Laboratory Procedure Manual

Analyte: **Herpes Simplex Virus Type 1 & 2**

Matrix: **Serum**

Method: **Solid-Phase Enzymatic Immunodot**

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Important Information for Users

Emory University periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for NHANES 2003–2004 data.

A tabular list of the released analytes follows.

Dataset name	Variable name	Description
l09_c	LBXHE1	Herpes simplex virus 1
	LBXHE2	Herpes simplex virus 2

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Although extensive antigenic cross-reactivity exists between the two viral types of herpes, a viral glycoprotein specific for herpes simplex virus type 2 (HSV-2) (designated gG-2) and a glycoprotein specific for herpes simplex virus type 1 (HSV-1) (designated gG-1) have been identified. Monoclonal antibodies and affinity chromatography have been used to purify these glycoproteins and thus provide antigens for type-specific herpes serologic assays. Solid-phase enzymatic immunodot assays are used to detect antibodies reactive to these antigens. The purified glycoprotein, gG-1 or gG-2, is adsorbed to the center of a nitrocellulose disk. The rest of the disk surface is coated with bovine serum albumin (BSA) to prevent further nonspecific protein adsorption. Incubation of test serum with the disk allows specific antibodies, if present, to bind to the immobilized antigen. After extensive washing to remove nonreactive antibodies, the bound antibodies are detected by sequential treatment with peroxidase-conjugated goat anti-human IgG and the enzyme substrate (H_2O_2 with chromogen 4-chloro-1-naphthol). A positive reaction is demonstrated by the appearance of a blue dot at the center of the disk. Serum reactive to an immunodot charged with gG-1 indicates previous and probable latent HSV-1 infection. Serum reactive with gG-2 indicates previous and probable latent HSV-2 infection.

HSV-1 is typically associated with infection of the upper body. HSV-2 commonly infects the genitalia and is primarily transmitted sexually. Perinatal transmission, usually of HSV-2, is comparatively infrequent but results in a severe, often fatal disease in newborns. Assays that can detect and distinguish antibodies to these viruses are of clinical and epidemiological importance.

2. SPECIAL SAFETY PRECAUTIONS

All human serum specimens are pretreated with 0.5% (v/v) Triton X-100 to inactivate enveloped viruses, including the human immunodeficiency virus, which may be present. However, observe universal precautions. Wear gloves, a lab coat, and safety glasses when handling all human blood specimens. Place all plastic tips, sample cups, and gloves that contact blood in a biohazard waste container. Discard all disposable glassware into a sharps waste container. Place all liquid hazardous waste materials in closed containers labeled as hazardous waste and stating the composition of waste being contained. These materials are decontaminated by autoclaving at 250^{o}F, 19 pounds pressure, for 1 hour.

Protect all work surfaces by absorbent benchtop paper. Discard the benchtop paper into the biohazard waste container daily or whenever blood contamination occurs. Wipe down all work surfaces with 10% (v/v) sodium hypochlorite weekly.
Material Safety Data Sheets (MSDSs) for sodium hypochlorite, Triton X-100, 4-chloro-1-napthol, methanol, and hydrogen peroxide are maintained in the Emory School of Medicine.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Each shipment of specimens received from the NHANES III mobile unit contains a corresponding transmittal sheet and an ASCII data file (OUTPUT.TXT) on a 5¼" high-density (HD) floppy diskette. The data file, containing the specimen ID, collection date, and type of sample (i.e., whole blood, serum, or plasma) is checked against the information on the transmittal sheet and specimen label prior to the assay.

b. After the data are calculated and the final values are approved by the reviewing supervisor for release, the data entry clerk transcribes the results into the NHANES III data base using Rbase software; data entry is proofed by the supervisor and clerk. Periodically, data from the laboratory database are downloaded onto floppy diskettes and delivered to the data manager of the Centers for Disease Control and Prevention.

After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete) the NHANES laboratory supervisor transmits the values to the NCHS mainframe computer along with the other NHANES III data.

c. Files stored on the network or CDC mainframe are automatically backed up nightly to tape by EHLS LAN support staff or CDC Data Center staff, respectively.

d. Documentation for data system maintenance is contained in hard copies of data records.

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. No special instructions such as fasting or special diets are necessary. Blood is collected in a red-top Vacutainer tube by standard venipuncture procedures.

b. Specimens for HSV-1 and HSV-2 analysis should be fresh or frozen serum.

c. A 0.5-mL sample of serum is preferable. The minimum sample volume required for analysis is 50 µL. Specimens are rejected if insufficient quantity is available for analysis.

d. The appropriate amount of serum is dispensed into a Nalgene cryovial or other plastic screw-capped vial labeled with the participant's ID.

e. Specimens collected in the field should be frozen, then shipped on dry ice by overnight mail. Once received, specimens are stored at ≤ -20°C until analyzed. Portions of the specimen that remain after analytical aliquots are withdrawn should be refrozen at ≤ -20°C. Samples thawed and refrozen several times are not compromised, but multiple brief freeze/thaw cycles should be avoided.

5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable to this procedure.

6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. Instrumentation

(1) Multiple-head 96-hole puncher (Emory University, Atlanta, GA).

(2) TekPro rotating platform (American Hospital Supply Corp., Evanston, IL).

(3) Manual ELISA washer (Corning Glassworks, Corning, N.Y.)

b. Other Materials

(1) Nitrocellulose membrane sheets (Schleicher & Schuell, Inc., Keene, NH).

(2) Polyvinyl chloride plates, 96 well (Dynatech Laboratories, Inc., Alexandria, VA).

(3) Microsyringe fitted with a repeating dispenser (Hamilton Co., Reno, NV).

(4) Bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO).

(5) Hydrogen peroxide, H_2O_2, 30% (Sigma Chemical Co.).

(6) 4-chloro-1-napthol, $C_{10}H_7ClO$ (Sigma Chemical Co.).

(7) Methanol, CH_3OH (Sigma Chemical Co.).

(8) Horseradish peroxidase-conjugated goat anti-human IgG (Jackson Immunological Laboratory, West Grove, PA).

(9) 0.55% Triton X-100 in Tris-buffered saline.

(10) Antigens, gG-1 and gG-2 (Dr. Lenore Periera, University of California, San Francisco, CA).

(11) Tris-HCl (Sigma Chemical Co., St. Louis, MO).

(12) Trizma base (Sigma Chemical Co.).

(13) Sodium chloride (NaCl) (Sigma Chemical Co.).

(14) Distilled water (American Sterilizer Co., Erie, PA).

(15) In-house HSV-1 and HSV-2 positive and negative control serum (Emory University, Atlanta, GA).

c. Reagent Preparation

(1) gG-1 and gG-2 Antigens
The gG-1 and gG-2 antigens have been prepared by affinity chromatography using specific monoclonal antibodies (H1379-2 and H1206), respectively. The purified materials are diluted 1:64 in Tris-buffered saline (pH 7.2) before they are used.

(2) Conjugate solution
Horseradish peroxidase-conjugated goat anti-human IgG. Dilute 1:1000 in phosphate-buffered saline (pH 7.2) containing 3% bovine serum albumin and 1% goat serum.

(3) Buffer solution
Tris-buffered saline (pH 7.2) containing 3 g/dL bovine serum albumin.

(4) Substrate solution
6 mg 4-chloro-1-napthol ($C_{10}H_7ClO$) dissolved in 2 mL of methanol mixed with 10 mL of TBS and 5 µL of 30% (v/v) hydrogen peroxide(H_2O_2).

(5) Tris-buffered saline (TBS), pH 7.2
Dissolve 6.6 g of Tris-HCl, 1.0 g of Trizma base, and 11.6 g of NaCl and bring to volume with 1,000 mL of distilled water in a 1-L flask.

d. Standards Preparation

There are no standards used in this assay, since no calibration curve is generated as part of this method.

e. Preparation of Quality Control Materials

In-house HSV-1, HSV-2, and negative control serum pools were prepared at Emory University. High-titered serum samples from patients with primary HSV-1 infection were pooled and then diluted to be used as HSV-1 positive controls. Serum samples from convalescent patients with primary HSV-2 infections were pooled, diluted and used as HSV-2 positive controls. Both positive pools are

monospecific, i.e. they do not cross-react with the other virus type. Serum samples from healthy donors, nonreactive to both HSV types in the screening ELISA, were pooled, diluted, and used as negative controls. The dilution scheme for controls is shown in Table 1.

Table 1. Dilution for Controls

Control	Dilution(s)
HSV-1 positive pool 1	1:800, 1:3200
HSV-1 positive pool 2	1:50, 1:200
HSV-2 positive pool 1	1:400, 1:1600
HSV-2 positive pool 2	1:50. 1:200
HSV negative pool	1:50

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. Calibration Curve

No calibration curve is generated by the user as part of these assay methods.

b. Verification

Verification for this assay is not possible in the conventional manner. The investigators who read assay results are trained to analyze the positive and negative controls for each test series. If, within a testing series, positive or negative controls do not conform to specifications as defined in the protocol, the results for the entire series are invalidated, and the series is retested in duplicate to confirm the initial test result.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. Preliminaries

(1) Prepare dilutions of controls, conjugate, buffer, substrate, and antigens.

(2) Assay one negative and two positive controls in duplicate for each virus type with each run of specimens.

(3) Ensure that all disks and plates are subjected to the same process and incubation times.

(4) Once the assay has been started, complete all subsequent steps without interruption and within the recommended time limits.

b. Sample Preparation

(1) Bring serum specimens to 20–25°C.

(2) Mix serum samples gently before testing to eliminate stratification which may occur when serum is frozen or stored at 4°C for extended periods.

(3) Identify the reaction tray wells for each specimen or control.

(4) Dilute test serum initially 1:10 in 0.55% Triton X-100 in TBS. After the incubation at room temperature, further dilute with Tris-buffered saline (pH 7.2) containing 3% bovine serum albumin, to a final serum dilution of 1:50.

c. Instrument Setup

There is no instrument required for this solid-phase enzymatic immunodot assay. Purified HSV-1 or HSV-2 antigens are immobilized on a small disk and incubated with test serum. A positive reaction is demonstrated by the appearance of a bluish-purple dot at the center of the disk.

d. Operation of Assay Procedure

HSV-1 and HSV-2 assays are run simultaneously in separate wells. Half of each plate is precoated with gG-1 antigen for HSV-1; the other half of the plate is precoated with gG-2 antigen for HSV-2.

(1) Prepare and deposit small disks of nitrocellulose membrane directly in the 96-well polyvinyl chloride plates with a 96-hole punch.

(2) Wash nitrocellulose disks in each well once with distilled water. Dry the discs completely at 20–25°C.

(3) Onto the center of each disk, deliver 1 µL of appropriately diluted antigen with a microsyringe fitted with a repeating dispenser.

(4) After drying the disks at 20-25°C overnight, wash them twice with TBS for 10 min each.

(5) Add 100 µL of buffer containing 3% BSA to each well and incubate at 20–25°C for 30 min on a rotating platform.

(6) Remove the buffer by suction.

(7) Add 100 µL of diluted serum or control to duplicate wells and incubate at 20–25°C overnight on a rotating platform.

(8) Remove the serum from each well by suction using the manual washer. Add 100 µL of TBS and incubate at 20–25°C for 10 min on rotator. Remove the TBS by suction. Repeat this procedure two times. Add 100 µL of buffer (3% BSA) to each well and incubate for 30 min.

(9) Remove the buffer by suction. Add 100 µL diluted conjugate solution to each well and incubate at 20–25°C for 2 hours on a rotating platform.

(10) Remove the conjugate by suction. Add 100 µL TBS to each well and incubate at 20–25°C for 10 min on rotator. Remove TBS by suction. Repeat this procedure two times.

(11) Remove the TBS by suction. Add 100 µL of freshly prepared substrate solution to each well.

(12) After 15 min, stop the reaction by removing the substrate and washing the plate twice with distilled water.

(13) Dry the plates overnight at room temperature in the dark. Examine the disks for color development. A positive reaction is demonstrated by the appearance of a bluish-purple dot at the center of the disk.

e. Recording of Data

(1) Quality Control Data
Positive and negative controls are determined to be valid or invalid. Results of each dilution of assay controls are recorded in standard forms as the test results are read by the investigators. The sample data are then entered into the computer database.

(2) Analytical Results
Results of each assay sample are recorded in standard forms as the test results are read by the investigators. The sample data are then entered into the computer database.

f. Replacement and Periodic Maintenance of Key Components

(1) Monitor and document the refrigerator temperature, freezer temperature, and room temperature on a weekly basis.

(2) Pipettors

All micropipettors that are used in testing clinical specimens should be checked for calibration every six months. Pipettors that do not conform to specifications should be autoclaved and sent out for recalibration in accordance with the manufacturer's recommendations. Calibration records should be kept for each pipettor by serial number.

h. Special Procedure Notes - Emory University

(1) With the availability of mouse monoclonal antibodies, it has become possible to purify HSV-2 proteins that fail to express type-common antigenic determinants detectable in serological assays.

(2) The use of gG-2, purified from extracts of HSV-2-infected cells, led to the development of an assay of high sensitivity, specificity, and reproducibility.

(3) This immunodot assay is suitable for large numbers of serum samples because it requires a small amount of purified glycoprotein.

(4) Purified gG-2 retains antigenicity at $\leq -70^{\circ}C$ for over 5 years if stored in glass (but not plastic) ampules.

(5) BSA from different sources could cause significant reductions in the sensitivity of the gG-2 assay. This problem is overcome by testing different batches of BSA from several sources and choosing a large stock of the best batch.

9. REPORTABLE RANGE OF RESULTS

Final reports express results as positive or negative for the presence of anti-HSV-1 or HSV-2 antibody in the sample.

10. QUALITY CONTROL (QC) PROCEDURES

The method described in this protocol has been developed and used for more than 8 years in the Pediatric Infectious Disease Immunology and Epidemiology Laboratory. This method has proven to be accurate, precise, and reliable.

This quality control system uses "bench" quality control samples. One negative and two positive type-specific in-house controls are prepared in a controlled process and are included in each analytical run (a set of consecutive assays performed without interruption). The presence or absence of HSV-1 or HSV-2 antibody is determined by presence of a bluish-purple dot at the center of the nitrocellulose membrane disk.

The following serum controls are included in each HSV-1 assay: an HSV-1 antibody-positive serum pool 1 diluted 1:800 and 1:3200; an HSV-2 antibody-positive serum pool 1 diluted 1:50 and 1:200, and an antibody-negative serum pool (nonreactive to both HSV types) diluted 1:50. Results are accepted only when both dilutions of the HSV-1 serum pool test positive and all the other controls test negative.

The following serum controls are included in each HSV-2 assay: an HSV-2 antibody-positive serum pool diluted 1:400 and 1:1600; an HSV-1 antibody-positive serum pool diluted 1:50 and 1:200, and an antibody-negative serum pool (non-reactive to both HSV types) diluted 1:50. Results are accepted only when both dilutions of the HSV-2 serum pool test positive and all the other controls test negative.

Twenty characterization runs are performed on each pool. These pools are prepared in sufficient quantity to last throughout the survey. The pools are divided into 30-mL aliquots and stored at ≤ –70°C. As needed, one 30-mL sample is thawed, divided into 30-µL aliquots and refrozen at ≤ –70°C. The 30-µL aliquots are then removed as needed to provide controls. Serum HSV-1 and HSV-2 antibodies are stable indefinitely when stored at ≤ –70°C.

Each sample is tested in duplicate and each assay is read by two individuals independently. Any discrepancy in the results leads to repeat testing of the sample involved. The prevalence of repeat runs required because of unacceptable controls is no more than 1%.

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

a. Repeat the test if both readers do not agree about the results in the duplicate wells.

b. Repeat the run using new dilutions if both dilutions of the positive control serum pool do not test positive or all the other controls do not test negative.

c. If controls continue to fail, consult the supervisor for other appropriate actions.

d. Do not report results from runs in which the controls did not meet expected reactivities.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

The qualitative assays provide positive or negative results, not antibody titers. The use of different sources of BSA could cause significant reductions in the sensitivity of the assay if not properly tested.

13. REFERENCE RANGES (NORMAL VALUES)

A normal sample is negative for HSV-1 and HSV-2 antibodies.

14. CRITICAL CALL RESULTS (PANIC VALUES)

Not applicable to this assay method.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens are stored at ≤ –20°C until testing. After an aliquot of the thawed sample has been removed for testing, the residual is refrozen and stored at ≤ –20°C.

16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

There are no alternate methods for performing analysis for HSV-1 or HSV-2 antibodies. If the analytical system fails, specimens may be stored at ≤ –20°C until the system is returned to functionality.

17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

Not applicable to this assay method.

18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

Standard recording keeping involves using the mainframe computer, floppy disks, and the hard copy results themselves to track specimens. Records are maintained indefinitely. Only numerical identifiers (e.g., case ID numbers) should be used. All personal identifiers should be available only to the medical supervisor or project coordinator to safeguard confidentiality.

For the NHANES study, residual serum is retained at ≤ –70°C for 1 year and then returned to NCHS serum bank.

19. SUMMARY STATISTICS AND QC GRAPHS

Qualitative assays are qualitative assays with a positive, negative or borderline/indeterminate result. The absorbance or reactivity values of specimens are compared with a cutoff value that is a ratio of the negative control mean and the positive control mean. Since the controls are read as cutoff values, plots of these values are not generated for quality control purposes.

REFERENCES

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