

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 2007–2008 data.

A tabular list of the released analytes follows.

Dataset name	Variable name	Description
HSV_D	LBXHE1	Herpes simplex virus I
	LBXHE2	Herpes simplex virus II

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Although extensive antigenic cross-reactivity exists between the two viral types of herpes simplex viruses, 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. Affinity chromatography based on monoclonal antibodies has 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 6 mm 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 predominantly associated with infection of the upper body through nonsexual routes, although it can be sexually transmitted to the genitalia. 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°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-naphthol, 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 mobile unit contains a corresponding shipping manifest listing the samples in the box. An electronic data file (Excel worksheet), containing the specimen ID's, specimen locations in the box, collection dates and other relevant information concerning individual samples, is independently sent from Westat to the laboratory via email. From the data file, a worksheet for each assay run is generated. Each specimen is checked against the worksheet for correct sample ID on the label and acceptable condition of the specimen prior to the assay.
- b. After the test results have been obtained and the final values approved by the reviewing supervisor for release, the result codes were transcribed into the data file originally sent from Westat. Data entry is proofed by the supervisor and clerk. The completed data file is then uploaded to the NHANES Westat laboratory data management website. A copy is archived in the local computer, with weekly backup, to maintain an independent record. The new data are also appended to a local database, which includes all

the specimens with results obtained in the project to-date; hardcopies of data are generated periodically and filed.

- c. 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 0.2 mL sample of serum is preferable. The minimum sample volume required for analysis is 50 μ L. 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, and then shipped on dry ice by overnight mail. Once received, specimens are stored at $\leq -70^{\circ}\text{C}$ until analyzed. Portions of the specimen that remain after analytical aliquots are withdrawn should be refrozen at $\leq -70^{\circ}\text{C}$. Samples thawed and refrozen several times are not compromised, but extensively repeated 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) Single channel adjustable-volume micropipettors (1-10 μ L, 40-200 μ L, 100-1000 μ L)
- (2) Multichannel micropipettor (25-200 μ L)
- (3) Orbital rotator /platform shaker (TekPro; American Hospital Supply Corp., Evanston, 11.)
- (4) Microplate washer (Model ELx405, BioTek Instruments, VT)
- (5) Repeater microsyringe-pipets (Hamilton Pipets, Reno, Nev.)
- (6) Illuminated desk magnifier lens (Fisher Scientific, Cat# 12-071-1)
- (7) 96-hole puncher (96 well microplate format) – custom-made, Emory University

b. Materials

- (1) Pure nitrocellulose membrane sheets (0.45 micron pore, Osmonic Inc, Fisher Scientific).

- (2) Polyvinyl chloride plates, 96 well (Dynatech Laboratories, Inc., Alexandria, VA).
- (3) Bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO).
- (4) Hydrogen peroxide, H₂O₂, 30% (Sigma Chemical Co.).
- (5) 4-chloro-1-naphthol, C₁₀H₇ClO (Sigma Chemical Co.).
- (6) Methanol, CH₃OH (Sigma Chemical Co.).
- (7) Horseradish peroxidase-conjugated goat anti-human IgG (Jackson Immunological Laboratory, West Grove, PA).
- (8) 0.55% Triton X-100 in Tris-buffered saline.
- (9) Antigens, gG-1 and gG-2 of HSV, affinity-purified (Emory University).
- (10) Tris-HCl (Sigma Chemical Co., St. Louis, MO).
- (11) Trizma base (Sigma Chemical Co.).
- (12) Sodium chloride (NaCl) (Sigma Chemical Co.).
- (13) Distilled water (American Sterilizer Co., Erie, PA).
- (14) 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 (optimal dilution as predetermined by titration) in Tris-buffered saline (pH 7.2) before they are used.
- (2) Conjugate solution
Horseradish peroxidase-conjugated goat anti-human IgG. Dilute in phosphate-buffered saline (pH 7.2) containing 3% bovine serum albumin and 2% goat serum. Optimal dilution predetermined by titration with each new lot to match reactivity of previous lots.
- (3) Sample Diluent
Tris-buffered saline (pH 7.2) containing 3 g/dL bovine serum albumin.
- (4) Substrate solution
6 mg 4-chloro-1-naphthol (C₁₀H₇ClO) dissolved in 2 mL of methanol, diluted with 10 mL of TBS and 5 µL of 30% (v/v) hydrogen peroxide (H₂O₂).
- (5) 10X 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 final volume of 1,000 mL with distilled water in a 1-L flask. Dilute 1:10 and check pH before use (1X TBS).

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. Selected high-titered serum samples from patients with HSV-1 infection only 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 HSV virus type. Serum samples from healthy donors, nonreactive to both HSV types 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	1:50, 1:200; 1:3200, 1:12800
HSV-2 positive pool	1:50, 1:200; 1:800, 1:3200
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) For HSV-1 (gG1) antibody test, assay one dual negative (HSV1-HSV2-) serum control diluted at 1:50, one type-negative (HSV1-HSV2+) control at 1:50 and 1:200, and one type positive (HSV1+HSV2-) control at 1:3200 and 1:12800, in duplicate with each run of specimens.
- (3) For HSV-2 (gG2) antibody test, assay one dual negative (HSV1-HSV2-) serum control diluted at 1:50, one type-negative (HSV2-HSV1+) control at 1:50 and 1:200, and one type positive (HSV2+HSV1-) control at 1:1600 and 1:6400, in duplicate with each run of specimens
- (4) Ensure that all disks and plates are subjected to the same process and incubation times.

- (5) 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 room temperature (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 incubation at room temperature for 10 minutes, 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

Prime microplate washer with wash buffer (1x TBS)

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 using the microplate washer.
- (5) Add 100 µL of TBS buffer containing 3% BSA (blocking buffer) to each well and incubate at 20–25°C for at least 30 min on a rotating platform.
- (6) Remove the blocking buffer and wash the plate once with TBS.
- (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 and wash plate three times with TBS, with 10 minutes of soaking in TBS between the second and third wash.
- (9) Add 100 µL diluted conjugate solution to each well and incubate at 20–25°C for 2 hours on a rotating platform.
- (10) Wash assay wells three times with TBS.
- (11) Add 100 µL of freshly prepared substrate solution to each well.
- (12) After 30 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 under the illuminated magnifying lens 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 daily basis.

- (2) Pipettors

All micropipettors that are used in testing clinical specimens should be checked for calibration every twelve 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.

- (3) Microplate washer is visually inspected for correct dispensing and aspirating functions before each assay run.

h. Special Procedure Notes - Emory University

- (1) With the availability of mouse monoclonal antibodies, it has become possible to purify a HSV-2 glycoproteins (gG2) that does not express type-common antigenic determinants. Similarly, a type-specific glycoproteins for HSV-1 (gG1), has been purified.
- (2) The use of gG-2 and gG-1, purified from extracts of HSV-2 or HSV-1 infected cells, respectively, led to the development of assays of high sensitivity, specificity, and reproducibility.
- (3) The immunodot assays are suitable for high throughput evaluation of sera for HSV-1 and HSV-2 antibodies.
- (4) Purified gG-2 and gG-1 can be stored at $\leq -70^{\circ}\text{C}$ without lose of antigenicity for many years if stored in glass ampoules. The reactivity levels of the stock antigens are confirmed annually by serial titrations against monoclonal antibodies, H1379-2 and H1206 for gG1 and gG2, respectively.
- (5) BSA from different sources could cause significant reductions in the sensitivity of the immunodot assays. This problem is overcome by periodically 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 many years in the Pediatric Infectious Disease Immunology and Epidemiology Laboratory for various projects, including NHANES I, II, III and NHANES 1999-2008. This method has proven to be accurate, robust and reproducible.

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 microplate in every 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: a monospecific HSV-1 antibody-positive serum pool diluted 1:1600 and 1:12800; an monospecific HSV-2 antibody-positive serum pool 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 1:800 and 1:3200; 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 3-mL aliquots and stored at $\leq -70^{\circ}\text{C}$. As needed, one 3-mL sample is thawed, divided into 30- μL aliquots and refrozen at $\leq -70^{\circ}\text{C}$. The 30- μL aliquots are then removed as needed to provide controls. Serum HSV-1 and HSV-2 antibodies are stable indefinitely when stored sealed at $\leq -70^{\circ}\text{C}$ without repeated freezing and thawing. The reactivity levels of all of the serum control pools are confirmed annually in serial titrations against gG-1 and gG-2 antigens

Each sample is tested in duplicate and each assay is read by two individuals independently. 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, use new aliquot of control pool and 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. Commercially available BSA is not well enough standardized as to quality and purity. 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 $\leq -70^{\circ}\text{C}$ until testing. After an aliquot of the thawed sample has been removed for testing, the residual is refrozen and stored at $\leq -70^{\circ}\text{C}$.

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

Other available methods have different performance characteristics (for example, lower sensitivity or specificity). If the analytical system fails, it is preferable to store specimens at $\leq -70^{\circ}\text{C}$ 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 record keeping involves using the computerized database 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 >100:1 is retained at $\leq -70^{\circ}\text{C}$ for 1 year and then returned to NCHS serum bank.

19. SUMMARY STATISTICS AND QC GRAPHS

Qualitative assays are assays with a positive, negative or borderline/indeterminate result. Since the controls do not generate quantitative values, plots are not generated for quality control purposes.

REFERENCES

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