GLOBAL
POLIOMYEITIS ERADICATION
BY THE YEAR 2000

MANUAL FOR THE
VIROLOGICAL INVESTIGATION
OF POLIOMYELITIS

EXPANDED PROGRAMME ON IMMUNIZATION
AND DIVISION OF COMMUNICABLE DISEASES
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INTRODUCTION

With the adoption of Resolution WHA41.28 in May, 1988, the World Health Assembly committed WHO to the exciting challenge of the global eradication of poliomyelitis by the year 2000.¹

Success will require strengthening of many aspects of the Expanded Programme on Immunization (EPI). The most important tasks are increasing routine levels of immunization coverage and improving disease surveillance.

For poliomyelitis, surveillance relies heavily on laboratory services. This reliance increases as the number of cases decreases. In countries/areas which are now polio-free, or nearly so, each suspected case requires complete laboratory workup. In addition, there needs to be continuous monitoring of viruses isolated from patients and from the environment. In the final stages of the initiative, given competent laboratories and an active programme of laboratory surveillance, absence of wild poliovirus will be the criterion for global eradication.

While there are many highly competent individuals and laboratories currently engaged in the laboratory diagnosis of poliomyelitis, methods and reagents are not highly standardized, and no global system of proficiency testing of laboratories has yet been established. It is hoped that this manual will make a contribution in both areas.

Because of the variety of methods currently in use, universal consensus on the methods recommended here will of course be difficult to reach. It is hoped, however, that any laboratories which are not in a position to adopt the recommendations in this manual will at least be willing to include them along with their own methods. This will provide both a means to assure global consistency in reporting results and, where the superiority of other methods can be demonstrated, a means for improving the WHO recommendations.

The WHO methods will also provide the basis for instituting a global system of proficiency testing. While the credibility of the eradication effort will ultimately depend on such a system, its main function in the beginning phases of this initiative will be to identify areas in which laboratories are experiencing difficulties so that support and training can be appropriately offered.

We thank all who have contributed their time and effort to this manual. We hope you, the user, will find it useful. But please help us by sharing your comments and suggestions so that we can continue to revise and improve it. We look forward to collaborating with you in this great adventure of polio eradication.

A complementary effort has been made to upgrade and standardize vaccine potency testing including oral poliovaccine. Information on the laboratory methods used in vaccine potency tests is available from the Biologicals (BLG) Unit, WHO, Geneva.
1. POLIOMYELITIS: THE VIRUS, PATHOGENESIS, TRANSMISSION, IMMUNITY AND PREVENTION

1.1 THE VIRUS

Polioviruses comprise three serotypes within the genus Enterovirus of the family, Picornaviridae. The poliovirus virion is approximately 27 nm in diameter, and consists of a capsid of 60 subunits, each with 4 proteins (VP1 through VP4) arranged in icosahedral symmetry around a genome made up of a single strand of positive-sense RNA. The capsid and noncapsid proteins are formed by cleavage from large precursor polyproteins. X-ray diffraction studies have revealed the three-dimensional molecular structure of the polio virion. The three largest proteins (VP1–VP3), are similar in core structure; the peptide backbone of the protein loops back upon itself, forming a barrel of eight strands held together by hydrogen bonds (the beta-barrel). Between the beta-barrel and the amino and carboxyl terminal portions of the protein, the amino acid chain contains a series of loops, which include the chief antigenic sites found on the virion surface; these sites are involved in the neutralization of virus infection. The smallest (internal) protein, VP4, is associated with viral RNA.

Polioviruses, like other enteroviruses, are stable at acid pH for 1–3 hours, and have a buoyant density in caesium chloride of about 1.34 g/cm³. Since the virion has no lipid-containing envelope, it is not affected by lipid solvents such as ether or sodium deoxycholate. The virus infectivity is completely inactivated when heated at 56°C for 30 minutes. In the presence of molar magnesium chloride poliovirus is partially protected from inactivation, a property which has been utilized in stabilizing oral poliovirus vaccine.

Poliovirus replicates in the cytoplasm of the infected cell, the infectious cycle being complete in approximately 6 hours. Mature virus is released by lysis of the cell. In the laboratory, virus is cultivated in primary or continuous-line cell cultures from various human or monkey tissues. Natural isolates of polioviruses infect only primate cells that contain a specific membrane receptor for the virus on the cell surface.
Within each of the three antigenic types, intratypic differences can be distinguished by use of newly developed tools of molecular biology. These include reacting with strain-specific absorbed polyclonal or monoclonal sera, oligonucleotide mapping, and sequencing the bases in the viral genome.

To ensure clarity of communication and uniformity of nomenclature, all antigenically characterized poliovirus isolates should be identified by antigenic type, country (or city) of origin, strain number, and year of isolation. Thus, P1/Houston/23/62 designates a type 1 poliovirus strain, number 23, isolated in Houston in 1962.

1.2 PATHOGENESIS AND DISEASE

The portal of entry of the virus is the mouth. Primary multiplication takes place in the oropharynx or the intestine, and for a few days virus may appear in the blood. The virus can be isolated from the throat just before and at the first signs of illness. The incubation period is usually between 7 and 14 days, but may range from 3 to 35 days. One week after onset there is little virus in the throat, but large amounts of virus continue to be excreted in the faeces for several weeks even though humoral antibodies have usually developed during the same period.

Virus from the bloodstream can invade the central nervous system (CNS) unless sufficiently high levels of neutralizing antibodies have developed to block this pathway. Within the CNS, the virus spreads along nerve fibres, and in the process of its intracellular multiplication it may damage or completely destroy the invaded nerve cells, resulting in flaccid paralysis. The anterior horn cells of the spinal cord are most prominently involved, but in severe cases the posterior horn and dorsal root ganglia may also be affected. In the brain, the reticular formation, vestibular nuclei, and deep cerebellar nuclei are most often affected. The cortex is almost completely spared, except for the motor cortex along the precentral gyrus.

Poliovirus does not multiply in muscle tissue in vivo. The malfunctions that occur in peripheral nerves and voluntary muscles follow upon the replication of virus in nerve cells. Changes occur rapidly in nerve cells, from mild chromatolysis to neuronophagia and complete destruction. Some nerve cells that lose their function due to local oedema may ultimately recover completely. Inflammation, chiefly by lymphocytes, is a secondary result of the attack on nerve cells.
When a susceptible individual is exposed to infection by a virulent poliovirus, the response may be (1) inapparent infection without symptoms, (2) minor illness, (3) aseptic meningitis, or (4) paralytic poliomyelitis. Only about 1% of poliovirus infections are recognized clinically. Shedding of virus from the throat and faeces and resultant transmission of infection can take place without any invasion of the CNS.

The minor illness may include fever, malaise, drowsiness, headache, nausea, vomiting, constipation and sore throat, in various combinations. The patient recovers in a few days. Nonparalytic poliomyelitis (aseptic meningitis), in addition to some of the above signs and symptoms, includes stiffness and pain in the back and neck; the illness lasts 2–10 days, with rapid and complete recovery. It is important to emphasize that poliovirus is only one of the many enteroviruses and other viruses that produce aseptic meningitis.

In paralytic poliomyelitis, onset of the major disease may follow minor illness such as described above, or it may occur without any antecedent first phase. The predominant complaint is flaccid paralysis resulting from lower motor neurone damage. The maximal recovery usually occurs within 6 months, but residual paralysis usually lasts much longer.

1.3 MODE OF TRANSMISSION OF INFECTION

Human beings are the sole reservoir of polioviruses, and close human contact is the primary avenue of spread. Virus can be recovered from the oropharynx for several days, but is shed in the faeces for periods up to a month or longer. The usual source of infection is virus from the oropharynx or faeces spread by contaminated fingers. Young children are the main reservoir of infection with spread to other family members. By the time poliomyelitis is recognized in any member of a family, all susceptible members have already been infected.

When poliovirus is circulating within a community, virus can usually be found in sewage, which can serve as a source of contamination of flies or of water used for drinking, bathing, or irrigation.

In temperate climates, infection with enteroviruses, including poliovirus, occurs mainly during the summer and autumn. In tropical climates virus circulation tends to occur all the year round or is associated with the rainy season. Throughout the world there is a direct correlation between poor hygiene, poor sanitation, and overcrowding and the acquisition of infection and antibodies at an early age.
1.4 IMMUNITY

Passive immunity is transferred from mother to offspring. The maternal antibodies gradually disappear during the first 6 months of life. Passively administered antibody lasts only 3–5 weeks.

Virus-neutralizing antibody forms within a few days after exposure to the virus, often before the onset of illness, and persists, apparently, for life. Its formation early in the infection is a result of viral multiplication in the intestinal tract and deep lymphatic structures before invasion of the nervous system. Since antibodies must be present in the blood to prevent the dissemination of virus to the brain and are not effective after this has already occurred, immunization is of value only if it precedes the onset of symptoms referable to the nervous system.

Circulating serum antibody against poliovirus is not the only source of protection against infection. Local or cellular immunity is manifested by protection against intestinal reinfection after recovery from a natural infection or after immunization with oral polio vaccine. Local or secretory antibody is increasingly recognized as having an important role in defence against infections by polioviruses or by other enteroviruses.

Viral infections in general carry an increased risk for persons with various immunodeficiencies in either humoral or cell-mediated immunity. In such persons, poliovirus infection – either by wild virus or by vaccine strains (which have reverted on passage in humans) – may develop in an atypical manner, with an incubation period longer than 28 days, a high mortality rate after a long chronic illness, and unusual distribution of lesions in the central nervous system.

A decrease in resistance to poliovirus infection accompanies removal of tonsils and adenoids. Pre-existing secretory antibody levels in the nasopharynx decrease sharply following operation, without any change in serum antibody levels. Local antibody levels remain low or absent for as long as 7 months. In seronegative children, the nasopharyngeal antibody response to poliovirus vaccine develops significantly later and to lower titres in children previously tonsillectomized than in those with intact tonsils.

Other host factors which influence the severity of poliovirus infection are age (particularly the very young), pregnancy, and chronic undernutrition.
1.5 PREVENTION BY IMMUNIZATION

Although improved sanitation and hygiene help to limit the spread of polioviruses, the only specific means of preventing paralytic polio is immunization with live oral polio vaccine (OPV) or/and inactivated polio vaccine (IPV). Both of the vaccines available at present are excellent. Both contain viruses of all three poliovirus serotypes.

Formalin-inactivated polio vaccine (IPV, also known as Salk vaccine) is prepared from virus grown in monkey kidney cultures (previously in primary cultures, now chiefly in continuous-passage Vero monkey cells). Repeated booster inoculations have been required to maintain immunity with IPV that has been generally available up to the present time. New formulations of IPV with higher concentrations of antigen (enhanced or e-IPV) have been developed recently. The aim of these new vaccines is to induce satisfactory and lasting antibody responses in a large proportion of vaccinees after only two doses. However, field experiences to date have not yielded unequivocally clear results as to their clinical efficacy and overall, long-term effect.

Live, attenuated oral polio vaccine (OPV, also known as Sabin vaccine) contains virus grown in primary monkey cell cultures or in human diploid cell cultures. OPV can be stabilized by the addition of magnesium chloride or saccharose, the former being used more often. Stabilized OPV can be stored at 0°C to 8°C for 6 to 12 months without significant loss of titre. When the distribution and administration of the vaccine is imminent, OPV may be stored up to 2 years at −20°C. At elevated temperatures stabilized OPV retains the minimum potency for shorter periods of time: 7 to 14 days at 26°C and 2 days at 31°C. At 37°C the vaccine loses about 0.15 log10 every day. With this degradation rate, a vaccine with an assumed total virus content of 6.15 log10 will lose half of its potency during a two-day exposure at 37°C. Further efforts are needed to increase the heat stability of OPV.

Both IPV and OPV induce humoral antibodies that circulate in the blood and protect the central nervous system from subsequent invasion by wild virus. The immunity induced by IPV, however, has little effect on intestinal infection and carriage of virus, and thus IPV recipients remain potential vehicles for spread of wild virus to susceptible persons with whom they come in contact.
In contrast, OPV infects, multiplies, and thus immunizes in a manner that parallels natural infection. OPV induces not only long-lasting IgG antibodies in the blood but also secretory IgA antibodies in the pharynx and intestine, which then become resistant to infection by wild virus.

The live attenuated polioviruses, especially type 3, mutate to some degree during their multiplication in vaccinated children, but not to full neurovirulence, and only extremely rare cases of paralytic poliomyelitis have occurred in recipients or their close contacts. The risks of paralytic polio associated with reversion of OPV are exceedingly small. Based on several large studies, including a 10-year study conducted by WHO, it is estimated that one case of vaccine-associated paralysis has occurred for every 2 to 4 million doses of trivalent OPV distributed.

In developing countries, the primary immunization schedule should begin early and be completed early in infancy. The immunization schedule recommended by the WHO Expanded Programme on Immunization (EPI) is a series of three doses of OPV. These should be administered at 6, 10, and 14 weeks of age, or as soon as possible thereafter. Intervals greater than 4 weeks between doses do not require restarting the series.

Where children are born in hospitals (or other maternity institutions) or where children come in contact with health services early in life, these opportunities should be used to administer an extra dose of OPV. This is called “OPV Zero”, rather than “OPV1”, to show that it does not replace a dose from the regular OPV series. Although the serological response to OPV in the first week of life is less than observed with immunization of older infants, more than 70% of neonates benefit by developing local immunity in the intestinal tract. In addition, 30% to 50% of neonates develop serum antibodies to one or more poliovirus types. Many of the remaining infants become immunologically primed and respond promptly to additional doses later in life. A dose of OPV at birth is particularly important in cities, in other areas with high population density, and where cases occur in the first year of life. In large cities in many parts of the world 40% to 50% of polio cases occur in the first year of life. This illustrates the need to complete the series of polio immunizations as early in life as possible.

In some, but not all, developing countries children have demonstrated a lower-than-expected serological response to three or more doses of OPV, possibly because of difficulties in maintaining the cold chain during transit to target areas, interference with vaccine 'take' by the presence of other enteroviruses, or interference by nonspecific inhibitors in the gut. The EPI is currently sponsoring
several large clinical trials to examine whether a different ratio of the three types of attenuated poliovirus in trivalent OPV could improve the serological response. In one developing country a combined regimen of IPV and OPV seems to have been successful in overriding the factors interfering with the serological response (by use of IPV) and in increasing intestinal immunity (by use of OPV). EPI-sponsored studies are being initiated in several other developing countries to examine the response of children to combined or simultaneous regimens of IPV and OPV.

Further details on the topics covered in this chapter, plus extensive literature citations, may be found elsewhere.2-4
2. USE OF THE MANUAL: APPROPRIATE INVESTIGATIONS
BASED ON A COUNTRY’S STAGE OF POLIO ERADICATION;
LABORATORY NETWORK

2.1 STAGES OF POLIO ERADICATION

A Plan of Action for the Global Eradication of Poliomyelitis by the
Year 2000 was drafted in 1988 and endorsed by the World Health
Assembly in 1989. For planning purposes the Plan identifies four
stages of polio eradication:

• Countries/areas in STAGE A are considered to be polio-free.
  They have a reliable reporting system, have reported no indige-
nous cases of poliomyelitis for at least the previous three years
and have immunization coverage rates with a protective course of
polio vaccine among children reaching their first birthday of at
least 80%.

• Countries/areas in STAGE B have fewer than 10 cases of polio-
myelitis reported per year and have immunization coverage rates
exceeding 50%.

• Countries/areas in STAGE C report 10 or more cases of polio-
myelitis per year and have immunization coverage rates exceed-
ing 50%.

• Countries/areas in STAGE D have 10 or more cases of poliomyel-
itis reported per year or the number unknown and/or immuniza-
tion coverage rates of 50% or less or unknown.

At the end of 1989, most (62%) of the world’s population were
living in areas considered to be in stage C; 26% of the world’s popu-
lation were living in areas considered to be in stage A or B, while
12% were living in areas considered to be in stage D (Figure 1).

The Global Plan of Action identifies activities needed with re-
spect to immunization coverage, surveillance, outbreak investiga-
tion/control, laboratory services, vaccine quality control (including
the maintenance of an effective cold chain), training, social mobiliza-
tion, rehabilitation, and research and development. The relative
priority for each differs depending on the stage of eradication.
The Plan of Action has been used as the basis for the development of a Manual for Immunization Programme Managers on Activities Related to Poliomyelitis Eradication, which was issued in 1989.\textsuperscript{6} Policies outlined in that Manual are intended for adaptation to national programmes, and are themselves continually evolving as additional experience is gained.

2.2
GUIDE TO THE USE OF VIROLOGICAL TECHNIQUES DESCRIBED IN THIS MANUAL

The use of the virological techniques described in this manual can be related to a country's stage of polio eradication. Brief guidelines on which techniques are recommended for which stages appear below and in Figure 2.
FIGURE 2
STAGES OF POLIO ERADICATION AND RECOMMENDED LABORATORY INVESTIGATIONS AT EACH STAGE

<table>
<thead>
<tr>
<th>Tests</th>
<th>Level of Polio Eradication</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>STAGE A</td>
</tr>
<tr>
<td>Poliovirus isolation</td>
<td>All cases</td>
</tr>
<tr>
<td>Poliovirus identification</td>
<td>Yes</td>
</tr>
<tr>
<td>Poliovirus characterization</td>
<td>Yes</td>
</tr>
<tr>
<td>Other enterovirus isolation</td>
<td>Yes</td>
</tr>
<tr>
<td>Other enterovirus identification</td>
<td>Yes</td>
</tr>
<tr>
<td>Rapid serological assessment after OPV</td>
<td>Yes</td>
</tr>
<tr>
<td>Environmental sampling:</td>
<td></td>
</tr>
<tr>
<td>Sewage</td>
<td>Yes</td>
</tr>
<tr>
<td>Faeces of healthy individuals</td>
<td>Optional</td>
</tr>
<tr>
<td>Serological surveys</td>
<td>Optional</td>
</tr>
</tbody>
</table>

*When <50 cases reported per year, extend poliovirus isolation/identification tests to include all cases.

Isolation and identification of poliovirus (Chapter 4)

In areas where poliomyelitis is endemic or epidemic, i.e. countries in stages C and D, the laboratory should concentrate entirely on the polioviruses. To establish the poliovirus serotype circulating, only faecal specimens from a small number of representative cases need to be virologically investigated. Any viruses isolated should be identified using only type-specific poliovirus antisera.

Where high levels of immunization coverage have greatly reduced the number of poliomyelitis cases occurring annually in a country or geographical area, i.e. stages A and B, virus isolation should be attempted in every suspected case. All viruses isolated should be identified using not only poliovirus antisera, but also those prepared against most of the other enterovirus serotypes (Figure 3). In this situation, distinction between paralysis due to poliovirus infection and poliomyelitis-like illness due to infection by other enteroviruses becomes significant. If the cases are associated with infection by non-polio enteroviruses (NPEV), the serotype(s) must be identified otherwise these cases may mistakenly be interpreted as vaccine failures.
**FIGURE 3**
**ENTEROVIRUS SEROTYPES**

<table>
<thead>
<tr>
<th>Group</th>
<th>Name Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polioviruses 1–3</td>
<td>Coxsackie A23 is Echovirus 9</td>
</tr>
<tr>
<td>Coxsackieviruses A1–22, 24</td>
<td>Echovirus 10 is Reovirus 1</td>
</tr>
<tr>
<td>Coxsackieviruses B1–6</td>
<td>Echovirus 28 is Rhinovirus 1A</td>
</tr>
<tr>
<td>Echoviruses 1–9, 11–27, 29–34</td>
<td>Echovirus 34 is a variant of Coxsackie A24</td>
</tr>
<tr>
<td>Enteroviruses 68–72</td>
<td>Enterovirus 72 is Hepatitis A</td>
</tr>
</tbody>
</table>

**Intratypic differentiation (Chapter 5)**

For countries at stages A and B, it is not sufficient to isolate poliovirus and identify the poliovirus serotype responsible. Further virological investigations involving intratypic differentiation of the virus isolates must also be carried out to clarify their origin. The presence of indigenous, imported or vaccine-derived poliovirus strains has completely different implications from the epidemiological point of view in respect of what action needs to be taken.

**Environmental sampling (Chapter 4, Section 4.1.4)**

Where poliomyelitis eradication has progressed successfully, and no indigenous poliomyelitis cases due to wild poliovirus have been detected for some years, it is nevertheless essential that constant surveillance should continue. Environmental sampling for poliovirus can be carried out either by attempted poliovirus isolations from faecal samples taken from healthy individuals or from sewage samples located at community sites. Because polioviruses of vaccine origin circulate widely due to use of OPV, intratypic differentiation of a sample of poliovirus isolates is essential in evaluating the situation.

Environmental sampling for poliovirus should also be intensified if cases of poliomyelitis caused by wild poliovirus occur suddenly in a country or geographic area that has been free from poliomyelitis for many years. Under such conditions, the knowledge of the extent of virus circulation is important in order to take appropriate measures.
Serological tests for neutralizing antibodies (Chapter 6)

These tests are no longer recommended for routine use in the diagnosis of poliomyelitis. There are several reasons for this decision, the primary one being that such tests have rarely been useful in clarifying questionable virus diagnoses. Interpreting serum antibody titres has become difficult now that poliovirus immunization is widespread. Moreover current neutralizing antibody methods do not differentiate between antibodies against wild and vaccine virus strains.

The neutralizing antibody test is a useful method to assess the presence of protective levels of antibody. EPI has developed a method for the rapid serological assessment of the response of infants to 3–4 doses of OPV given in the recommended schedule. Detailed instructions appear in Annex 1. Less-than-optimal responses to OPV have been reported in some tropical countries and so it is important to document the global picture in the 1990s using standard methods (Annex 1). Such assessments are recommended for countries at stages A, B, and C.

Countries at stages A and B may wish to conduct more extensive serological surveys of different segments of the population. Neutralizing antibody surveys carried out using sera collected from healthy individuals in a number proportionate to the total population by age groups and geographical area, can reflect the immune status of the population. This type of study is important at advanced stages of eradication to detect potential gaps in immunity of the population in the absence of clinical cases of poliomyelitis.

2.3
LABORATORIES AND THEIR FUNCTIONS

The complexity of the virological investigation of cases of poliomyelitis and of poliovirus surveillance depends on the laboratory facilities and expertise available. Laboratories functioning at various levels are envisaged. 7

National laboratories

The main tasks of these laboratories are confirmation of clinical cases of poliomyelitis by the isolation and identification of virus from patients. These laboratories will also be responsible for the isolation and identification of polioviruses and for antibody surveys arising from epidemiological investigations. In those national laboratories with appropriate facilities, poliovirus isolates may also be characterized as wild or vaccine-derived using a panel of centrally supplied monoclonal antibodies.
Reference laboratories

Not all countries will have available national poliovirus diagnostic laboratories. Suitable laboratories in other countries will be invited to provide the necessary support and these are described as “reference laboratories”. They may be responsible for all the necessary work from a given country, and for certain of the more complex tests, including intratypic differentiation of poliovirus isolates. Reference laboratories may also take part in training programmes. Though most of the reference laboratories are expected to be in the same part of the world as the countries they serve, there may be instances in which, for example, laboratories in Europe may function as reference laboratories for countries in Africa or Asia.

Reference laboratories with specialized functions

A small number of reference laboratories will be identified to perform one or more specialized functions in lieu of or in addition to those described above. One function will be to provide definitive identification of virus isolates, using monoclonal antibody panels and/or cDNA probes to differentiate wild and vaccine strains and to determine the origins of wild strains. Other responsibilities might include: (1) obtaining and distributing relevant standard reagents and reference materials, (2) acting as sources for the development and use of training materials, (3) obtaining and distributing panels of unknowns to other designated laboratories, and (4) serving as participants in collaborative studies to assess proposed standards and reference materials and to answer specific research questions.
3. COLLECTION, STORAGE AND SHIPMENT OF SPECIMENS

Effective diagnostic virology depends upon the correct collection and timing of clinical specimens and their proper transport to the laboratory under optimal conditions. A typical process of collection and transport of specimens from laboratory to laboratory is illustrated in Figure 4.

**FIGURE 4**
TYPICAL SPECIMEN COLD CHAIN SYSTEM

- **Health Centre/Local Hospital**
  - identifies suspected case:
  - Notifies National Surveillance Centre with name/address of case

- **Visit by national polio epidemiologist with kit**

- **National Surveillance Centre**
  - National Laboratory
    - Receive, unpack, register faecal specimens
    - Store in dedicated refrigerator at 0°C to 8°C
    - Process the faeces
    - Conduct virus isolation and identification tests
    - Dispatch faeces
    - Dispatch isolates

- **Visit to case**
  - National epidemiologist classifies as probable case or discard.
  - If probable case:
    1. allocate EPId number
    2. initiate case investigation form
    3. initiate case laboratory report form (to accompany all samples related to this case)

- **Collect faecal specimen**

- **Collect second faecal specimen**
  - 48 hours after first
  - (applies only to countries at Stages A or B of polio eradication)

- **Reference Laboratory**
  - Receive, unpack, register
  - Store at 0°C to 8°C (short term) or at −20°C (long term)
  - Confirm virus isolation and identity of the isolates. If more definitive characterization required or results of these confirmed, dispatch to specialized laboratory

- **Specialized Reference Laboratory**
  - Receipt, registration and analysis by monoclonal antibodies and/or by c-DNA probes
  - Store in dedicated −20°C freezer (long term)
3.1
COLLECTION OF SPECIMENS

For non-hospitalized patients, obtain faeces.

For hospitalized patients, the following samples should be collected:

- flaccid paralysis: faeces, throat swabs
- meningo-encephalitis: faeces, throat swabs, and cerebrospinal fluid
- fatalities: necropsy specimens (tissues from brain stem, spinal cord, descending colon) and serum

3.1.1
Specimens for virus isolation

Because polioviruses multiply in the intestinal tract for several weeks after infection, samples of faeces are those most suitable for virus isolation. Throat swabs are less useful since polioviruses are present in the oropharynx for only 7–10 days after onset of illness. Paradoxically, despite the neurotropism of polioviruses, they are rarely detected in cerebrospinal fluid (CSF); however other enteroviruses can be isolated from this site with relative ease. All specimens for virus isolation studies should be collected as soon as possible after onset of illness. In fatal cases, necropsy specimens must be taken within hours of death.

All specimens for virus isolation must be taken with care to exclude contamination from other types of specimens or material from other patients.

Faeces

Samples of faeces should be collected as soon as possible (preferably within seven days) after the onset of illness. Since virus shedding may be intermittent, the isolation rate can be increased by collecting two samples 24–48 hours apart. In countries at stage C and stage D of polio eradication (Figure 2) virus diagnosis should be concentrated on a single sample of faeces. A faecal sample about the size of an adult thumbnail (i.e. 4–8 g) is desirable. This should be placed in a dry, clean, leakproof container; standardized plastic containers with a spoon are preferable. In the absence of such containers, clean, dry plastic ointment/medicinal boxes can be used.

If faeces cannot readily be obtained, as may be the case with outpatients or those diagnosed under field conditions, rectal straw samples provide an alternative method for obtaining faeces.
**Faecal specimens obtained by rectal straw**

Rectal straws are plastic tubes made and packaged to EPI/WHO specifications; these and a list of suppliers are available from EPI/WHO, Geneva. The straw is inserted gently into the rectum and with slight movement, an appropriate amount of faeces is usually obtainable. After removal, place the straw in a securely sealed bottle or protective tube.

**Throat swabs**

A sterile wooden-shafted swab is rubbed gently across the tonsils and posterior wall of the pharynx. The swab shaft is then broken into a screw-capped vial/bottle containing VTM (see Annex 2). Note that poliovirus is present in the oropharynx for 7–10 days after onset of illness and it is reasonable to obtain throat swabs only during this period.

**Cerebrospinal fluid (CSF)**

2–3 ml of CSF is transferred directly into a sterile screw-capped vial/bottle (without the addition of VTM).

**Necropsy specimens**

In fatal cases, samples from the central nervous system, (especially from cervical and lumbar cord, medulla and pons) as well as from the descending colon, are desirable. The specimens should be taken as soon after death as possible. The samples should be excised using separate sets of sterile instruments and placed in separate sterile plastic or glass containers with sufficient VTM present to keep the specimens moist. The size of the CNS samples should be about 1 cm³; from the colon, take a segment approximately 3–5 cm long which contains faecal material.

### 3.1.2

**Specimens of blood for serological studies**

The serological diagnosis of poliovirus infection in patients with poliomyelitis is no longer routinely recommended due to difficulties in interpreting the tests, particularly in the light of high coverage rates with polio vaccine.

For serological surveys, only one sample is required from each selected person; the optimal amount is 5–10 ml of blood. If venepuncture is not feasible, an amount sufficient for most important serological tests can be obtained by the finger-prick method; this yields 0.3–0.4 ml of blood which can be collected aseptically into a small stoppered or screw-capped vial/bottle using a pipette. For infants, a heel-stick may also be used.
All blood samples should be placed in sterile containers free of anticoagulants or preservatives. The blood is allowed to clot at room temperature for 2 hours or, if this is not practicable, the sample is refrigerated (approximately 0°C to 8°C) as soon as possible after collection and the serum removed within the following 24 hours. Serum is separated by low-speed centrifugation (500 g* for 5 minutes) and transferred to a sterile vial using a sterile disposable pipette. If a centrifuge is not available, or large numbers of samples await processing, centrifugation can be omitted by careful removal of the serum after retraction of the clot; avoid mixing red blood cells with the serum as these haemolyze during storage and may interfere with subsequent serological tests.

**Filter paper discs**

Blood samples of less than 0.3 ml volume can be collected by impregnating filter paper discs. These discs are first calibrated to absorb 0.025 ml of blood and sterilized by autoclaving at 10 PSI for 15 minutes. All subsequent manipulations must be carried out using sterile technique, instruments and containers, since polio neutralizing antibody studies are performed in cell cultures. Collect a total of 0.1 ml of blood (i.e. 4 discs x 0.025 ml) and place either (a) each disc immediately into a clearly labelled bottle/tube containing 0.7 ml maintenance medium (see Annex 2). Keep samples at 0°C to 8°C until arrival at the laboratory; store at −20°C until ready for testing. Alternatively, (b) place the discs with blood into a clearly labelled dry container, leave overnight to dry and store at −20°C until ready to test; elution is carried out by adding 0.7 ml of sterile PBS (see Annex 2) and incubating overnight at 0°C to 8°C. Both methods give a dilution of 1/8 which is the starting dilution of the micro-neutralization test.

### 3.1.3 Labelling of specimens

Specimens taken from a suspected case of polio are identified nationally and internationally by a standard case investigation number known as the **EPId number**. This identification number is used as a common reference to the case by all laboratories.

The EPId number can immediately be established in any country beginning to collect specimens for analysis by the senior epidemiologist in charge of this activity who will keep a single national register of case investigations. An EPId number will be issued by the epidemiologist (or by a trained designate) to each new investigation of a suspected case of polio (see Figure 5).

---

* g = Relative Centrifugal Force; to convert to RPM use the following formula: 
  \[ g = (11.7 \times 10^{-2}) \times R \times N^2 \]
  where:
  - \( R \) = radius in mm from centrifuge spindle to extreme point on the tube.
  - \( N \) = speed of centrifuge spindle in RPM.
FIGURE 5
EPID NUMBER: A WORLD-WIDE SYSTEM FOR IDENTIFICATION OF PATIENTS WITH SUSPECTED POLIOMYELITIS

<table>
<thead>
<tr>
<th>3-Letter Country Code</th>
<th>Year of Onset of Paralysis</th>
<th>Suspected Polio Patient Number for That Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL</td>
<td>90</td>
<td>008</td>
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*THREE LETTER COUNTRY CODES

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<th>Europe</th>
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<tr>
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<td>Saint Christopher and John</td>
<td>NJ</td>
<td>NJ</td>
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</table>
An example of a specimen label, incorporating the EPId number, which should always appear prominently in non-water soluble ink at the top of the label, is illustrated in Figure 6.

**FIGURE 6**
**EXAMPLE OF A SPECIMEN LABEL**

<table>
<thead>
<tr>
<th>EPId No.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient Name</td>
<td></td>
</tr>
<tr>
<td>Date Specimen collected</td>
<td>/ / /</td>
</tr>
<tr>
<td></td>
<td>day mo yr</td>
</tr>
</tbody>
</table>

Laboratories must not obscure the data given on the top part of the form; use the relevant space on the lower half of the form for recording individual laboratory numbers, dates, etc.

### 3.1.4

**Laboratory report form**

A case investigation form is initiated for each suspected case of polio investigated; this is held by the national epidemiologist responsible for polio eradication activities. Some of the information from the case investigation form is transferred to the laboratory report form (Figure 7). The laboratory report form accompanies all specimens/isolates relating to a single case from time of collection through all stages of virological analysis until completion.
FIGURE 7
EXPANDED PROGRAMME ON IMMUNIZATION – GLOBAL POLIO ERADICATION LABORATORY REPORT FORM ON SPECIMEN ANALYSIS

<table>
<thead>
<tr>
<th>Patient Name:</th>
<th>EPId Number:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Country:</td>
<td>State/Region:</td>
</tr>
<tr>
<td></td>
<td>Town/District:</td>
</tr>
</tbody>
</table>

Clinical Diagnosis:
Date of onset: da__/mo__/yr___
Poliomyelitis History: Date of last OPV dose: da__/mo__/yr___
Specimen type: From patient/Contact No.: Date collected: da__/mo__/yr___
Date sent: da__/mo__/yr___ See reverse for name/address of investigator.

Laboratory 1
□ National □ Reference □ Specialized
Name of laboratory: See reverse for address.
Date specimen received: da__/mo__/yr___
Specimen type: Temperature monitor index on receipt: 🔩
Isolate(s) sent to Reference Laboratory: Virus isolation:
Date sent: da__/mo__/yr___
Original specimen sent to Reference Laboratory: Date sent: da__/mo__/yr___
Comments: Date reported: da__/mo__/yr___

Laboratory 2
□ National □ Reference □ Specialized
Name of laboratory: See reverse for address.
Date specimen/isolate(s) received: da__/mo__/yr___
Temperature monitor index on receipt: 🔩
Virus identification:
If isolate(s) sent to Specialized Laboratory: Date sent: da__/mo__/yr___
Comments: Date reported: da__/mo__/yr___

Laboratory 3
□ National □ Reference □ Specialized
Name of laboratory: See reverse for address.
Date isolate(s) received: da__/mo__/yr___
Temperature monitor index on receipt: 🔩
Virus identification:
Virus characterization:
Comments: Date reported: da__/mo__/yr___
3.2  
STORAGE AND SHIPMENT OF SPECIMENS

3.2.1  
Temperature considerations

The quantity of virus or antibody in original clinical samples or in virologically processed specimens can decline during storage and shipment. This can seriously affect the virus diagnostic result. Therefore, special care must be taken before and during transit of materials to the laboratory to protect them from heating and desiccation.

Specimens should not be repeatedly frozen and thawed. Specimens should be frozen only when it is certain that at all future stages of transport and storage a maximum temperature of $-20^\circ C$ can be assured. This is not usually the case in the national specimen cold chain. For this reason it is recommended that specimens are transported and kept refrigerated at temperatures of $0^\circ C$ to $8^\circ C$.

3.2.2  
Shipping materials

The materials and equipment used to cool polio specimens can become contaminated with the virus. It is therefore recommended that materials which are used to transport specimens are either destroyed or sterilized after each use and that polio specimens do not share space in refrigeration equipment with vaccines.

In order to ensure that specimens remain cold during the various stages of transport and shipment to the laboratory and between laboratories, icepacks or dry-ice and heavily insulated containers are required. To minimize weight and bulk and to prolong the cold life of this packaging, the volume occupied by specimens should be minimized.

The set of specimen containers relating to a single case investigation should be placed in a single box or plastic bag (with an effective seal) just large enough to hold the containers and enough absorbent material to ensure that, if all the containers should leak, any liquid is absorbed. The laboratory report form should be sealed within a separate plastic bag and wrapped around or attached firmly to the box of specimens.

It is recommended that, where practicable, a single set of specimens for one case investigation is packed in a single insulated carton or carrier for transport to the laboratory. The EPI uses vaccine carriers and plastic bottles known as icepacks which can be allocated for this
However, once used for specimen collection, they should never again be used for vaccine transport. Examples of suitable carriers illustrated in Figure 8 have a maximum internal capacity of about 1.5 litres, excluding the volume required for ice packs. When the box or bag of specimens has been placed inside a vaccine carrier, the maximum number of frozen ice packs which can be fitted around the specimens should be inserted. No less than four ice packs should be used to transport specimens in a vaccine carrier and these should be arranged to surround the specimens. Specimens can be kept at least 36 hours, even in high external temperatures of 43°C in this way.

FIGURE 8
EXAMPLES OF VACCINE CARRIERS AND ICEPACKS USED IN THE EPI

Provided these specimen carriers and ice packs are not visibly contaminated with faecal material they can be reused after thorough washing in water and/or hypochlorite solution.

Since the virus content of specimens deteriorates when partially frozen, pack specimen carriers with ice packs where the ‘frost’ is no longer visible on their surfaces.

If vaccine carriers or other specially made materials are not available, it will be necessary to make up an insulated container from locally available materials. The main features of this packaging and a guide to the selection of these materials follow (Figures 9 and 10).
FIGURE 9
IMPROVISING SHIPPING MATERIALS FOR SPECIMENS

a) 30-60 ml faeces container with external screw cap
b) sealed polyethylene bag to hold faeces containers
c) sealed polyethylene bag to hold report form
d) absorbent material (cotton wool absorbs 8-10 times its own weight)
e) icepacks obtainable from national EPI
f) high density (30-35 kgs/m³) polystyrene (small bubbles and firm when squeezed)
g) infectious substance label
h) outer carton of double-ply corrugated cardboard or plastic
FIGURE 10  
MATERIALS FOR SHIPPING SPECIMENS AT 0°C TO 8°C OR FROZEN

<table>
<thead>
<tr>
<th>Materials</th>
<th>Journey Time</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Less than 24 hours</td>
</tr>
<tr>
<td>1. Maximum volume of packed specimens</td>
<td>0.5 lts</td>
</tr>
<tr>
<td>2. Minimum thickness of insulation</td>
<td>40 mm</td>
</tr>
<tr>
<td>(low density polystyrene)</td>
<td>30 mm</td>
</tr>
<tr>
<td>(high density polystyrene)</td>
<td>25 mm</td>
</tr>
<tr>
<td>3. Minimum weight of icepacks</td>
<td>0.8 kgs</td>
</tr>
<tr>
<td>(transport at 0°C to 8°C)</td>
<td></td>
</tr>
<tr>
<td>OR</td>
<td></td>
</tr>
<tr>
<td>Minimum weight of dry ice</td>
<td>1.5 kgs</td>
</tr>
<tr>
<td>(transport frozen; use slabs if possible)</td>
<td></td>
</tr>
</tbody>
</table>

**Insulation**

*Polystyrene foam*: is usually white and is made in a variety of densities. The low density foam is generally used for packing materials, has large bubbles and is easily dented with the finger. Low density polystyrene has a poorer insulation performance than high density polystyrene (30–35 kgs/m³) which is also white but which has small bubbles and is harder to dent.

*Polyurethane foam*: is a yellowish colour and should be rigid to touch. (Soft polyurethane foams found in cushions and mattresses are not suitable for insulating packages for specimen shipment). Rigid polyurethane is also made in different densities and the higher densities with the smallest bubbles are the best insulators. Polyurethane is the best insulation which you are likely to find locally and is often used to ship perishables.

Whichever material you use, the insulation should form a box around the icepacks which themselves surround the specimen samples. All joints between the walls and the lids of this box must be well sealed. Good sealing can be achieved by pressing the walls and lid together firmly within a strong cardboard box and by sealing the joints with tape so that no air can pass from the outside to the inside of the box. If air can pass, the cold life of the box will be dramatically shortened, particularly when the outside temperature is high.
Icepacks

Icepacks are water/ice-filled flat plastic bottles with a seal in the lid to prevent them from leaking when the contents melt. They can usually be obtained from the national EPI but they should NEVER be used again for cooling vaccines after they have been used to transport specimens to the laboratory. If flat bottles cannot be found, any well-sealed plastic bottle can be used if, by experiment, it can survive freezing and thawing without cracking. Used icepacks should be thoroughly washed down in water or in hypochlorite solution after use and before freezing.

Dry-ice

Dry-ice should only be used to cool specimens when it is certain that at all future stages of transport and storage a maximum temperature of \(-20^\circ\text{C}\) can be assured. This is not usually the case in the national specimen cold chain; the transport of specimens in the frozen state will usually apply to reference and specialized laboratories (see Figure 10 for details).

3.2.3

Airfreight Shipment

If the specimens are to be shipped airfreight, correct labelling and dispatch procedures will be essential to ensure safe and timely arrival of the specimens at the laboratory.\(^\text{9,10}\) A list of the most important considerations follows:

- Choose the most direct route.
- Plan arrival to avoid weekends and holidays.
- Give advance warning one week ahead by telexing the laboratory the following information:
  - number of specimens
  - estimated number of cartons and weight
  - flight and arrival date/time
  - airwaybill number
  - "PLEASE TELEX IF NOT RECEIVED"
- Mark the airwaybill with the following information:
  - name, address, telephone/telex of consignee
  - number of specimens
  - "HIGHLY PERISHABLE"
  - "TELEPHONE CONSIGNEE UPON ARRIVAL" (repeat telephone number)
- Airwaybill handling information:
  - "URGENT: DO NOT DELAY: Biological specimens – highly perishable – store at 0°C to 8°C."
- Mail a copy of the airwaybill to the laboratory at the time of shipping.
- Label the package clearly with:
  - an infectious substance warning label (Figure 11)
  - name/address/telephone/telex of consignee

**FIGURE 11**  
EXAMPLE OF AN INFECTIONOUS SUBSTANCE WARNING LABEL APPROVED BY WHO AND IATA
Corrigendum, July 1990

Page 29, Section 4.1.1 Isolation of Polioviruses, Specimen Preparation, Faeces and rectal straw samples.

It is recommended that all faeces and rectal straw samples are treated with chloroform. The chloroform treatment of specimens is described on page 31. This procedure should be done after step 2, page 29 (shaking) and prior to step 3, page 29 (centrifuging).

IMPORTANT: The centrifuge tube should be resistant to chloroform.
4.
ISOLATION AND IDENTIFICATION OF POLIOVIRUSES

4.1
ISOLATION OF POLIOVIRUSES

It is essential that all clinical specimens or other materials for laboratory investigations be allocated a specific identification number (see 3.1.3) which is entered in the laboratory "day book", on the corner of the accompanying report form and on the specimen container.

Most specimens require pretreatment before inoculation to remove gross debris and bacteria/fungi which might interfere with virus isolation attempts.

Since most virus laboratories are liable to receive many specimens from various sources within the working day it is essential that great care is taken in handling the specimens, not only for the protection of the investigator but also to avoid viral cross-contamination between specimens which could result in false-positive findings. Careful labelling of all containers, centrifuge tubes and vials throughout all the procedures listed below is paramount.

4.1.1
Specimen preparation

Faeces and rectal straw samples

1. Using a hard-wall screw-capped centrifuge tube, prepare a 20% suspension of faecal sample in “complete” PBS (see Annex 2) containing 200 units penicillin and 200 μg streptomycin (or other available antibiotics). Retain a small portion of untreated, original sample at −20°C for possible future transfer to a specialized reference laboratory.

2. Secure tightly and homogenize the suspension by shaking vigorously by hand or on a mechanical shaker for a standard period.

3. Centrifuge at 500 g for 20 minutes at 0°C to 8°C or at room temperature.

4. Remove the supernate carefully and transfer to a fresh clean centrifuge tube and spin again at 500 g for 20 minutes. Again remove the supernate carefully and add extra antibiotic solution to give a
final concentration of 1000 units of penicillin and 200 µg of streptomycin. Divide the supernate equally into 4 appropriately labelled screw-capped vials: 2 for virus isolation tests and 2 remain unopened in case equivocal results are obtained or transfer of original extracts to specialized laboratories is required; store vials at −20°C.

**Throat swabs in VTM**

1. Shake the tube/bottle containing the swab thoroughly by hand or on a mechanical mixer.
2. Using sterile forceps, press the swab firmly against the wall of the tube/bottle to squeeze out the fluid from the swab, then discard the swab.
3. Transfer fluid to 2 labelled vials. Add extra antibiotics (100 units penicillin, 100 µg streptomycin); store at −20°C.

**Cerebrospinal fluid (CSF)**

CSF specimens are inoculated into cell cultures without prior treatment; store at −20°C or below.

**Necropsy samples**

A. **CNS tissue (brain, medulla, spinal cord) in VTM**

1. Prepare a 10% extract of each tissue in complete PBS containing 200 units penicillin and 200 µg streptomycin.
2. Homogenize in a TenBroeck grinder or if this is unavailable, use a pestle and mortar and sterile sand.
3. Transfer fluid to a hard-wall screw-capped centrifuge tube and centrifuge at 500 g for 10 minutes.
4. Carefully transfer the supernate to labelled vials; store at −20°C or at −70°C where possible.

B. **Colon with contents in VTM**

1. Remove colon from VTM and transfer to a petri dish; cut open colon and scrape off sufficient contents to prepare a 20% extract as described for faeces.
2. Transfer harvested supernate into 4 labelled vials and store at −20°C.
Chloroform treatment of specimens

Some specimens, particularly faecal and necropsy samples, even after processing, still contain bacteria and/or fungi which multiply in the inoculated cell cultures rendering the assessment of virus growth impossible. Since enteroviruses are chloroform-resistant, this problem is readily solved by mixing the contents of the vials (previously stored) with one tenth their volume of chloroform and shaking for 10 minutes. The mixtures are then centrifuged at 500 g for 15 minutes and the supernates transferred to appropriately labelled fresh vials ready for inoculation of cultures or storage at temperatures previously recommended.

4.1.2

Recommended cell lines for the isolation of polioviruses

Although primary monkey kidney cell cultures are among the most sensitive for the detection of small amounts of polioviruses, they are often difficult to obtain on a regular weekly basis. Countries far from the source of these cells experience the added problem of poor cell survival due to inevitable long delays during transit. Fortunately, polioviruses grow readily in a wide variety of human continuous cell lines.

WHO recommends that all specimens suspected of containing polioviruses should be inoculated into the following two cell lines:

1. RD cells, derived from a human rhabdomyosarcoma, and
2. HEP-2 (Cincinnati) cells, derived from a human epidermoid carcinoma.

The selection of only two cell lines for the laboratory diagnosis of poliomyelitis permits the standardization of techniques and the comparability of results among various virus laboratories.

These two cell lines are particularly suitable, as both are highly susceptible to polioviruses. The RD cell line has the added advantage that it can also support the growth of other enteroviruses (e.g. some Coxsackie group A viruses and many Echoviruses). If detection of a wide range of enteroviruses is considered important, i.e. countries at stages A and B of polio eradication, other cell lines or primary cultures, particularly of monkey kidney origin, may also be included in the virus isolation regimen.
It is important to monitor any alteration in the sensitivity of the RD and HEp-2 (Cincinnati) cell lines to polioviruses by titrating reference poliovirus strains after every 8 to 10 cell passages.

The maintenance of the cell lines in the laboratory and their preparation for virus isolation are described later in this manual (Chapter 7).

### 4.1.3 Poliovirus isolation

**Procedure**

1. Change recently monolayered, healthy (check microscopically) tube cultures to 1 ml maintenance medium (see Annex 2).

2. Label tubes with patient's laboratory number, year, cell culture type, and passage number (e.g. 100/89/RD/1 and/or 100/89/HEp-2/1).

3. Inoculate a minimum of two culture tubes of each cell type with 0.2 ml of specimen extract and incubate in the stationary sloped (5°) position at 36°C. Include one uninoculated tube of each cell type as controls.

4. Examine cultures daily, using a standard microscope, for the appearance of cytopathic effects (CPE) for at least 10-14 days before discarding as negative. The CPE produced by all the enteroviruses is characteristic of this group, i.e. rounding of cells and detachment of cells from the culture tubes. Tubes showing these morphological changes (or the culture fluids harvested from these tubes) are stored at −20°C for further study.

   Record all details concerning specimen (laboratory number, dates of inoculation, passage, etc.) in a laboratory record book.

   If cell cultures show rapid degeneration within 1 or 2 days of inoculation this may be due to nonspecific toxicity of the specimen. These tubes should be frozen at −20°C, thawed and 0.2 ml volumes passaged (i.e. now second passage) in cultures of the same cell type. If toxic appearances recur, return to the original specimen extract and dilute this in PBS at 1/10 and re-inoculate cultures as described above.

5. Control cultures should maintain their healthy appearance. However, if at the end of one week “ageing” of the cultures becomes evident (this is common in cultures of continuous cell lines), freeze the tubes at −20°C, thaw and passage 0.2 ml of culture fluid to freshly monolayered tubes of the same cell type and examine daily for a further 7 days. The total observation period for virus isolation should be a minimum of 14 days; if cultures show no CPE by this stage, the result is regarded as negative.
Some virologists use a virus isolation procedure which differs from that described above. Instead of transferring specimen extract directly into cultures containing maintenance medium, the cell growth medium is decanted, the cell layer rinsed with PBS and the specimen extract allowed to adsorb to the cell layer directly for 1 hour at room temperature before the addition of cell maintenance medium. Studies have shown that using this latter method CPE may be detected at least 1 day earlier. Against this benefit must be weighed the possibility of viral (if not bacterial) cross-contamination of tubes due to the extra opening/closing of cell cultures during this procedure. Investigators are encouraged to use this latter technique if laboratory space and facilities are good or where poliovirus-related paralysis is sporadic or rare.

Since viral cross-contamination of cultures during inoculation, medium changes and passage procedures is a hazard in any busy virus diagnostic laboratory, the utmost care e.g. thorough flaming of the necks of tubes before decanting medium or stoppering, must be taken to minimize this. If there is reason to suspect this has occurred, repeat the virus isolation procedure using the original specimen extract. Repeat testing is also recommended for all epidemiologically important cases, e.g. the index case in an outbreak or the rare case following vaccination or contact with a vaccinee.

Normally poliovirus isolation studies are complete (including serotype identification) within 2 weeks of receipt of specimen. Where virus mixtures are detected, the results may not be available for at least one month.

4.1.4
Isolation of polioviruses from environmental specimens

Circulation of polioviruses in a population can be monitored by looking for the presence of the virus in the environment. This is usually achieved by sampling sewage. The search for a source or vehicle of an outbreak may also require examination of drinking and bathing water for the presence of enteroviruses.

Preparation of environmental specimens for virological examination differs from that of clinical specimens in that concentration of the sample is a necessary step. Several methods can be used for this. The following procedure is technically relatively simple, does not require expensive equipment, and can easily be performed by any virological laboratory; it provides a 100-fold concentration, which is usually adequate for sewage specimens. Other methods with greater concentration potential may be preferred for analyzing samples of drinking/bathing water.
Collection of sewage specimens

A site chosen for sample collections should contain typical household sewage. Sewage which also contains industrial output may have toxic compounds present which can compromise attempts to isolate viruses from the sample. One litre of sewage fluid is taken into a clean vessel and transported, preferably at 0°C to 8°C, to the virus laboratory.

Pre-treatment of sewage specimens for virus isolation: concentration by two-phase method

1. Stock solutions:
   A. 20% Dextran (MW 40,000) in distilled water (w/v).
   B. 30% Polyethylene glycol (MW 6,000) in distilled water (w/v).

   It is advisable to prepare both solutions A and B by slowly mixing the pre-weighed amount of the dry material with part of the water using a magnetic stirrer. When the powder has dissolved, add the rest of the water. Both solutions may be sterilized by autoclaving.

2. Mix 15 ml of solution A and 100 ml of solution B in an Erlenmeyer flask, add 12 ml of 5M NaCl and fill with the specimen to 600 ml. (Any multiples of these volumes can also be used.) Check pH; if below 7, adjust to pH 7–8 with 1N NaOH.

3. Agitate for one hour at room temperature (20°C to 25°C) in a horizontal shaker (or use a magnetic stirrer). After agitating, the material should be allowed to settle for 5 minutes. Use only the supernate as any coarse solid material in the sewage would interfere with the phase separation step.

4. Transfer the mixture into a separation funnel; leave at 0°C to 8°C for 20 hours.

5. Collect the small bottom phase (2–3 ml) and also separate a few millilitres of the opaque interphase. Treat with chloroform as described in Section 4.1.1, except that equal volumes of specimen and chloroform should be used, and analyze the two samples for cytopathogenic viruses.

   Note that only half of the collected sewage specimen was used. The rest should be kept at 0°C to 8°C for possible re-investigation.

Inoculation of sewage specimens into cell cultures

1. Use cells monolayered in 75 cm² disposable plastic flasks (or corresponding glass bottles) rather than regular tubes, to improve the sensitivity of the isolation attempts.
2. Decant the growth medium, rinse the cell layer with PBS and inoculate with 0.5 ml of the concentrated, chloroform-extracted sample.

3. After one hour adsorption at 36°C, remove the inoculum and add 20 ml of maintenance medium. Removal of the inoculum is important because sewage specimens are often toxic for cell cultures.

4. Incubate at 36°C for 7–10 days and examine for CPE as described for the tube cultures (see Section 4.1.3).

Since isolates from sewage often contain several enterovirus serotypes (and viruses of other groups) identification procedures are necessarily more complex (see Section 4.1.5 below). The use of several human and monkey cell lines with different sensitivities to polio and non-polio enteroviruses will improve the possibility of finding polioviruses among other enteroviruses.

The Polymerase Chain Reaction (PCR) technique may soon supersede these time-consuming cell culture methods for the detection of viruses in general and polioviruses in particular in sewage and other environmental samples. Since PCR is so exquisitely sensitive, its use must necessarily be confined to those laboratories with sophisticated virological facilities.

4.1.5 Isolation of polioviruses from specimens containing other enteroviruses

Enteroviruses other than polioviruses may sometimes cause paralytic disease. However, identification of non-polio enteroviruses (NPEVs) in a specimen from a suspected case of poliomyelitis does not exclude the possibility that the specimen also contains poliovirus. Laboratory contamination may also produce apparent multiple virus isolates. Various techniques are available to separate suspected virus mixtures.

A. Plaque separation

Most enteroviruses produce plaques in infected cell cultures under agar. These plaques are more or less characteristic for particular enteroviruses, e.g. large round plaques of poliovirus type 1 and small irregular plaques of Echovirus type 1.

1. Prepare nutrient overlay medium, i.e. Eagle’s maintenance medium (Annex 2) incorporating 1.5% Bacto-Agar and 1/30,000 neutral red.

2. Drain growth medium from freshly monolayered bottle (120 cm²) cultures of HEP-2 cells.
3. Inoculate bottle with 0.1 ml to 0.5 ml of specimen extract and tilt bottle to distribute inoculum evenly over the cell monolayer.

4. Incubate at 36°C for 1 hour to allow virus adsorption.

5. Add approximately 10 ml pre-warmed nutrient agar overlay medium to each bottle.

6. Invert bottle after agar has solidified and incubate at 36°C, avoiding exposure to light since neutral red may induce photosensitization.

7. Check bottles daily for appearance of plaques, recognizable as colourless areas surrounded by viable cells stained with neutral red. Using sterile Pasteur pipettes carefully pick out single representative plaques of various sizes (a fresh pipette for each plaque) and passage in tube cultures.

B. Re-isolation in the presence of antibodies to the identified virus

Usually if polioviruses are present in the patient’s specimen, these will outgrow and mask the presence of any other usually slower-growing enterovirus which may also be present. However, the reverse may also occur. For example, Echovirus 9, another fast-growing enterovirus, if identified in the specimen may conceal the presence of poliovirus. To overcome this, use the following procedure, illustrated below using an example where Echovirus 9 has been isolated from the faeces of a patient suspected to have poliomyelitis.

1. Mix 0.5 ml of original specimen extract (NOT the isolate material) with an equal volume of Echovirus 9 antiserum containing at least 50 neutralizing antibody units/0.1 ml (e.g. if the homotypic antibody titre of Echovirus 9 antiserum is 1/1000, use at 1/20).

2. Incubate specimen/antiserum mixture for 2 hours at 36°C.

3. Inoculate 2 tubes of RD cell culture with 0.2 ml of specimen/antiserum mixture.

4. Examine tubes daily for CPE and harvest positive tubes as described above for primary isolation.

5. If the virus isolated is again identified as Echovirus 9, this indicates that this virus was present in high titre in the original specimen and the antiserum failed to neutralize all of the virus. Repeat steps 1-4 using Echovirus 9 antiserum >50 units/0.1 ml; alternatively repeat these steps using original specimen extract diluted to 1/10 and 1/100 and mix with 50 units of Echovirus 9 antiserum. If no further virus is isolated using this antibody-blocking procedure, this indicates Echovirus 9 was the only virus present in the original specimen. If enterovirus CPE is again detected, this indicates that there is a mixture of enteroviruses present and virus identification procedures should be instigated as described in Section 4.2.
4.1.6

Titration of polioviruses

Previously it was customary to titrate all virus isolates before their identification. Now, those viruses which produce rapid CPE on passage (i.e. within 2 days) are used at two arbitrarily selected dilutions (e.g. $10^{-3}$ and $10^{-4}$) for identification purposes. However, if problems arise, or serum antibody levels are to be determined, titration of virus must be carried out. For this, a series of 10-fold dilutions of the isolate (or stock poliovirus) are made in virus dilution fluid. Thereafter the test procedure can be performed either by microtechnique (using wells in microtitre plates) or by macro-technique (using tube cultures). The titre ($\text{TCD}_{50}$) of the virus is determined as the highest dilution giving CPE in 50 per cent of inoculated cell cultures.

Microtechnique

1. Thaw virus preparation to be titrated.
2. Add 1.8 ml of virus diluent (i.e. Eagle's maintenance medium, see Annex 2) to each of a set of 8 sterile test tubes.
3. Add 0.2 ml of virus to the first tube ($=10^{-1}$ dilution) using a sterile graduated pipette. Take a fresh pipette, mix thoroughly but avoid 'frothing' of fluid, as this disseminates virus into the atmosphere.
4. Transfer 0.2 ml of $10^{-1}$ dilution into the next tube and discard pipette; take a fresh pipette and mix thoroughly. Continue until all 8 serial 10-fold steps have been completed.

Note: To avoid extra carry-over of virus on the outside of the graduated pipette follow this maxim:

MIX — TRANSFER — DISCARD.

5. Add 0.05 ml of each virus dilution to a row of 4 wells in a microtitre plate already containing 0.05 ml of maintenance medium (see Figure 12). Use an automatic pipette with disposable tips if available; transfer the most dilute virus first and the other dilutions in order thereafter.
6. Add 2 x 0.05 ml of maintenance medium to 4 wells which will act as uninoculated cell controls (see Figure 12).
7. Add 0.1 ml of appropriate cell suspension (approximately $1-2 \times 10^4$ cells/0.1ml) in growth medium (see Annex 2) to all the wells containing virus plus the cell control wells.
8. Seal wells with adhesive tape and incubate at 36°C for 3–5 days. Examine the plates daily, using an inverted microscope, for the appearance of CPE.

**FIGURE 12**
EXAMPLE OF TITRATION OF A POLIOVIRUS ISOLATE USING MICROTECHNIQUE

Interpretation of Results
Titre of poliovirus = \(10^{4.25}\) (calculated by the Kärber formula)
Cell controls = normal growth
Wells 5–8 of Rows A–H may be used for the titration of a second virus

9. Calculate the virus titre by the Kärber formula:
\[
\log \text{TCD}_{50} = L - d (S - 0.5),
\]
where
\(L\) = log of lowest dilution used in the test
\(d\) = difference between log dilution steps
\(S\) = sum of proportion of 'positive' tests
(i.e. cultures showing CPE)
Example (also see Figure 12):

<table>
<thead>
<tr>
<th>Virus dilution</th>
<th>Proportion of cultures infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-1}$</td>
<td>$4/4 = 1$</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>$4/4 = 1$</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>$4/4 = 1$</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>$2/4 = 0.5$</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>$1/4 = 0.25$</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>$0/4 = 0$</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>$0/4 = 0$</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>$0/4 = 0$</td>
</tr>
</tbody>
</table>

\[
\log_{10} \text{TCD}_{50} = L - d (S - 0.5)
\]
\[
= -1 - 1 (3.75 - 0.5)
\]
\[
= -4.25
\]

Note that the Kärber formula gives a 'negative log' term: $10^{-4.25}$ is the TCD$_{50}$ endpoint dilution. The TCD$_{50}$ titre is more correctly expressed as $10^{4.25}$ per inoculum volume, in this case per 0.05 ml.

Macrotechnique

1. Thaw frozen virus preparation to be titrated.
2. Add 1.8 ml PBS to each of a set of 8 sterile test tubes.
3. Add 0.2 ml of virus to the first tube using a sterile graduated pipette ($10^{-1}$ dilution). Take a fresh pipette and mix thoroughly but avoid 'frothing' of fluid, as this disseminates virus into the atmosphere.
4. Transfer 0.2 ml of $10^{-1}$ dilution into the next tube; take a fresh pipette and mix thoroughly. Continue until all 8 serial ten-fold steps have been completed.

Note: To avoid extra carry-over of virus on the outside of the graduated pipette follow this maxim:

MIX — TRANSFER — DISCARD.

5. Label a set of healthy cell tube cultures containing fresh maintenance medium; use a minimum of two tubes for each dilution.
6. Inoculate these tubes with 0.1 ml of appropriate dilutions; transfer the most dilute virus first and the other dilutions in order thereafter using an automatic pipette.

7. Include 2 tubes inoculated with undiluted virus and two uninoculated tubes to serve as cell controls.

8. Incubate at 36°C in the stationary sloped position. Read titration when endpoint (i.e. CPE) reasonably stabilized (i.e. days 3–5). One tissue (= cell) culture infectious dose (TCD$_{50}$) per 0.1 ml is present in the highest dilution producing CPE in 50 per cent of the tubes inoculated.

9. Calculate the virus titre using the convenient simple Kärber formula:

$$\log \text{TCD}_{50} = L - d (S - 0.5),$$

where

- $L$ = log of lowest dilution used in the test
- $d$ = difference between log dilution steps
- $S$ = sum of proportion of ‘positive’ tests (i.e. cultures showing CPE)

Example

<table>
<thead>
<tr>
<th>Virus dilution</th>
<th>Proportion of cultures infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-1}$</td>
<td>$2/2 = 1$</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>$2/2 = 1$</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>$2/2 = 1$</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>$2/2 = 1$</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>$2/2 = 1$</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>$0/2 = 0$</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>$0/2 = 0$</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>$0/2 = 0$</td>
</tr>
</tbody>
</table>

$$\log \text{TCD}_{50} = L - d (S - 0.5)$$

$$= -1 - 1 (5 - 0.5)$$

$$= -5.5$$

Virus titre = $10^{-5.5}/0.1$ ml

Although the accuracy of the titration can be increased by using 4 culture tubes per virus dilution, the titre results obtained from the use of 2 tubes is usually adequate for virus identification purposes. Examples of the interpretation of typical poliovirus titrations are given in Figure 13.
It is important to remember that any alteration in the cell system used, volumes of virus used or days on which titres are read necessitate re-titration of the original virus material. Viruses which have necessitated titration are used at 100 TCD₅₀ concentrations per inoculum volume for virus identification and serological studies. Fortunately, polioviruses (and enteroviruses in general) are very stable when aliquots are stored at -20°C and their titres are usually reproducible when thawed in preparation for these tests.

**FIGURE 13**
EXAMPLE OF TITRATION OF POLIOVIRUS ISOLATES USING MACROTECHNIQUE

<table>
<thead>
<tr>
<th>Virus Dilution</th>
<th>Isolate 1</th>
<th>Isolate 2</th>
<th>Isolate 3</th>
<th>Isolate 4</th>
<th>Isolate 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻¹</td>
<td>+/+</td>
<td>+/+</td>
<td>o/o</td>
<td>+/+ +/+ +</td>
<td>+/+ +/+ +</td>
</tr>
<tr>
<td>10⁻²</td>
<td>+/+</td>
<td>+/+</td>
<td>o/o</td>
<td>+/+ +/+ +</td>
<td>+/+ +/+ +</td>
</tr>
<tr>
<td>10⁻³</td>
<td>+/+</td>
<td>+/+</td>
<td>o/o</td>
<td>+/+ +/+ +</td>
<td>+/+ o/o/o</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>o/o</td>
<td>o/o</td>
<td>o/o</td>
<td>+/+ o/o/o</td>
<td>o/o/o/o</td>
</tr>
<tr>
<td>TCD₅₀/0.1 ml</td>
<td>10⁴</td>
<td>10⁴.5</td>
<td>No virus</td>
<td>10².5</td>
<td>10².75</td>
</tr>
</tbody>
</table>

+ = CPE (virus present)
0 = no CPE (virus absent)

**4.2**
IDENTIFICATION OF POLIOVIRUS ISOLATES

For the identification of poliovirus isolates, samples of diluted isolate are mixed with equal volumes of a selected set of polyclonal antisera raised in animals against known virus serotypes. The serum/virus mixtures are incubated for two hours to allow the antibodies to bind to the virus. Subsequently, suspensions of cells are added or alternatively the mixtures are inoculated into cell culture tubes. These are examined daily for the presence of CPE. The antiserum which prevents the development of CPE indicates the identity of the virus serotype.
The test is performed using polyclonal antiserum of high titre, which is then mixed with 100 TCD₅₀ (range 32–320 TCD₅₀) of unknown virus. Previously it was customary to titrate all virus isolates before attempting their identification. Now the procedure recommended for those viruses which on passage produce rapid CPE (within 2 days) is to use these at two arbitrarily selected dilutions (i.e. 10⁻³ and 10⁻⁴). Should problems in identification of the isolate arise, then titration of the virus is required (Section 4.1.6).

The procedure for virus isolate identification can be performed using microtitre plates (microtechnique) or cell culture tubes (macrotechnique). While microtechnique is more economical in requirements for precious viral antisera, in inexperienced hands the possibilities of viral cross-contamination between isolates is considerable. Macrotechnique requires larger volumes of reagents but individual tube cultures are opened only at the serum/virus inoculation stage. If difficulties in identifying the isolates are encountered or virus mixtures are suspected, neutralization tests using tube cultures should be used.

If virus isolates are obtained in both RD and in HEP-2 cell cultures, identification of both isolates is essential since the virus susceptibility of these two cell lines varies.

4.2.1
Antisera

The primary aim of the virus laboratory participating in the poliomyelitis eradication initiative is to identify polioviruses isolated from clinical specimens. First attempts at virus isolate identification should concentrate on this aspect alone, using monospecific polyclonal poliovirus types 1, 2, and 3 antisera individually and/or combined as poliovirus antiserum pools. Requests for these antisera should be submitted to CDS/EPI, WHO, Geneva.

4.2.2
Identification using microtechnique

The following equipment is needed: (1) sterile, disposable, flat-bottomed (cell-culture grade) microtitre plates; (2) adhesive film or tape for sealing plates; (3) adjustable automatic pipette with sterile disposable plastic tips, capable of delivering volumes of 0.05–0.1 ml.

1. Prepare four pools of poliovirus antisera (diluted according to the supplier’s instructions) as follows:
   i. Pool P₁ + P₂ + P₃ = anti-type 1, type 2, type 3
   ii. Pool P₁ + P₂ = anti-type 1 and type 2
   iii. Pool P₁ + P₃ = anti-type 1 and type 3
   iv. Pool P₂ + P₃ = anti-type 2 and type 3
Label each bottle clearly, listing the pool contents and date of preparation. Once the 4 pools have been made, those required for frequent use can be stored at 0°C to 8°C; stock preparations should be distributed in several vials and stored at -20°C. Each time a batch of pools is prepared it is essential that their ability to identify correctly poliovirus isolates is confirmed by testing each pool against prototype poliovirus types 1, 2 and 3.

2. Unwrap sufficient plates to accommodate the number of isolates to be tested. Label the plates with the sample laboratory number, the date and the antisera (pools) used. It may be more convenient to write these details on a sheet of paper which accompanies the numbered plate.

3. Distribute 0.05 ml of each of the 4 antiserum pools into wells of the microtitre plate (4 wells/pool) using separate tips for each pool. Add 0.05 ml of maintenance medium (see Annex 2) into a further 4 wells per virus dilution, which will serve as virus controls (Figure 14).

4. Dilute the isolates in maintenance medium at 10⁻³ and 10⁻⁴, the arbitrarily selected dilutions as discussed earlier.

5. Add 0.05 ml of the virus isolates diluted at 10⁻³ to 2 wells and the same volume of 10⁻⁴ dilution to the other two wells of each antiserum pool and to the wells acting as virus controls, using a separate tip for each virus dilution.

6. Prepare a back titration of the isolate in tubes/bottles containing maintenance medium, beginning from the strongest dilution used (usually 10⁻³) and continue in serial ten-fold steps (i.e. to 10⁻⁷, see Figure 14).

7. Distribute 0.05 ml maintenance medium into 20 empty wells and then add 0.05 ml samples of the above dilutions into 4 wells/dilution, beginning with the most dilute virus.

8. Cover the plates with their lids and incubate at 36°C for 2 hours.

9. During this incubation period, prepare a cell suspension from a cell culture flask (i.e. the cell type in which the virus was isolated) and dilute this in growth medium to contain 1–2 x 10⁴ cells/0.1 ml.

10. Distribute 0.1 ml of cell suspension into all wells containing serum/virus mixtures or virus and 4 wells containing 2 x 0.05 ml volumes of maintenance medium which act as cell controls.

11. Seal the plates with adhesive tape and incubate at 36°C.
12. Examine plates daily (using an inverted microscope) for development of CPE, usually for 3–5 days. When virus controls show complete CPE, record final results on the same day. The antisera pools which prevent the development of CPE indicate the identity of the virus isolate or mixtures of polioviruses.

13. At this stage all cell control wells should have a complete monolayer of cells; the back titration of virus should confirm that the virus dilutions used in the test were acceptable (i.e. 100 TCD$_{50}$ with a range of 32–320 TCD$_{50}$).

Figure 14 shows the typical lay-out of a test plate with interpretation of results. It is also recommended that 10$^{-3}$ and 10$^{-4}$ TCD$_{50}$ of poliovirus 1, 2 and 3 Sabin strains should be included in each batch of identification tests.

**FIGURE 14**
IDENTIFICATION OF POLIOVIRUS ISOLATES USING THE MICROTECHNIQUE

<table>
<thead>
<tr>
<th>Pool</th>
<th>Pool</th>
<th>Pool</th>
<th>Pool</th>
<th>Controls</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_1+P_3$</td>
<td>$P_1+P_3$</td>
<td>$P_1+P_3$</td>
<td>$P_1+P_3$</td>
<td>Virus</td>
<td>Cell</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>

Virus isolate X at -3
-4

Virus isolate Y at -3
-4

Back titrations:
- isolate X
- isolate Y

- = CPE  
- = no CPE  
- = unused wells

**Interpretation of results:**

Virus titre of isolate $X = 10^{6.5}$; isolate neutralized by Polio pools $1+2+3$;
$1+2$; $1+3$

= Poliovirus type 1

Virus titre of isolate $Y = 10^3$; isolate neutralized by Polio pools $1+2+3$;
$1+2$; $2+3$

= Poliovirus type 2

Cell controls = Normal growth
4.2.3

**Identification using macrotechnique**

1. Prepare the 4 pools of poliovirus antisera and store exactly as described for use in the microtechnique method.

2. Distribute 0.2 ml of each pool into labelled test tubes (or into the wells of 22 cm x 14 cm plastic disposable plates) using separate pipettes for each pool. Use 2 tubes (wells) per pool.

3. Dilute the isolate in PBS at $10^{-3}$ and $10^{-4}$, the arbitrarily selected dilutions discussed earlier. At the same time prepare a back titration of the isolate ranging from $10^{-3}$ to $10^{-7}$.

4. Using a separate pipette each time, add 0.2 ml of virus dilution to each tube (well) containing antisera; mix serum virus mixtures gently to avoid 'fothing'.

5. Incubate these capped/covered mixtures for 2 hours at 36°C.

6. During this incubation period, select freshly monolayered tubes of cell cultures corresponding to the cell type in which the virus was isolated. Label these, using 2 tubes per antisera/virus mixture, 2 tubes each for the virus controls ($10^{-3}$ and $10^{-4}$), and 6 additional tubes for the remaining back titration of the isolate from $10^{-2}$ to $10^{-7}$, and two tubes to act as uninoculated cell controls.

7. Transfer the serum/virus mixtures (total volume 0.2 ml per tube) to the correspondingly labelled tubes, using a separate pipette for each inoculation.

8. Inoculate 0.1 ml of the back titrations into the cell cultures. The same pipette can be used throughout, providing the most dilute virus is added first and ending with the two tubes each of $10^{-4}$ and $10^{-3}$, which will also serve as the virus controls. Add 0.1 ml of PBS to each tube culture containing diluted virus (to equate to the antiseraum volume).

9. Incubate culture tubes in the stationary, sloped position at 36°C.

10. Examine virus control tubes daily using a standard microscope until complete CPE observed (usually 3 days). Tubes containing antisera are read on the same day. The antiseraum pools which prevent the development of CPE at that time indicate the identity of the isolate(s).

11. Figure 15 illustrates the interpretation of the results.
FIGURE 15
IDENTIFICATION OF POLIOVIRUS ISOLATES USING THE MACROTECHNIQUE

<table>
<thead>
<tr>
<th>Pool $P_1+P_2+P_3$</th>
<th>Pool $P_1+P_2$</th>
<th>Pool $P_1+P_3$</th>
<th>Pool $P_2+P_3$</th>
<th>Virus Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>Poliovirus type 1</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>Poliovirus type 2</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>Poliovirus type 3</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>Mixture of poliovirus types 1 &amp; 2</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>Mixture of poliovirus types 1 &amp; 3</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>Mixture of poliovirus types 2 &amp; 3</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Mixture of all three poliovirus types</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>No poliovirus or mixture of poliovirus with other enterovirus(es)</td>
</tr>
</tbody>
</table>

$+$ = CPE
0 = no CPE

With most isolates some “breakthrough” of virus is expected 1–2 days after reading the result. If this “breakthrough” is rapid (i.e. complete CPE) this can be due to: too high a dose of virus used in the test; presence of a mixture of viruses; or some antigenic variation in the isolate. It is therefore recommended that all results be confirmed using individual poliovirus antisera.
5. INTRATYPIC DIFFERENTIATION OF POLIOVIRUS STRAINS

For countries at stage A or stage B of polio eradication, intratypic differentiation becomes important. This technique will demonstrate whether poliovirus isolated from a case is a wild or a vaccine-derived strain. If wild poliovirus is implicated, is the strain already circulating in the area or has it been imported from elsewhere?

Two methods for intratypic differentiation are described in this manual, the first using monoclonal antibody panels and the second using nucleic acid probe hybridization. Both techniques are currently being used today in a small number of international reference laboratories.

5.1 INTRATYPIC DIFFERENTIATION OF POLIOVIRUS STRAINS USING MONOCLONAL ANTIBODY PANELS

Monoclonal antibodies are produced by hybridoma cells derived from immune lymphocytes rendered immortal by fusion with a myeloma cell line. Monoclonal antibodies are exquisitely sensitive with respect to the sites they recognize on an antigen and a single amino-acid change can block antibody binding. The panels of antibodies described here are primarily intended to distinguish OPV strains from wild type poliovirus strains. They have been devised to identify strains as being: (a) identical to Sabin vaccine strains, (b) related to, but drifted from the Sabin vaccine strains, (c) typical wild strains, or (d) antigenically unusual wild strains.

The antibodies of the type 1 panel are numbered 11–16, of the type 2 panel 21–26 and of the type 3 panel 31–36. Their reactions with wild type and vaccine-related viruses from a collection at the National Institute of Biological Standards and Control, London, UK, are shown in Figure 16. Some of the antibodies are specific for the Sabin vaccine strain or strains derived from it. Antibodies 11, 12 and 15 do not usually react with wild type 1 strains, antibody 24 does not react with wild type 2 strains and antibodies 33 and 34 do not react with most wild type 3 strains tested. In some cases, these antibodies do not react with isolates known to be derived from the vaccine strains and obtained from vaccinees or contacts. This is due to change on an antigenic site of the virus as a result of replication in the host.
However, change in all strain-specific sites is unlikely, and if the virus reacts with any of the strain specific antibodies, it is likely to be derived from the Sabin vaccine strain.

Requests for these panels of monoclonal antibodies should be submitted to CDS/EPI, WHO, Geneva.

### FIGURE 16
PERCENTAGE OF WILD AND VACCINE-RELATED POLIOVIRUSES REACTING WITH MONOCLONAL ANTIBODY PANELS*

<table>
<thead>
<tr>
<th>Antibody Panels</th>
<th>Poliovirus Wild Type</th>
<th>Poliovirus Live Vaccine-related</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0%</td>
<td>88%</td>
</tr>
<tr>
<td>12</td>
<td>0%</td>
<td>88%</td>
</tr>
<tr>
<td>13</td>
<td>49%</td>
<td>100%</td>
</tr>
<tr>
<td>14</td>
<td>51%</td>
<td>100%</td>
</tr>
<tr>
<td>15</td>
<td>0%</td>
<td>65%</td>
</tr>
<tr>
<td>16</td>
<td>97%</td>
<td>100%</td>
</tr>
<tr>
<td>Type 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>95%</td>
<td>100%</td>
</tr>
<tr>
<td>22</td>
<td>81%</td>
<td>75%</td>
</tr>
<tr>
<td>23</td>
<td>93%</td>
<td>100%</td>
</tr>
<tr>
<td>24</td>
<td>0%</td>
<td>80%</td>
</tr>
<tr>
<td>25</td>
<td>67%</td>
<td>100%</td>
</tr>
<tr>
<td>26</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Type 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>92%</td>
<td>100%</td>
</tr>
<tr>
<td>32</td>
<td>24%</td>
<td>100%</td>
</tr>
<tr>
<td>33</td>
<td>8%</td>
<td>80%</td>
</tr>
<tr>
<td>34</td>
<td>8%</td>
<td>100%</td>
</tr>
<tr>
<td>35</td>
<td>71%</td>
<td>100%</td>
</tr>
<tr>
<td>36</td>
<td>25%</td>
<td>70%</td>
</tr>
</tbody>
</table>

* Results of studies at the National Institute of Biological Standards and Control, UK, on over fifty viruses for each serotype.
Procedure

Using poliovirus isolates which have previously been serotyped and titrated:

1. Prepare a series of 8 ten-fold dilutions of virus from $10^{-1}$ to $10^{-8}$ (i.e. 0.2 ml of virus plus 1.8 ml of Eagle's assay medium (Annex 2), using a separate graduated pipette at each dilution stage).

2. Add 0.05 ml volumes of these dilutions to each well of a row in two microtitre plates, and four wells of a row in a third microtitre plate; i.e. row A for the first dilution, row B for the second, etc. The plates should be previously labelled (code to identify the samples, type and number of monoclonal antibody, etc.) (see Figure 17).

3. Dilute each monoclonal antibody 1/100 in Eagle's assay medium and add 0.05 ml to 4 wells of each row of a microtitre plate. In this way, the monoclonal antibody panel is accommodated on two microtitre plates. Finally, add 0.05 ml of assay medium to the 4 wells of the third microtitre plate. This plate is the control titration of the poliovirus isolate.

4. Cover plates with lids and incubate at 36°C for 2 hours.

5. Add 0.1 ml of HEP-2 cells, resuspended in growth medium to give a concentration of about 1-2 x 10⁴ cells to each well.

6. Prepare a number of wells on the third microplate to serve as cell controls by adding 2 x 0.05 volumes (= total 0.1 ml) assay medium to the wells and then add 0.1 ml of cell suspension to each.

7. Seal plates with adhesive tape and incubate at 36°C for a total of 7 days.

8. Examine plates with an inverted microscope at 4 and 7 days; wells showing complete CPE are scored as positive. The log titre of virus in the presence and absence of antibody is calculated by the method of Kärber and the numerical value of the difference is taken as the Neutralization Index for each serum using the following formula:

$$\text{Neutralization Index} (NI) = \log V_{To} - \log V_{Tma},$$

where

$V_{To}$ = titre of virus in the absence of monoclonal antibody

$V_{Tma}$ = titre in the presence of monoclonal antibody

A Neutralization Index of 1 or greater indicates that the isolate is sensitive to the antibody.

It is recommended that the reference strains of poliovirus (attenuated) be included in all tests with monoclonal antibodies. Requests for these strains should be submitted to CDS/EPI, WHO, Geneva.
FIGURE 17
INTRATYPIC DIFFERENTIATION OF POLIOVIRUS STRAINS USING MONOCLONAL ANTIBODIES

PLATE 1

<table>
<thead>
<tr>
<th></th>
<th>0.05 ml diluted Antibody 1</th>
<th>0.05 ml diluted Antibody 2</th>
<th>0.05 ml diluted Antibody 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1  2  3  4</td>
<td>5  6  7  8</td>
<td>9  10 11 12</td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

0.05 ml Virus dilution [-1, -2, -3, -4, -5, -6, -7, -8]

PLATE 2

<table>
<thead>
<tr>
<th></th>
<th>0.05 ml diluted Antibody 4</th>
<th>0.05 ml diluted Antibody 5</th>
<th>0.05 ml diluted Antibody 6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1  2  3  4</td>
<td>5  6  7  8</td>
<td>9  10 11 12</td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

0.05 ml Virus dilution [-1, -2, -3, -4, -5, -6, -7, -8]

= test wells
FIGURE 17 (Continued)
INTRATYPIC DIFFERENTIATION OF POLIOVIRUS STRAINS USING MONOCLONAL ANTIBODIES

**PLATE 3**

<table>
<thead>
<tr>
<th>0.05 ml virus iteration + 0.05 ml diluent</th>
<th>Cell controls + 2 x 0.05 ml diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>A</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td></td>
</tr>
</tbody>
</table>

*0.05 ml Virus dilution*

-1

-2

-3

-4

-5

-6

-7

-8

(○) = test wells
(□) = unused wells

**Layout of Test Plates and Control Plate**

Virus dilutions are distributed over three plates. The $10^{-1}$ dilution is distributed in 0.05 ml volumes to each well of row A of Plate 1 and Plate 2, and to four wells of row A in Plate 3. The $10^{-2}$ dilution is similarly distributed in 0.05 ml volumes over row B and so on. Antibody dilutions are distributed over Plate 1 and Plate 2. For type 1, the panel consists of antibodies 11, 12, 13, 14, 15, and 16. For type 2, the panel consists of antibodies 21, 22, 23, 24, 25, and 26. For type 3, the panel consists of antibodies 31, 32, 33, 34, 35, and 36. A 0.05 ml volume of the first antibody (11, 21, or 31) is distributed to wells of lines 1, 2, 3, and 4 of Plate 1. The second antibody (12, 22, 32) is distributed to wells of lines 5, 6, 7, and 8, and so on. The wells of Plate 3 receive 0.05 ml diluent. Plate 3 also contains 8 cell control wells, each containing two volumes of diluent.

**Interpretation of results**

Some of the patterns of reaction which have been encountered are illustrated in Figure 18 together with their interpretations.
FIGURE 18
EXAMPLES OF THE INTERPRETATION OF REACTIONS OF MONOCLONAL ANTIBODIES WITH TYPE 1, TYPE 2, AND TYPE 3 STRAINS OF POLIOVIRUS

<table>
<thead>
<tr>
<th>Virus</th>
<th>Type 1 Monoclonal Antibody</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>Sabin 1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VW1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VW4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hong Kong 15/8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Greece 5/82</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Spain 459/82</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Virus</th>
<th>Type 2 Monoclonal Antibody</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>21</td>
<td>22</td>
</tr>
<tr>
<td>Sabin 2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>KT228/II</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>KT428/II</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5503/Isr/8014</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A2969/Kuw/8017</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>II-1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>II-299</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Virus</th>
<th>Type 3 Monoclonal Antibody</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>31</td>
<td>32</td>
</tr>
<tr>
<td>Sabin 3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DM4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EM25</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DM35</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DM66</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>III-715</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>III-374</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>III-1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Fin/23127/84</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ = neutralized  
- = not neutralized
5.2 INTRATYPIC DIFFERENTIATION OF POLIOVIRUS ISOLATES USING NUCLEIC ACID PROBE HYBRIDIZATION

Nucleic acid probe hybridization is a rapid, reliable, and simple method for differentiating poliovirus isolates. Synthetic DNA probes have been prepared that recognize either all human enteroviruses, or specific poliovirus strains, such as isolates derived from the Sabin vaccine strains. Recognition is by specific base-pairing of the probes to sequences within the genomic RNAs of poliovirus isolates. The enterovirus group probe binds to sequences conserved among enteroviruses and polioviruses, while the strain-specific probes bind to sequences that are highly variable across and within poliovirus serotypes. Although limited variation of the sequences targeted by the Sabin strain-specific probes occasionally occurs during vaccine replication in humans, the hybridization conditions described in this chapter permit recognition of such variants as being vaccine-related.

The probes have been designed to be used in concert with primary isolation and typing with hyperimmune sera. Virus isolation in cell culture is essential for resolving mixtures of viruses that may exist in clinical specimens and for increasing virus titres to levels giving good RNA yields for hybridization tests. Accurate typing data are critical for the correct identification of polioviruses and for reducing the overall work load in intratypic differentiation by early exclusion of non-polio enteroviruses from further routine testing.

Probes specific to several wild poliovirus genotypes have been prepared, such that important epidemiological information regarding the distribution of specific wild genotypes is obtained through routine diagnostic procedures. It appears feasible to prepare probes for the wild poliovirus genotypes endemic to each region of the world. Such a collection would necessarily be large, since numerous wild poliovirus genotypes currently exist worldwide. The probe set presently available for distribution consists of (1) a probe to the enterovirus group, which will confirm an enterovirus (including poliovirus) is present in the isolate and give some indication of the amount of RNA present (2) three Sabin vaccine strain-specific probes, which will recognize vaccine-related isolates of each serotype. Many variations in probe design and experimental procedures have been successfully applied. This chapter describes conditions for use of short synthetic DNA probes covalently linked to a molecule of alkaline phosphatase. A simple histochemical reaction is used to detect hybrids.

Probe hybridization reactions are discussed below in five steps (1) preparation of samples using formaldehyde treatment, (2) immobilization of poliovirus RNAs onto membrane filters, (3) hybridization by incubation of the filters in buffers containing specific probes, (4) washing the filters to remove unbound probes, and (5) detection of hybrids.
5.2.1
Preparation of samples using formaldehyde treatment

Materials
1. Healthy monolayers of poliovirus-sensitive cells (RD or HEp-2, in culture tubes or microtitre plates).
2. Dry ice/ethanol bath or freezer (−70°C or −20°C).
3. 20X Saline Sodium Citrate (20X SSC). (see 5.2.6).
4. 37% formaldehyde solution (Reagent Grade).

CAUTION:
CARCINOGEN. AVOID INHALING FUMES.
WEAR PROTECTIVE GLOVES.

Procedure

CAUTION:
PERFORM ALL OPERATIONS WITH OPEN SAMPLES OF INFECTIOUS VIRUS IN A BIOLOGICAL SAFETY CABINET.

1. For each poliovirus isolate, inoculate rinsed cell monolayer cultures with 0.1 ml of infected culture fluid from clinical isolates to produce high-titre stocks. RD cells give high virus yields.
2. After adsorption (36°C for 1 hour), add only sufficient cell culture medium (lacking foetal calf serum) to completely cover the monolayers.
3. Incubate the cultures at 36°C until complete CPE is obtained.
4. Suspend the infected cells by agitation. Freeze (dry ice/ethanol bath or freezer) and thaw (36°C) twice.
5. Transfer 0.5 ml of culture fluid to a 1.5 ml microcentrifuge tube. Pellet cellular debris in a microcentrifuge (>10,000 g for 1 minute).
6. Transfer the supernate to a new 1.5 ml tube. For each 0.5 ml of culture supernate add:
   - 20X SSC 0.3 ml
   - 37% formaldehyde 0.2 ml
Vortex to thoroughly mix and centrifuge briefly (15 seconds).
7. Seal the tube with parafilm to prevent the cap from popping open, and heat at 60°C for 15 minutes.

*Comments:* The heat step disrupts the virion proteins, releasing the viral RNA. The 6X SSC (final concentration) provides the high salts necessary for efficient binding of RNA to membrane filters. Formaldehde disrupts base-pairing within RNA molecules, causing native RNAs to form extended chains. *The heat and formaldehyde combine to completely inactivate virus infectivity so that subsequent steps can be performed on the open laboratory bench.*

Since RNA molecules are degraded upon prolonged exposure to formaldehde, it is best to prepare only enough formaldehde-treated samples as will be used immediately. Untreated infected culture medium or clarified supernates can be stored indefinitely at −20°C.

### 5.2.2

**Immobilization of poliovirus RNAs onto membrane filters**

Samples may be spotted directly onto membrane filters using a micropipette. However, when the number of samples is large, it is convenient to use a blot filtration manifold. Plexiglass manifolds, containing 96 wells in exactly the same size and arrangement as the wells in a microtitre plate, are commercially available. The blot manifold is assembled from three parts. The base is connected to a vacuum source, which can be provided by the central vacuum system, a water aspirator, or an electric vacuum pump.

**Materials**

1. Nitrocellulose membrane filters.

2. Thic filter paper.

3. Blot filtration manifold.

4. Vacum source and filter flasks.

5. Filter forceps.
Procedure

1. Cut the corners from the sheets of filter paper and membrane filters so that the alignment pins in the manifold will be clear. Mark the corner of the membrane filter with a pencil to identify well A1. Do not touch the membrane filters with your fingers (wear gloves and use filter forceps), as oils from the skin will interfere with RNA binding to the filter. Nitrocellulose membrane filters are fragile and must be handled with care.

2. Saturate a piece of thick filter paper with 6X SSC and place it on the manifold.

3. Float the membrane filter in a shallow container of distilled water to wet. The filter becomes darker when wet. After 30 seconds invert the filter to thoroughly wet it.

4. Drain off excess water and briefly soak filter in 6X SSC by the method described in step 3.

5. Position the membrane filter on top of the filter paper. Clamp the “sandwich” together and gently apply vacuum.

6. To ensure that none of the wells is blocked, filter a small amount of 6X SSC through all wells. Air bubbles prevent complete filtration of the sample and can be removed by gently tapping the manifold on the bench. After the wells are cleared, turn off vacuum.

7. Apply samples (up to 0.2 ml) to the filter with a micropipette by dispensing down the wall of each well.

8. Spot samples in replicate sets. Samples should include Sabin vaccine strain and wild poliovirus strain RNAs for reference. At least two sets are needed:
   - 1 set for the group probe, and
   - 1 set for each strain-specific probe.

   If the serotypes of the isolates are unknown, it is necessary to prepare four replicate sets: one set for the group probe and one set for each Sabin strain-specific probe. Samples should be grouped in such a way that separate areas may be cut from the filter to give equivalent patterns containing all RNA samples. Take care to unambiguously mark all filter pieces for proper orientation.

9. After all samples have been dispensed, gently apply vacuum until all wells are empty. Disassemble the manifold and carefully remove the membrane filter.

10. Place the membrane filter between two pieces of filter paper and allow to air dry completely (usually 30 to 60 minutes). Do not heat filters. The filter is now ready for hybridization.

Note: Volumes of treated culture fluid greater than 0.2 ml may clog filters. Volumes less than 0.05 ml may not distribute the samples evenly at the bottom of the well and should be further diluted to 0.2 ml using 6X SSC.
5.2.3

Hybridization

To reduce background spots caused by non-specific binding of probes, membrane filters are first incubated in hybridization buffer in the absence of probe. This step is called "pre-hybridization". Pre-hybridization is unnecessary with many synthetic DNA probes, but with others some background remains despite all precautions. Resolution of background problems must often be approached empirically.

Materials

1. Hybridization buffer (see 5.2.6).
2. Sealable plastic sandwich boxes.
3. Shaking water bath.

Procedure

1. Place filters in plastic sandwich boxes containing 1 ml of hybridization buffer for each square centimeter of membrane filter. Several filters may be placed in the same box.
2. Seal the sandwich boxes and incubate at 45°C for 2 hours in a shaking water bath (120 RPM).
3. After pre-hybridization, transfer filters to a separate sandwich box for each probe.
4. Add 0.1 ml of hybridization buffer for each square centimeter of membrane filter (one-tenth the volume used for pre-hybridization).
5. Add the probe to the hybridization buffer using a micropipette.
6. Seal each box and transfer to a 45°C shaking water bath.
7. Incubate 1 to 2 hours.
8. Transfer filters to shallow containers, such as sealable plastic food containers, for washing.

Comments: Hybridization reactions are generally performed in small volumes to increase probe concentrations. To assure that all accessible target sequences can hybridize, probes are added at stoichiometric excess over the "target" sequences immobilized on the membrane filters. The high concentrations of the probes drive the hybridization reactions to completion. The optimal temperatures for hybridization reactions represent a balance between increasing the rate of strand association and thermal dissociation of the hybrids. For most hybridizations, the optimal incubation temperatures are 15°C to 25°C lower than the dissociation temperatures (T_d) of the
hybrids. The dissociation temperatures of perfectly matched hybrids formed by synthetic DNA probes in 6X SSC are determined primarily by chain length and composition (G–C pairs which have three hydrogen bonds are stronger than A–T or A–U pairs, which have two hydrogen bonds), and can be roughly estimated from the following formula:

\[
T_d = 4^\circ C \times \text{(No. of G+C bonds)} + 2^\circ C \times \text{(No. of A+T bonds)}
\]

5.2.4 Washing the filters to remove unbound probes

Materials
1. Wash Buffers A and B (see 5.2.6).
2. Sealable plastic food containers, 200 ml capacity or larger.
3. Shaking water bath.

Procedure
1. Wash filters twice for 5 minutes at room temperature with shaking in 100 ml Wash Buffer A.
2. Wash filters twice for 5 minutes at 45^\circ C with shaking in 100 ml Wash Buffer A.
3. Wash filters twice for 5 minutes at 45^\circ C with shaking in 100 ml Wash Buffer B (Note: No SDS).

Comments: Non-specifically bound probes are removed by washing conditions described as being "stringent". Stringency is increased by raising the temperature and reducing the salt concentrations in the wash buffers. Both of these conditions disrupt the hydrogen bonds forming the base pairs of the hybrids.

Careful choice of the washing conditions can be used to select for hybrids having varying degrees of base-pair mismatch with the target sequences. For short probes, having less than 30 nucleotides, 3 to 4 mismatches with the target sequences virtually eliminate hybridization. Under certain conditions, even a mismatch at a single nucleotide position may be detected by a reduction in the thermal stabilities of the hybrids. Conditions described here have been optimized to distinguish Sabin vaccine-related isolates from wild polioviruses.
5.2.5
Detection of hybrids

Materials
1. Clean plastic sandwich box
2. Alkaline phosphatase substrate buffer
3. Nitro blue tetrazollium (NBT)
4. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) see 5.2.6

Procedure
1. Transfer each washed filter to a clean sandwich box.
2. To each box add 0.033 ml NBT and 0.025 ml BCIP in 7.5 ml
   alkaline phosphatase substrate buffer.
3. Incubate at room temperature in the dark for up to 4 hours.
   Check at 30 minutes for colour development.
4. Wash filters at room temperature in distilled H₂O. Air dry at
   room temperature.
5. Colour develops at sites of hybridization.

5.2.6
Reagents

HYBRIDIZATION BUFFER
6X SSC/0.1% Sodium Dodecyl Sulphate (SDS)/5X Denhardt's Solution
20X SSC .............................................................. 30 ml
10% SDS .............................................................. 1 ml
50X Denhardt's Solution ............................................. 10 ml
Distilled H₂O ......................................................... to 100 ml

WASH BUFFER A
1X SSC/0.1% SDS
20X SSC .............................................................. 50 ml
10% SDS .............................................................. 10 ml
Distilled H₂O ......................................................... to 1000 ml
WASH BUFFER B

1X SSC

20X SSC ................................................................. 50 ml
Distilled H₂O ........................................................... to 1000 ml

20X SSC

3.0 M NaCl/300 mM Sodium Citrate (ph 7.0)

Sodium chloride (NaCl F.W.58.4) ....................... 876 g
Sodium citrate (Na₃C₄H₅O₆·H₂O F.W.294.1) .......... 441 g
Distilled H₂O .......................................................... to 5000 ml

Weigh out each component and add 4000 ml of distilled water. Stir until dissolved. Titrate to pH 7.0 with a few drops of 10 N NaOH. Adjust volume to 5000 ml with distilled water. Dispense 1000 ml amounts into screw-cap bottles. Sterilize by autoclaving for 20 minutes.

DENHARDT’S SOLUTION (50X)

Ficoll ................................................................. 5 g
Polyvinylpyrrolidone .............................................. 5 g
BSA Pentax (Fraction V) ......................................... 5 g
Distilled H₂O ........................................................... to 500 ml

Sterilize by filtration. Store 25 ml aliquots at −20°C.

ALKALINE PHOSPHATASE SUBSTRATE BUFFER

100 mM Tris·HCl (pH 8.5)/100 mM NaCl/50 mM MgCl₂

Tris Base ................................................................. 1.21 g
NaCl ................................................................. 0.58 g
MgCl₂·6H₂O ............................................................ 1.02 g
Distilled H₂O ........................................................... to 100 ml

Adjust pH to 8.5 with HC1. Add 20 mg NaN₃ as preservative. Store at 4°C.
**NITRO BLUE TETRAZOLIUM (NBT)**

- Nitro Blue Tetrazolium ........................................ 375.0 mg
- Dimethylformamide ............................................. 3.5 ml
- Distilled H₂O ..................................................... 1.5 ml

Store 1 ml aliquots *in dark* at or below −20°C.

**5 BROMO-4-CHLORO-3-IODOLYL PHOSPHATE (BCIP)**

- BCIP ........................................................................ 250 mg
- Dimethylformamide ............................................. 5 ml

Store 1 ml aliquots *in dark* at or below −20°C.
6. DETERMINATION OF NEUTRALIZING ANTIBODIES TO POLIOVIRUSES

Tests for neutralizing antibodies are considered to be the most specific for determining the antibody response to poliovirus infections. Consequently they are invaluable for the serological surveillance of target groups of the population to ascertain their immune status to polioviruses.

Since current methods do not allow differentiation between antibodies against wild and vaccine strains, their use is no longer recommended in the routine laboratory diagnosis of patients with poliomyelitis.

6.1 SEROLOGICAL SURVEILLANCE OF POLIOVIRUS IMMUNITY

Immunity to poliovirus is measured by determining the ability of a human serum sample to neutralize the infectivity for cell cultures \textit{in vitro} of each of the three types of poliovirus. Neutralizing antibody to polioviruses can be demonstrated in either of two ways: (1) incubating a fixed amount of serum sample with a range of dilutions of the challenge viruses; (2) incubating a standard dose of virus, with dilutions of serum. The first method gives a quantitative estimate for the amount of antibody present. The second is somewhat less precise and depends to some extent on the avidity of the antibody. Nevertheless, it is this latter test that is most widely used in the diagnostic virus laboratory and allows the antibody level to be expressed as a titre.

Titration of the serum sample is simple, as the required serial dilutions can be carried out using one pipette, and the dilutions prepared in the wells of a microtitre plate. The same procedure \textit{must not} be used for preparing virus dilutions. Viruses, unlike serum, are particulate and frequently exist in clumps which are carried over in an unpredictable manner if a single pipette is used resulting in errors of a 1000-fold between the theoretical and observed concentrations of virus. Therefore, for each virus dilution step a change of pipette after transfer is absolutely essential.
Cell culture assays are technically more demanding than other methods of measuring antibody-antigen binding such as agglutination, immune precipitation, and ELISA. However, these latter techniques have not been found to be generally suitable as they measure both neutralizing and non-neutralizing antibody and the value of the latter in protection against poliomyelitis is unknown, and may be irrelevant.

The test to be described has been found to be convenient, but variations are possible and indeed may be necessary to accommodate local circumstances. The essential element is to control the test using challenge viruses whose serotype identities have been confirmed using reference antisera and, most importantly, each test must include a reference serum; this may be an in-house standard, i.e. any human serum available in large amounts for long-term use, the titre of which has been established against a national or international reference antiserum.

The method describes the use of microtitre plates with flat-bottomed wells, and the preparation of the serial serum dilutions by means of an automatic pipette. This is most conveniently achieved for a large number of samples by using a multichannel pipette with disposable tips. Alternatively, 1 ml graduated pipettes may be used to prepare dilutions of the test sample in test tubes.

No attempt has been made to give a figure for the cell concentration per test well. This is a matter of experience according to local conditions and cell vigour, but it has been found that harvesting a confluent cell sheet from a 75 cm² flask into 20 ml of medium provides approximately 10⁶ cells per 0.1 ml and is a suitable inoculum for a microtitre well; other workers prefer up to 5 x 10⁴ cells/0.1 ml. Once a routine has been established, it should not always be necessary to count each time the cells in the cell suspension. Experience should tell whether the culture to be harvested will provide the anticipated cell yield.

After inoculation, the plates may be sealed with nontoxic adhesive film and incubated at 36°C in a normal incubator, or covered with a lid and incubated in a CO₂ incubator. Either method is equally satisfactory.

The periods used for the incubation of serum/virus mixtures vary in different virus laboratories. Where the immune status of individuals is being determined, the detection of even low levels of neutralizing antibody is important. Long incubation periods, i.e. 3 hours at 36°C and overnight at 0°C to 8°C can increase by fourfold to eightfold the detection of neutralizing antibody with the added benefit of increased reproducibility of the tests. Standardized samples of sera, to act as reference points, must be included in each batch of tests.
The essence of successful laboratory practice is attention to
detail in preparing and performing the test and constant monitoring
of the results to detect trends that may warn of impending difficul-
ties. Points to note in this test system are differences from previ-
ously established norms for reference viruses and sera, delays in
achieving a confluent cell layer or complete viral CPE, and unusual
pH changes in the cell culture medium.

6.1.1 Materials required

Serum to be tested

The minimum amount of serum needed for an assay is 0.1 ml. A
finger prick will yield 0.3–0.4 ml of blood which may be collected in
a small stoppered sterile glass or plastic tube or on filter paper discs
(see section 3.1.2). Before testing, the serum is diluted (generally
1/4) and inactivated at 56°C for 30 minutes. Because of increasing
concern that serum samples, particularly for surveillance studies,
may contain human immunodeficiency virus (HIV) it is becoming
common practice to heat-inactivate all serum samples before storage
at −20°C. This heat treatment can seriously reduce any IgM which
may be present in the samples; new techniques for the assay of
poliovirus IgM are imminent. This should be borne in mind when
preparing precious serum collections for storage (e.g. sera from
infants).

Reference antiserum

A reference antiserum of known neutralizing activity must be in-
cluded in each test to control reproducibility. Requests for interna-
tional reference anti-poliovirus sera should be submitted to CDS/
EPI, WHO, Geneva. In-house, working reference sera can usually be
obtained by pooling serum samples from adults who have recently
received a booster dose of oral polio vaccine, or from patients with
paralytic poliomyelitis. Potency of these sera must be determined by
assay in parallel with the international reference antisera.

Poliovirus challenge samples

Types 1, 2 and 3 of the Sabin strains of known titre are diluted to
a dose of 100 TCD₉₀/0.025 ml and in sufficient volume for the size of
the test. Requests for the seed poliovirus strains of each type (Sabin
strains) should be submitted to CDS/EPI, WHO, Geneva. Seed
strains should be stored at −20°C or below. These viruses should be
used for the preparation of virus stocks of reference strains which
will be held within the laboratory (see Annex 4).

> GREAT CARE MUST BE TAKEN IN THE PREPARATION
> OF THESE REFERENCE STOCK VIRUSES.
**Hep-2 (Cincinnati) cells**

This cell line is recommended for use in serological surveys. The cells should be suspended in culture medium at a concentration found to produce a confluent cell sheet within 2–3 days in a microtitre well.

**Cell culture medium**

Eagle's growth medium (see Annex 2) containing sufficient foetal calf serum to provide minimal cell growth has been found to be suitable. The intention is to permit the early growth of a cell sheet but without overgrowth of cells with consequent excess production of acid during the 3–5 days' observation period. Serum and virus should be diluted in maintenance medium (see Annex 2).

**Trypsin**

To prepare the cell suspension see section 7.3.

**Microtitre plates (flat bottomed)**

Sufficient for the number of samples to be tested.

**Micro-pipettes (with disposable plastic tips)**

Capable of delivering 0.025 ml. For making the serum dilutions, it is convenient to have pipettes that suck up and deliver 0.025 ml and for delivering the virus challenge dose, to have pipettes with a reservoir so that a series of 0.025 ml amounts can be delivered in quick succession. Volumes of 0.05 ml are equally satisfactory but require larger quantities of serum sample and virus. Non-sterile new tips direct from the manufacturer may be used without sterilization provided they are kept covered in a clean area and used as if they were sterile.

**Equipment**

No special equipment is necessary beyond that normally available in a basic virus laboratory.

**6.1.2 Microneutralization procedure**

Because it is too time-consuming and wasteful in reagents and reference antisera to perform poliovirus neutralizing antibody surveys by macrotechnique using tube cultures, microtechnique, using 0.025 ml volumes in wells of microtitre plates, is recommended.
Two wells of each of 3 microtitre plates (one plate for each of type 1, 2 and 3 polioviruses) are inoculated with each serum sample and 2 wells per serum to act as serum controls; therefore, a minimum of 0.2 ml of diluted serum sample (i.e. 8 x 0.025 ml) is required for each test. It is prudent to retain sufficient samples for at least one repeat test. Seven dilutions of 5 serum samples plus a homotypic reference antiserum (which must always be used to control each test) can be tested on each plate and it is advisable to keep one plate for each virus type, therefore 3 plates will accommodate 5 serum samples and an extra control plate is required to confirm that the challenge virus was of the required dose and the cells monolayered at an acceptable rate.

If each plate is covered with a sterile lid/cover between each operation and if normal good laboratory practice is followed, the procedure described below can be conducted on the open bench, without problems of bacterial contamination. This is true even in laboratories without controlled ventilation and in conditions of high humidity and temperature as long as a room free of draughts is available. If bacterial/fungal contamination continues to be a problem, use the Class 1 safety cabinet (see Section 8.3) with the air-flow switched off. Thoroughly wash down the cabinet with Cidex (i.e. activated, 2% gluteraldehyde, non-corrosive) before and after use.

Procedure

1. Prepare 1/4 dilution of each serum sample to be tested in maintenance medium; inactivate at 56°C for 30 minutes.

2. Prepare sufficient virus challenge suspension in maintenance medium for the number of samples to be tested. For this, dilute each virus (poliovirus types 1, 2 and 3) to contain 100 TCD<sub>50</sub>/0.025 ml; each plate requires approximately 2.5 ml of diluted challenge virus.

3. Prepare the back titration of virus in tubes/bottles using a separate graduated pipette at each dilution stage. Titrate each virus from 100 TCD<sub>50</sub> as used in the test ("undiluted"), a further 3 tenfold steps using maintenance medium as diluent.

4. Unwrap sufficient plates to accommodate the number of samples to be tested and label them with a code to identify plate, virus used and samples tested. Protect plates with lid supplied or cover with sterile foil.

5. At this stage, prepare a similarly coded sheet of paper, recording precisely the corresponding plate code, cells, virus and serum samples used and date of test. The final readings are recorded on this document and filed for later access if required (see Figure 19).

6. Add 0.025 ml of maintenance medium to each well in plates to be used for the assay of serum.
7. Add 0.025 ml of serum sample at 1/4 to wells A1 and A2 of each of 3 plates (i.e. the type 1, 2 and 3 virus plates, Figure 19) and to wells H1 and H2 of the polio type 1 plate only; these latter wells will serve as serum toxicity controls.

8. Add 0.025 ml of the second serum sample at 1/4 to wells A3 and A4 and to H3 and H4. Repeat this process for the other three serum samples. Thus all wells in Rows A1–10 and Rows H1–10 of the polio 1 plate have serum samples at 1/4 added to them. For the polio 2 and polio 3 plates only wells in Rows A1–10 have serum samples at 1/4 added to them.

9. Add 0.025 ml of diluted homotypic reference antiserum (of previously determined titre) to wells of Row A11 and A12 and to wells of Row H11 and H12 of the poliovirus 1, 2 and 3 plates, respectively.

10. Prepare serial dilutions of all these sera using a micropipette with disposable tips to mix and withdraw 0.025 ml volumes from each well in Row A and transfer these to the wells in Row B. Repeat this until Row G (7th row) is reached when the final volume is mixed and 0.025 ml discarded. Similarly for Row H, using a fresh tip for each serum sample, the volumes are mixed and 0.025 ml discarded.

11. Fit fresh tips to the pipette and treat the second and third plates similarly (Rows A–G) and Row H11–12 for reference antisera. Each of the 5 test serum samples have now been diluted from 1/8 to 1/512 in each of the 3 plates and the serum toxicity controls at dilutions of 1/8 in the first plate; the reference antisera of known titre (the dilution range of which is usually 1/80 to 1/5120) must be included in these tests.

12. Add 0.025 ml of poliovirus type 1 challenge virus containing 100 (range 32–320) TCD₅₀ of virus to every well of the type 1 plate except Row H (the serum control wells), which instead receives 0.025 ml of maintenance medium. Repeat this with the type 2 and type 3 polioviruses, i.e. adding 0.025 to all the wells except Row H11 and H12 wells, which instead receive 0.025 ml of maintenance medium.

13. Back titrate each of the three 100 TCD₅₀ challenge virus suspensions a further 3 steps, using maintenance medium as diluent and a fresh pipette for each dilution step.

14. Add to each of wells A1–A4 of the control plate 0.025 ml of poliovirus at its highest (10⁻²) dilution. To the next row (B1–4) add the 10⁻² dilution, to row C1–4 the 10⁻¹ dilution and to row D1–4 the challenge virus suspension as used in the test.
Repeat these steps for poliovirus types 2 and 3 virus dilutions. Thus each challenge virus occupies a block of 4 x 4 wells (Figure 19).

15. To all these wells add 0.025 ml of maintenance medium (to equate to the serum volume).

16. Cover with a lid or tin foil, rock plates very gently by hand to mix contents and incubate for 3 hours at 36°C and at 0°C to 8°C overnight. To avoid evaporation of moisture from plates during this long incubation period it is advisable to stack plates carefully and wrap with tin foil.

17. The following day prepare the cell suspension (i.e., HEP-2) according to the previously established procedure.

18. Add 0.1 ml of cell suspension (1-2 x 10⁴ cells/0.1 ml) in growth medium to every well containing serum/virus mixtures, the serum toxicity controls and the virus titrations.

19. Add 0.1 ml of cell suspension to a further 8 wells in the control plate and to these add 2 x 0.025 ml volumes of maintenance medium (to equate to serum/virus mixture volumes); these 8 wells serve as cell controls.

20. Seal the plates with adhesive tape and incubate at 36°C.

21. Examine plates every day (using an inverted microscope) to confirm that the cells are monolayering, that the sera are not toxic, and the challenge viruses are showing CPE. It is usual to take the definitive readings on the third day by which time CPE is complete in the virus control wells.

22. Record whether CPE is present or not and calculate the endpoint of neutralization using the Kärber formula (and transcribing the serum dilutions into log terms), i.e. the serum antibody titre is the highest dilution of serum which protects 50% of the cultures against 100 TCD₅₀ of challenge virus. Antibody titre results are normally expressed as reciprocals (e.g. titre of 512, not 1/512).

23. Confirm that the challenge virus titres were 100 (range 32-320) TCD₅₀ using the Kärber formula (see section 4.1.6).

24. If toxicity of serum is evident at 1/8 dilution, re-titrate sera to determine at which dilution toxicity absent. If toxicity is still evident at dilutions higher than 1/64, the serum is unsuitable for poliovirus antibody determinations.

25. If reference antiserum titres are not within the range recommended by the supplier, repeat tests.
FIGURE 19
MICRONEUTRALIZATION PROCEDURE FOR DETERMINING NEUTRALIZING ANTIBODIES TO
POLIOVIRUSES

Poliovirus type 1 Plate

Serum dilutions:
1/8
1/16
1/32
1/64
1/128
1/256
1/512
Serum controls

1/80
1/5120

Conclusions:
Serum titre against Polio 1 = >512 512 8 16 96 160
Serum/antiserum controls = no toxicity
Antibody titre of Polio 1 Reference Antiserum = within accepted range

Poliovirus type 2 Plate

Serum dilutions:
1/8
1/16
1/32
1/64
1/128
1/256
1/512

1/80
1/5120

Conclusions:
Serum titre against Polio 2 = 256 128 128 32 64 320
Antiserum controls = no toxicity
Antibody titre of Polio 2 Reference Antiserum = within accepted range
FIGURE 19 (Continued)
MICRONEUTRALIZATION PROCEDURE FOR DETERMINING NEUTRALIZING ANTIBODIES TO POLIOVIRUSES

**Poliovirus type 3 Plate**

<table>
<thead>
<tr>
<th>Serum Dilution</th>
<th>Serum 1</th>
<th>Serum 2</th>
<th>Serum 3</th>
<th>Serum 4</th>
<th>Serum 5</th>
<th>Polio 3 Reference Antiserum</th>
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<tbody>
<tr>
<td>1/8</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>1/16</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>10</td>
<td>11</td>
<td>12</td>
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<td>1/32</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1/80</td>
</tr>
<tr>
<td>1/64</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1/80 antiserum control</td>
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<tr>
<td>1/128</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>1/5120</td>
</tr>
<tr>
<td>1/256</td>
<td></td>
<td></td>
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<td></td>
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<td>1/512</td>
<td></td>
<td></td>
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</tbody>
</table>

**Conclusions:**
- Serum titre against Polio 3 = 512, 64, 256, 128, 256, 640
- Antiserum controls = no toxicity
- Antibody titre of Polio 3 Reference Antiserum = within accepted range

**Virus and Cell Control Plate**

<table>
<thead>
<tr>
<th>Polio 1</th>
<th>Polio 2</th>
<th>Polio 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>100 TCD&lt;sub&gt;50&lt;/sub&gt; x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>100 TCD&lt;sub&gt;50&lt;/sub&gt; x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>100 TCD&lt;sub&gt;50&lt;/sub&gt; x 10&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>100 TCD&lt;sub&gt;50&lt;/sub&gt; undiluted</td>
<td></td>
<td></td>
</tr>
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</table>

**Conclusions:**
- Titres of Polioviruses 1, 2 and 3 as used in the test = within the range 32–320 TCD<sub>50</sub>
- Cell controls = normal growth

- = CPE
- = no CPE
- = unused wells
7.
CELL CULTURE TECHNIQUES AND THEIR APPLICATION TO THE LABORATORY DIAGNOSIS OF POLIOMYELITIS

The quality of cell cultures used in the virological investigation of poliomyelitis is important for the standardization of poliovirus isolation and its characterization. This chapter provides some guidance on the correct handling of cell cultures used for virus diagnostic procedures. More detailed information is provided in the manual of basic techniques used in the culture of animal cells written by Freshney.\textsuperscript{33}

7.1
BASIC REQUIREMENTS FOR CELL CULTURE

Although the laboratory space and the equipment necessary for the handling of cell cultures in a virus diagnostic laboratory can be reduced to relatively modest expense, a minimum of facilities is required. These are listed in Figure 20.

FIGURE 20
MINIMUM EQUIPMENT FOR CELL CULTURE

- Stainless steel autoclave with clean source of steam
- Drying and sterilizing ovens
- Refrigerators, 0°C to 8°C
- Deep freezers, -20°C and -70°C
- Gaseous/liquid nitrogen storage facilities
- Source of pure (cell culture grade) water
- Cell culture incubator
- Racks for cell culture tubes
- Cell culture quality glassware and/or plastic ware
- Stoppers (caps) for sealing cell cultures
- Coarse and precision scales
- pH meter
- Micropore sterilizing filtration systems
- Magnetic stirrer
- Low-speed centrifuge for cell spinning
- Vacuum pump
- Microscopes, standard and inverted with x4 and x10 objectives
Briefly, a number of important conditions must be satisfied for successful cell culture. The temperature should be 36°C or lower. The pH for growth should be between 7.2 and 7.4. Fortunately, most cells can survive a wider pH range (6.8–7.8) and temperatures of 33°C or lower. The osmotic pressure of cell medium is critical and can be severely affected by increases in glucose concentration. Inorganic ions, e.g. sodium, potassium, calcium, magnesium, iron, carbonate and phosphate are essential for cell survival. Cell cultures require amino acids and vitamins in the form of serum or as a synthetic mixture. Glucose is usually added as a source of usable carbohydrate. Both oxygen and carbon dioxide are essential and are provided either as a mixture of CO₂ and air supplied to the culture vessel or by sealing the vessel tightly to retain the CO₂ produced by cell metabolism.

**ASEPTIC TECHNIQUE AND THE SCRUPULOUS PREPARATION OF GLASSWARE, MEDIA AND REAGENTS ARE ESSENTIAL.**

Because of the risks of contamination and cross-infection, cell culture in the virus diagnostic laboratory is carried out in closed vessels, usually stoppered/capped tubes and flat-sided bottles. Cultures are initially set up in growth medium supplemented with serum. Once the cells have formed a confluent monolayer, cultures are changed to maintenance medium which is designed to maintain cultures in a healthy state for as long as possible without stimulating growth; this is usually achieved by reducing the serum content.

**7.2 SELECTION OF CELL CULTURE SYSTEMS**

Many cell culture systems support the growth of polioviruses and other enteroviruses.

WHO RECOMMENDS THAT ALL SPECIMENS SUSPECTED OF CONTAINING POLIOVIRUSES SHOULD BE INOCULATED INTO THE FOLLOWING TWO CELL LINES:

1. RD CELLS, DERIVED FROM A HUMAN RHABDOMYOSARCOMA, AND
2. HEp-2 (CINCINNATI) CELLS, DERIVED FROM A HUMAN EPIDERMAL CARCINOMA.

THE SELECTION OF ONLY TWO CELL LINES FOR THE LABORATORY DIAGNOSIS OF POLIOMYELITIS PERMITS THE STANDARDIZATION OF TECHNIQUES AND THE COMPARABILITY OF RESULTS AMONG VARIOUS VIRUS LABORATORIES.
Reference laboratories are advised to obtain cell cultures from the official collections. Requests for these cell lines should be submitted to CDS/EPI, WHO, Geneva.

National poliomyelitis laboratories can in turn apply to their nearest reference laboratory for supplies of these cell lines. For additional information on the origins of RD and HEp-2 cell lines see Annex 3.

7.3

PREPARATION OF CELL CULTURES

In handling cell cultures, laboratory personnel must be concerned with not only preventing microbial contamination of the cultures, but also with avoiding contamination of the working environment with cell culture materials. All cultures must be considered potentially hazardous whether inoculated or uninoculated. All cultures and their fluids should finally be decontaminated by autoclaving. Cross contamination between different cell types, especially continuous cell lines is an ever present hazard. To obviate this, DIFFERENT CELL TYPES SHOULD NEVER BE PROCESSED AT THE SAME TIME. All working areas should be thoroughly washed down between preparation of different cell types. The necks of all culture flasks/tubes should be passed through a Bunsen burner flame after opening and again before closing. Perform all cell culture work in a draught-free room (or in a clean, still-air cabinet).

Cell culture media employed in virology can be divided into two main categories, growth media and maintenance media. These contain various combinations of synthetic and natural ingredients (see Annex 2).

Growth media (GM), high in serum content, promote rapid cell growth. After a monolayer has formed and prior to inoculation with virus, the growth medium is removed and replaced with maintenance medium.

Maintenance media (MM), low in serum content, are intended to keep the cell cultures in a slow, steady state of metabolism during the period of viral replication.

Foetal calf serum is the serum of choice – it is good for promoting cell growth and it lacks viral inhibitors. If serum from other sources is used, it must be pretested for the presence of inhibitors to the viruses being studied. All sera for cell culture use are inactivated at 56°C for 30 minutes.

Either of the following two methods (Method A and Method B) can be used to prepare cultures of RD or HEp-2 cell lines, but Method A requires fewer manipulations.
Method A (see Annex 2 for composition of various reagents)

1. Decant growth medium (GM) from the cell culture bottle and gently wash the confluent cell layer in PBS (without calcium and magnesium components).

2. Add 0.25% trypsin solution (or equal parts of 0.25% trypsin and 1:5000 Versene solution) in PBS (without calcium and magnesium components) to the monolayer; leave in contact with the cells for 15-30 seconds and decant.

3. Incubate at 36°C until all the cells detach from the bottle (check with inverted microscope).

4. Resuspend cells in GM to desired concentration based either upon counting the cells or upon an arbitrary “split” of 1:2 or greater. The serum in the GM halts the action of the trypsin still present. Seed fresh culture vessels/tubes.

5. Change tubes to maintenance medium (MM) when nearly monolayered (2–3 days). Bottles are usually subcultured every 5–7 days, a 1:8 “split” being adequate.

Method B

1. Decant growth medium (GM) from the cell culture bottle and gently wash the confluent cell layer with PBS (without the calcium and magnesium components).

2. Add 0.25% trypsin solution (or equal parts of 0.25% trypsin and 1:5000 Versene solution) in PBS, sufficient to cover the cell monolayer.

3. Incubate at 36°C until all the cells detach from the bottle (check with inverted microscope).

4. Centrifuge the cell suspension at 100g for 10 minutes and remove the supernate.

5. Resuspend cell pellet in GM to desired concentration based either upon counting the cells or use a 1:2 to 1:8 “split” and seed fresh culture vessels/tubes. Change tubes to maintenance medium (MM) when nearly confluent (2–3 days). Bottles are usually subcultured every 5–7 days, a 1:8 “split” being adequate.

Alternatively, step 2 of methods A and B can be carried out using Versene solution alone.

KEEP A CAREFUL RECORD OF ALL THE PASSAGES CARRIED OUT AFTER RECEIPT OF ORIGINAL RD AND HEP-2 (CINCINNATI) CELL LINES. LABEL EACH CULTURE BOTTLE WITH CELL TYPE, PASSAGE NUMBER AND DATES OF SEEDING BOTTLE AND ANY MEDIUM CHANGES.
Cell counting

Accurate numbers in a cell suspension can be calculated by counting the cells in a haemocytometer (e.g. improved Neubauer); it is important to disperse the cells thoroughly by pipetting up and down.

1. Dilute 0.1 ml of the cell suspension in 0.9 ml of trypan blue (i.e. 0.1% solution in PBS); non-viable cells are stained blue.
2. Mix well with fine Pasteur pipette and aspirate sufficient volume to fill haemocytometer immediately.
3. Count cells in each corner square of both chambers, omitting cells lying on the lines (see Figure 21).
4. If cell “clumping” (aggregation) is observed, discard and resuspend original cell suspension.
5. Calculate the mean count of the cells in the total of 8 corners.
6. Calculate the total number of cells in the suspension using the following formula:

\[
N = \frac{m \times tb \times V \times 10^4}{V} = m \times tb \times 10^4 = \text{cell concentration per ml}
\]

7. Calculate the dilution factor (d) to obtain the working cell concentration per ml (C).

\[
d = \frac{C}{m \times tb \times 10^4}
\]

Example: \( m = 24 \); \( tb = 10 \); \( C = 2 \times 10^5 \)

Then: \( d = \frac{2 \times 10^5}{24 \times 10^4} = \frac{2}{24} = \frac{1}{12} \)

The working concentration can be obtained by mixing 1 volume of the original cell suspension with 11 volumes of the growth medium.

8. Dispense the cells in growth medium, seed into bottles/tubes and incubate at 36°C.
FIGURE 21
CELL COUNTING USING A HAEMOCYTOMETER (BASED ON FRESHNEY13)

a) Cell suspension

b) 0.1 mm deep

c) 1 mm

d) Count all cells within corner areas (unshaded)
Most continuous cell lines monolayer well. Cross contamination of one cell line with another is a constant hazard; never process different cell lines at the same time and wash down bench with disinfectant (e.g. Cidex) between each cell passage procedure. Mycoplasma (and L-form) infection of continuous cell lines is almost unavoidable. Alteration in appearance and growth rate of cells and rapid development of acidity of growth medium often indicate gross mycoplasma infection of the cells which can seriously affect their virus susceptibility. When this occurs fresh stocks of cells should be obtained.

It is important to monitor the sensitivity of these cell lines to polioviruses after 8 to 10 passages; the titration of standard reference polioviruses 1, 2 and 3 is recommended.

7.4

PRESERVATION OF CELL CULTURES

It is possible to maintain stocks of cells in a viable state for long periods at low temperatures by the addition of preservatives such as dimethyl sulphoxide (DMSO) to the cell growth medium. The essential features of the method are to freeze the cells slowly, keep them at a temperature below -70°C while frozen and to thaw them rapidly ready for the preparation of fresh cell culture stocks. The method is as follows:

Freezing

1. Use only bottle cultures of cells that are in a healthy state.
2. Remove cells by trypsinization-Versenation as described earlier. Use sufficient bottles to yield a minimum of 4 x 10^6 cells/ml.
3. Resuspend cells in growth medium; centrifuge at 100g for 10 minutes.
4. Discard supernate and resuspend thoroughly the cell pellet in growth medium containing 10% dimethyl sulphoxide.
5. Dilute 0.1 ml cell suspension in trypan blue and count cells in a haemocytometer.
6. Adjust cell concentration to 4–8 x 10^6 cells/ml in growth medium containing DMSO.
7. Dispense in 1 ml or 2 ml volumes in clearly labelled (cell type, passage number and date of freezing) screw-capped vials, or polypropylene-sealed/glass-sealed ampoules. The former are suitable for storage in gaseous nitrogen, the latter for storage in liquid nitrogen.

**WHEN USING GASEOUS PHASE OR LIQUID NITROGEN CONTAINERS, VIZORS AND HEAVY-DUTY GLOVES MUST BE WORN TO AVOID INJURIES FROM NITROGEN SPLASHES OR EXPLOSION OF IMPERFECTLY-SEALED AMPOULES.**

8. Freeze vials/ampoules slowly. Ideally the temperature should drop at 1°C/minute. Place vials/ampoules in the special container which holds them in the gaseous phase of the liquid nitrogen vessel; a formula is supplied by the manufacturer for the level vials/ampoules are held, number to be stored and length of time required to achieve this temperature drop (see Figure 22). Alternatively, place vials/ampoules wrapped in cotton wool in a polystyrene container with a wall thickness of ~15mm and place this in the −70°C freezer overnight.

9. Transfer the vials to the gaseous phase (−150°C to −180°C) and polypropylene or glass-sealed ampoules to the liquid nitrogen (−196°C) storage containers (See Figure 23). For long-term storage of cells (i.e. a period of years) store cells in liquid nitrogen.

**FIGURE 22**
**APPARATUS FOR CONTROLLED COOLING OF CELLS (FROM FRESHNEY**

The retaining ring is used to set the height of the ampoules within the neck of the liquid N_{2} container and thus the rate of freezing.
Thawing

1. Remove vial/ampoule from gaseous/liquid nitrogen and transfer immediately to a water bath or a beaker of water at 36°C.

2. When contents are completely thawed, wipe outside of vial/ampoule with alcohol to reduce bacterial contamination, transfer cell suspension to culture bottle. Add very slowly, drop by drop, sufficient growth medium for the production of a cell monolayer. The viability of the thawed cells is severely affected if growth medium is added rapidly at this delicate stage.

3. Incubate bottle overnight at 36°C.

4. Carefully decant medium (to get rid of DMSO present) and add fresh growth medium.

5. Alternatively spin thawed cell suspension (made up to 10 ml slowly with growth medium) at 80g for 10 minutes; discard supernatant and resuspend cell pellet in sufficient growth medium for production of a cell monolayer and incubate at 36°C.

An outline of the procedures used for the freezing and thawing of cells using polypropylene-sealed ampoules is illustrated in Figure 24.
FIGURE 24
PREPARATION OF CELLS FOR FREEZING USING POLYPROPYLENE-SEALED AMPOULES AND SUBSEQUENT RECOVERY AFTER STORAGE (BASED ON FRESHNEY\textsuperscript{12})

1. Trypsinize healthy cell layer.

2. Resuspend cells at $4 \times 10^4 \text{cells/ml}$ in growth medium containing DMSO.

3. Using a Pasteur pipette, add cell suspension to sterile, labelled ampoule. Avoid wetting inside neck of ampoule.

4A. Flame neck of ampoule until opaque colour becomes transparent (1–2 secs).

4B. Tightly squeeze neck twice with flat forceps until opaque colour returns. Check seal perfect by inverting ampoule and squeezing lightly.

5. Place sealed ampoules on rods; transfer to insulated box (or place ampoules in neck plug apparatus, see Figure 22).

6. Place insulated box at $-70^\circ C$ and allow ampoules to cool slowly ($-1^\circ C$/minute) for minimum of 2 hours.
FIGURE 24 (Continued)
PREPARATION OF CELLS FOR FREEZING USING POLYPROPYLENE-SEALED AMPOULES AND SUBSEQUENT RECOVERY AFTER STORAGE (BASED ON FRESHNEY\textsuperscript{19})

7. Quickly transfer rods to cannisters.

8. Quickly place cannisters in liquid N\textsubscript{2} container.

9. Record details of cell source, passage history and location in liquid N\textsubscript{2} container.

10. Thaw ampoule rapidly at 36°C in a covered beaker (or waterbath). Remove ampoule, wipe with alcohol, and cut open neck end with sterile scissors.

11. Seed culture flask with minimum 1 ml of cell suspension.

12. Very slowly – add growth medium with constant gentle rocking of culture flask.
7.5

ESTABLISHMENT OF CELL BANKS

The RD and HEp-2 (Cincinnati) continuous cell lines recommended for use by all virus laboratories studying poliomyelitis have been well characterized as regards species identity and lack of contamination by bacteria, mycoplasma and infectious virus.

Those poliomyelitis reference laboratories which propose to set up a master cell bank (MCB) are strongly advised to obtain their original cell cultures of RD and HEp-2 (Cincinnati) directly from official sources. Requests for these rigorously passaged cells should be submitted to CDS/EPI, WHO, Geneva. Using scrupulous laboratory techniques of cell culture passage and storage, these reference laboratories can supply cell cultures directly to the national laboratories which come within their ambit. If they have the facilities, national laboratories are also encouraged to store supplies of these cells, in gaseous or liquid nitrogen, i.e. they will act as the working cell bank (WCB). Figure 25 outlines the formation of the MCB and WCB.

FIGURE 25

ESTABLISHMENT OF A CELL BANK

*Requests for cell lines held in official collections should be submitted to CDS/EPI, WHO, Geneva.
The advantages of initiating a cell bank are: (1) the expense of transport of original cell lines from official sources is minimized; (2) the reference laboratory acts as the MCB repository of well-controlled cell culture stocks; (3) the national laboratory is nearer a supply of good viable cell cultures held at the MCB, but is also encouraged, if the facilities are available, to be self-supporting by producing and storing cells in its own bank (WCB). Thus, from 1 or 2 original bottles of stock cultures, both MCB and WCB can, theoretically at least, be self-sufficient in approved continuous cell culture systems for some years. It is advisable that cell stocks be monitored for their continued susceptibility to poliovirus infection.

When passaging these cell lines for eventual storage in cell banks, several criteria must be observed:

1. Check that the identity of the cells received at the laboratory corresponds to those ordered.

2. Initiate a record card listing cell type, source of cells, passage number and date received at the laboratory. Keep a detailed record of all subsequent culture procedures carried out at the laboratory.

3. *Never process different cell types simultaneously;* ideally process one day apart.

4. Process cell cultures in clean areas distant, if possible, to those where routine cell culture work or virus isolation studies are being carried out. This minimizes the risk of contamination by mycoplasma and extraneous viruses.

5. Keep all cell culture flasks firmly sealed; open these one at a time only when absolutely necessary; pass necks of flasks through the flame of a Bunsen burner after opening and again before closing.

6. Ensure all growth media used are sterile and that the serum used in their composition has been inactivated at 56°C for 30 minutes.

7. Label all culture flasks clearly with cell type, passage number and dates of passage and medium changes.

8. *Never,* if adding (or replacing) growth medium, touch the neck of the culture flasks with the bottle containing the growth medium or use the same pipette for transfer of growth medium to each bottle. Ideally, aliquot the amount of growth medium required for each culture bottle; store the remainder at 0°C to 8°C.

9. Because of the ever-present possibility of mycoplasma contamination of cell cultures, even in well-equipped laboratories, sub-culturing of cells beyond 15 passages is not recommended.

10. All monolayered cell cultures should be in a healthy state before using for cell bank purposes. Healthy cell cultures are those which have monolayered at their normal rate, have clear growth medium, and are not excessively yellow (i.e. acid).
8. LABORATORY BIOSAFETY

Good laboratory technique is the best guarantee of safety. Informed awareness of potential hazards leads to acceptably safe procedures which affect the worker, his/her colleagues, other laboratory personnel and the work itself from cross-infection and contamination. Each laboratory should devise its own code of practice which should be periodically reviewed and revised. An example of a basic code of practice is given in Figure 26.

The immunization history of all personnel commencing work in a virus laboratory should be recorded on a personal information card. If a full course of polio immunization has not been completed or this is in doubt, this should be instigated; otherwise a booster dose is sufficient.

8.1 NATURE OF INFECTIOUS RISKS

All specimens submitted for virus investigation may harbour not only viruses, but also pathogenic bacteria or parasites. In order to infect man, these agents must gain access to the body by inhalation, ingestion, through broken skin or through the conjunctiva or other mucous membrane. Techniques which minimize these risks should be practised.

1. Aerosols

   These are produced in the course of many laboratory procedures, e.g., pouring, pipetting, homogenization. Not only is the worker at risk, cross-infection or contamination of specimens and cell cultures is also a strong possibility.

2. Oral infection

   This can result from the now unacceptable technique of mouth-pipetting. Eating, drinking, smoking and applying cosmetics in work areas, i.e. known sources of infection, should be prohibited. Unwashed, contaminated hands are another hazard; hand washing between each laboratory procedure is essential.

3. Eye infection

   The eye presents a large receptive target for splashes, airborne infection and rubbing with infected fingers and should be protected by appropriate shielding of the work or the eye and careful technique.
8.2
HAZARDOUS PROCEDURES

Accidents are usually due to carelessness and lack of forethought. Breakages, leaks and spills should be minimized by avoiding the use of defective, cracked, chipped containers, by firm closures or sealing and by placing tubes and bottles in racks for support. Do not overfill bottles/vials to be frozen as the contents expand and can cause breakage. The tips of sharp instruments should be kept covered in sterile tubes while not being handled. Gloves can protect the skin from contamination, but not from accidental needlestick. The disposal of sharp instruments and syringes/needles should be planned to avoid accidental pricks/cuts to oneself and others.

Hypodermic needles and syringes should not be used as substitutes for automatic pipetting devices in the manipulation of infectious fluids.

1. Breakage of cell culture tubes
   Cuts due to tube breakage while handling cell cultures, particularly when stoppering tubes, are common in cell culture and virus isolation procedures. Tubes should be flawless, strengthened by a rolled rim and stoppers inserted gently but firmly to give a good air-tight closure. Alternatively, tubes with screw-caps may be used.

2. Pipetting
   Fluid transfers can cause dangerous aerosols and spills. Vigorous pipetting, causing frothing and bubbling of fluids, must be avoided. Fluid manipulation should be slow and gentle using a rubber teat or bulb (propipette) or automatic pipette. NEVER PIPETTE BY MOUTH.

3. Grinding, homogenization
   Aerosols are generated during these procedures which should preferably be carried out using a Class I safety cabinet. After homogenization, a period for the aerosol to settle should be allowed to elapse before opening the apparatus. When tissues are ground, the grinder should be held in a wad of absorbent material in a gloved hand.

4. Centrifugation
   The potential hazards in centrifugation are obvious, i.e. aerosol dispersion due to leakage of over-filled or insecurely capped containers or their breakage due to the use of flawed containers or careless balancing of contents. Anglehead centrifuges are no longer considered safe. Small, modern, horizontal-head centrifuges with lids, which cannot be opened during operation, are now recommended. The use of sealable plastic or nitrocellulose tubes effectively reduces hazards associated with centrifugation.
FIGURE 26
BASIC CODE OF PRACTICE IN THE VIRUS LABORATORY

**Clothing**
- Cover your clothes with protective gowns and/or plastic aprons.

**Hair**
- Long hair should be held back away from face and machinery.

**Food, Drink, Smoking, Applying Cosmetics**
- These are prohibited in laboratory areas.

**Mouth**
- NEVER mouth-pipette any materials, infected or uninfected. Do not put pens/pencils in mouth, nor lick labels.

**Hands**
- Keep unwashed hands/fingers away from mouth and eyes.
  - Always wash your hands after each laboratory procedure, and before leaving laboratory.

**Working Bench**
- If available, cover working area with “Benchkote” to limit splashes from spills. Keep required reagents handy. Support bottles, tubes, etc. to avoid spillage. Keep containers closed and working tops covered when not in use. Clear up and wash working area with Cidex (activated, 2% glutaraldehyde) when finished. Clearly label all specimens and reagents.

**Disposal of Infected Materials, Equipment, etc**
- Autoclave or place in Chlorox or hypochlorite solutions as appropriate (see Figure 27).

**Spills and Accidents**
- Report these to a senior member of the laboratory and clear up speedily. Personal injuries must be dealt with immediately.

**Absence from Work**
- Inform laboratory and tell doctor where you work.

**Rest Areas**
- Never wear laboratory gowns or take infected materials into these areas.

**Access**
- Limit this to those who need to enter the laboratory. Exclude dogs and other animals.
8.3 METHODS TO MINIMIZE INFECTIOUS HAZARDS

Protective clothing

The wearing of a gown supplemented where possible by a disposable plastic apron and gloves is recommended. Hand basins should be present in the work area with adjacent disposable towels. Gowns should be autoclaved after use and plastic aprons/gloves placed in large plastic bags and incinerated. Protective clothing must be removed before leaving the laboratory.

Biological safety cabinets

Most procedures associated with the investigation of poliovirus infection can be carried out with the use of a Class I biological safety cabinet and a draught-free room. Class I cabinets which provide for the flow of room air into the cabinet away from the operator prevent the spread of infectious aerosols and the exhausted air from the cabinet is passed through a HEPA filter. The cabinet can be used open-fronted or with a front closure panel. The cabinet must be installed by the manufacturer or exactly according to their instructions. Regular maintenance, including checks on air-flow and changes of air filters, is essential. The preparation of specimen extracts for virus isolation studies should be carried out in a Class I cabinet. Always wash down the interior of the cabinet with Cidex (activated, 2% gluteraldehyde) after use.

THE USE OF A SAFETY CABINET IS NO SUBSTITUTE FOR GOOD LABORATORY TECHNIQUE.

8.4 DISPOSAL OF INFECTED MATERIALS

It is the responsibility of the laboratory staff to ensure that no exposed, infected items reach the disinfection/autoclaving/incineration department. All open items should be securely closed or enclosed and sharp instruments sheathed or enclosed in lidded receptacles.

Heat is the most effective means of sterilization; this is provided either by autoclaving with steam under 15 PSI at 120°C for at least 20 minutes, by dry heat at 160°C minimum, or by incineration. Wherever possible an autoclave should be readily accessible to obviate the need to transport infected materials through other rooms or corridors. It is usually sufficient to disinfect glass pipettes by total immersion in a hypochlorite solution.

Ultraviolet (UV) light is effective in decontaminating air surfaces; however, its usefulness is limited where organic material is present. UV lights should be cleaned periodically and their radiation intensity checked by a UV meter. UV lights must be installed so that they do not shine directly into the eyes of workers and personnel should be warned about the danger of eye exposure to UV irradiation.
Decontamination of rooms and buildings used for poliovirus investigations is rarely required. However, if indicated, this can be achieved by the use of vapourized formaldehyde (usually supplied as commercially available vapourizers).

An outline of recommended disposal techniques is given in Figure 27. Additional information is available in the *WHO Laboratory Biosafety Manual* and also in the book written by Collins.

**FIGURE 27**
**DISPOSAL OF LABORATORY MATERIALS**

<table>
<thead>
<tr>
<th>Material</th>
<th>Disposal Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory glassware bottles, tubes, vials, culture tubes</td>
<td><strong>Firmly</strong> close, place in autoclave basket, → autoclave → recycle, except vials</td>
</tr>
<tr>
<td>Pipettes, glass</td>
<td><strong>Totally</strong> immerse in Chloros or hypochlorite solution, → recycle</td>
</tr>
<tr>
<td>Broken, infected glassware</td>
<td>Use forceps, place in plastic bag → seal → use additional wrapping, place in separate autoclave drum → autoclave → discard</td>
</tr>
<tr>
<td>Laboratory plastics</td>
<td>Plastic bag → seal → incinerator bucket* → incinerate</td>
</tr>
<tr>
<td>plates, cellulose material, tubes, petri dishes, culture vessels, pipette tips</td>
<td></td>
</tr>
<tr>
<td>Plastic syringes/needles</td>
<td>Place in thick-walled plastic or cardboard container (e.g., “Cinbin”) → incinerate</td>
</tr>
<tr>
<td>Rubber/Silicone Stoppers for cell culture tubes</td>
<td>Place in autoclavable bowl → cover with tinfoil → autoclave → recycle</td>
</tr>
<tr>
<td>Instruments</td>
<td>Immerse in water in autoclavable container (e.g. fishkettle) → autoclave → recycle</td>
</tr>
<tr>
<td>forceps, scissors, mortars, pestles, grinders, homogenizer units</td>
<td></td>
</tr>
<tr>
<td>Centrifuge/ultracentrifuge components</td>
<td>Wash and sterilize according to manufacturer’s instructions</td>
</tr>
<tr>
<td>Contaminated tissues necropsy specimens</td>
<td><strong>Firmly</strong> seal in container → autoclave drum → autoclave → discard</td>
</tr>
<tr>
<td>Protective clothing, cloth plastic</td>
<td>Place in autoclave drum → autoclave → launder plastic bag → seal → incinerator bucket → incinerate</td>
</tr>
<tr>
<td>Paper Products paper towels, tissues, Benchkote</td>
<td>Plastic bag → seal → incinerator bucket → incinerate</td>
</tr>
</tbody>
</table>

* Incinerator bucket is lined with a large plastic bag which is removed when full and tied securely ready for transport to the incinerator.
9.
REFERENCES


* References 6 and 8 may be ordered from: Expanded Programme on Immunization, World Health Organization, 1211 Geneva 27, Switzerland
ANNEX 1
EPI PROTOCOL FOR RAPID ASSESSMENT OF SEROLOGICAL RESPONSE TO THREE DOSES OF TRIVALENT ORAL POLIO VACCINE

PURPOSES
- To obtain data on the immunogenicity of trivalent OPV in children less than one year of age when administered under actual field conditions.
- To determine the potential geographic extent of less-than-optimal rates of seroconversion observed in some countries.
- To increase overall awareness of poliomyelitis control programmes.

METHODS
- Enroll 30 to 60 children 7 to 12 months of age who present for routine measles immunization at local health clinics; restrict study to those children with history (by written record) of having received OPV1, OPV 2 and OPV3. It should be at least 30 days after each child received OPV3. Infants who also received OPV Zero should be considered eligible. Minimum sample size per country = 30 children; Maximum = 60 children.
- Record the following information on a list: name; ID number; age; immunization status determined by immunization card (Use sample form shown overleaf).
- Collect blood sample by finger-stick or by venepuncture (minimum volume = 1 ml) and label tube with the child’s name or ID number. Consult laboratory to determine preferred method of collection.
- Submit blood samples to the laboratory (preferably within 48 hours) for processing. Sera should be separated by centrifugation and stored at -20°C until tested.
- Measure neutralizing antibody against poliovirus types 1, 2, and 3 in the laboratory using a standardized method (see Chapter 6). Record as positive: antibody titre of 8 or higher. Record as negative: antibody titre of less than 8.
- Report results according to the percentage of children with neutralizing antibody (a titre of 8 or higher) against poliovirus types 1, 2 and 3. Also report percentage of children who are triple seropositive and the percentage who are triple seronegative.

RATIONALE FOR (AND LIMITATIONS OF) STUDY DESIGN
Objectives of Proposed Design
- to carry out the assessment as rapidly as possible.
- To maximize the quality and ease of interpretation of the data by using a simple, but standardized approach.

Advantages of Proposed Design
- Simulates seroprevalence of antibody following recommended EPI schedule (in children who have already received 3 doses of trivalent OPV).
- Removes age as confounder in the analysis.
- Simplifies selection criteria.
- Small sample size (minimum sample size per country = 30 children).
Disadvantages of Proposed Design

- Since sample will consist solely of children who have complied with the recommended immunization schedule, there is a greater likelihood of biases towards higher socioeconomic status, better nutrition, fewer instances of exposure to enteric pathogens and other factors which may affect serological responses to OPV.
- Since sample is not representative of the general population, low seroprevalence of antibody to one or more types suggests a severe problem which may apply to the population as a whole.

**NOTE:** Please report study results to Regional WHO Office (Attention of EPI, WHO, 1211 Geneva 27, Switzerland). This will contribute to the global assessment of serological responses to OPV.
SAMPLE FORM FOR EPI RAPID ASSESSMENT OF SEROLOGICAL RESPONSE TO THREE DOSES OF TRIVALENT OPV

COUNTRY: ____________________________ YEAR: __________
Name of Clinic: _______________________________________
Address: _____________________________________________
Contact Person: _______________________________________

<table>
<thead>
<tr>
<th>Name/ID No.</th>
<th>Date of Birth</th>
<th>OPV Zero Date</th>
<th>OPV 1 Date</th>
<th>OPV 2 Date</th>
<th>OPV 3 Date</th>
<th>Date Serum Obtained</th>
<th>Antibody ≥ 8 Titers</th>
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ANNEX 2
COMPOSITION OF MEDIA AND OTHER REAGENTS USED IN CELL AND VIRUS CULTURE

NOTE: ANALYTICAL GRADE CHEMICALS MUST BE USED THROUGHOUT

1. PHOSPHATE BUFFERED SALINE, pH 7.2 to 7.4 (PBS)
This is the simplest of basic salt solutions and is used for cell disaggregation and washing cells. PBS in the incomplete and complete form is available commercially. An incomplete solution of PBS contains no calcium or magnesium ions. A complete solution of PBS is used mainly in the preparation of specimen extracts and as diluent for viruses; the presence of calcium and magnesium ions stabilizes viruses, particularly poliovirus and other enteroviruses.

Solution A

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.00 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.20 g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>1.15 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.20 g</td>
</tr>
</tbody>
</table>

Dissolve in distilled H₂O. Add 2 ml of 0.4% phenol red as pH indicator. Make up to 800 ml and autoclave at 10 PSI for 15 minutes. This gives a working solution of incomplete PBS (i.e. no calcium or magnesium ions present).

Solution B

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl₂·6H₂O</td>
<td>0.10 g</td>
</tr>
</tbody>
</table>

Dissolve in 100 ml distilled H₂O. Autoclave at 10 PSI for 15 minutes.

Solution C

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂</td>
<td>0.10 g</td>
</tr>
</tbody>
</table>

Dissolve in 100 ml distilled H₂O. Autoclave at 10 PSI for 15 minutes.

Working Solution of complete PBS

Add 8 parts of Solution A to 1 part of Solution B and 1 part of Solution C.
2. HANKS' BASIC SALT SOLUTION (HANKS' BSS)

This is often the basis of many growth media, but its main use is in the preparation of virus transport
tissue extracts or dilutions of viruses or antisera which may be in contact with the air for long periods. In
In those cases, complete PBS is more suitable. Hanks' BSS is available commercially but may be prepared
in the laboratory as follows:

Solution A (Stock)

<p>| | |</p>
<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>40.00 g</td>
</tr>
<tr>
<td>KCl</td>
<td>2.00 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.50 g</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>0.50 g</td>
</tr>
</tbody>
</table>

Dissolve in 200 ml distilled H₂O. Dissolve separately 0.70 g CaCl₂ in 30 ml distilled H₂O. Mix with
Solution A and make up to 250 ml with distilled H₂O. Add 0.5 ml chloroform. Store at 0°C to 8°C. Stable
for 1 year.

Solution B (Stock)

<p>| | |</p>
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Na₂HPO₄·12H₂O</td>
<td>0.76 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.30 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>5.60 g</td>
</tr>
</tbody>
</table>

Dissolve in 200 ml distilled H₂O. Make up to 250 ml with distilled H₂O. Add 0.5 ml chloroform. Store
at 0°C to 8°C. Stable for 1 year.

Working Solution

<p>| | |</p>
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Solution A</td>
<td>50 ml</td>
</tr>
<tr>
<td>Solution B</td>
<td>50 ml</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>870 ml</td>
</tr>
<tr>
<td>0.4% Phenol red</td>
<td>2 ml</td>
</tr>
</tbody>
</table>

Mix solutions A and B as indicated and add 870 ml distilled H₂O and phenol red as pH indicator.
Distribute in bottles in desired volumes and autoclave at 10 PSi for 15 minutes. Before use, adjust pH as
required by addition of 7.5% solution of sodium bicarbonate (see below).

3. SODIUM BICARBONATE SOLUTION

Together with gaseous CO₂, this provides the buffering system for many cell culture media; it is also an
essential metabolite.

NaHCO₃     7.5 g

Dissolve in 50 ml distilled H₂O; add 0.2 ml of 0.4% phenol red. Make up to 100 ml with distilled H₂O,
saturate with CO₂ until orange in color. Dispense in approximately 5 ml volumes in tightly capped
bottles. Autoclave at 10 PSI for 15 minutes.
4. **EAGLE'S GROWTH MEDIUM (GM) AND EAGLE'S MAINTENANCE MEDIUM (MM)**

Used for culturing RD and HEP-2 (Cincinnati) Cell Lines

<table>
<thead>
<tr>
<th></th>
<th>GM</th>
<th>MM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eagle's Minimum Essential Medium (Earle's Salts Base)</td>
<td>89.0 ml</td>
<td>91.5 ml</td>
</tr>
<tr>
<td>L-Glutamine 200 mM</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Foetal Calf Serum</td>
<td>5.0 ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>NaHCO₃ solution 7.5%</td>
<td>1.5 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>HEPES 1M</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>Penicillin/Streptomycin solution*</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>0.4% Phenol red</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
</tr>
</tbody>
</table>

* Dissolve $1 \times 10^4$ units crystalline penicillin G and 1g streptomycin sulphate in 100 ml sterile PBS; distribute into 5 ml volumes and store at $-20^\circ$C. For use, add 1 ml of this stock solution to 100 ml medium to give a final concentration of 100 units penicillin and 100 µg streptomycin.

5. **EAGLE'S ASSAY MEDIUM**

Used for intratypic differentiation of polioviruses with monoclonal antibody panels

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<table>
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<tbody>
<tr>
<td>Eagle's Minimum Essential Medium (Earle's Salts Base)</td>
<td>93.0 ml</td>
</tr>
<tr>
<td>Foetal Calf Serum</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>NaHCO₃ solution 7.5%</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>Penicillin/streptomycin solution</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>0.4% Phenol red</td>
<td>0.2 ml</td>
</tr>
</tbody>
</table>

6. **VIRUS TRANSPORT MEDIUM (VTM)**

Used for the collection of swabs and necropsy specimens. It consists of Hanks' BSS buffered to pH 7.4 with HEPES, 0.2% bovine albumin, penicillin/streptomycin or gentamicin**, and phenol red as indicator. It is distributed in 2.5 ml amounts in firmly closed bottles and stored at 0°C to 8°C.

** Dissolve 1g Gentamicin sulphate in 200 ml sterile PBS, distribute into 5 ml volumes and store at $-20^\circ$C. For use, add 1 ml of this stock solution to 100 ml medium to give a final concentration of 30–50 µg/ml.

7. **OTHER ANTIBIOTICS**

Penicillin and streptomycin are the antibiotics most commonly used in cell and routine virus culture work; they are also the least expensive. Gentamicin is more expensive, but it is inhibitory to a wider range of bacteria and it is autoclavable. Mycostatin may be used at 25 units/ml to counteract fungal and yeast contaminants; however, it is only fungistatic and not fungicidal, is rapidly inactivated at 36°C (usual cell culture incubation temperature) and some batches are slightly cytotoxic. Fungizone also often produces cytotoxic effects.

Mycoplasma contamination of continuous cell cultures is common. Special detection kits are available commercially. Kanamycin and Tylasin can remove mycoplasma infections, but the treatments involved can result in altered susceptibility of the cells for viruses. It is advisable therefore to discard all mycoplasma-infected cultures and procure fresh mycoplasma-free cell lines.
8. CELL DISPERSING AGENTS

Trypsin and Versene are commonly used either separately or combined. The proteolytic enzyme trypsin is particularly suitable for the digestion of cells from whole organs. It is also used for the removal of cells from glass or plastic, but the chelating agent Versene is probably as good. Solutions of trypsin and/or Versene should be prepared in PBS free of calcium and magnesium, as the presence of these ions increases the stability of the intercellular matrix thereby making detachment of the cells from the glass/plastic difficult.

**Trypsin**
Dissolve 1 g of Difco 1:250 Trypsin in 400 ml PBS (no Ca, Mg) by agitation with a magnetic stirrer for 30 minutes at 36°C. Membrane filter (pore size 0.22 μm), dispense into approximately 5 ml volumes in sealed bottles and store at −20°C.

**Versene**
(EDTA = Disodium salt of ethylenediaminetetra-acetic acid). Dissolve 0.1 g of Versene in 10 ml distilled-water. Distribute in 0.5 ml volumes in sealed bottles; autoclave at 10 PSI for 15 minutes and store at room temperature. For use add 0.4 ml of this 1% solution to 20 ml PBS (without Ca, Mg) to give a final concentration of 1/5000.

9. PHENOL RED INDICATOR

This indicator of pH is used in cell culture media. Prepare stock 0.4% solution in distilled H₂O. Distribute into sealed bottles; autoclave at 10 PSI for 15 minutes and store at room temperature. For media preparation, 1–2 ml of this solution per litre is usually adequate.
ANNEX 3

INFORMATION ON THE ORIGINS OF CELL LINES RD AND HEP-2
(from Catalogue of American Type Culture Collection, ATCC)

ATCC CCL 136 RD (Rhabdomyosarcoma, embryonal, Human)

Current medium for propagation: ATCC-CRCM 30, 90%; fetal bovine serum, 10%.

The RD cell line was established by R.M. McAllister from a malignant embryonal rhabdomyosarcoma of the pelvis of a 7-year-old Caucasian female in February, 1968 (Cancer 24: 520-526, 1969). The cells grew as monolayers in liquid medium and formed colonies in agar in minimum essential medium (Eagle) supplemented with 10% fetal bovine serum or in Freemen medium (J. Virol. 1: 326, 1967). The culture consists of two cytologic types resembling those of the original tumor-spindle cells and large multinucleated cells. No myofibrils could be demonstrated in the cells by light or by electron microscopy nor were virus particles detected. The cells contain myoglobin and myosin-ATPase activity. The RD cell line is the first human rhabdomyosarcoma cell line characterized and studied in detail. The cells were submitted to the American Type Culture Collection in the 22nd passage in September, 1969. It has been demonstrated that the cells grow well in culture through at least 56 serial subcultures. NOTE: This line has also been designated TE32 and 130T in the literature.

DESCRIPTION OF REPOSITORY REFERENCE SEED STOCK

Number of Serial Subcultures from Tissue of Origin: 32

Freeze Medium: Culture medium, 95%; dimethyl sulfoxide (DMSO), 5%; antibiotic-free.

Viability: Approximately 80% (dye-exclusion).

Culture Medium: Minimum essential medium (Eagle) with twice the standard concentrations of amino acids and vitamins with Hanks' BSS, 90%; fetal bovine serum (not inactivated), 10%; antibiotic-free.

Growth Characteristics of Thawed Cells: An inoculum of 10^5 viable cells/ml in the above culture medium at 37° C, multiplies 34 fold in 8 days.

Plating Efficiency: Less than 1% in the above culture medium.

Morphology: Spindle cells and large multinucleated cells.

Karyotype: Chromosome Frequency Distribution 51 Cells: 2n = 46

Cells: 1 2 3 4 9 10 4 3 1 1 1 1 1 1 1 1 6

Chromosomes: 45 46 47 48 49 50 51 52 53 54 -58 -60 61 -68 -84 -87 88 91 -97

Karyotype unstable within a hyperdiploid bimodal stemline number of 49 and 50. Twenty-two cells had chromosome associations, 15 cells microchromosomes, two cells had fragments, two cells with breaks, two cells with acentric gaps and one cell with a secondary constriction.

Sterility: Tests for mycoplasma, bacteria, and fungi were negative.

Species: Confirmed as human by the cytotoxic-antibody dye-exclusion test.

Virus Susceptibility: Susceptible to poliovirus type 1, vesicular stomatitis (Indiana Strain), herpes simplex and vaccinia viruses.

Reverse Transcriptase: Not detected.

Isoenzymes: G6PD type B.

Submitted by: R.M. McAllister, Professor of Pediatrics, U.S.C. School of Medicine and Head, Division of Virology, Children's Hospital of Los Angeles, California

Prepared and characterized by: Institute for Medical Research, Camden, New Jersey and American Type Culture Collection, Rockville, Maryland
ATCC CCL23  HEp-2* (Epidermoid carcinoma, larynx, Human, Hela Markers)

Current medium for propagation: Eagle's MEM with Earle's BSS, 90%; fetal bovine serum, 10%.

The HEp-2 cell line was established by A.E. Moore, L. Sabachewsky, and H.W. Toolan in 1952, (Cancer Res. 15: 598, 1955) from tumors that had been produced in irradiated-cortisonized weanling rats after injection with epidermoid carcinoma tissue from the larynx of a 56-year-old Caucasian male (H. Toolan, Cancer Res. 14: 660, 1954). The in vitro isolation was accomplished in each of several mixtures of bovine amniotic fluid, embryo extracts, human and horse sera, and balanced salt solution, and the epithelial-like cells subsequently grew well in several types of culture media. It is a hardy cell line which resists temperature, nutritional, and environmental changes without loss of viability.


A subculture of the line (passage number approximately 240) in basal medium (Eagle), 85%; calf serum, 15%; was submitted to the Institute for Medical Research in January, 1958. The line was authorized for inclusion in the American Type Culture Collection in 1961. It was found to be contaminated with mycoplasma which was subsequently eliminated by treatment with kanamycin.

DESCRIPTION OF REPOSITORY REFERENCE SEED STOCK

Number of Serial Subcultures from Tissue of Origin: Approximately 350.
Freeze Medium: Culture medium, 95%; glycerol, 5%; antibiotic-free.
Viability: Approximately 85% (dye-exclusion).
Culture Medium: Basal medium (Eagle) with Hanks' BSS, 85%; calf serum, 15%; antibiotic-free.
Growth Characteristics of Thawed Cells: An inoculum of approximately 10^3 cells/ml in the above culture medium multiplies 10-fold in 5 to 6 days.
Plating Efficiency: Approximately 44% in basal medium (Eagle), 80%; calf serum, 20%.

Cell type: Chromosome Frequency Distribution 47 Cells: 2n = 46

<table>
<thead>
<tr>
<th>Cells</th>
<th>1</th>
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<th>1</th>
<th>2</th>
<th>1</th>
<th>8</th>
<th>14</th>
<th>9</th>
<th>4</th>
<th>2</th>
<th>1</th>
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</tr>
</thead>
</table>
Chromosomes: 59-66-70-73 74 75 76 77 78 79 -85-135 -149 -195

Occasional polyploids. Several marker chromosomes were observed along with frequent minutes, and often 2 large chromosomes with subterminal centromeres.

HeLa Marker Chromosomes: One copy of No. 2, two-four copies of No. 3 and one copy of No. 4 as revealed by G-banding patterns. This line probably was contaminated with HeLa cells. See discussion in introduction to Cell Cultures.

Sterility: Tests for mycoplasma, bacteria and fungi were negative.
Species: Confirmed as human by immunofluorescence and by cytotoxic-antibody tests (dye-exclusion).
Virus Susceptibility: Supports the growth of both virulent and avirulent poliovirus type 1, adenovirus type 3, and vesicular stomatitis (Indiana Strain) virus.

Reverse Transcriptase: Not detected.
Isoenzymes: G6PD type A, not the expected type B.
Submitted by: A.E. Moore, Sloan-Kettering Institute, New York, New York.
Prepared and characterized by: Institute for Medical Research, Camden, New Jersey and American Type Culture Collection, Rockville, Maryland.

* Note: The HEp-2 (Cincinnati) cell line recommended in this manual is more susceptible to poliovirus infection than the standard HEp-2 cell line described above.
ANNEX 4
PREPARATION OF VIRUS STOCKS

For neutralizing antibody tests, stock virus suspensions of all three poliovirus serotypes (Sabin strains) should be prepared in cell cultures. Prototype strains of poliovirus must be used for this purpose. Requests for these should be submitted to CDS/EPI, WHO, Geneva.

Before preparing stocks of prototype virus, initiate a record card/book listing virus serotype, its source and previous cell culture passage history. To this can be added its current passage history, dates and place of storage, virus titre and confirmation of serotype identity.

METHOD

1. Select bottle cultures of RD or HEp-2 (Cincinnati) showing confluent healthy monolayers.
2. Carefully decant the growth medium and rinse the cell layers gently in PBS ( Annex 2).
3. Add 1 ml of the prototype poliovirus strain at approximately TCD₅₀.
4. Allow to adsorb to cell monolayer for 1 hour at 36°C.
5. Add Eagle’s maintenance medium ( Annex 2).
6. Incubate at 36°C until almost complete CPE develops.
7. Check visually that the cultures are not bacteriologically/fungally contaminated; pool all the cultures inoculated with the same serotype.
8. Clarify by centrifugation at 500g for 20 minutes. Remove the supernate.*
9. Clearly label vials stating virus serotype and source, culture passage number, date of freezing. Also, update the record card/book each time new virus stocks are prepared.
10. Distribute the supernate, which constitutes the stock virus suspension, into working volumes, e.g. 1-2 ml amounts in labelled screw-capped vials and store at -20°C or lower.
11. Titrate the stock virus and check its correct identity using homotypic reference antisera. Enter results in record card/book.
12. Each time a working stock of virus is made it must be titrated and its identity confirmed.

NEVER PREPARE STOCKS OF DIFFERENT VIRUS SEROTYPES SIMULTANEOUSLY NOR IN THE SAME WORK AREA

* The virus can be further stabilized at this stage of preparation by adding 1 part 4M MgCl₂ to 3 parts virus supernate. Since this concentration of MgCl₂ is toxic for cell cultures, dilute the virus suspension 1/10 in PBS before passage.
LIST OF ABBREVIATIONS

CNS  Central Nervous System
CPE  Cytopathic Effect
CSF  Cerebrospinal Fluid
DMSO Dimethylsulphoxide
ELISA Enzyme-Linked Immunosorbent Assay
EPI  Expanded Programme on Immunization
g  Relative Centrifugal Force*
GM  Growth Medium
MM  Maintenance Medium
NPEV Non-polio Enterovirus
PBS Phosphate Buffered Saline
PCR Polymerase Chain Reaction
PSI Pounds per Square Inch (50 PSI = 3.50 kg/cm²)
RPM Revolutions Per Minute*
SSC Sodium Saline Citrate
TCD₅₀ 50 percent Tissue (Cell) Culture Infecting Dose (= TCID₅₀ = CCID₅₀)
VTM Virus Transport Medium

* Calculation of g and RPM given in section 3.1.2.