Antiviral Susceptibility Testing: Herpes Simplex Virus by Plaque Reduction Assay; Approved Standard

This document provides a protocol for the performance of the plaque reduction assay for phenotypic antiviral susceptibility testing of herpes simplex virus.

A standard for global application developed through the NCCLS consensus process.
NCCLS...  
Serving the World’s Medical Science Community Through Voluntary Consensus

NCCLS is an international, interdisciplinary, nonprofit, standards-developing, and educational organization that promotes the development and use of voluntary consensus standards and guidelines within the healthcare community. It is recognized worldwide for the application of its unique consensus process in the development of standards and guidelines for patient testing and related healthcare issues. NCCLS is based on the principle that consensus is an effective and cost-effective way to improve patient testing and healthcare services.

In addition to developing and promoting the use of voluntary consensus standards and guidelines, NCCLS provides an open and unbiased forum to address critical issues affecting the quality of patient testing and health care.

PUBLICATIONS
An NCCLS document is published as a standard, guideline, or committee report.

Standard A document developed through the consensus process that clearly identifies specific, essential requirements for materials, methods, or practices for use in an unmodified form. A standard may, in addition, contain discretionary elements, which are clearly identified.

Guideline A document developed through the consensus process describing criteria for a general operating practice, procedure, or material for voluntary use. A guideline may be used as written or modified by the user to fit specific needs.

Report A document that has not been subjected to consensus review and is released by the Board of Directors.

CONSENSUS PROCESS
The NCCLS voluntary consensus process is a protocol establishing formal criteria for:

- the authorization of a project
- the development and open review of documents
- the revision of documents in response to comments by users
- the acceptance of a document as a consensus standard or guideline.

Most NCCLS documents are subject to two levels of consensus—“proposed” and “approved.” Depending on the need for field evaluation or data collection, documents may also be made available for review at an intermediate (i.e., “tentative”) consensus level.

Proposed An NCCLS consensus document undergoes the first stage of review by the healthcare community as a proposed standard or guideline. The document should receive a wide and thorough technical review, including an overall review of its scope, approach, and utility, and a line-by-line review of its technical and editorial content.

Tentative A tentative standard or guideline is made available for review and comment only when a recommended method has a well-defined need for a field evaluation or when a recommended protocol requires that specific data be collected. It should be reviewed to ensure its utility.

Approved An approved standard or guideline has achieved consensus within the healthcare community. It should be reviewed to assess the utility of the final document, to ensure attainment of consensus (i.e., that comments on earlier versions have been satisfactorily addressed), and to identify the need for additional consensus documents.

NCCLS standards and guidelines represent a consensus opinion on good practices and reflect the substantial agreement by materially affected, competent, and interested parties obtained by following NCCLS’s established consensus procedures. Provisions in NCCLS standards and guidelines may be more or less stringent than applicable regulations. Consequently, conformance to this voluntary consensus document does not relieve the user of responsibility for compliance with applicable regulations.

COMMENTS
The comments of users are essential to the consensus process. Anyone may submit a comment, and all comments are addressed, according to the consensus process, by the NCCLS committee that wrote the document. All comments, including those that result in a change to the document when published at the next consensus level and those that do not result in a change, are responded to by the committee in an appendix to the document. Readers are strongly encouraged to comment in any form and at any time on any NCCLS document. Address comments to the NCCLS Executive Offices, 940 West Valley Road, Suite 1400, Wayne, PA 19087, USA.

VOLUNTEER PARTICIPATION
Healthcare professionals in all specialties are urged to volunteer for participation in NCCLS projects. Please contact the NCCLS Executive Offices for additional information on committee participation.
Antiviral Susceptibility Testing: Herpes Simplex Virus by Plaque Reduction Assay; Approved Standard

Ella M. Swierkosz, Ph.D., Co-Chairholder
Richard L. Hodinka, Ph.D., Co-Chairholder
Barbara M. Moore
Stephen Sacks, M.D.
David R. Scholl, Ph.D.
D. Kathleen Wright

Abstract

NCCLS document M33-A—Antiviral Susceptibility Testing: Herpes Simplex Virus by Plaque Reduction Assay; Approved Standard, the first to address phenotypic antiviral susceptibility testing, standardizes susceptibility testing of herpes simplex virus (HSV). This standard contains information about performance of the plaque reduction assay (PRA) for HSV susceptibility testing, the test method to which other antiviral susceptibility testing methods are usually compared.

This standard provides detailed instructions for preparation of the viral inoculum, preparation and dilution of antiviral agents, inoculation of cell cultures, preparation of the overlay medium, incubation of inoculated cell cultures, fixation and staining of cell cultures, counting of plaques, calculation of endpoints, and interpretation of results. A quality control section addresses procedures for evaluation of cell lines, selection of reference strains and quality control ranges, standardization of inocula, and other quality control issues.


THE NCCLS consensus process, which is the mechanism for moving a document through two or more levels of review by the healthcare community, is an ongoing process. Users should expect revised editions of any given document. Because rapid changes in technology may affect the procedures, methods, and protocols in a standard or guideline, users should replace outdated editions with the current editions of NCCLS documents. Current editions are listed in the NCCLS Catalog, which is distributed to member organizations, and to nonmembers on request. If your organization is not a member and would like to become one, and to request a copy of the NCCLS Catalog, contact the NCCLS Executive Offices. Telephone: 610.688.0100; Fax: 610.688.0700; E-Mail: exoffice@nccls.org; Website: www.nccls.org
This publication is protected by copyright. No part of it may be reproduced, stored in a retrieval system, transmitted, or made available in any form or by any means (electronic, mechanical, photocopying, recording, or otherwise) without prior written permission from NCCLS, except as stated below.

NCCLS hereby grants permission to reproduce limited portions of this publication for use in laboratory procedure manuals at a single site, for interlibrary loan, or for use in educational programs provided that multiple copies of such reproduction shall include the following notice, be distributed without charge, and, in no event, contain more than 20% of the document’s text.


Permission to reproduce or otherwise use the text of this document to an extent that exceeds the exemptions granted here or under the Copyright Law must be obtained from NCCLS by written request. To request such permission, address inquiries to the Executive Director, NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA.

Copyright ©2004. The National Committee for Clinical Laboratory Standards.

Suggested Citation


Proposed Standard
November 2000

Approved Standard
February 2004

ISBN 1-56238-522-4
ISSN 0273-3099
Committee Membership

Area Committee on Microbiology

Mary Jane Ferraro, Ph.D., M.P.H.
Chairholder
Massachusetts General Hospital
Boston, Massachusetts

James H. Jorgensen, Ph.D.
Vice-Chairholder
University of Texas Health Science Center
San Antonio, Texas

Donald R. Callihan, Ph.D.
BD Diagnostic Systems
Sparks, Maryland

David L. Sewell, Ph.D.
Veterans Affairs Medical Center
Portland, Oregon

Thomas R. Shryock, Ph.D.
Lilly Research Laboratories
Greenfield, Indiana

Jana M. Swenson, M.M.Sc.
Centers for Disease Control and Prevention
Atlanta, Georgia

Michael L. Wilson, M.D.
Denver Health Medical Center
Denver, Colorado

Advisors

Ellen Jo Baron, Ph.D.
Stanford Univ. Hospital & Medical School
Stanford, California

Lynne S. Garcia, M.S.
LSG & Associates
Santa Monica, California

Subcommittee on Antiviral Susceptibility Testing

Richard L. Hodinka, Ph.D.
Co-Chairholder
Children’s Hospital of Philadelphia
Philadelphia, Pennsylvania

Ella M. Swierkosz, Ph.D.
Co-Chairholder
St. Louis University
St. Louis, Missouri

Barbara M. Moore
Newport, Minnesota

David R. Scholl, Ph.D.
Diagnostic Hybrids, Inc.
Athens, Ohio

D. Kathleen Wright
FDA Center for Devices/Rad. Health
Rockville, Maryland

Angela M. Caliendo, M.D., Ph.D.
Emory University Hospital
Atlanta, Georgia

Lorraine Clarke, Ph.D.
New York State Department of Health
Albany, New York

W. Lawrence Drew, M.D.
University of California
San Francisco, California

Shirley Floyd-Reising, Ph.D.
Children’s Hospital Medical Center
Cincinnati, Ohio

Michael Forman, Ph.D.
Johns Hopkins Hospital
Baltimore, Maryland

Frederick G. Hayden, M.D.
University of Virginia
Charlottesville, Virginia

Myron Levin, M.D.
University of Colorado Health Sciences Center
Denver, Colorado

Prof. Raimundo Diogo Machado
Sociedade Brasileira de Analises Clinicas
Rio de Janeiro, Brazil

James B. Mahony, Ph.D.
St. Joseph’s Hospital
Hamilton, ON, Canada

Stephen Sacks, M.D.
Viridae Clinical Sciences, Inc.
Vancouver, BC, Canada

Sally Selepak, M.T.(ASCP)
FDA Center for Devices/Rad. Health
Rockville, Maryland

Steven Specter, Ph.D.
University of South Florida
Tampa, Florida

Stephen A. Spector, M.D.
University of California, San Diego
La Jolla, California

Staff

Tracy A. Dooley, M.L.T.(ASCP)
Staff Liaison
NCCLS
Wayne, Pennsylvania

Donna M. Wilhelm
Editor
NCCLS
Wayne, Pennsylvania

Melissa A. Lewis
Assistant Editor
NCCLS
Wayne, Pennsylvania
## Contents

Abstract....................................................................................................................... i

Committee Membership............................................................................................... iii

Foreword....................................................................................................................... vii

### 1 Introduction and Scope ...................................................................................... 1

### 2 Antiviral Agents for Treatment of HSV ............................................................... 3
   2.1 Acyclovir .............................................................................................................. 3
   2.2 Valacyclovir........................................................................................................... 3
   2.3 Famciclovir/Penciclovir........................................................................................ 3
   2.4 Foscarnet.............................................................................................................. 4
   2.5 Idoxuridine and Trifluridine .............................................................................. 4
   2.6 Vidarabine........................................................................................................... 4

### 3 Definitions .......................................................................................................... 4

### 4 Variables of Antiviral Susceptibility Testing....................................................... 5
   4.1 Host Cell Line, Viral Inoculum, Incubation Time, and Range of
       Concentration of the Antiviral Agent................................................................... 5
   4.2 Heterogeneity of Virus Population and Strain of Virus Used (“Wild” versus
       “Laboratory Adapted”) ....................................................................................... 6
   4.3 Reference Strains ............................................................................................... 6
   4.4 Assay Method .................................................................................................... 6
   4.5 Endpoint Criteria............................................................................................... 6
   4.6 Calculation of Endpoints .................................................................................. 7
   4.7 Interpretation of Endpoints .............................................................................. 7

### 5 Plaque Reduction Assay (PRA) ......................................................................... 8
   5.1 Cells, Media, and Reagents............................................................................... 8
   5.2 Supplies............................................................................................................... 11
   5.3 Equipment ......................................................................................................... 12
   5.4 Storage Instructions .......................................................................................... 12
   5.5 Preparation of Cell Culture Plates, HSV Isolates, and Antiviral Agents........... 13
   5.6 Performance of the Plaque Reduction Assay.................................................... 24

### 6 Quality Control Procedures ................................................................................. 26
   6.1 Purpose .............................................................................................................. 26
   6.2 Quality Control Responsibilities....................................................................... 27
   6.3 Reference Strains for Quality Control ............................................................. 27
   6.4 Storing Quality Control Strains ....................................................................... 28
   6.5 Medium Quality Control .................................................................................. 28
   6.6 Cell Line Quality Control ................................................................................. 28
   6.7 Antiviral Drug Quality Control......................................................................... 28
   6.8 Antiviral Cytotoxicity Control.......................................................................... 28
   6.9 Inoculum Control ............................................................................................. 29
   6.10 Quality Control Ranges .................................................................................. 29
   6.11 Frequency of Quality Control Testing ............................................................. 29
Contents (Continued)

7 Results: Interpretation and Reporting .............................................................. 29
   7.1 Data Analysis ......................................................................................... 29
   7.2 Interpretation and Reporting ............................................................... 29
   7.3 Limitations of Procedure ................................................................. 30

8 Safety ........................................................................................................ 30

References ......................................................................................................... 32

Summary of Comments and Subcommittee Responses ......................................... 34

Summary of Delegate Comments and Subcommittee Responses ......................... 36

The Quality System Approach ............................................................................. 38

Related NCCLS Publications ............................................................................ 39
Foreword

The NCCLS Subcommittee on Antiviral Susceptibility Testing was formed in 1997 and is composed of representatives from the professions, government, and industry, including virology and microbiology laboratories, healthcare providers and educators, and diagnostic microbiology and pharmaceutical industries. Our ultimate goal is to develop standards that provide specific details for the materials, methods, and practices necessary for the correct performance and appropriate reporting of phenotypic antiviral susceptibility tests. Although antiviral agents have been used in clinical practice for two decades, in vitro testing methods have never been standardized. This has hampered comparison of susceptibility testing results from different laboratories as well as development of definitive interpretive breakpoints denoting susceptibility and resistance of viral isolates to tested drugs. As more patients fail to respond to appropriate therapy and additional antiviral agents are produced, it has become increasingly important for diagnostic laboratories to provide antiviral susceptibility testing to assist physicians in defining drug resistance and choosing alternative therapies.

Because many variables can affect antiviral susceptibility testing, including the cell line, virus inoculum, and assay method, this subcommittee has chosen to restrict the focus of this document to testing of herpes simplex virus (HSV). There are three major reasons for our decision. First, many antiviral agents have been approved for treatment of HSV, and a large body of literature exists regarding emergence of drug-resistant HSV and management of patients with resistant virus. Second, many of the variables that affect antiviral susceptibility testing have been defined for HSV. Third, because of the relative ease of culturing HSV, it should be possible to complete validation studies of this standard in a timely manner.

The plaque reduction assay (PRA) was the first antiviral susceptibility testing method performed to determine the susceptibility of cytopathic viruses to antiviral agents and traditionally has been the standard to which newer methods are compared. For this reason, this NCCLS approved standard will limit its discussion to the performance of the PRA. This is necessary as a first step to standardizing other methodologies, interpretive criteria, and quality control parameters so that results from different assays can be correlated with PRA.

The methods described herein are generic reference procedures that can be used for routine antiviral susceptibility testing by clinical laboratories, or that can be used by clinical laboratories to evaluate commercial devices for possible routine use. Results generated by the NCCLS reference methods may be used by the United States Food and Drug Administration to evaluate the performance of commercial systems before clearance is given for marketing in the United States. Clearance by the FDA indicates that the agency concludes that commercial devices provide antiviral susceptibility results that are substantially equivalent to results generated using the NCCLS reference methods for the viruses and antiviral agents described in the manufacturer’s approved package insert.

Key Words

Antiviral agents, plaque reduction assay, susceptibility testing
Antiviral Susceptibility Testing: Herpes Simplex Virus by Plaque Reduction Assay; Approved Standard

1 Introduction and Scope

During the past decade, safe and effective antiviral therapy has been developed for treatment of a number of viral infections. While the overwhelming majority of clinical viral isolates from drug-naive patients are susceptible to antiviral agents, widespread use of some antiviral agents has led to the emergence of drug-resistant strains. Most drug resistance has been observed in the immunocompromised host, especially transplant recipients and AIDS patients who fail to respond to therapy for herpes simplex virus (HSV), cytomegalovirus (CMV), and human immunodeficiency virus (HIV) infections.

This standard, the first to address antiviral susceptibility testing, will limit its scope to a description of antiviral agents, the FDA-approved uses of these drugs for the treatment and prophylaxis of HSV infections, and antiviral susceptibility testing for HSV. Detailed discussions of antiviral susceptibility testing for other viruses, including HSV, have been recently published. We have restricted our focus to HSV for three reasons. First, effective antiviral therapy for HSV has been available for over a decade, and there is widespread experience regarding development of resistance and management of patients with resistant virus. Second, there is also abundant laboratory experience with in vitro susceptibility testing of HSV. Many of the variables that affect antiviral susceptibility testing have been defined for HSV so that a standard can be developed. Third, because HSV is relatively easy to culture, validation of this standard can proceed in a straightforward and timely manner.

A number of antiviral agents have been developed for the management of HSV infections, including acyclovir, valacyclovir, famciclovir, foscarnet, idoxuridine, trifluridine, and vidarabine (Table 1). Of these drugs, acyclovir has been widely used as an effective treatment for and prophylactic against HSV infections. However, resistance of HSV clinical isolates to acyclovir has emerged with the occurrence of chronic, progressive, debilitating disease in immunocompromised patients receiving prolonged courses of continuous or intermittent suppressive therapy. The development of viral resistance to acyclovir and the morbidity and mortality associated with these virus strains is a problem of concern. Foscarnet has been employed successfully as an alternative antiviral agent for treating acyclovir-resistant HSV. Resistance to this drug, however, has also been documented and clinical isolates resistant to both acyclovir and foscarnet have been reported. With the rapid advance in the development and use of additional antiviral agents and the continued emergence of drug resistance, there is a definite need for diagnostic laboratories to provide rapid and practical antiviral susceptibility testing.

This document provides a brief discussion of the major antiviral agents with activity against HSV, their mechanisms of action and development of resistance, the clinical use of these drugs, and detailed instructions for the performance of the plaque reduction assay as the standard for antiviral susceptibility testing of HSV.
<table>
<thead>
<tr>
<th>Drug Class/ Mechanism of Action</th>
<th>Antiviral Agent(s)</th>
<th>Clinical Indications for Use*</th>
<th>Route of Administration</th>
<th>Mechanism(s) of Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleoside analog/ inhibitor of viral DNA polymerase</td>
<td>Acyclovir</td>
<td>Treatment of localized and systemic herpes simplex virus (HSV) infections; suppression of genital HSV; prophylaxis of transplant recipients</td>
<td>Intravenous, oral, topical</td>
<td>Altered or deficient viral thymidine kinase (TK); altered viral DNA polymerase (pol)</td>
</tr>
<tr>
<td></td>
<td>Famciclovir (active metabolite: penciclovir)</td>
<td>Treatment or suppression of recurrent genital HSV; treatment of recurrent mucocutaneous HSV in HIV-infected patients</td>
<td>Oral</td>
<td>Altered or deficient viral TK; altered viral DNA pol</td>
</tr>
<tr>
<td></td>
<td>Penciclovir (cream)</td>
<td>Topical treatment of recurrent cold sores due to HSV</td>
<td>Topical</td>
<td>Mutations in viral TK and viral DNA pol genes</td>
</tr>
<tr>
<td></td>
<td>Valacyclovir (active metabolite: acyclovir)</td>
<td>Treatment or suppression of genital herpes in immunocompetent adults; treatment of cold sores</td>
<td>Oral</td>
<td>Mutations in viral TK and viral DNA pol genes</td>
</tr>
<tr>
<td>Nucleoside analog/ inhibitor of DNA synthesis (topical only)</td>
<td>Idoxuridine</td>
<td>HSV keratitis</td>
<td>Topical</td>
<td>Mutations in viral TK gene; Unknown in clinical isolates; Unknown in clinical isolates</td>
</tr>
<tr>
<td></td>
<td>Trifluridine</td>
<td>HSV keratitis</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vidarabine</td>
<td>HSV keratitis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrophosphate analog/ inhibitor of viral DNA polymerase</td>
<td>Foscarnet</td>
<td>Treatment of mucocutaneous acyclovir-resistant HSV infections in immunocompromised patients</td>
<td>Intravenous</td>
<td>Mutations in viral DNA pol gene</td>
</tr>
</tbody>
</table>

* U.S. FDA-approved uses
2 Antiviral Agents for Treatment of HSV

The discussion of the following antiviral agents includes information on only the FDA-approved uses of these drugs for the treatment and prophylaxis of HSV infections. Information on unapproved usage of these agents and their approved uses for other infections is beyond the scope of this document.

2.1 Acyclovir

Acyclovir (9-[(2-hydroxyethoxymethyl)]-9-guanine; acycloguanosine), a guanosine analog, has in vitro activity against a number of herpes group viruses but is used clinically for HSV and varicella-zoster virus (VZV) infections. Acyclovir has largely supplanted vidarabine because of its ease of administration, low toxicity, and greater efficacy. Acyclovir is phosphorylated by a viral-specific thymidine kinase (TK) to its monophosphate form which is subsequently phosphorylated to acyclovir triphosphate by cellular enzymes. Acyclovir triphosphate acts as a competitive inhibitor of viral DNA polymerase and results in termination of DNA synthesis. Acyclovir has efficacy for the treatment of primary and recurrent HSV infections, HSV encephalitis, neonatal herpes, and the management of mucocutaneous HSV infections. Acyclovir resistance arises as a result of mutations in either the viral TK or DNA polymerase genes. Mutation in the TK gene can produce strains deficient in TK (TK-), which results in reduced or absent phosphorylation of acyclovir. A second type of TK mutation results in altered substrate binding properties for acyclovir (TKA). The majority of acyclovir-resistant clinical isolates of HSV are TK-, although rare TKA or DNA polymerase mutants have been recovered.

2.2 Valacyclovir

Valacyclovir (L-valine, 2-(2-amino-1,6-dihydro-6-oxo-9H-purin-9-yl) methoxyl ethyl ester) is the 1-valyl ester of acyclovir recently approved for treatment or suppression of genital HSV infections in immunocompetent adults. The drug is rapidly and extensively converted to acyclovir after oral administration and results in a three- to five-fold increase in acyclovir bioavailability compared to oral acyclovir. The mechanism of viral resistance to valacyclovir is the same as that for acyclovir, and cross-resistance occurs between the two drugs.

2.3 Famciclovir/Penciclovir

Famciclovir, 2-(2-(2-amino-9H-purin-9-yl)ethyl]-1,3-propanediol diacetate, is a synthetic acyclic guanine derivative that is the orally administered prodrug of the active antiviral compound penciclovir. Following oral administration, famciclovir undergoes rapid biotransformation to penciclovir which has inhibitory activity against HSV-1, HSV-2, and VZV. Like acyclovir, penciclovir requires phosphorylation by viral TK to a monophosphate form that is converted to penciclovir triphosphate by cellular enzymes. Penciclovir triphosphate is a competitive inhibitor of HSV DNA polymerase, thus inhibiting viral DNA synthesis and ultimately viral replication. Penciclovir-resistant mutants of HSV can result from mutations in viral TK or DNA polymerase genes. Acyclovir-resistant HSV mutants deficient in TK are also penciclovir-resistant; penciclovir is active in vitro, however, against some acyclovir-resistant isolates of HSV with altered thymidine kinase or DNA polymerase genes. Famciclovir has been approved for the treatment or suppression of recurrent episodes of genital herpes in adults and for the treatment of recurrent mucocutaneous HSV in HIV-infected adults. The drug has a much higher oral bioavailability than acyclovir and its active form is phosphorylated much more efficiently and has a significantly longer intracellular half-life in herpes-infected cells. However, the triphosphate form of penciclovir is less active than acyclovir in inhibiting the viral DNA polymerase. Penciclovir cream has been approved for topical treatment of recurrent cold sores due to HSV.
2.4 Foscarnet

Foscarnet (trisodium phosphonoformate) is an organic analog of inorganic pyrophosphate that has antiviral activity against HSV, VZV, CMV, Epstein-Barr Virus (EBV), Human Herpesvirus-6 (HHV-6), hepatitis B virus (HBV), and HIV-1.²⁵ It serves as a noncompetitive inhibitor of viral DNA polymerases and reverse transcriptases by acting at the pyrophosphate-binding site on these specific enzymes. Phosphorylation by thymidine kinase or other kinases is not required for activation of foscarnet. Therefore, acyclovir-resistant HSV isolates that are TK⁻ or TKA mutants may be susceptible to foscarnet. Resistance to foscarnet is due to mutations in the viral DNA polymerase gene; acyclovir-resistant isolates with altered DNA polymerase activity may be resistant to the drug. Clinical isolates of foscarnet-resistant HSV have been recovered; these isolates are usually susceptible or borderline susceptible to acyclovir.² Foscarnet has been used for treatment of acyclovir-resistant mucocutaneous HSV infections in immunocompromised patients.

2.5 Idoxuridine and Trifluridine

Idoxuridine (5-iodo-2'-deoxyuridine) and trifluridine (5-trifluoromethyl-2'-deoxyuridine; trifluorothymidine) are thymidine nucleoside analogs that represent some of the earliest antiviral compounds developed.² Their use is limited because of toxicity and insufficient potency. These drugs are phosphorylated to the monophosphate form by viral thymidine kinase and to the triphosphate form by cellular enzymes. Viral replication is thought to be disrupted by inhibition of the viral DNA polymerase, although the exact mechanism of action of these drugs is unknown. Trifluridine is indicated for the topical treatment of primary HSV keratoconjunctivitis and recurrent epithelial keratitis. In the U.S., idoxuridine is approved for the treatment of HSV keratitis. Viral resistance to idoxuridine and trifluridine following clinical use has not been reported, although resistance to either drug has been demonstrated in vitro. Resistance is thought to be due to deficient or altered viral thymidine kinase activity. Isolates resistant to idoxuridine are cross-resistant to trifluridine.

2.6 Vidarabine

Vidarabine (9-beta-D-arabinofuranosyladenine; adenine arabinoside), an adenosine nucleoside analog, was the first systemic antiviral agent licensed for use in the United States. It was shown to be effective for treatment of herpes simplex virus (HSV) encephalitis, neonatal infection,² herpes keratitis, and mucocutaneous HSV infection. Although the exact mechanism of action is not completely understood, it is known that vidarabine is phosphorylated by cellular kinases to its triphosphate form and incorporated into newly synthesized viral and cellular DNAs, thus inhibiting DNA synthesis. Vidarabine also is a competitive inhibitor of cellular and viral DNA polymerases.²⁶ Vidarabine-resistant HSV can be generated in vitro but is not a problem clinically. Despite its in vitro activity against acyclovir-resistant HSV, vidarabine has not been effective for treatment of these isolates in HIV-1-infected patients.²⁶ The drug also is not easy to use due to its toxicity, poor solubility in water, and rapid deamination in vivo to a less active compound. Currently, vidarabine is seldom used and has largely been replaced by acyclovir. Vidarabine’s use in the U.S. is now restricted to treatment of HSV keratitis.³

3 Definitions

**Antiviral resistance** – A decrease in susceptibility to an antiviral drug that can be clearly established by in vitro testing and can be confirmed by genetic analysis of the virus and biochemical study of the altered enzymes; **NOTES:** a) In vitro drug resistance must be distinguished from treatment failure in which the viral infection fails to respond to therapy. This failure may or may not be due to the presence of a drug-resistant virus, but may be related to the pharmacokinetics of the drug in an individual patient and the patient’s immunologic status; b) For HSV, in vitro resistance to antiviral agents such as acyclovir and foscarnet has been correlated with clinical resistance.¹⁵
IC$_{50}$ – Concentration of antiviral agent which inhibits virus production by 50% as measured, for example, by plaque formation, DNA synthesis, or antigen-production; **NOTE:** Antiviral susceptibility results have been traditionally expressed as IC$_{50}$ values.$^{15}$

**Precision (of measurement)** – The closeness of agreement between independent test results obtained under stipulated conditions; **NOTE:** Precision is not typically represented as a numerical value but is expressed quantitatively in terms of imprecision—the standard deviation (SD) or the coefficient of variation (CV) of the results in a set of replicate measurements (ISO 3534-1).$^{27}$

**Reproducibility (of results of measurements)** – Closeness of the agreement between the results of measurements of the same measurand carried out under changed conditions of measurement (VIM93).$^{28}$

**Validation** – Confirmation through the provision of objective evidence, that requirements for a specific intended use or application have been fulfilled; (ISO 9000)$^{29}$; **NOTES:** a) WHO defines validation as the action {or process} of proving that a procedure, process, system, equipment, or method used works as expected and achieves the intended result (WHO-BS/95.1793)$^{30}$; b) The components of validation are quality control, proficiency testing, validation of employee competency, instrument calibration, and correlation with clinical findings.

### 4 Variables of Antiviral Susceptibility Testing

To date, no standards exist for antiviral susceptibility testing. The major obstacle to standardization of antiviral susceptibility testing is that many variables influence the final result. These include:

- host cell line;
- viral inoculum titer;
- heterogeneity of virus population;
- strain of virus examined;
- incubation time;
- range of concentration of the antiviral agent tested;
- reference strains;
- assay method;
- endpoint criteria;
- calculation of the endpoint; and
- interpretation of endpoint.

#### 4.1 Host Cell Line, Viral Inoculum, Incubation Time, and Range of Concentration of the Antiviral Agent

The type of host cell line employed, the age and confluence of the cells when used in the assay, and the cell passage level will influence the results of any antiviral susceptibility assay. A number of cell types can be used for susceptibility testing of HSV, including primary rabbit kidney, primary human neonatal kidney, mink lung, continuous African green monkey kidney (CV-1 or Vero), continuous lung carcinoma (A549), or human diploid fibroblast (MRC-5 or WI-38) cells. Testing of a single virus isolate in different cell culture lines may lead to drastically different IC$_{50}$ values.$^{31}$ The size of the virus inoculum is also critical; too high an inoculum can make a susceptible isolate appear resistant, while too low an inoculum can make all isolates appear susceptible. The length of time the virus is exposed to an antiviral agent can also affect results. A longer incubation time may allow replication of a resistant subpopulation of virus which would be manifested by a higher IC$_{50}$ value. The concentration range of the drug tested also affects the IC$_{50}$ value, as does the dilution sequence chosen (e.g., using twofold versus fourfold dilutions of the drug). As the number of drug concentrations tested increases, the precision of the dose-response curve, and therefore the validity of endpoint calculation, improves.
4.2 Heterogeneity of Virus Population and Strain of Virus Used (“Wild” versus “Laboratory Adapted”)

A given isolate of HSV may be a heterogeneous population containing both susceptible and resistant viruses, which may make in vitro results difficult to interpret. Also, continuous subpassage of HSV isolates prior to performing susceptibility testing may result in a viral population that no longer represents the wild-type virus initially identified in a clinical specimen.

4.3 Reference Strains

Reference viral strains for testing each virus-antiviral agent combination should include genetically and phenotypically well-characterized drug-susceptible and drug-resistant isolates that are representative of those most commonly encountered. Drug-resistant strains chosen for reference strains should include relevant drug-resistant phenotypes based on the mode of action of the drug to be tested. For example, when testing nucleoside analogues such as acyclovir against HSV, which require phosphorylation by viral thymidine kinase, TK-negative or deficient viral control strains should be included. Because foscarnet resistance is mediated by mutations in viral DNA polymerase, DNA polymerase mutants should be included when testing these drugs. Drug-susceptible virus isolates are critical for verifying the potency of antiviral stocks and for determining assay-to-assay variability. An acceptable range of IC50s for each strain is determined after repetitive testing of each strain. Antiviral susceptibility assays are valid only if IC50s are within range for each reference strain (see Section 6.3).

4.4 Assay Method

A number of phenotypic assays have been described for testing the susceptibility of HSV to antiviral agents. The most common antiviral susceptibility assays in use include plaque reduction (PRA), dye-uptake, enzyme immunoassay, and DNA hybridization. Detailed descriptions of these methods are presented elsewhere.1,12-15,32,33 The underlying principle for each of these assays is the same. Each utilizes viral-infected cell cultures to which various concentrations of the antiviral agent is added. After appropriate periods of incubation, viral replication is measured by a variety of endpoints such as inhibition of virus-induced cytopathic effect (CPE) or plaque formation; a decrease in the production of viral antigens, enzyme activities, or total virus yield; a reduction in viral nucleic acid synthesis; or the inhibition of cell transformation.

The principle of the PRA is the inhibition of viral plaque formation in the presence of an antiviral agent. Prior to performing the antiviral assay per se, HSV isolates must be titered to ensure an inoculum of approximately 50 to 100 plaque-forming units (pfu) per cell well of a 12-well tissue culture plate. Cell monolayers within wells are infected with titered virus, and subsequently overlaid with medium containing serial twofold dilutions of the antiviral agent to be tested. After an appropriate incubation time (two to three days), infected plates are fixed and stained, and the monolayers are examined to determine the number of plaques. The IC50 is calculated based on the concentration of antiviral agent that reduces the plaque number by 50% as compared to the virus-infected, no-drug control.

4.5 Endpoint Criteria

Susceptibility results have been traditionally expressed as IC50 values because of a greater mathematical precision of the 50% endpoint versus a 90% or 99% endpoint. IC90 values may eventually be shown to correlate better with clinical response, but they are less precise and reproducible than IC50 measurements. An appreciation that clinical isolates represent mixtures of drug-susceptible and drug-resistant phenotypes is critical to understanding how the endpoint criterion can affect results. Resistant virus may be present in low levels which might not be reflected in the IC50 value but would manifest its presence in higher IC90 or IC99 values.34,35 Furthermore, DNA polymerase mutations of HSV may confer only marginal shifts in IC50 values which could go undetected in a mixed population of wild-type and mutant virus. Good definition of such
mixtures can be obtained only by testing appropriate concentrations of drug and by testing a sufficiently large fraction of the population to detect resistant strains. At this time, it is unknown whether a small fraction of drug-resistant virus is important to the behavior of the virus in vivo or how it affects the response of the infection to therapy in a normal host.

### 4.6 Calculation of Endpoints

A variety of linear-regression analyses have been used as well as simple extrapolation from arithmetically plotted graphs for calculation of endpoints. For consistency of results, the laboratory performing HSV antiviral susceptibility tests should adopt a single method of calculating \( IC_{50} \) values. Some variation in absolute \( IC_{50} \) values may be observed when using different methods for endpoint calculations.

### 4.7 Interpretation of Endpoints

The concentration of antiviral drug to which the virus is considered susceptible or resistant has generally been based on median susceptibilities of large numbers of clinical isolates from patients prior to, during, and after antiviral therapy. Because of the variables that affect antiviral susceptibility results, the absolute \( IC_{50} \) value can vary from assay to assay and from laboratory to laboratory. Moreover, in vitro results indicating susceptibility or resistance may not correlate with response of the infection to therapy in vivo. The clinical response of the patient depends upon a number of other factors such as immunological status and pharmacokinetics of the drug in that particular patient (e.g., dose or route of administration could be inappropriate). A poor clinical response may occur even though the antiviral susceptibility testing denotes in vitro susceptibility. For example, oral administration of an antiviral in an AIDS patient with underlying absorption problems due to gastroenteritis may result in a poor clinical response even though the \( IC_{50} \) denotes in vitro susceptibility.

Englund, et al\(^{17}\) and Safrin, et al\(^{26}\) have documented HSV infections in patients with AIDS and other immunological disorders who failed to respond to therapy despite in vitro \( IC_{50} \) values indicating susceptibility to vidarabine or acyclovir. Conversely, HSV isolates with elevated \( IC_{50} \) values for acyclovir occasionally can be recovered from normal hosts who have responded to acyclovir therapy. Thus, a high \( IC_{50} \) alone may not be sufficient to predict treatment failure. Neither can in vitro susceptibility to a drug a priori predict successful clinical outcome. Nevertheless, in vitro resistance often does correlate with treatment failure in immunocompromised patients. For example, patients with AIDS and other immune disorders who have HSV infections refractory to acyclovir frequently shed HSV strains with elevated \( IC_{50} \) values, and those that have been tested for TK activity have been shown to have deficient or altered TK activity.\(^{17}\)

Another approach to interpreting susceptibility endpoints is to compare the ratio of \( IC_{50} \) values of an isolate obtained prior to therapy (or a well-characterized reference control strain) with that of an isolate obtained during therapy; a significant increase in the ratio of \( IC_{50} \) values would denote resistance. However, pretherapy isolates are often unavailable, and the ratio of \( IC_{50} \)s considered clinically significant is unknown.

The antiviral susceptibility testing method itself can markedly affect \( IC_{50} \) values. For example, it has been consistently documented that the dye uptake assay for HSV results in \( IC_{50} \) values two to three times higher than those of the PRA. Furthermore, ELISA or DNA hybridization assays which measure viral antigen and nucleic acid production, respectively, may produce lower \( IC_{50} \) values than the PRA. This occurs with PRA because antiviral activity, at low drug concentrations, may be manifested not as a reduction in plaque number but a decrease in size of countable plaques. Only at relatively higher concentrations of drug will the actual plaque number decrease.
5 Plaque Reduction Assay (PRA)

5.1 Cells, Media, and Reagents

The following are the media and reagents used in the PRA:

- Three 12-well cell culture plates containing monolayers of Vero cells in Eagle’s minimum essential medium (EMEM) with 10% fetal bovine serum (FBS). Vero cells (CCL 81, American Type Culture Collection, Manassas, VA) can be propagated in the laboratory. Stock flasks of these cells can be maintained and used for preparing monolayers in tissue culture tubes and 12-well plates. 37

- Overlay medium. Three different media may be used to overlay antiviral agents onto infected cell monolayers. The medium allows for the formation of discrete plaques without secondary spread of the virus to the remainder of the monolayer. The choice of medium should be made by the individual laboratory based on availability and logistics of use.
  - 0.8% agarose (w/v) in distilled, deionized water. Prior to virus adsorption, prepare and autoclave for 15 minutes at 15 psi and 121 °C. Autoclaved agarose should be equilibrated to 60 °C. After virus adsorption, mix twofold concentrations of drug in twofold EMEM (without phenol red and supplemented with 4 mM L-glutamine and 4% FBS) with equal volumes of 0.8% agarose and hold mixture at 45 °C. The agarose used in the assay must be of high quality and purity to avoid toxicity of the cell monolayer. Molecular biology grade agarose containing no more than 0.15% sulfate is usually acceptable but it should be tested for toxicity to the cell monolayer prior to performing the plaque reduction assay.
  - Human immune globulin (HIG), diluted to 1% v/v in EMEM (with phenol red and supplemented with 2 mM L-glutamine and 2% FSBS) also can be used for the overlay medium. The HIG used must contain group-specific HSV neutralizing antibodies to prevent secondary spread of virus from the primary plaques that form. A 1% (v/v) concentration of HIG reliably contains sufficient concentration of neutralizing antibody to HSV to prevent secondary plaque formation.
  - 2% Methylcellulose (w/v) in distilled, deionized water. Use methylcellulose 4,000 cps viscosity. To prepare, slowly add methylcellulose to water heated to near boiling in a large bottle containing a heavy stir bar. Stir until well dispersed. Autoclave immediately (while hot) for 20 minutes at 15 psi and 121 °C. Stir in cold water (2 to 8 °C in refrigerator) until cool (overnight) and methylcellulose has gone into solution. Store at 2 to 8 °C for up to six months. Warm to room temperature before use and mix with twofold concentrations of drug in twofold EMEM supplemented with L-glutamine (4 mM final concentration) and fetal bovine serum (4% final concentration, v/v).

- Twofold EMEM without phenol red or L-glutamine when using an agarose or methylcellulose overlay. Prior to use in the PRA, the medium should be supplemented with L-glutamine (4-mM final concentration) and fetal bovine serum (4% final concentration, v/v). If HIG is used as the overlay, EMEM with phenol red (without L-glutamine) is used and is supplemented with L-glutamine (2 mM final concentration), 2% FBS and HIG to 1%.

- 10% neutral buffered formalin, pH 6.9 to 7.1 (commercially available).

- Absolute methanol; 50% methanol v/v in distilled or deionized water.

- Stock solutions of acyclovir ([9-(2-hydroxyethoxymethyl)-9H-guanine] and foscarnet (trisodium phosphonoformate)).
— Source. Reference powders of antiviral agents can be obtained commercially or directly from the drug manufacturer. Acceptable powders bear a label that states the drug’s generic name, lot number, potency, and its expiration date. The powders should not contain preservatives or stabilizers that may inhibit viral growth. Patient-use formulations should not be used for susceptibility testing. The powders are to be stored as recommended by the manufacturer.

— Weighing antiviral powders. Either of the following formulas may be used to determine the amount of powder or diluent needed for a standard solution:

\[
\text{Weight (mg)} = \frac{\text{Volume (mL)} \cdot \text{Concentration (µg/mL)}}{\text{Potency (µg/mg)}}
\]

\[
\text{Volume (mL)} = \frac{\text{Weight (mg)} \cdot \text{Potency (µg/mg)}}{\text{Concentration (µg/mL)}}
\]

The antiviral powders should be weighed on an analytical balance that has been calibrated with National Institute of Standards and Technology (NIST) weights (or other approved reference weights). If possible, more than 100 mg of powder should be weighed. It is advisable to accurately weigh a portion of the antimicrobial agent in excess of that required and to calculate the volume of diluent needed to obtain the final concentration desired as in Formula 2 above.

**NOTE:** Because the concentration of the active compound depends on the particular formulation of drug used, it is important to state the exact formulation used and the formula weight. For example, commercially available foscarnet consists of the trisodium salt of phosphonoformate with 6 equivalents of water of hydration with a formula weight of 300. Each gram of foscarnet contains only 423 mg of the active agent, phosphonoformic acid.

**Example:** To prepare 100 mL of a stock solution containing 640 µg/mL of antiviral agent with a potency of 825 (µg/mg), (70 to 75 mg) 70,000 to 75,000 µg of the antiviral powder should be accurately weighed. If the actual weight is (72 mg) 72,000 µg, the volume of diluent needed is then as follows:

\[
\text{Volume (mL)} = \frac{72,000 \mu g (72 \text{ mg})}{640 \mu g/mL} \cdot \frac{825 (\mu g/\text{mg})}{(\text{Potency})} = 92.8 \text{ mL}
\]

Therefore, the (72 mg) 72,000 µg of antiviral powder is to be dissolved in 92.8 mL of diluent.

— Preparing stock solutions. Stock solutions of acyclovir and foscarnet should be prepared in sterile, distilled, deionized water at concentrations (normally ten times the highest concentration to be tested) which are convenient for preparing a range of drug dilutions to be used in the susceptibility assay. Final stock concentrations of 640 µg/mL of acyclovir and 2,000 µg/mL of foscarnet are suggested. The concentrations of stock solutions, however, may vary depending on the range of drug concentrations selected for testing and on the antiviral agent employed. See Table 2 for the recommended range of antiviral drug concentrations for use in a plaque reduction assay.
**Table 2. Recommended Range of Antiviral Drug Concentrations for Use in the Plaque Reduction Assay**

<table>
<thead>
<tr>
<th>Virus Type</th>
<th>Antiviral Agent</th>
<th>Suggested Drug Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV (Susceptible control)</td>
<td>Acyclovir</td>
<td>0, 0.12, 0.25, 0.5, 1.0, 2.0</td>
</tr>
<tr>
<td>HSV (Resistant control)</td>
<td></td>
<td>0, 1.0, 2.0, 4.0, 8.0, 16</td>
</tr>
<tr>
<td>Patient isolate</td>
<td></td>
<td>0, 0.06, 0.12, 0.25, 0.50, 1.0, 2.0, 4.0, 8.0, 16, 32, 64</td>
</tr>
<tr>
<td>HSV (Susceptible control)</td>
<td>Foscarnet</td>
<td>0, 16.7, 25, 33.3, 50, 100</td>
</tr>
<tr>
<td>HSV (Resistant control)</td>
<td></td>
<td>0, 50, 66.7, 100, 200, 400</td>
</tr>
<tr>
<td>Patient isolate</td>
<td></td>
<td>0, 8.35, 16.7, 25, 33.3, 50, 66.7, 100, 200, 400</td>
</tr>
</tbody>
</table>

**NOTE:** The concentration range of drug tested affects the quality of the dose-response curve, and therefore, the validity of endpoint calculations.

Sterilization of stocks through a 0.2-µm filter is optional, since microbial contamination is extremely rare, solutions that have not been sterilized are generally acceptable. Since some filters can bind the antiviral or antimicrobial agent, thus reducing the calculated concentration and potency, it may be necessary to pretest filters to determine their binding capacity, or use filters recommended by the manufacturer to be low binding filters.

Aliquots of the prepared stock concentrations of acyclovir and foscarnet should be dispensed into sterile glass, polypropylene, polystyrene, or polyethylene vials, and carefully labeled with the name of the drug, its concentration, the date prepared, and the expiration date. Stock concentrations must be stored at -70 °C and can be used for up to one year. Vials should be thawed as needed and used the same day. Any unused drug should not be refrozen and should be discarded after the testing for the day is completed.

- EMEM with Earle's balanced salt solution supplemented with 2 mM L-glutamine, 20 mM HEPES, and either 10% (cell growth medium) or 2% (cell maintenance medium) heat-inactivated (56 °C for 30 minutes) FBS.38
- Fresh or frozen (-70 °C) virus isolates, including acyclovir- and foscarnet-susceptible and acyclovir- and foscarnet-resistant control strains of HSV (See Table 3). Drug-susceptible and drug-resistant control strains of HSV can be obtained as gifts from drug manufacturers and from colleagues. Well-characterized, drug-susceptible and drug-resistant clinical isolates may also be used. HSV-F can be obtained from the ATCC® as number VR-733.
Table 3. Recommended IC50 Limits for Quality Control Strains for Plaque Reduction Assay

<table>
<thead>
<tr>
<th>Strain Designation</th>
<th>HSV Type</th>
<th>Acyclovir</th>
<th>Foscarnet</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1 F†</td>
<td>1</td>
<td>0.25 - 0.50</td>
<td>30.0 - 50.0</td>
</tr>
<tr>
<td>SLU-360 ACV†</td>
<td>1</td>
<td>≥ 2.0</td>
<td>15.0 - 30.0</td>
</tr>
<tr>
<td>PAA5‡</td>
<td>1</td>
<td>≥ 2.0</td>
<td>≥ 100.0</td>
</tr>
</tbody>
</table>

HSV-1 F, acyclovir susceptible, was originally isolated from a facial lesion. It is available from the ATCC®: ATCC® VR-733.

SLU 360 ACV† was derived from an acyclovir-susceptible HSV-1 isolate cultured from a finger. It was generated by sequential passage in the presence of 10 µg of acyclovir per mL.39

PAA5‡ is a DNA polymerase mutant of HSV-1 strain KOS generated by passage in the presence of phosphonoacetic acid. It is both acyclovir- and foscarnet-resistant.40

- Staining solutions
  - 0.8% crystal violet in 50% ethanol.
  - 4.0% Giemsa stain (alternative stain). A saturated solution of Giemsa stain is prepared in absolute methanol. On the day of use, a 4.0% (v/v) working solution of Giemsa is prepared in distilled water from the saturated Giemsa stock.

- Distilled, deionized water.

- Phosphate buffered saline (PBS), pH 6.9-7.1 (commercially available).

5.2 Supplies

The following supplies are needed for the plaque reduction assay:

- Sterile 1-, 5-, 10-, and 25-mL serological pipettes. Disposable plastic or glass pipettes are commercially available and convenient to use.

- Sterile, unplugged pipettes.

- Sterile 15-mL and 50-mL centrifuge tubes for dilution of HSV isolates and antiviral agents.

- Tissue culture plates, 12-well, flat-bottom.

- Tissue culture flasks, 75 cm².

- 16 x 125-mm tissue culture tubes.

- Freezer vials tolerant to -70 °C for freezing HSV isolates and stock solutions of antiviral agents.

- 0.5% hypochlorite solution (1:10 dilution of bleach) for cleaning work surfaces.

- Racks designed to hold tissue culture tubes at a 5 to 7° angle.
• Safety pipetting devices, protective clothing, latex gloves, infectious-waste disposal containers, and adequate sterilization facilities.

• Linear regression probability program for calculation of IC\textsubscript{50} values (dose-effect analysis).

5.3 Equipment

The following equipment is needed for the plaque reduction assay:

• Inverted and standard light microscopes capable of 100x to 200x magnification.

• Stereo dissecting microscope.

• Class II biological safety cabinet.

• Refrigerator at 2 to 8 °C.

• Freezer at -70 °C or lower.

• Vacuum source and collection trap containing 0.5% sodium hypochlorite to be used for collecting aspirated infectious waste.

• Humidified incubator at 35 to 37 °C with 5% CO\textsubscript{2}.

• Water baths at 56 °C for heat inactivation of FBS; 60 °C and 45 °C for storing autoclaved 0.8% agarose prior to use; 37 °C for rapid thawing of frozen isolates.

5.4 Storage Instructions

The storage instructions noted below should be followed:

• Twelve-well cell culture plates containing monolayers of Vero cells in EMEM with 10% FBS should be prepared and incubated at 35 to 37 °C in a humidified atmosphere of 5% CO\textsubscript{2} for 24 to 72 hours or until the cell monolayers are just confluent. Stock flasks of these cells can be maintained and used for preparing monolayers in tissue culture tubes and 12-well plates.\textsuperscript{37}

• The acyclovir and foscarnet stock solutions should be stored in single-use aliquots at -70 °C for a period of one year. The antiviral agents should be used immediately after thawing; repeated freeze-thaws or storage under conditions other than those recommended should be avoided.

• EMEM and twofold EMEM supplemented with L-glutamine and FBS should be stored at 2 to 8 °C for a period of up to one month. For unsupplemented media, observe the expiration date established by the manufacturer.

• All other reagents should be stored at room temperature (20 to 25 °C) or otherwise specified by the manufacturer.

• Expiration dates should be indicated on the labels of all media and reagents, or observe the expiration date established by the manufacturer.
5.5 Preparation of Cell Culture Plates, HSV Isolates, and Antiviral Agents

5.5.1 Collection, Preparation, and Storage of HSV Isolates

Clinical isolates of HSV are required to perform the PRA. First or low-passage isolates are used, because they are more likely to be representative of the original mixed population of the clinical isolate than a higher passaged stock. Continuous subpassage of isolates should be avoided. Frozen isolates are stored at -70 °C or freshly isolated virus can be used for testing. Clinical isolates or control viral strains should be grown in 16 x 125-mm tissue culture tubes of appropriate host cells. The isolates are harvested when 50 to 100% of the cell monolayer is infected. To harvest an isolate, collect both infected cells and cell culture medium by using a pipette to scrape the cells from the tube surface. Because HSV is efficiently released from infected cells, cell-free supernatant is prepared by centrifuging the harvest at low speed (approximately 1000 x g) for 10 minutes at 4 °C. Clarified supernatants can be used fresh or should be frozen at -70 °C or colder in single-use aliquots of 0.5 mL to 0.75 mL. Manipulations of virus should be performed in a Class II biological safety cabinet.

5.5.2 Dilution and Titration of HSV Isolates and Control Strains

The steps outlined below should be followed for preparing titered stocks of clinical HSV isolates and control strains:

(1) The optimum virus inoculum for the PRA can range from 50 to 100 plaque-forming units (PFU) per well in a 12-well plate format. Six- and 24-well plates are also acceptable with appropriate adjustments in the number of PFUs and volumes of reagents and antiviral agents used.

(2) To determine the dilution factor required to achieve 50 to 100 PFU per well in a 12-well format, virus stocks must first be titrated:

(a) Refer to Section 5.5.3 for preparation of 12-well culture plates and Section 5.5.1 for preparation and storage of HSV isolates.

(b) If using frozen clarified supernatants of HSV isolates, thaw virus stocks quickly in a 37 °C water bath, and maintain on ice. Use aseptic technique when handling the virus stock and preparing dilutions.

(c) Make tenfold serial dilutions of the virus stock from $10^{-1}$ to $10^{-7}$ in EMEM with 2% FBS.

(d) Remove the medium from each well prior to inoculation. Inoculate each well with 0.4 mL of diluted virus. Inoculate two wells per tenfold virus dilution.

(e) Allow virus to adsorb at 35 to 37 °C in a humidified incubator with 5% CO₂ for 75 minutes ± 15 minutes. Gently rock the plate back and forth every 15 minutes to maximize virus adsorption and to prevent drying of the monolayer. Prior to virus adsorption, autoclave the agarose stock and equilibrate to 60 °C, if an agarose overlay will be utilized.

(f) Remove the inoculum and replace it with 3.0 mL of agarose, methylcellulose, or HIG (see Section 5.1 for preparation depending on the overlay medium chosen to use). When using agarose, allow the agarose overlay to gel at room temperature before placing plates in the incubator.

(g) Incubate plates at 35 to 37 °C for 72 hours in a humidified atmosphere of 5% CO₂. Examine the plates daily for the development of plaques. HSV strains forming very large plaques may require staining at 48 hours.
(h) Refer to Section 5.6.3 for fixation, staining, and counting of plaques. A plaque is a focal area of cytopathic effect that may or may not surround a clear area of cell lysis.

(i) Calculate the titer of virus in 0.4 mL. Using the plaque counts of the wells that contain between 50 and 100 PFU, calculate the average plaque count of the two wells, and multiply the average plaque count by the dilution factor. For example, if the $10^{-3}$ wells contain an average plaque count of 100 PFU, then the titer in 0.4 mL is $10^5$ PFU ($10^3 \times 100$). Record this titer for future use when performing the actual antiviral susceptibility assay.

5.5.3 Preparation of 12-Well Cell Culture Plates

The following is the procedure for preparing a 12-well culture plate:

(1) Determine the number of 12-well plates of Vero cells needed to perform a given susceptibility assay. See Figures 1 and 2 for the number and configurations of 12-well plates when one clinical HSV isolate and two control virus strains are examined for acyclovir or foscarnet susceptibility. To prepare cell monolayers, the 12-well plates should be seeded with Vero cells and incubated at 35 to 37 °C in a humidified atmosphere of 5% CO$_2$ until the cell monolayers are just confluent (usually 24 to 72 hours). Stock flasks of these cells can be maintained and used for preparing monolayers in tissue culture tubes and 12-well plates. Cell culture manipulations should be performed in a Class II biological safety cabinet.

(2) For each antiviral agent employed, two 12-well plates are needed for each clinical HSV isolate, and an additional plate is required for each control strain.

(3) The cell monolayers should be microscopically examined before use to determine the quality of the cells. At the time of virus inoculation, the cell monolayers should be freshly confluent and not overgrown.

5.5.4 Preparation of Antiviral Agent Dilutions

The steps outlined below are to be followed in preparing dilutions of antiviral agents:

(1) The dilutions and volumes of drug needed to perform a susceptibility test are dependent on the range drug concentrations employed and the total number of HSV isolates to be tested. See Table 2 for the recommended range of acyclovir and foscarnet concentrations and Figures 1 and 2 for the number and configuration of 12-well plates when one clinical isolate and two control virus strains are examined. See Tables 4 through 7 for suggested schemes for preparing dilutions of acyclovir or foscarnet when using HIG, agarose, or methylcellulose overlay medium. Include a sufficient number of drug concentrations in an appropriate range of concentrations for optimal calculation of the IC$_{50}$. The proposed susceptibility “breakpoints” for the PRA for HSV in Vero cells are $\geq 2$ µg/mL for acyclovir and $\geq 100$ µg/mL for foscarnet. The concentrations to be tested for a particular antiviral agent should encompass the interpretive breakpoints.

(2) Use aseptic technique when preparing dilutions of antiviral agents. Use sterile polypropylene tubes to perform drug dilutions. Prepare working dilutions of the drugs from stock solutions on the day of use. Use a separate pipette for each dilution in the set. Mix each dilution thoroughly before making the next dilution.

(3) Use a “no drug” control for each virus tested.

(4) Test all drug concentrations in duplicate.
When using HIG as overlay medium, prepare drug dilutions in EMEM-2% FBS-2 mM L-glutamine with 1% human immunoglobulin. Tables 4 and 5 illustrate the preparation of the recommended drug dilutions when using 1% HIG as overlay medium. Volumes are sufficient to test one clinical isolate and two control viruses in a 12-well plate format (3 mL of media per well, two wells per concentration). Six- and 24-well plates are also acceptable with appropriate adjustments in the volume of media.

When using agarose or methylcellulose as overlay media, prepare drug dilutions in 2 x EMEM-4% FBS-4 mM L-glutamine. Mix drug dilutions with an equal volume of 0.8% agarose or 2% methylcellulose just prior to use. Since there will be a 1:1 dilution of the drug when combined with the agarose or methylcellulose, prepare working antiviral solutions at twice the desired final concentration (illustrated in Tables 6 and 7). Once agarose or methylcellulose is added to the drug dilutions and mixed, immediately add the prepared mixtures to the appropriate wells of the 12-well plate.
Figure 1. Configuration of 12-well Plates When One Clinical HSV Isolate and Two Control Virus Strains are Examined for Acyclovir Susceptibility
Patient Isolate

Acyclovir (µg/mL)

Figure 1. (Continued)

An NCCLS global consensus standard. ©NCCLS. All rights reserved.
Figure 2. Configuration of 12-well Plates When One Clinical HSV Isolate and Two Control Virus Strains are Examined for Foscarnet Susceptibility
Patient Isolate

Foscarnet (µg/mL)

Figure 2. (Continued)
Table 4. Scheme for Preparing Dilutions of Acyclovir to Be Used With 1% Immunoglobulin Overlay Medium. (NOTE: Volumes are sufficient to test one clinical isolate and two control viruses in a 12-well plate format.)

<table>
<thead>
<tr>
<th>Step</th>
<th>Conc. (µg/mL)</th>
<th>Source</th>
<th>Volume (mL)</th>
<th>+ Medium* (mL)</th>
<th>Final Conc. (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>640</td>
<td>stock</td>
<td>2</td>
<td>18</td>
<td>64</td>
</tr>
<tr>
<td>2</td>
<td>64</td>
<td>step 1</td>
<td>12</td>
<td>12</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>32</td>
<td>step 2</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>step 3</td>
<td>18</td>
<td>18</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>step 4</td>
<td>20</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>step 5</td>
<td>20</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>step 6</td>
<td>20</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>step 7</td>
<td>20</td>
<td>20</td>
<td>0.5</td>
</tr>
<tr>
<td>9</td>
<td>0.5</td>
<td>step 8</td>
<td>20</td>
<td>20</td>
<td>0.25</td>
</tr>
<tr>
<td>10</td>
<td>0.25</td>
<td>step 9</td>
<td>15</td>
<td>15</td>
<td>0.12</td>
</tr>
<tr>
<td>11</td>
<td>0.12</td>
<td>step 10</td>
<td>10</td>
<td>10</td>
<td>0.06</td>
</tr>
</tbody>
</table>

* EMEM-2% FBS-2 mM L-glutamine + 1% HIG.

NOTE: An additional 18 mL of EMEM-2% FBS-2 mM glutamine + 1% HIG is needed for the “no drug” control wells.
Table 5. Scheme for Preparing Dilutions of Foscarnet to Be Used With 1% Immunoglobulin Overlay Medium. (NOTE: Volumes are sufficient to test one clinical isolate and two control viruses in a 12-well plate format.)

<table>
<thead>
<tr>
<th>Step</th>
<th>Conc. (µg/mL)</th>
<th>Source</th>
<th>Volume (mL)</th>
<th>+ Medium* (mL)</th>
<th>Final Conc. (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2,000</td>
<td>stock</td>
<td>7</td>
<td>28</td>
<td>400</td>
</tr>
<tr>
<td>2</td>
<td>400</td>
<td>step 1</td>
<td>22</td>
<td>22</td>
<td>200</td>
</tr>
<tr>
<td>3</td>
<td>200</td>
<td>step 2</td>
<td>20</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>200</td>
<td>step 2</td>
<td>10</td>
<td>20</td>
<td>66.7</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>step 3</td>
<td>15</td>
<td>15</td>
<td>50</td>
</tr>
<tr>
<td>6</td>
<td>66.7</td>
<td>step 4</td>
<td>15</td>
<td>15</td>
<td>33.3</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>step 5</td>
<td>10</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>8</td>
<td>33.3</td>
<td>step 6</td>
<td>15</td>
<td>15</td>
<td>16.7</td>
</tr>
<tr>
<td>9</td>
<td>16.7</td>
<td>step 8</td>
<td>15</td>
<td>15</td>
<td>8.35</td>
</tr>
</tbody>
</table>

*EMEM-2% FBS-2 mM L-glutamine + 1% HIG.

NOTE: An additional 18 mL of EMEM-2% FBS-2 mM glutamine + 1% HIG is needed for the “no drug” control wells.
Table 6. Scheme for Preparing Dilutions of Acyclovir to Be Used With Agarose or Methylcellulose Overlay Medium. (NOTE: Volumes are sufficient to test one clinical isolate and two control viruses in a 12-well plate format.)

<table>
<thead>
<tr>
<th>Step</th>
<th>Conc. (µg/mL)</th>
<th>Source</th>
<th>Vol. (mL)</th>
<th>Medium* Vol. (mL)</th>
<th>Final Conc. ‡ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>640</td>
<td>stock</td>
<td>1</td>
<td>4</td>
<td>64</td>
</tr>
<tr>
<td>2</td>
<td>640</td>
<td>stock</td>
<td>2</td>
<td>18</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>64</td>
<td>step 2</td>
<td>10</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>32</td>
<td>step 3</td>
<td>10</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>step 4</td>
<td>12</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>step 5</td>
<td>14</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>step 6</td>
<td>12</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>step 7</td>
<td>10</td>
<td>10</td>
<td>0.5</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>step 8</td>
<td>10</td>
<td>10</td>
<td>0.25</td>
</tr>
<tr>
<td>10</td>
<td>0.5</td>
<td>step 9</td>
<td>10</td>
<td>10</td>
<td>0.12</td>
</tr>
<tr>
<td>11</td>
<td>0.25</td>
<td>step 10</td>
<td>10</td>
<td>10</td>
<td>0.06</td>
</tr>
</tbody>
</table>

*Twofold EMEM-4% FBS-4 mM L-glutamine.

† Mix this volume of working solution with an equal volume of 0.8% agarose or 2% methylcellulose.

‡ Following addition of solid overlay medium.

NOTE: An additional 9 mL of twofold EMEM-4% FBS-4 mM L-glutamine mixed with an equal volume of 0.8% agarose or 2% methylcellulose is needed for the “no drug” control wells.
Table 7. Scheme for Preparing Dilutions of Foscarnet to Be Used With Agarose or Methylcellulose Overlay Medium. (NOTE: Volumes are sufficient to test one clinical isolate and two control viruses in a 12-well plate format.)

<table>
<thead>
<tr>
<th>Step</th>
<th>Conc. (µg/mL)</th>
<th>Source</th>
<th>Vol. (mL)</th>
<th>Working Sol. Conc. (µg/mL)</th>
<th>Working Sol. Vol. (mL)</th>
<th>Final Conc. (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2,000</td>
<td>stock</td>
<td>10</td>
<td>800</td>
<td>7</td>
<td>400</td>
</tr>
<tr>
<td>2</td>
<td>800</td>
<td>step 1</td>
<td>15</td>
<td>400</td>
<td>7</td>
<td>200</td>
</tr>
<tr>
<td>3</td>
<td>400</td>
<td>step 2</td>
<td>12</td>
<td>200</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>400</td>
<td>step 2</td>
<td>6</td>
<td>133.3</td>
<td>7</td>
<td>66.7</td>
</tr>
<tr>
<td>5</td>
<td>200</td>
<td>step 3</td>
<td>10</td>
<td>100</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>6</td>
<td>133.3</td>
<td>step 4</td>
<td>10</td>
<td>66.7</td>
<td>7</td>
<td>33.3</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>step 5</td>
<td>5</td>
<td>50</td>
<td>7</td>
<td>25</td>
</tr>
<tr>
<td>8</td>
<td>66.7</td>
<td>step 6</td>
<td>10</td>
<td>33.3</td>
<td>7</td>
<td>16.7</td>
</tr>
<tr>
<td>9</td>
<td>33.3</td>
<td>step 8</td>
<td>10</td>
<td>16.7</td>
<td>4</td>
<td>8.35</td>
</tr>
</tbody>
</table>

* Twofold EMEM-4% FBS-4 mM L-glutamine.

† Mix this volume of working solution with an equal volume of 0.8% agarose or 2% methylcellulose.

‡ Following addition of solid overlay medium.

NOTE: An additional 9 mL of twofold EMEM-4% FBS-4 mM L-glutamine mixed with an equal volume of 0.8% agarose or 2% methylcellulose is needed for the “no drug” control wells.
5.6 Performance of the Plaque Reduction Assay

5.6.1 Dilution of Clinical HSV Isolates and Control Strains

The steps outlined below should be followed for preparing dilutions of titered clinical HSV isolates and control strains:

1. The optimum virus inoculum for the PRA susceptibility assay can range from 50 to 100 PFU per well in a 12-well format. Six- and 24-well plates are also acceptable with appropriate adjustments in the number of the PFUs and volumes of reagents and antiviral agents used.

2. Refer to Section 5.5.2 for the virus titration procedure.

3. Use aseptic technique and a Class II biosafety cabinet for preparing all dilutions of virus stocks. Thaw the titered virus stock quickly in a 37°C water bath, mix thoroughly, and dilute with EMEM-2% FBS-2 mM L-glutamine to a final concentration of 50 to 100 PFU per 0.4 mL. The dilutions required will vary depending on the titer of the stock virus. For example, if the virus titration showed that 0.4 mL of stock virus contains 1 x 10⁵ PFU, use the material at a dilution of 1:1000 (10⁻³). Keep all viral dilutions on ice. Each well of a 12-well plate will be inoculated with 0.4 mL of diluted virus stock (in this example, 100 PFU). A total of 5.0 mL each of diluted drug-susceptible and drug-resistant control strains and 10.0 mL of diluted virus stock of the patient isolate will be needed when testing one patient isolate and two control viruses. To ensure a sufficient volume of diluted virus to compensate for volume lost during pipetting, a minimum of 6.0 mL of diluted drug-susceptible and drug-resistant controls strains and 12.0 mL of diluted patient isolate should be prepared.

5.6.2 Virus Inoculation and Drug Addition

The following steps should be performed for virus inoculation and drug addition:

1. Prepare and autoclave the agarose stock and equilibrate to 60°C if an agarose overlay will be used. If methylcellulose is used, warm to room temperature.

2. Remove a 12-well plate of freshly confluent Vero cells from the incubator and gently aspirate the medium from each of the 12 wells, taking care not to disturb the cell monolayer.

3. Inoculate the wells with 0.4 mL of the diluted HSV suspension. Clearly label the plate and wells with an appropriate identification number.

4. Incubate the plate for 75 minutes ± 15 minutes at 35 to 37°C in a humidified incubator with 5% CO₂ to allow for virus adsorption. Gently rock the plate back and forth every 15 minutes to prevent drying of the monolayer.

5. If agarose will be used in the overlay, mix the twofold concentrations of drug in twofold EMEM with equal volumes of 0.8% agarose and hold at 45°C. If methylcellulose is used, mix the twofold concentrations of drug in twofold EMEM with equal volumes of 2% methylcellulose.

6. Gently aspirate the virus inoculum from each well and overlay duplicate wells with 3.0 mL each of the diluted antiviral agent. Two wells should receive 3.0 mL of overlay (twofold EMEM/0.8% agarose, twofold EMEM/2% methylcellulose, or EMEM + 1% HIG) to which no drug has been added. These serve as virus-infected “no drug” controls. Allow the agarose overlay to gel at room temperature.

7. Incubate the plates at 35 to 37°C in a humidified incubator with 5% CO₂ for 72 hours. Best results are obtained when plaque formation in the “no drug” control wells develops within this time. Occasionally,
clinical isolates that produce large plaques must be stained at 48 hours. The monolayers should be examined daily for progression of the plaques and possible deterioration of the monolayers. If the cell monolayers deteriorate, the PRA should be repeated.

5.6.3 Fixation, Staining, and Counting of Plaques

For fixation, staining, and counting of plaque formation, the following steps should be performed:

1. **Wells overlaid with agarose**: Following the three-day incubation, add 2.0 mL of 10% buffered formalin onto the top of the agarose plugs in each well of the 12-well plate and maintain the plates at room temperature for 60 minutes. Following the one-hour incubation with formalin, aspirate the formalin into a suitable trap for the collection of formalin. Immediately remove the agarose plug from each well. Be careful not to disturb the cell monolayers. Gently rinse the wells with tap water to remove any remaining pieces of agarose. Flood each well of the 12-well plate with 0.8% crystal violet in 50% ethanol for 30 to 60 seconds at room temperature. Aspirate off the stain, gently rinse the wells in tap water, and invert the plates to dry.

2. **Wells overlaid with HIG**: Following the three-day incubation, aseptically aspirate the HIG overlay from each well of the 12-well plate. Flood each well with 50% methanol in water for 30 seconds and then aspirate the methanol. Flood each well with absolute methanol for an additional 30 seconds and again aspirate. Stain the methanol-fixed wells with 4% Giemsa stain for 20 minutes at room temperature. Aspirate off the staining solution and invert the plates to dry.

3. **Wells overlaid with methylcellulose**: Following the three-day incubation, aseptically aspirate the methylcellulose overlay from each well of the 12-well plate. Gently wash each well with PBS to remove any remaining methylcellulose and then aspirate off the PBS. Be careful not to disturb the cell monolayers during the washing step. Add 2.00 mL of 10% buffered formalin in each well of the 12-well plate and maintain the plates at room temperature for 60 minutes. Following the one-hour incubation with formalin, aspirate the formalin into a suitable trap for the collection of formalin. Gently rinse the wells in tap water. Flood each well of the 12-well plate with 0.8% crystal violet in 50% ethanol for 30 to 60 seconds at room temperature. Aspirate off the stain, gently rinse the wells in tap water, and invert the plates to dry.

4. Count the plaques under a dissecting microscope (20x to 30x). A plaque is a focal area of cytopathic effect that may or may not surround a clear area of cell lysis.

5. Record counts in duplicate and analyze data to determine the 50% inhibitory concentration (IC50) for each drug and virus isolate tested (see Section 7).

5.6.4 Procedural Notes

The following procedural items should be noted when performing the PRA:

1. Bring all reagents and medium to room temperature (20 to 25 °C) before use and return all of them to appropriate storage temperatures immediately after use.

2. Do not use materials and reagents beyond the assigned expiration dates, and do not interchange or mix different lots of reagents.

3. Accurate pipetting and careful dilution of antiviral drugs and virus isolates is required for quality performance of this procedure. Use separate, sterile pipettes when preparing each dilution and during the inoculation of each virus isolate.
Care must be taken to use an appropriate virus inoculum in the PRA. As previously stated, optimum results are achieved with 50 to 100 PFUs per well when using a 12-well plate. If the virus inoculum is too low or too great, one may have the false impression that the antiviral agent is more or less effective than what might be expected if the appropriate inoculum was employed.

When aspirating culture medium and inoculum from the wells of the cell culture plates, use a sterile, disposable pipette attached to the tubing from a vacuum aspirator and trap. The vacuum should be such that it yields a gentle aspiration without disturbing the cell monolayer. Hold the cell culture plate at a 20 to 30° angle and aspirate at the meniscus, directing the tip of the pipette at a 45° angle and moving down the side of each well to the bottom. Completely aspirate all the residual material from the wells. To avoid drying out the monolayer, do not aspirate the medium or inoculum from more than one plate at a time.

Strictly adhere to incubation temperatures and times to avoid false results.

Plaque development is optimal when cell monolayers are just confluent at the time of infection.

Agarose of high quality and purity is important for good plaque formation. The agarose should also be prepared in high-quality distilled, deionized water.

Before overlaying infected monolayers with the appropriate mixtures of drug, be certain that the agarose is cooled to 45 °C. Otherwise the virus or the cells may be killed when the overlay is added.

The HIG used as an overlay medium should contain group-specific HSV neutralizing antibodies to prevent secondary spread of virus from the primary plaques that form.

Microscopic examination of stained monolayers is critical for detection of small plaques which may indicate drug-resistant virus.

6 Quality Control Procedures

6.1 Purpose

The goals of a quality control program for antiviral susceptibility testing are to assist in the monitoring of the following:

- precision of the PRA;
- the performance of reagents used in the test; and
- the performance of persons who carry out the tests and read the results.

Performance of antiviral susceptibility testing is a complex procedure which involves the interaction of virus, antiviral agents, and host cell lines. These and other component interactions may significantly influence test results. Consequently, close attention must be paid to performance of quality control procedures to ensure reliable test results.

The goals are best accomplished by, but not limited to, the use of reference strains selected for their genetic stability and for their usefulness in the particular method and with the antiviral agent being controlled.
6.2 Quality Control Responsibilities

6.2.1 Manufacturers (Commercial and/or “In-House” Products)

Manufacturers are responsible for the following:

- antiviral drug stability;
- antiviral drug identification;
- potency of antiviral drug stocks;
- integrity of commercially available host cell lines and stock strains of virus controls;
- compliance with current Good Manufacturing Practices (cGMP) and/or applicable quality system guidelines;
- integrity of the product; and
- accountability and traceability to the consignee.

Manufacturers should design and recommend a quality control program that allows users to evaluate those variables (host cell line condition, inoculum density, storage/shipping conditions) that most likely could cause user performance problems and to determine that the test is performing correctly when used according to established protocols.

6.2.2 Laboratory (User)

The laboratory is responsible for the following:

- drug storage to prevent deterioration;
- maintenance of host cell line;
- operator proficiency; and
- adherence to the established procedure, e.g., preparation of viral inoculum, reagents, and media, incubation conditions, calculation, and interpretation of endpoints.

6.3 Reference Strains for Quality Control

Phenotypically and genotypically well-characterized acyclovir- and foscarnet-susceptible and acyclovir- and foscarnet-resistant reference strains of HSV should be tested with each run of susceptibility testing. The IC₅₀ values for each control should fall within an expected range for the assay to be valid. Ideal drug-susceptible reference strains have IC₅₀ values that fall near the middle of the concentration range tested for the antiviral agent. An ideal control strain is inhibited at the fourth dilution of a seven-dilution series, but strains with IC₅₀ values at either the third or fifth dilution would be acceptable. Before a strain is accepted as a reference, it should be tested for as long as is necessary to demonstrate that its antiviral susceptibility pattern is genetically stable. NCCLS document M23—Development of In Vitro Susceptibility Testing Criteria and Quality Control Parameters provides guidelines for the selection of appropriate quality control strains and the determination of acceptable IC₅₀ ranges.
Reference strains should also include drug-resistant strains containing mutations relevant to the mode of action of the drug to be tested. For example, when testing acyclovir, both TK-positive and TK-negative or deficient strains should be included. For foscarnet testing, include a foscarnet-resistant DNA polymerase mutant. Recommended quality control strains for antiviral susceptibility testing of acyclovir and foscarnet are listed in Table 3. The pharmaceutical manufacturers that market acyclovir and foscarnet provide a number of strains upon request, or these may be obtained as gifts from colleagues. Well-characterized, drug-susceptible, and drug-resistant clinical isolates may also be used. HSV F can be obtained from the ATCC® as number VR-733.

6.4 Storing Quality Control Strains

Continuous subpassage of control strains should be avoided to prevent selection of subpopulations of virus lacking the desired phenotype. Isolates should be frozen at -70 °C or below in single-use aliquots and be passaged only when needed.

6.5 Medium Quality Control

Cell culture medium must be checked for sterility and toxicity to the cell culture type (e.g., Vero cells) being used after all supplements have been added. In addition, cell culture medium should be checked for the formation of precipitates, crystals, or sediments and the ability to support cell growth and viral infection.

6.6 Cell Line Quality Control

Cell cultures require meticulous care for optimum performance. Cells should be observed routinely for indications of instability or deterioration. Cell line passages made by the laboratory user must be checked for microbial contamination, including mycoplasma contamination. Bacterial and fungal contamination of cell lines becomes apparent by visual inspection of the cell culture medium for turbidity, toxicity, abnormal pH changes, and development of fungal elements. Continuous cell lines should be monitored quarterly for mycoplasma contamination.

The ability of the cell line to support the growth of virus should also be monitored. Decreased sensitivity is apparent when the pretitered inoculum for PRA, calculated to obtain 50 to 100 PFU per cell culture well, actually produces 10% or less of the target PFU. Decreased susceptibility of cell line to virus may indicate mycoplasma contamination.

6.7 Antiviral Drug Quality Control

When preparing stock solutions of acyclovir and/or foscarnet, the potency of the drugs should be validated by performing antiviral susceptibility testing on the newly made antiviral agents using appropriate susceptible and resistant control strains. This procedure should also be done when changing lot numbers of antiviral drugs. Samples of the new lot or batch of antiviral agent should be tested against the old lot/batch number to determine any difference in potency. Any significant problem in the preparation of the drugs or any difference in the potency of the antiviral agents between lots should be reflected in the results of the susceptibility testing using quality control strains.

6.8 Antiviral Cytotoxicity Control

Determine if the antiviral agents are cytotoxic to the Vero cells. To do this, examine the effect that the various concentrations of drug have on normal cell morphology when drug is incubated with uninfected cells for 72 hours. Cytotoxicity is defined as a microscopically visible disruption of normal cell morphology. Cytotoxicity in cells treated with drug can also be measured by performing viable cell counts using a 0.4% solution of trypan blue and a hemacytometer. The drug concentration required to reduce the viable count by
50% is determined by comparison with cell counts in control cultures without drug. The range of drug concentrations used in the PRA must be below the cytotoxic concentration. This information can often be obtained from the pharmaceutical manufacturer supplying the antiviral agent. For example, the IC<sub>50</sub> of acyclovir for Vero cells is approximately 74 mg/mL (300 mM)<sup>45</sup>.

6.9 Inoculum Control

The growth control well (no drug control), serves as the control for sufficient inoculum. Each well must contain between 50 and 100 PFUs or the test is not valid. Too low an inoculum can make an isolate appear falsely susceptible; too high an inoculum can make an isolate appear falsely resistant.

6.10 Quality Control Ranges

The IC<sub>50</sub> values for each control strain should fall within the range listed in Table 3 for the PRA to be valid. An IC<sub>50</sub> value above the control ranges may indicate deterioration of the drug.

6.11 Frequency of Quality Control Testing

Cell culture medium must be tested by batch or lot. Cell culture lines should be monitored for bacterial and fungal contamination when microscopically examining cells. Monitoring for mycoplasma contamination should be performed quarterly. Antiviral drug cytotoxicity needs to be determined only once for each cell line used. Reference strains must be run with each assay. The no-drug control must be performed with each assay for each virus strain tested including reference strains. The no-drug control also serves as the inoculum control.

7 Results: Interpretation and Reporting

7.1 Data Analysis

Analyze results by linear regression analysis to calculate the dose of antiviral drug producing 50% inhibition of plaque formation compared to the virus control without drug. A linear regression probability program can be used to assist with plotting the data points. In some cases the curve may be sigmoidal rather than a straight line making the determination of the IC<sub>50</sub> difficult. If this occurs, use only the linear part of the curve. If the IC<sub>50</sub> controls do not fall within a prescribed range, then the test must be repeated.

7.2 Interpretation and Reporting

HSV replication in vitro is significantly reduced by acyclovir and foscarnet. HSV isolates with in vitro IC<sub>50</sub> values of ≥2 µg/mL for acyclovir<sup>1,18,40,42,43</sup> and ≥100 µg/mL for foscarnet<sup>18,40</sup> are considered to be resistant to these antiviral agents. HSV isolates with in vitro IC<sub>50</sub> values of <2 µg/mL for acyclovir and <100 µg/mL for foscarnet are considered to be susceptible to these antiviral agents. These breakpoints were established by testing large numbers of HSV isolates from drug-naïve individuals to establish a susceptible range and by testing HSV isolates with alterations in the enzymes targeted by the antiviral agents to establish a threshold denoting resistance.

Susceptible HSV-1 isolates typically have mean IC<sub>50</sub> values of 0.12 µg/mL for acyclovir and 35 µg/mL for foscarnet, while susceptible HSV-2 isolates have mean IC<sub>50</sub> values of 0.5 µg/mL for acyclovir and 30 µg/mL for foscarnet.

<table>
<thead>
<tr>
<th></th>
<th>Susceptible</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acyclovir</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; &lt; 2 µg/mL</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; ≥ 2 µg/mL</td>
</tr>
<tr>
<td>Foscarnet</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; &lt; 100 µg/mL</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; ≥ 100 µg/mL</td>
</tr>
</tbody>
</table>
NOTE: Uninfected monolayers incubated without antiviral drug in the overlay medium (cell culture controls) should not exhibit CPE, toxicity, or other conditions that may render the cultures uninterpretable.

7.3 Limitations of Procedure

Because numerous methodological factors (including the tissue culture cell line used, the size of virus inoculum, and the adsorption and incubation conditions used) can affect the IC$_{50}$ values obtained in the test, strict adherence to the protocol must be followed. Control HSV strains with known IC$_{50}$ values must be included in each test.

As with other antimicrobial susceptibility testing, in vitro antiviral test results are not absolute predictors of either clinical response or failure. The results of susceptibility testing should therefore be interpreted in conjunction with patient clinical information. Antiviral resistance tends to occur in immunocompromised individuals, especially transplant recipients and AIDS patients. Acyclovir-resistant HSV infection has, however, been documented in an immunocompetent individual. Clinical conditions which favor the development of resistance may include long-term suppressive therapy, recurrent intermittent therapy, and the use of less-than-optimal doses of antiviral agent. HSV isolates resistant to acyclovir in vitro have responded to antiviral treatment, and patients with isolates that remain susceptible to the drug in vitro have failed therapy. Acyclovir-resistant isolates of HSV have also been obtained from individuals before therapy has been initiated and from healthy individuals on chronic suppressive therapy. In some patients, recurrent infections are caused by drug-susceptible isolates after successful treatment of infection with a resistant virus.

The absolute IC$_{50}$ value for a single isolate of HSV may be less important than the relative change in IC$_{50}$ values between paired isolates.

The optimum antiviral susceptibility testing conditions have not been determined for penciclovir. Cell-dependent differences in HSV susceptibility testing for penciclovir as compared to acyclovir are significant and would need to be resolved before a procedure can be recommended.$^{44}$

8 Safety

The following are safety and procedural comments to be adhered to when performing the plaque reduction assay:

- Because it is often impossible to know what might be infectious, all patient specimens are to be handled with “standard precautions.” Standard precautions are guidelines that combine the major features of “universal precautions and body substance isolation” practices. Standard precautions cover the transmission of any pathogen and thus are more comprehensive than universal precautions which are intended to apply only to transmission of blood-borne pathogens. Standard precaution and universal precaution guidelines are available from the U.S. Centers for Disease Control and Prevention.$^{46}$ NCCLS document M29—Protection of Laboratory Workers from Occupationally Acquired Infections deals specifically with this issue.

- Do not eat, drink, smoke, or apply cosmetics or contact lenses in areas where specimens, virus isolates, or cell cultures are handled.

- Preparation of cell culture medium and cell culture lines, and manipulation of virus should be conducted in a Class II biosafety cabinet.

- Local/state regulations regarding the disposal of all hazardous material must be followed.
• Appropriate precautions should be taken when handling infectious materials. Do not mouth pipette. Avoid contact with broken skin or mucous membranes. Wear disposable gloves, laboratory coats, and other appropriate protective devices. Wash hands thoroughly after handling these materials.

• Use an absorbent material and suitable disinfectant to clean spills from involved surfaces and to wipe work areas before and after handling infectious material. Dispose of all infectious materials properly.

• Arrange materials and equipment to provide easy access and to minimize the number of manipulations.

• Use the appropriate aseptic technique throughout the procedure.
References


43 Field HJ, Darby G. Pathogenicity in mice of strains of herpes simplex virus which are resistant to acyclovir in vitro and in vivo. Antimicrob Agent Chemother. 1980;17:209-216.


Summary of Comments and Subcommittee Responses

M33-P:  *Antiviral Susceptibility Testing: Proposed Standard*

**General**

1. Consider the use of $\mu$M concentrations rather than $\mu$g/mL to determine the susceptibility of HSV. Although acyclovir is less of a problem, the use of $\mu$g/mL for foscarnet may cause serious problems in interpretation of resistance with HSV. For the sake of standardization, I think that these guidelines should adopt molar concentrations for the HSV susceptibility testing.

   - The antiviral literature is clearly evenly divided in its usage of $\mu$g/mL and $\mu$M. Furthermore, clinical laboratorians and clinicians are more familiar with $\mu$g/mL for drug concentrations, particularly antibiotic concentrations. However, the importance of stating the complete chemical name and formula weight of the compound has been noted in Section 5.1: “NOTE: Because the concentration of the active compound depends on the particular formation of drug used, it is important to state the exact formulation used and the formula weight. For example, commercially available foscarnet consists of the trisodium salt of phosphonoformate with 6 equivalents of water of hydration with a formula weight of 300. Each gram of foscarnet contains only 423 mg of the active agent, phosphonoformic acid.”

**Section 4.1, Host Cell Line, Viral Inoculum, Incubation Time, and Range of Concentration of the Antiviral Agent, and Section 6, Quality Control Procedures**

2. I want to point out the usual misinterpretation (always occurring in U.S. papers) of the term “accuracy,” used in lieu of “trueness.” As pointed out in the Edma terminology paper, “accuracy” is covering both “precision” and “trueness.”

   - This has been revised as suggested.

**Section 5, Plaque Reduction Assay (PRA)**

3. I recommend the use of the more common 24-well plates rather than the 12-well plates for the PRA. Although standard, the guidelines state that 6 and 24 wells are acceptable; the use of 24-well plates would simplify the dilution scheme. One of the inherent problems with the PRA is the limited number of plaques that one can count. The presence of a minority population of resistant virus may be missed using the PRA; however, doubling the number of plaques by using 12-well plates will probably not solve this problem. I suggest that the recommendation be to use 24-well plates with fewer concentrations of the drug being tested. The use of 24-well plates will also allow the patient isolate to be performed on a single plate rather than on multiple plates. The use of one 24-well plate for the two control strains and one 24-well plate for the patient strain may reduce the chance of errors.

   - Use of 12-well plates is preferred to 24-well plates for the PRA. In a 24-well plate, plaques are more difficult to visualize. In addition, the inoculum that can be accommodated in a 12-well plate is greater than that for a 24-well plate. It is important to ensure a sufficient sample size of the virus population being examined to maximize detection of a resistant subpopulation that may be present in low titer.

**Section 5.5, Preparation of Cell Culture Plates, HSV Isolates, and Antiviral Agents, and Table 7, Recommended IC_{50} Limits for Quality Control Strains for Plaque Reduction Assay (Now Table 3)**

4. Our virologists would like to see more detail on the selection of reference strains and properties and manipulations of the culture plates. For example, methods for manipulating the cells, counting them, determining seeding density, and performing the calculations are not well known by most virology laboratories, and they should be presented in detail in the document so the laboratories can do it correctly.

   - Additional detail of reference strains has been added to the table as suggested. Methods for manipulating cell cultures (including determining seeding density) are described in detail in references 33 and 34. It is beyond the scope of this standard to reiterate these procedures.
Tables 3 through 6 (Now Tables 4 through 7)

5. The dilution scheme for both acyclovir and foscarnet should be changed. The same drug concentrations should be used for all strains tested: susceptible, resistant, and patient strains. Errors could be made if people use a different drug dilution scheme for each of the viruses being tested. I would suggest using 0.5, 1.0, 2.5, 5.0, and 10.0 µM concentrations of acyclovir for all strains. If a 24-well plate is used, duplicate control wells can be used along with duplicate test wells for these five concentrations of acyclovir using one-half of a plate. In addition, both the susceptible and resistant control strains could be tested on a separate 24-well plate using the same concentrations of ACV.

- It is unnecessary to use the same dilution scheme for susceptible and resistant control strains and patient isolates. The dilution schemes, as listed, allow efficient testing of each control strain on a single 12-well plate. Instructions in Tables 3 through 6 clearly prescribe preparation of all necessary concentrations of antivirals, which should minimize technical errors in antiviral preparation. A competent technologist should be capable of transferring the correct concentration of antiviral drug from the dilution tubes to the appropriate wells of the culture plates.
Summary of Delegate Comments and Subcommittee Responses

M33-A:  *Antiviral Susceptibility Testing: Herpes Simplex Virus by Plaque Reduction Assay; Approved Standard*

**General**

1. I would encourage the application of these standards to other anti-HSV agents when the appropriate reference HSV strains become available.

   - **In future editions of this document, the intent is to apply the standard to other anti-HSV agents, but only after we clearly understand how these agents will perform according to the criteria outlined in this standard. This was discussed at length at many of our committee meetings, and it was decided by vote of the members to keep the standard as is for the first edition. This has been addressed in the Foreword of the document.**

   We know that Vero cells should not be used when testing the susceptibility of HSV to penciclovir as erroneous ID₅₀ values are obtained. Therefore, in order to incorporate penciclovir into the standard, we would need to modify the standard to accommodate the differences observed with this antiviral. This will no doubt hold true for other antiviral agents as well. This issue will be addressed over time as the standard evolves.

**Section 5.1, Cells, Media, and Reagents**

2. In Section 5.1, it is suggested to add the phrase, “Sterilization of stocks through a 0.2-μm filter is optional, but encouraged.”

   In the research environment, any solution being applied to cell culture—even antibiotics or antivirals—is so filtered, and therefore, this reviewer feels a similar practice is also warranted in the clinical laboratory.

   - **Text has been added to page 10, 1st paragraph to address sterilization of stocks through a 0.2-μm filter as suggested.**

**Section 6.7, Antiviral Drug Quality Control**

3. In Section 6.7, it is suggested to add the phrase, “Samples of the new lot or batch of antiviral agent should be tested against the old lot/batch number to determine any difference in potency.” This process is analogous to testing for interbatch variation in antibiotic stock potency, and although everybody probably understands the intent, an explicit statement may help clarify that intent.

   - **This phrase has been added to Section 6.7 as suggested.**
The Quality System Approach

NCCLS subscribes to a quality system approach in the development of standards and guidelines, which facilitates project management; defines a document structure via a template; and provides a process to identify needed documents through a gap analysis. The approach is based on the model presented in the most current edition of NCCLS HS1—*A Quality System Model for Health Care*. The quality system approach applies a core set of “quality system essentials (QSEs),” basic to any organization, to all operations in any healthcare service’s path of workflow. The QSEs provide the framework for delivery of any type of product or service, serving as a manager’s guide. The quality system essentials (QSEs) are:

- **Documents & Records**
- **Organization**
- **Personnel**
- **Equipment**
- **Purchasing & Inventory**
- **Process Control**
- **Information Management**
- **Occurrence Management**
- **Process Improvement**
- **Assessment**
- **Service & Satisfaction**
- **Facilities & Safety**

M33-A addresses the quality system essentials (QSEs) indicated by an “X.” For a description of the other NCCLS documents listed in the grid, please refer to the Related NCCLS Publications section.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td>M7</td>
<td>M23</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Adapted from NCCLS document HS1—*A Quality System Model for Health Care*.

Path of Workflow

A path of workflow is the description of the necessary steps to deliver the particular product or service that the organization or entity provides. For example, GP26-A2 defines a clinical laboratory path of workflow which consists of three sequential processes: preanalytical, analytical, and postanalytical. All clinical laboratories follow these processes to deliver the laboratory's services, namely quality laboratory information.

M33-A addresses the clinical laboratory path of workflow steps indicated by an “X.” For a description of the other NCCLS documents listed in the grid, please refer to the Related NCCLS Publications section.

<table>
<thead>
<tr>
<th>Preanalytic</th>
<th>Analytic</th>
<th>Postanalytic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient Assessment</td>
<td>Test Request</td>
<td>Specimen Collection</td>
</tr>
<tr>
<td>Specimen Transport</td>
<td>Specimen Receipt</td>
<td>Testing Review</td>
</tr>
<tr>
<td>Laboratory Interpretation</td>
<td>Results Report</td>
<td>Post-test Specimen Management</td>
</tr>
<tr>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

Adapted from NCCLS document HS1—*A Quality System Model for Health Care*. 

An NCCLS global consensus standard. © NCCLS. All rights reserved.
### Related NCCLS Publications

**M7-A6**  
Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Sixth Edition (2003). This newly revised standard provides updated reference methods for the determination of minimal inhibitory concentrations (MICs) for aerobic bacteria by broth macrodilution, broth microdilution, and agar dilution. This document contains MIC interpretive criteria and quality control parameters tables updated for 2003.

**M23-A2**  

**M29-A2**  
Protection of Laboratory Workers from Occupationally Acquired Infections—Second Edition; Approved Guideline (2001). Based on U.S. regulations, this document provides guidance on the risk of transmission of hepatitis viruses and human immunodeficiency viruses in any laboratory setting; specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials; and recommendations for the management of blood-borne exposure.

* Proposed- and tentative-level documents are being advanced through the NCCLS consensus process; therefore, readers should refer to the most recent editions.
Active Membership (as of 1 January 2004)

Abbott Laboratories
Abbot Laboratories, MediSense Products
AABB Biocompatibility Corporation
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Abbott Laboratories
Achilles S.P.A.
Grady Memorial Hospital (GA)
Guthrie Clinic Laboratories (PA)
Hagerstown Medical Laboratory (MD)
Hahnemann University Hospital (PA)
Harri Methordist Fort Worth (TX)
Hartford Hospital (CT)
Headwaters Health Authority (Alberta, Canada)
Health Network Lab (PA)
Health Partners Laboratories (VA)
Highlands Regional Medical Center (FL)
Hinsdale Hospital (IL)
Hoag Memorial Hospital Presbyterian (CA)
Holms Regional Medical Center (FL)
Holy Cross Hospital (MD)
Hôpital du Sacré-Cœur de Montréal (Montreal, Quebec, Canada)
Hôpital Maisonneuve - Rosemont (Montreal, Canada)
Hôpital Saint-Luc (Montreal, Quebec, Canada)
Hospital for Sick Children (Toronto, ON, Canada)
Hospital Sousa Martins (Portugal)
Hotel Dieu Grace Hospital (Windsor, ON, Canada)
Huddinge University Hospital (Sweden)
Hunter Area Pathology Service (Australia)
Hurley Medical Center (MI)
Indiana University
Innova Fairfax Hospital (VA)
Institute of Medical and Veterinary Science (Australia)
International Health Management Associates, Inc. (IL)
Jackson Memorial Hospital (FL)
Jacobi Medical Center (NY)
John C. Lincoln Hospital (AZ)
John F. Kennedy Medical Center (NJ)
Johns Hopkins Medical Institutions (MD)
Kadlec Medical Center (WA)
Kaiser Permanente (MD)
Kangnam St. Mary's Hospital (Korea)
Kantoshospital (Switzerland)
Kenora-Rainy River Regional Laboratory Program (Ontario, Canada)
Kimbull Medical Center (NJ)
King Faisal Specialist Hospital (Saudi Arabia)
LabCof (NC)
Laboratoire de Santé Publique du Québec (Canada)
Laboratorio Dr. Echevarne (Spain)
Laboratório Fleurys S/C Ltda. (Brazil)
Laboratory Corporation of America (NJ)
LAC and USC Healthcare Network (CA)
Lakeland Regional Medical Center (FL)
Lancaster General Hospital (PA)
LeBonheur Children's Medical Center (TN)
Lewis-Gale Medical Center (VA)
L'Hôtel-Dieu de Québec (Canada)
Libero Instituto Univ. Campus BioMedico (Italy)
Loma Linda Mercantile (CA)
Louisiana State University Medical Center
Lourdes Health System (NJ)
Maccabi Medical Care and Health Fund (Israel)
Magnolia Regional Health Center (MS)
MaineHealth Medical Center (NY)
Malcolm Grow USAF Medical Center (MD)
Marion County Health Department (IN)
Martin Luther King/Drew Medical Center (CA)
Massachusetts General Hospital (Microbiology Laboratory)
MDS Metro Laboratory Services (Burnaby, BC, Canada)
Medical College of Virginia Hospital
Medicare/Medicaid Certification, State of North Carolina
Memorial Medical Center (LA)
Jefferson Davis Hwy
Memorial Medical Center (Jefferson Davis Pkwy, New Orleans, LA)
Memorial Medical Center (LA)
Napoleon Avenue
Mercy Hospital (ME)
Methodist Hospital (TX)
Michigan Department of Community Health
Mid America Clinical Laboratories, LLC (IN)
Middlesex Hospital (CT)
Mississippi Baptist Medical Center
Montreal Children's Hospital (Canada)
Montreal General Hospital (Canada)
National Serology Reference Laboratory (Australia)
National University Hospital (Singapore)
The Nebraska Medical Center
New Britain General Hospital (CT)
New England Fertility Institute (CT)
New England Medical Center (MA)
New Mexico VA Health Care System
New York University Medical Center
NorDx (ME)
North Carolina State Laboratory of Public Health
North Central Medical Center (TX)
North Shore - Long Island Jewish Health System Laboratories (NY)
North Shore University Hospital (NY)
Northwestern Memorial Hospital (IL)
Ochsner Clinic Foundation (LA)
O.L. Vrouwziekenhuis (Belgium)
Ordre professionnel des technologistes médicaux du Québec
Ospedale Riuniti (Italy)
The Ottawa Hospital (Ontario, ON, Canada)
OU Medical Center (OK)
Our Lady of the Resurrection Medical Center (IL)
Pathology Associates Medical Laboratories (WA)
The Permanente Medical Group (CA)
Piedmont Hospital (GA)
Pocoma Medical Center (PA)
Presbyterian Hospital of Dallas (TX)
Providence Health Care
Provincial Laboratory for Public Health (Edmonton, AB, Canada)
Queen Elizabeth Hospital (Prince Edward Island, Canada)
Queensland Health Pathology Services (Australia)
Quest Diagnostics Incorporated (CA)
Quintiles Laboratories, Ltd. (GA)
Regions Hospital
Research Medical Center (MO)
Rex Healthcare (NC)
Rhode Island Department of Health Laboratories
Riverside Medical Center (IL)
Riyadh Armed Forces Hospital (Saudi Arabia)
Robert Wood Johnson University Hospital (NJ)
Royal Columbian Hospital (New Westminster, BC, Canada)
Saad Specialist Hospital (Saudi Arabia)
Sahlgrenska Universitetssjukhuset (Sweden)
Saint Mary's Regional Medical Center (NV)
St. Alexius Medical Center (ND)
St. Anthony Hospital (IL)
St. Anthony's Hospital (FL)
St. Barnabas Medical Center (NJ)
St. Eustache Hospital (Quebec, Canada)
St. Francis Medical Ctr. (CA)
St. John Hospital and Medical Center (CA)
St. John's Hospital & Health Center (CA)
St. Joseph's Hospital - Marshfield Clinic (WI)
St. Joseph's Hospital & Medical Center (CA)
St. Jude Children's Research Hospital (TN)
St. Luke's Regional Medical Center (LA)
St. Mary of the Plains Hospital (TX)
St. Michael's Hospital (Toronto, ON, Canada)
Ste. Justine Hospital (Montreal, PQ, Canada)
San Francisco General Hospital (CA)
Santa Clara Valley Medical Center (CA)
Sentara Williamsburg Community Hospital (VA)
Seoul National University Hospital (Korea)
Shands at the University of Florida
So. California Permanente Medical Group
South Bend Medical Foundation (IN)
South Western Area Pathology
Service (Australia)
Southern Maine Medical Center
Southwest Texas Methodist Hospital (TX)
Spartanburg Regional Medical Center (SC)
Speciality Laboratories, Inc. (CA)
State of Connecticut Dept. of Public Health
State of Washington Department of Health
Stony Brook University Hospital (NY)
Stromont-Vail Regional Medical Center (KS)
Sun Health-Boswell Hospital (AZ)
Sunnybrook Health Science Center (ON, Canada)
Swedish Medical Center - Providence Campus (WA)
Temple University Hospital (PA)
Tenet Odessa Regional Hospital (TX)
The Toledo Hospital (OH)
Tufts Inflammatory (LA)
Tripler Army Medical Center (HI)
Truman Medical Center (MO)
Tuensum Hospital (Hong Kong)
UCLA Medical Center (CA)
UCSF Medical Center (CA)
UNC Hospitals (NC)
Unidad de Patología Clínica (Mexico)
Union Clinical Laboratory (Taiwan)
University Hospitals of Cleveland (OH)
University of Alabama-Birmingham Hospital
University of Chicago Hospitals (IL)
University of Colorado Hospital
University of Debremer Medical Health and Science Center (Hungary)
University of Illinois Medical Center
University of the Ryukyus (Japan)
The University of the West Indies
University of Virginia Medical Center
University of Washington
UnivCor, A Division of Duion Systems, Inc. (OK)
UZ-KUL Medical Center (Belgium)
VA (Hampton) Medical Center (VA)
VA (Hines) Medical Center (IL)
VA (Kansa City) Medical Center (MO)
VA (San Diego) Medical Center (CA)
VA (Tuskegee) Medical Center (AL)
Valley Children's Hospital (CA)
Vejle Hospital (Denmark)
Virginia Department of Health
VitaMed Laboratories (MN)
Warren Hospital (NJ)
Washington Adventist Hospital (MD)
Washoe Medical Center Laboratory (NV)
Waterford Regional Hospital (Ireland)
Wellstar Health Systems (GA)
West Jefferson Medical Center (LA)
West Shore Medical Center (MI)
Willford Hall Medical Center (TX)
William Beaumont Army Medical Center (TX)
William Beaumont Hospital (MI)
William Osler Health Centre (Brampton, ON, Canada)
Wom Army Community Hospital (GA)
Winthrop Regional Health Authority (Winnipeg, Canada)
Wisard Memorial Hospital (IN)
Yonsei University College of Medicine (Korea)
York Hospital (PA)

OFFICERS
Donna M. Meyer, Ph.D., President
CHRISTUS Health
Thomas L. Hearm, Ph.D., President Elect
Centers for Disease Control and Prevention
Emil Voelker, Ph.D., Secretary
Roche Diagnostics GmbH
Gerald A. Hoeltge, M.D., Treasurer
The Cleveland Clinic Foundation
F. Alan Andersen, Ph.D., Immediate Past President
Cosmetic Ingredient Review
John V. Bergen, Ph.D., Executive Director

BOARD OF DIRECTORS
Susan Blonshine, RRT, RPFT, FAARC
Wayne Brister
Kurt H. Davis, FCSMLS, CAE
Mary Lou Ganzter, Ph.D
Dade Behring Inc.
Lillian J. Gill, M.S.
FDA Center for Devices and Radiological Health
Robert L. Habig, Ph.D.
Abbott Laboratories

Carolyn D. Jones, J.D., M.P.H.
J. Stephen Kroger, M.D., MACP
J. Stephen Kroger, M.D., MACP
Willie E. May, Ph.D.
Gary L. Myers, Ph.D.
Gary L. Myers, Ph.D.
Kiyoshi Watanabe, M.D.
Judith A. Yost, M.A., M.T.(ASCP)

AdvMed
AdvMed
COLA
Institute of Standards and Technology
Centers for Disease Control and Prevention
Keio University School of Medicine
Centers for Medicare & Medicaid Services