**CMV IgM
Capture Enzyme
Immunoassay**

Laboratory Name		
Adopted		
Reviewed		
Reviewed		
Revised		
Supercedes		

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

Cytomegalovirus (CMV) is a herpes virus which is responsible for a range of infections in humans of all ages. The vast majority of healthy children and adults who acquire CMV infection remain asymptomatic. Symptoms, if they are present, include fever, lethargy and atypical lymphocytosis that can mimic the symptoms caused by Epstein-Barr virus. However, the incidence and spectrum of disease in newborns, in organ transplant recipients and in AIDS and HIV-symptomatic individuals establish this virus as an important and significant human pathogen (1). CMV infections can be acquired before birth (congenital), at birth (perinatal) or later in life (postnatal). Fewer than 5% of congenitally infected infants develop symptoms during the newborn period; possible manifestations range from severe disease with intrauterine growth retardation, jaundice, hepatosplenomegaly, petechiae, central nervous system abnormalities, and chorioretinitis, to more limited involvement. Symptomatic infants may die of complications within the first month of life; more commonly, they survive but are neurologically damaged. Newborns can also acquire infection at birth by contact with the virus in the birth canal. Such infants begin to excrete virus at 3 to 12 weeks of age but usually remain asymptomatic. Most postnatal infections are acquired by close contact with individuals who are shedding virus. Since CMV has been detected in several body fluids including saliva, urine, breast milk, tears, stool, vaginal or cervical secretions and semen, transmission can occur in a number of ways. CMV can also be transmitted by blood transfusion or organ transplantation (1,2,3,4).

CMV infections are frequent and occasionally severe in immunosuppressed individuals such as patients with AIDS, HIV-virus, cancer patients and organ donor recipients. Such infections may represent reactivation of latent virus or primary infection introduced by blood transfusion or transplanted organ (2,3,4,5).

Serologic procedures are useful in detecting CMV antibodies in patient sera. CMV IgG antibodies generally appear 1 to 2 weeks after infection, reach peak levels in 6-10 weeks, and persist at various levels for life (6). IgM antibodies may appear as early as 5 days after infection, rise sharply and fall to low levels or disappear within a few weeks or months.

Traditional methods for determining levels of CMV antibody such as the complement fixation (CF) or IHA (indirect hemagglutination) tests have been replaced by enzyme immunoassays (EIAs) which are more sensitive, easier to perform and more amenable to automation for screening large numbers of samples. Capture EIAs offer the additional advantage of avoiding interference due to rheumatoid factor and competing IgG antibodies.

The Diamedix Immunosimplicity® (Is)-CMV IgM Capture Test Kit is a Capture EIA procedure intended for the qualitative detection of IgM antibodies to CMV antigen. The test can be performed either manually or in conjunction with one of the Diamedix Automated EIA Systems.

Principle of the Procedure

The Is-CMV IgM Capture Test Kit utilizes ELISA based on the antibody-capture technique. Diluted patient sera are incubated with mouse monoclonal antibody against human IgM bound to the solid surface of a microtiter well. Patient IgM is 'captured' by the surface bound antibody. Unbound serum components are washed away. Patient anti-CMV IgM antibodies are 'detected' and bound by an immunocomplex, Enzyme Tracer, consisting of CMV antigen which is linked to a mouse monoclonal anti-CMV antibody conjugated to horseradish peroxidase. Unbound tracer 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 IgM antibodies to CMV antigen 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 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. The NCCLS provides recommendations for collecting and storing blood specimens (8).

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

Reagents

Anti-IgM Coated Wells Twelve, 8-well microwell breakapart strips, color-coded pink, coated with mouse monoclonal anti-human IgM (heavy chain). Antibody designated IgM 1.1.

Cut-Off Calibrator One vial with blue cap containing 0.25 ml of human serum, preserved with 0.1% sodium azide, weakly reactive for CMV IgM antibodies. 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 preserved with 0.1% sodium azide. Assigned range printed on label. The Low Positive Control is used to control the low range of the assay.

Negative Control One vial with black cap containing 0.25 ml of human serum preserved with 0.1% sodium azide, non-reactive for CMV IgM antibodies. The Negative Control is used to control the negative range of the assay.

> *Note: The Cut-Off Calibrator and Controls are prepared from different serum lots.*

Lyophilized Antigen Six vials of lyophilized CMV antigen, (partially purified by sucrose gradient centrifugation, Davis Strain, prepared from infected human fibroblast cells).

30X Tracer One vial with red cap containing 0.8 ml mouse monoclonal anti-CMV antibody conjugated to horseradish peroxidase (30X concentrate) in stabilizer. The mouse monoclonal anti-CMV antibody (designated 2.5.5.6) recognizes an antigen present in the nucleus of cells 1 week post-infection (an early antigen).

Tracer Diluent One bottle with red cap containing 25 ml borate buffer. Also includes protein stabilizers, gentamycin and Proclin™ 300 as preservatives. Color-coded pink.

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.

Substrate G   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 serum 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, Cut-Off Calibrator, controls and the materials that contact them as potential biohazards. Each donor unit in the Cut-Off Calibrator and controls has been found negative for Hepatitis B surface antigen, HCV and HIV-1 and 2 antibodies by FDA-approved third generation tests. However, because no method can offer complete assurance that HIV-1 and 2, Hepatitis B virus or Hepatitis C 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 the immunocomplex 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 (Cut-Off Calibrator). The Cut-Off 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 CMV IgM antibodies and samples whose absorbances are less than this value are considered negative for CMV IgM antibodies. To account for the inherent variations in enzyme immunoassays an equivocal range of $\pm$ 10% has been included at the assay cut-off.

Quality Control

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

b) The absorbance of the Blank must be < 0.100.

c) The absorbance of the Cut-Off Calibrator must be $\geq$ 0.150 when read against the blank.

d) The Low 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.

NOTES: Additional controls may be tested according to guidelines or requirements of local, state, and/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_2O.

Each vial of lyophilized antigen is sufficient for at least 2 strips. Reconstitute only the number of vials required. Discard any unused Enzyme Tracer after the day's testing is completed.

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 tip 2 or 3 times or by vortex mixing for 2 or 3 seconds. Transfer 100 µl of diluted Calibrator, controls and patient samples, to the 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. DO NOT ADD ENZYME TRACER TO THE BLANK WELL.

3. Allow the wells to incubate uncovered at 37 $\pm$ 3°C for 60 ± 5 minutes.

4. As soon as the sample incubation has commenced, prepare the Enzyme Tracer by adding 2.9 ml of Tracer Diluent to each vial of lyophilized antigen required for the run. Mix until all the lyophilized material is reconstituted. Then add 100 µl 30X Tracer to each antigen vial and mix well. Allow the prepared Enzyme Tracer to sit at room temperature (18–30°C) for at least 30 minutes.

5. 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 each of Wash Solution. After the 3rd volume of Wash Solution, allow the wells to "soak" for at least one minute prior to final aspiration/emptying. When using an automated washer, follow the manufacturer's instructions and set up the same wash procedure as described.

6. Place 100 µl of Enzyme Tracer into each well (except the blank), avoiding bubble formation.

7. Add 100 µl Sample Diluent to the Blank well.

8. Allow the wells to incubate uncovered at 37 $\pm$ 3°C for 60 ± 5 minutes.

9. Wash the wells as described in Step 5 above.

10. Place 100 µl of Substrate into each well, avoiding bubble formation.

11. Allow the wells to incubate uncovered at 37 ± 3°C for 20 ± 2 minutes.

12. Place 100 µl of Stop Solution into each well, avoiding bubble formation.

13. Read the absorbance of the wells at 450 nm using a reference wavelength of 600-630 nm. The plate should be read within 30 minutes of adding Stop Solution.

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, procedure, and accessories/consumables needed.

Calculation of Results

Calculate the MEAN absorbance of the Cut-Off Calibrator. Note: When calculating the mean absorbance exclude any absorbance value that deviates by more than 20% from the mean absorbance value. Calculate the mean absorbance value from the two remaining absorbances. Exclusion of more than one of the 3 absorbance values invalidates the run. Determine the Index Value for each patient sample or control using the following formula:

$$\frac{\text{Absorbance of Sample}}{\text{Mean Absorbance of Cut-Off Calibrator}} = \text{Index Value}$$

Example: Absorbance values obtained for Cut-Off Calibrator: 0.356, 0.345, 0.368 (after subtraction of the Blank)
Mean Absorbance of the Cut-Off Calibrator = 0.356
Sample Absorbance = 0.959
Index Values are then calculated as follows:
Sample Absorbance / Mean Absorbance of Cut-Off Calibrator = 2.69

When using the Diamedix Automated EIA Systems, results are automatically calculated and expressed as Positive, Equivocal or Negative.

Reference Ranges

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

Index Value	Interpretation
Index < 0.90	Negative for anti-CMV IgM.
Index ≥ 1.10	Positive for anti-CMV IgM.
Index 0.90-1.09	Equivocal for anti-CMV IgM.*

Note that the magnitude of the Index Value has no significance and results should be reported as under 'Interpretation' above.

In addition, note that a sample may have been collected too early during the course of the disease for antibodies to have appeared and that an IgM antibody response may not occur or be below detectable levels during reactivation.

* When equivocal results are obtained, another specimen should be collected at least fourteen days later and tested in parallel with the initial specimen. If the second sample is also equivocal, antibody status cannot be determined. Other clinical and serological evidence should be sought in those cases. If the second sample is positive, seroconversion has occurred and may be indicative of a current or recent infection.

Reporting Results

When reporting results the following statement should be included: "The following results were obtained with the Diamedix Immunosimplicity Is-CMV IgM Capture EIA Test Kit. The magnitude of the measured result, above the cut-off, is not indicative of the total amount of antibody present."

Procedure Notes

1. The concentrations of anti-CMV IgM in a given specimen determined with assays from different manufacturers can vary due to differences in assay methods and reagent specificity.

2. Do not interchange reagents from different reagent lots except for Sample **A** Diluent, Wash **S** Concentrate, Substrate **G** and Stop **M** Solution.

3. Do not use reagents beyond their expiration date.

4. Store unused reagents at 2 to 8° C.

5. Incubations above or below the recommended temperatures or times may give erroneous results.

6. The Capture ELISA 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.

7. Coated microwells should be stored with the desiccant in the resealable bag provided and returned to the refrigerator immediately after use.

8. *(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.*

Limitations

1. Is-CMV IgM Capture Test Kits are not intended to replace viral isolation and/or identification.

2. Assay performance characteristics have not been established for visual result determination.

3. Assay performance characteristics for the use of specimen matrices other than serum have not been established.

4. A negative result does not always exclude the possibility of active CMV infection. The sample may have been collected before the appearance of IgM antibody. If an infection is suspected, a second sample should be obtained at least 14 days later and tested in parallel with the first specimen to look for seroconversion or a significant rise in titer of IgM which is indicative of a primary infection.

5. Specific IgM antibodies are usually detected in patients with recent or primary infection. In some cases, however, low levels of antibodies may persist for more than 12 months.

6. Assay performance characteristics have not been established with single wavelength spectrophotometers.

7. Re-infections or reactivation of latent infections may not be positive for specific IgM antibodies. Therefore a negative IgM result does not necessarily preclude a current re-infection or reactivation.

8. Isolation of the virus is the preferred method for diagnosing congenital CMV infection since such infections are often asymptomatic and serological evidence of CMV IgM antibody may be difficult to obtain.

9. Performance characteristics have not been established for newborns, for cord blood or for immunosuppressed individuals (including HIV-positive and pre-and post-transplant patients).

10. Performance characteristics of the Diamedix Is-CMV IgM Capture Test Kit with automated equipment other than the Diamedix Automated EIA Systems have not been established.

References

1. Hodinka, R. L. and Friedman, H. M. 1995. Human Cytomegalovirus. In: Manual of Clinical Microbiology. Baron, E., Pfaller, M. A., Tenover, F. C. and Yolken, R. H. (eds). 6th Edition, ASM Press, Washington, DC, p.884-894.

2. Tegtmeier, G. E. 1989. Posttransfusion Cytomegalovirus Infections. Arch. Pathol. Lab. Med. 113: 236-245.

3. Fiala, M., Payne, J.E., Berne, T. V., Moore, T. C., Henle, W., Montgomerie, J. Z., Chatterjee, S. N. and Guze, L. B. 1975. Epidemiology of Cytomegalovirus Infection After Transplantation and Immunosuppression. J. Infect. Dis. 132: 421-433.

4. Prince, A.M., Szmuness, W., Millian, S. J. and David, D. S. 1971. A Serologic Study of Cytomegalovirus Infections Associated with Blood Transfusions. N. Engl. J. Med. 284: 1125-1131.

5. Drew, W. L. 1992. Cytomegalovirus Infection in Patients with AIDS. Clin. Infect. Dis. 14: 608-615.

6. Horwitz, C. A., Henle, W. and Henle, G. 1979. Diagnostic Aspects of the Cytomegalovirus Mononucleosis Syndrome in Previously Healthy Persons. Postgrad. Med. 66: 153-158.

7. Procedures for the Handling and Processing of Blood Specimens: Approved Guideline-Second Edition NCCLS Document H18-A2, Vol. 19, No. 21.1999.

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