For Individual Laboratory to Complete:

Laboratory Name		
Adopted		
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HSV 1 & 2 IgG Enzyme Immunoassay

Method: **Diamedix Corp., Immunosimplicity®**
Manual or in conjunction with one of the Diamedix Automated EIA Systems such as the MAGO Plus, the DSX or the DS2. For *In Vitro* Diagnostic Use.

Clinical Significance

Herpes simplex virus (HSV) is classified in the alphaherpesvirus subfamily of herpesviruses and is a linear DNA virus. Two antigenic types, HSV type 1 and HSV type 2, have been identified. The DNAs of both HSV types share approximately 50% of their base pairs. Due to this extensive sequence homology between the antigens from type 1 and type 2, subtyping analysis can only be performed if specific proteins or fragments of each of the subtypes are used in the assays (1,2,3,4).

HSV infections are extremely common and widespread and can involve mucocutaneous surfaces, internal organs and the central nervous system. HSV type 1 is usually acquired through contact with infectious salivary secretions whereas HSV type 2 infection is primarily transmitted by sexual contact. The initial infection caused by HSV type 1 or type 2 is followed by latent infection of neuronal cells in the dorsal root ganglia. Subsequent viral reactivation is accompanied by viral excretion from the original mucocutaneous sites of infection with or without concomitant appearance of clinical signs and symptoms (1,5). HSV transmission can result from direct contact with infected secretions from a symptomatic or an asymptomatic host. Although previous infection with HSV type 1 does not prevent infection upon exposure to HSV type 2, preexisting HSV type 1 immunity may modify the severity of HSV type 2 infection rendering it clinically mild or asymptomatic (1). The prevalence of HSV type 1 infections increases gradually from childhood reaching 70 to 80% in adult years. The prevalence of type 2 antibodies ranges from 15 to 50%,depending on a number of demographic variables (6,7).

The potential adverse outcome of HSV infection during pregnancy underscores the importance of determining the HSV immunological experience of the mother. Congenital and neonatal HSV can occur with primary or recurrent, symptomatic or aysmptomatic, maternal HSV infection. Infections usually result from exposure of neonates to virus being excreted by mothers at the time of vaginal delivery. If the neonate is exposed at delivery to a mother with a recurrent infection the attack rate, is probably less than 5%.

However, if the mother is experiencing a primary infection at delivery the attack rate is probably greater than 50%. Neonates may present with infection localized to the skin, eyes and mucosa or the central nervous system, or with a disseminated infection (1,8).

Because of the high prevalence of past HSV infections in the general population, many patients who develop malignancy, an immunodeficiency such as AIDS or other diseases that require immunosuppressive therapy, may experience HSV infection. These infections, which may be primary or arise from reactivations, can be severe (1).

Both clinical and laboratory criteria are useful in establishing the diagnosis of HSV infection. Laboratory diagnosis is usually accomplished by isolating the virus in cell culture or by determining serologically the presence of HSV specific antigens or antibodies. IgG antibodies to HSV usually appear 1 to 2 weeks after the onset of infection and persist at various levels for life. Determination of HSV antibody status is diagnostically useful in several situations, including the demonstration of recent seroconversion after acute infection, the documentation of symptomatic past infections and the identification of allograft recipients or patients with cancer who are at risk for reactivation of latent HSV infection. Perhaps the greatest value of serologic tests lies in their ability to identify asymptomatic carriers (4). Serological testing is most often performed using enzyme immunoassays (EIAs) which are easy to perform and more amenable to automation for screening large numbers of samples than other methodologies.

The Diamedix Immunosimplicity® Is-HSV 1 & 2 IgG Test Kit is an EIA procedure intended for the qualitative and semi-quantitative detection of antibodies to HSV type 1 and type 2 antigens. The test can be performed either manually or in conjunction with one of the Diamedix Automated EIA Systems. The results are objective and reported in EU/ml (ELISA Units per ml) which are traceable to in-house reference materials.

Principle of the Procedure

Diluted samples are incubated with HSV 1 & 2 antigens bound to the solid surface of a microtiter well. If IgG antibodies against HSV are present in the samples, they will bind to the antigen forming antigen-antibody complexes. Residual sample is eliminated by aspirating and washing. Conjugate (horseradish peroxidase-labeled anti-human IgG) is added and will bind to these complexes. Unbound conjugate is removed by aspiration and washing. Substrate is then added and incubated. In the presence of bound enzyme the substrate is converted to an end product. The absorbance of this end product can be read spectrophotometrically at 450 nm (reference 600-630 nm) and is directly proportional to the concentration of IgG antibodies to HSV 1 & 2 antigens present in the sample.

Specimen Collection

Whole blood should be collected by accepted medical techniques. Separated serum should remain at 22°C for no longer than 8 hours. If assays are not completed within 8 hours, serum should be refrigerated (2 to 8°C). If

assays are not completed within 48 hours, or the separated sample is to be stored beyond 48 hours, samples are to be frozen at -20°C. Avoid multiple freeze-thaw cycles. Prior to testing, bring frozen sera to room temperature slowly and mix gently, avoiding foam formation. Specimens containing visible particulate matter should be clarified by centrifugation before testing. Grossly contaminated, hemolyzed, lipemic, or icteric specimens should not be used. The NCCLS provides recommendations for collecting and storing blood specimens, (Approved Standard-Procedures for the Handling and Processing of Blood Specimens, H18A).

For the diagnosis of recent HSV infection, paired sera should be obtained at least 7-10 days apart.

CAUTION: Serum samples must not be heat-inactivated prior to use.

Reagents

Antigen Wells	Twelve, 8-well microwell breakapart strips, color-coded light green, coated with sucrose density gradient purified HSV-1 (MacIntyre strain) and HSV-2 (G-strain) antigens produced in Vero cells.
Calibrator	One vial with blue cap containing 1.8 ml of pre-diluted human serum, highly reactive for HSV 1 & 2 IgG antibodies, 0.2% sodium azide and Proclin™ 300, 90 ppm active ingredient. Assigned EU/ml value printed on label.
Positive Control	One vial with white cap containing 1.8 ml of pre-diluted human serum, moderately reactive for HSV 1 & 2 IgG antibodies, 0.2% sodium azide and Proclin™ 300, 90 ppm active ingredient. Assigned EU/ml range printed on label.
Negative Control	One vial with black cap containing 1.8 ml of pre-diluted human serum, non-reactive for HSV 1 & 2 IgG antibodies, 0.2% sodium azide and Proclin™ 300, 90 ppm active ingredient.

Note: *Calibrator and controls are prepared from different serum lots.*

Sample A Diluent	One bottle with blue cap containing 60 ml Phosphate buffer with protein stabilizers. Contains 0.2% sodium azide and Proclin™ 300, 90 ppm active ingredient. Color-coded blue.
Wash S Concentrate	Two bottles with clear caps containing 50 ml of Phosphate buffered saline with Proclin™ 300, 15 ppm active ingredient. Color-coded light blue/green. Each bottle is sufficient to make 1 liter of wash solution.
Conjugate	One bottle with red cap containing 25 ml goat anti-human immunoglobulin G labeled with horseradish

peroxidase. Also includes protein stabilizers and preservatives. Color-coded pink.

Substrate HRP — One amber bottle with brown cap containing 25 ml buffered TMB solution (3,3',5,5' tetramethylbenzidine).

Stop M Solution — One bottle with white cap containing 30 ml of 1 N Phosphoric and 1N Hydrochloric acids. **CAUTION:** Acids are corrosive. Avoid contact with skin or eyes. If contact is made, flush area with copious amounts of water. See Precautions section.

These reagents should be stored at 2 to 8° C.

Other Materials Required

Manual Users:

1. Wash bottle or automated microplate washer
2. Pipettors capable of dispensing appropriate volumes
3. Timer
4. One liter graduated cylinder
5. One liter wash solution reservoir
6. Deionized or distilled water
7. Absorbent toweling
8. Tubes or microwell plate for sample dilution
9. Reader capable of reading absorbance at 450nm, reference at 600-630 nm.
10. Incubator capable of maintaining temperature of 37 $\pm$ 3°C

Diamedix Automated EIA System Users:

1. One liter graduated container
2. Deionized or distilled water
3. Dilution containers as appropriate to system
4. Sample and Reagent tips required by system
5. Reagent containers required by system

Warnings:

1. Handle samples, Calibrator, controls and the materials that contact them as potential biohazards. Each donor unit in the Calibrator and controls has been found negative for Hepatitis B surface antigen and HIV-I antibodies by FDA-approved third generation tests. However, because no method can offer complete assurance that HIV-1, Hepatitis B virus, or other infectious agents are absent, these materials should be handled at the Biosafety Level 2 as recommended for any potentially infectious serum or blood specimen in the Centers for Disease Control/ National Institutes of Health manual, "Biosafety in Microbiological and Biomedical Laboratories", 1993.
2. Never pipette by mouth.
3. Avoid contact with open skin and mucous membranes.

4. Certain of the test reagents contain Proclin™ 300 as a preservative. When disposing of reagents containing Proclin™ 300, flush drains with copious amounts of water to dilute the active components below active levels.
5. Serum components contain sodium azide as preservative. Azides are reported to react with lead and copper in plumbing to form compounds that may become explosive. When disposing of solutions containing sodium azide, flush with copious amounts of water to minimize the build up of metal azide compounds.
6. Sodium azide inhibits conjugate activity. Care must be taken to ensure that azide is not carried over from other reagents into conjugate and substrate steps.
7. Avoid contamination of the TMB substrate solution with conjugate or other oxidants which will cause the solution to change color prematurely.
8. The substrate contains 3,3' 5,5' Tetramethylbenzidine (TMB) which has shown possible mutagenic effects in laboratory experiments.

Calibration

This test uses an in-house reference Calibrator. This Calibrator has been prepared from a pool of sera strongly positive for the antibody under investigation. The Calibrator functions as an internal reference preparation and is assigned a unitage in ELISA units (EU) per ml. The Calibrator is included in every test run.

These tests have been optimized to permit the use of single point calibration. This is possible because the dose response curves are sufficiently linear and pass near to, or through the origin. The linearity of the dose response has been validated by the manufacturer during quality control testing.

Patient samples which contain very high levels of antibody may produce absorbance values greater than the Calibrator absorbance. Patient sample results greater than the Calibrator value should be reported as "Greater than Calibrator value EU/ml". If numerical results are required for such samples, dilute the sample using Sample Diluent and re-assay. Several dilutions (for example 1/10, 1/50 and 1/100) of the pre-diluted sample may be re-assayed simultaneously. Select the dilution that has an absorbance reading about 50% of the absorbance reading of the Calibrator; calculate the EU/ml for this dilution and multiply by the dilution factor to obtain estimated values.

Quality Control

a) The Positive and Negative Controls must be included in each test run.

b) The absorbance of the Blank must be < 0.2.

c) The Positive Control must be within its assigned range.

d) The Negative Control must be < 16.0 EU/ml.

If any of these criteria is not met, the run is invalid and must be repeated.

NOTE: The Negative and Positive Control are intended to monitor substantial reagent failure. The controls will not control all parts of the procedure such as technical dilution of patient specimens. The Positive Controls will not ensure precision at the assay cut-off. Users may wish to establish an in-house control having a quantitative value determined by replicate testing, at or near the cut-off to monitor the precision of the assay cut-off. Additional controls may be tested according to guidelines or requirements of local, state, and/or federal regulations or accrediting organizations.

Procedure

Allow all test components and patient samples to warm to room temperature before use. Invert reagent bottles gently several times before use. Return promptly to the refrigerator after use.

Prepare Wash Solution by adding 50 ml of Wash Concentrate(20X) to one liter of deionized or distilled H_2O.

Manual Users:
The Calibrator and Controls are provided ready to use: DO NOT DILUTE FURTHER.

1. Prepare 1:101 dilutions of the patient samples in Sample Diluent. (e.g., by addition of 2 µl sample to 200 µl Sample Diluent or 5 µl sample to 500 µl Sample Diluent)
2. Mix sample dilutions gently by withdrawing and expelling in a pipette tip 2 or 3 times or by vortex mixing for 2 or 3 seconds. Transfer 100 µl of Calibrator, controls and diluted patient sample, to the antigen wells. Avoid formation of bubbles when transferring diluted samples.

   NOTE: *Include one well which contains 100 µl of Sample Diluent as a reagent blank. This will ultimately be used to "zero" the photometer before reading test results.*
3. Allow the wells to incubate uncovered at 37 $\pm$ 3° C for 60 ± 5 minutes.
4. Aspirate or discard the contents of the wells. Remove any excess moisture in the wells by tapping on paper toweling. Wash the wells by rinsing 3 times with at least 300 µl of Wash Solution. Remove excess moisture from the wells after washing. When using an automated washer, follow the manufacturer's instructions.
5. Place 100 µl of Conjugate into each well, avoiding bubble formation.
6. Allow the wells to incubate uncovered at 37 $\pm$ 3° C for 60 ± 5 minutes.
7. Wash the wells as described in Step 4 above.
8. Place 100 µl of Substrate into each well, avoiding bubble formation.
9. Allow the wells to incubate uncovered at 37 $\pm$ 3° C for 20 ± 2 minutes.
10. Place 100 µl of Stop Solution into each well, avoiding bubble formation.

11. Read the absorbance of the wells at 450 nm using a reference wavelength of 600-630 nm. The plate should be read within 60 minutes of adding Stop Solution.

Refer to the BP-96 Plate Reader Operation Manual for complete instructions on set-up and operating procedures.

Diamedix Automated EIA System Users:
If using one of Diamedix's Automated EIA Systems, please refer to the corresponding Operating Manual for the test setup, procedures, and accessories/consumables needed.

Calculation of Results
Determine the EU/ml (ELISA Units/ml) for each patient specimen or control using the following formula:

$$\frac{\text{EU/ml of Calibrator}}{\text{Absorbance of Calibrator}} \times \text{Absorbance of Sample} = \text{EU/ml of sample}$$

The Diamedix Automated EIA Systems will calculate results using the above formula and will print them automatically.

Reference Ranges
The following is only a guide to interpretation. **Each laboratory can establish its own "normal" ranges based on populations encountered.**

Less than 16.0 EU/ml Index < 0.80	Negative for anti-HSV 1&2 IgG.
Greater than/equal to 20.0 EU/ml Index $\geq$ 1.0	Positive for anti-HSV 1&2 IgG.
16.0 to 19.9 EU/ml Index 0.8-0.99	Equivocal for anti-HSV 1&2 IgG*.

* When equivocal results are obtained, another specimen should be collected ten to fourteen days later and tested in parallel with the initial specimen. If the second sample is also equivocal, the patient is negative for primary or recent infection, and equivocal for antibody status. If the second sample is positive, the patient can be considered to have a primary infection. The conversion of an individual patient's serum from negative to positive for antibodies to the infectious agent in question, is defined as seroconversion, and indicates primary or recent infection.

A single positive result only indicates previous immunologic exposure. The level of antibody response or class of antibody may not be used to determine active infection or disease stage.

A negative result does not always exclude the possibility of active HSV infection. The sample may have been collected before appearance of IgG antibody. If infection is suspected, a second sample should be collected at least 7 days later and tested concurrently with the first sample to determine if seroconversion has occurred. In addition, a negative result

does not exclude the possibility of past infection, since, in some cases, antibody levels may fall to undetectable levels after primary HSV infection.

Reporting Results

When the EU/ml value is reported for a single specimen the following statement should be included: "The following results were obtained with the Diamedix Immunosimplicity Is-HSV 1 & 2 IgG EIA Test System. The magnitude of the measured result, above the cut-off, is not indicative of the total amount of antibody present. The magnitude of the reported IgG level cannot be correlated to an end-point titer."

When the assay is used semi-quantitatively, the following statement should be included when reporting results:"Timing of specimen collection for paired sera may be critical. In some patients, antibody titers may rise to significant levels and fall again to lower or undetectable levels within a month. Other patients may not develop significant antibody levels. Culture results, serology and antigen detection methods should all be appropriately used along with clinical findings for diagnosis."

Paired Sera

To determine a significant difference between acute/convalescent sera, both specimens should be run within the same assay. Paired sera should be evaluated within the reportable range of the assay. In addition, the EU/ml value for the acute serum should be less than 60. Studies by the manufacturer performed both manually and using Diamedix Automated EIA Systems have shown that a 1.9-fold or greater increase in the EU/ml or Index ratio (convalescent serum EU/ml or Index Value / acute serum EU/ml or Index Value) corresponds to a four-fold increase in HSV IgG antibody level. The mean ratio obtained for two-fold dilutions was 1.4. Therefore, ratios in the range of 1.4 to 1.9 may be considered equivocal for significant increase status. If paired sera controls are desired, it is recommended that 1:2, 1:4 and 1:8 dilutions of the Calibrator be made first in Sample Diluent. The 1:2 and 1:8 dilutions will provide a simulated serum pair. The ratio of this simulated serum pair can then be compared against the established ratio.

Procedure Notes

1. Do not interchange reagents from different reagent lots except for Sample **A** Diluent, Wash **S** Concentrate, Substrate **HRP** and Stop **M** Solution.
2. Do not use reagents beyond their expiration date.
3. Store unused reagents at 2 to 8 ^{o}C.
4. Incubations above or below the recommended temperatures or times may give erroneous results.
5. The EIA method is a very sensitive technique. Maintain consistent pipetting technique, incubation times, and temperature conditions throughout the test procedure. Cross contamination between reagents can invalidate the test.

6. Antigen coated microwells should be stored with the desiccant in the resealable bag provided and returned to the refrigerator immediately after use.
7. *(Manual Procedure Only)* The washing procedure is very important and requires special attention. (Please refer to the Procedure section)

   NOTE: *Improperly washed* wells *may* give *erroneous results.*
8. The concentration of anti-HSV IgG in a given specimen determined from assays from different manufacturers can vary due to differences in assay methods and reagents.

Limitations

1. The results obtained with the Is-HSV 1 & 2 IgG Test Kit serve only as an aid to diagnosis and should not be interpreted as diagnostic in themselves.
2. Assay performance characteristics have not been established for visual result determination.
3. HSV 1 & 2 IgG EIAs are not intended to replace virus isolation and/or identification.
4. This test is not intended to be used for the diagnosis of current herpes simplex infection in pregnant women. The presence of HSV should be demonstrated by isolation of live virus.
5. The test should be performed on serum. The use of whole blood or plasma has not been established.
6. Performance of this assay has not been established on spectrophotometry utilizing a single wavelength.
7. The continued presence or the level of antibody cannot be used to determine the success or failure of therapy.
8. The presence of HSV IgG does not imply protection from disease with one type-specific herpes (type 1 or 2) and does not provide protection from a second infection with the other type, however, it may lessen the severity of the second infection. In addition, individuals with past HSV infection may exhibit recurrent (reactivated) episodes of the past HSV infection.
9. Interpretation of serologic data for HSV must be completed with the understanding of cross-reactivity due to shared antigens and the chronic nature of HSV infections. Due to commonly shared antigens, infections with one type of HSV in the presence of antibody to the heterologous type, may produce an anamnestic response with the pre-existing antibody to become more elevated than the antibody titer of the infective agent of the current infection. Therefore, definitive diagnosis of HSV typing should be made by viral isolation.
10. A significant increase in HSV antibody titer may not accompany recurrent herpes simplex disease. In addition, a significant increase in HSV antibody titer may not accompany first episode infection of the

other type-specific HSV (e.g. HSV-1 infection followed by HSV-2 infection).

11. The performance characteristics have not been established for neonates, infants or on cord blood.
12. Results from immunosuppressed patients should be interpreted with caution.
13. The performance characteristics of the Diamedix Is-HSV 1 & 2 IgG Test Kit with automated equipment other than Diamedix Automated EIA Systems have not been established.

References

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