For Individual Laboratory to Complete:

Laboratory Name		
Adopted		
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Supercedes		

EBV-EA-D IgM 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

Epstein-Barr virus (EBV) is a member of the *herpesvirus* family that infects human lymphocytes (1, 2). It is known to cause infectious mononucleosis (IM) and is transmitted primarily by saliva. EBV has been detected in oropharyngeal secretions of healthy, asymptomatic adults, and is a possible source of infection for susceptible individuals (1,2,3). As with other herpesviruses, EBV causes a persistent latent infection with intermittent reactivations. EBV infection is usually asymptomatic in infants and young children. In adolescents and young adults infection usually results in IM (1,3,4,5,6). Diagnosis is generally based on the characteristic symptoms of sore throat, lymphadenopathy, fever, splenomegaly and possibly the presence of heterophile antibodies (1,6). Because not all symptoms may be present and, since other infectious agents such as *Toxoplasma gondii* and cytomegalovirus may cause similar symptoms, serological detection of circulating antibodies is an important step in the diagnosis of EBV infection (1,6).

Humoral response to primary EBV infections appears to be quite rapid. Antibodies to EBV are made to various viral proteins, with specific antibodies correlating to disease state. In acute infection, IgM and then IgG antibodies are sequentially made to EA-D, VCA and EBNA. Current or recent infection is marked by the presence of IgM antibodies to VCA, EA-D and EBNA. IgG antibodies to VCA and EA-D are normally present in current infection, while IgG antibodies to EBNA are absent. Post-EBV infection is indicated by sustaining IgG antibody to VCA and EBNA and the absence of IgM antibodies (1,3,7,8). Thus, the monitoring of EBV antibody patterns may assist in the diagnosis of EBV infection since individual levels of specific antibodies may not necessarily be indicative of disease but can be of diagnostic importance when monitored as a profile.

The indirect fluorescent antibody (IFA) assays for detecting antibodies to EBV antigens have been largely replaced by enzyme-linked immunosorbent assays (ELISA or EIA) which are easier to perform, easier to interpret and amenable to automation.

The Immunosimplicity® Is-EBV-EA-IgM Test Kit is an EIA procedure intended for the qualitative detection of EA-D IgM antibodies and can be performed either manually or in conjunction with one of the Diamedix Automated EIA Systems.

Principle of the Procedure

Recombinant EA-D antigen is bound to microwells. Diluted patient sera, Cut-Off Calibrator and controls are placed in the microwells and incubated. Anti-EA-D IgM antibodies, if present, will bind to the antigen forming antigen-antibody complexes. Residual sample is eliminated by aspirating and washing. Conjugate (horseradish peroxidase-labeled anti-human IgM) 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). Color development above a certain level denotes the presence of IgM antibodies to EA-D 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 (2-8°C). If assays are not completed within 48 hours, or the separated sample is to be stored beyond 48 hours, samples should 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. If paired sera analysis is to be performed, obtain the second sample at least two weeks after the first sample. Test both samples within the same assay.

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

Reagents

Antigen Wells Twelve, 8-well microwell breakapart strips, color-coded pink, coated with purified recombinant (*E. coli* as vector) EA-D antigen (a 28kd protein from the c-terminal half of EA-D).

Cut-off Calibrator One vial with blue cap containing 0.25 ml of human serum or defibrinated plasma, weakly reactive for EA-D IgM antibodies, 0.1% sodium azide. The Cut-Off Calibrator is used to determine the cut-off of the assay.

Low Positive Control One vial with white cap containing 0.25 ml of human serum or defibrinated plasma, 0.1% sodium azide. Assigned range printed on label. The positive control is used to control the low range of the assay.

Negative Control One vial with black cap containing 0.25 ml of non-reactive human serum or defibrinated plasma. Assigned range printed on the label. The Negative Control is used to control the negative range of the assay and to control the removal of IgG antibodies.

Note: The Cut-Off Calibrator and controls are prepared from different serum lots.

Sample C Diluent One bottle with green cap containing 60 ml of Phosphate buffer with goat anti-human IgG and protein stabilizers. Contains Proclin™ 300, 15 ppm active ingredient. Color-coded green.

Wash T Concentrate (20X) Two bottles with clear caps containing 50 ml of Tris buffer with detergent and Proclin™ 300, 15 ppm active ingredient. Each bottle is sufficient to make 1 liter of wash solution.

Conjugate One bottle with red cap containing 25 ml goat anti-human immunoglobulin M 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' Tetramethyl-benzidine).

Stop N Solution One bottle with white cap containing 30 ml of 1 N Sulfuric Acid. **CAUTION:** Acids are corrosive. Avoid contact with skin or eyes. If contact is made, flush area with copious amounts of water.

Store these reagents 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 serum dilution
9. Reader capable of reading absorbance at 450nm, reference at 600-630 nm (Performance characteristics have not been established for a single wavelength reader.)

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-1 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 horseradish peroxidase 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 standard (or Calibrator). The Calibrator has been derived from weakly positive sera and is titrated to an absorbance value equivalent to the cut-off of the assay. Samples whose absorbances exceed this value are considered positive for EA-D IgM antibodies and samples whose absorbances are less than this value are considered negative for EA-D IgM antibodies. To account for the inherent variations in enzyme immunoassays an equivocal range of ±10% has been included at the assay cut-off.

Quality Control

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

b) The absorbance of the Blank must be < 0.25.

c) The absorbance of the Cut-Off Calibrator must be > 0.10.

d) The Positive and Negative Controls must be within their assigned ranges.

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

Note: Additional controls may be tested according to guidelines or requirements of local, state, or federal regulations or accrediting organizations. For guidance on appropriate quality control practices, please refer to NCCLS document C24-A, Internal Quality Control Testing: Principles and Definitions.

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_20.

Manual Users:

1. Prepare 1:101 dilutions of the Cut-Off Calibrator (in triplicate), controls and 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 2 or 3 times or by vortex mixing for 2 or 3 seconds. Transfer 100 µl of Calibrator, controls and diluted patient samples, 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 at room temperature (18-30°C) for 30 ± 5 minutes.
4. Aspirate or discard the contents of the wells. Remove 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 room temperature (18-30° C) for 30 ± 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 room temperature (18-30°C) for 30 ± 5 minutes.
10. Place 100 µl of Stop Solution into each well, avoiding bubble formation.
11. Read the absorbance of each well at 450 nm using a reference wavelength of 600-630 nm. The plate should be read within 60 minutes of adding Stop Solution.

Diamedix Automated EIA System Users:

When using one of Diamedix's Automated EIA Systems, refer to the corresponding Operating Manual for the test setup, procedure, and accessories/consumables needed.

Calculation of Results

Calculate the MEAN absorbance of the Cut-off Calibrator. **Note:** When calculating the mean absorbance value for the Cut-Off Calibrator exclude any absorbance value that deviates by more than 15% from the mean of the three absorbance values. Use the mean of the remaining two replicates in calculations. Exclusion of more than one of the three absorbance values invalidates the run.

$$\frac{\textbf{Absorbance of Sample}}{\textbf{Mean Absorbance of Cut-off Calibrator}} = \textbf{Index Value}$$

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

Example: Absorbance values obtained for the Calibrator: 0.276, 0.288, 0.258 (after subtraction of Blank)

Mean Absorbance of Cut-off Calibrator = 0.274

Sample Absorbance = 1.150

Index Value 1.150/ 0.274 = 4.2

Reference Ranges

Index < 0.90	No detectable EA-D IgM antibody; result does not exclude EBV infection. An additional sample should be tested within 4-6 weeks if early infection is suspected. Other EBV serology tests are necessary to rule out acute infection.
Index ≥ 1.10	EA-D IgM antibody detected. Other EBV serology tests are necessary for confirmation of acute EBV-associated infectious mononucleosis.
Index 0.90-1.09	Equivocal for antibodies to EA-D. Sample can be retested, tested by another method or a new sample can be tested.

Reporting Results

When the Index Value is reported for a single specimen the following statement should be included: "The following results were obtained with the Is-EBV-EA-IgM Test Kit. 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 IgM level cannot be correlated to an endpoint titer".

Procedure Notes

1. Do not interchange reagents from different reagent lots except for Sample **C** Diluent, Wash **T** Concentrate, Substrate **HRP** and Stop **N** Solution.
2. Do not use reagents beyond their expiration date.
3. Store unused reagents at 2 to 8°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 reported concentration of anti-EA-D IgM in a given specimen determined with assays from different manufacturers can vary due to differences in assay methods and reagent specificity.

Limitations

1. The results obtained with the Is-EBV-EA-D-IgM 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. The test should be performed on serum. The use of whole blood or plasma has not been established.
4. There is a possibility of assay cross-reactivity with specimens containing anti-*E.coli* antibody.
5. The performance characteristics have not been established for patients with nasopharyngeal carcinoma, Burkitt's lymphoma, other EBV-associated lymphadenopathies, and other EBV-associated diseases other than EBV-related mononucleosis.
6. A single result cannot be used for diagnosis. Accurate interpretation of EBV infection is based on the results from EA-D IgG, EA-D IgM, EBNA IgG, EBNA IgM, VCA IgG, VCA IgM, and heterophile antibody testing.
7. Screening of the general population should not be performed. The positive predictive value depends on the likelihood of Epstein Barr Virus being present. Testing should only be performed when clinical symptoms are present or exposure is suspected.
8. Results from immunosuppressed patients should be interpreted with caution.
9. The performance characteristics of the Is-EBV-EA-D-IgM Test Kit with automated equipment other than the Diamedix Automated EIA Systems have not been established.
10. Since rheumatoid factor (RF) binds to IgG in immunocomplexes, false positive results may arise in sera with RF and specific IgG. False negatives may arise due to specific IgG competing with the specific IgM. The goat anti-human IgG in the sample diluent diminishes RF interference and minimizes competing specific IgG in the samples. The sample diluent removes >95% of the IgG levels of 1400 mg/dl. Samples with IgG levels >1400 mg/dl should be interpreted with caution.
11. Performance characteristics have not been established for diagnosing adolescents with infectious mononucleosis.

References

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