolve 1×10^6 units of penicillin and 1g streptomycin sulphate in 100ml sterile PBS. Store 5ml aliquots at -20°C .

One ml of this solution in 100ml medium gives a final concentration of 100 units of penicillin and 100mg of streptomycin per ml.

Oral fluid Extraction buffer

Extraction Buffer	100 ml volume
10% fetal calf serum	10 ml
0.2% Tween 20 (Sigma)	200 μl
Phosphate buffered saline	90 ml
0.5% Gentamicin (50 mg/ ml stock)	500 μl
0.2% Fungizone (250 μg / ml stock)	200 μl
Red food dye *	50 μl

* The addition of red food dye is optional. It can be used to help confirm that sample has been added to the ELISA plate.

10. Suggested further reading

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