Appendix C. IgM and IgG Serologic Assay Protocols

IgM ANTIBODY CAPTURE ENZYME-LINKED IMMUNOSORBENT ASSAY PROTOCOL

Introduction Assays that detect viral specific immunoglobulin M (IgM) are advantageous because they detect antibodies produced during the first few days after onset of clinical symptoms in a primary infection, obviating the need for convalescent-phase specimens in many cases. IgM capture is the optimum approach to IgM detection because it is simple, sensitive, and applicable to serum and cerebrospinal fluid (CSF) samples from a variety of animal species (*e.g.* human, equine, avian). False-positive reactions due to rheumatoid factor are minimized.

Principle IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) provides a useful alternative to immunofluorescence for documentation of a serologic response. ELISA is less subjective than immunofluorescence and large numbers of samples can be processed. However, the principle of ELISA is similar to that of immunofluorescence. In our laboratory, anti-IgM (the capture antibody) is coated on 96-well plates. This is followed sequentially by the patient's serum, then known non-infectious viral antigen. The presence of antigen is detected by using enzyme-conjugated anti-viral antibody, and a colorimetric result is generated by the interaction of the enzyme and a chromogenic substrate. This constitutes the MAC-ELISA.

Safety The procedure should be performed under laboratory safety conditions that take into consideration the potential infectious nature of the serum specimens involved. Lab coat, gloves, and a laminar flow hood is recommended.

Materials and Reagents

Coating buffer: Carbonate/bicarbonate buffer pH 9.6
1.59g Na_2CO_3 + 2.93g $NaHCO_3$ diluted in 1L water.

Wash buffer: Phosphate buffered saline (PBS), 0.05% Tween 20, pH 7.2. PBS is available in powdered form from multiple commercial sources.

Blocking buffer: PBS/5% milk/ 0.5% Tween 20

Stop solution: 1 N H_2SO_4

Coating antibody: Goat anti-human IgM
Kirkegaard and Perry Laboratories cat# 01-10-03

Viral antigen: Sucrose-acetone extracted suckling mouse brain viral antigens, non-infectious, previously titrated

Normal antigen: Sucrose-acetone extracted suckling mouse brain antigen from mock-infected animals.

Detecting antibody conjugate: Horseradish peroxidase conjugated monoclonal antibody, previously titered

Substrate: 3,3'5, 5' tetramethylbenzidine base (TMB-ELISA), Gibco cat# 15980-0414

Plates: Immulon II HB flat-bottomed 96 well plates Dynatech Technologies cat# 3455

Microplate washer
Microplate reader
Incubator
Single and multi-channel pipettors
Reagent reservoirs
Ziploc bags, paper towels

Clinical specimens

Acute and convalescent human serum and/or cerebrospinal fluid (CSF) specimens
Previously tested antibody-positive and antibody-negative human sera for controls

NOTE: STORE ALL DIAGNOSTIC SPECIMENS AT 4EC PRIOR TO TESTING, AND -20EC AFTER ALL ANTICIPATED TESTING HAS BEEN COMPLETED. AVOID REPEATED FREEZE-THAW CYCLES.

Procedure

NOTE: THE FOLLOWING PROCEDURE INCLUDES INFORMATION ON QUALITY CONTROL AND INTERPRETATION. EACH SERUM SPECIMEN IS TESTED IN TRIPLICATE ON BOTH VIRAL AND NORMAL ANTIGENS. 8 TEST SPECIMENS CAN BE ANALYZED PER PLATE. CSF SPECIMENS ARE USUALLY TESTED ONLY SINGLY.

1. Using a fine-tipped permanent marker, number and label the plates (use Fig. 1. as a guide). Identify the location of each clinical specimen (S1-S8) by using the appropriate laboratory code number. *To keep timing of reagent addition consistent, process plates in the order that they are numbered during all steps of the procedure.* Plates should be kept in an enclosed, humidified environment during all incubation times with the exception of the coating step. A large ziploc bag containing a moist paper towel works well for this purpose.

2. Coat the inner 60 wells of 96 well plates with 75 microliters per well of goat anti-human IgM diluted 1:2000 in coating buffer pH 9.6. **Incubate at 4^0 C overnight.**

3. Dump out the coating antibody and blot plates on paper towels.
Block plates with 200:l blocking buffer per well. **Incubate at room temperature for 30 minutes.**

4. Wash wells 5X with wash buffer by using an automatic plate washer. Wells should be filled to the top each cycle.

5. Add 50:l per well of the patient's serum (S) diluted 1:400 in wash buffer to a block of 6 wells, or add patient's CSF undiluted to two wells only, so that the CSF will be tested singly against the viral and normal antigens. Note: CSF can be diluted to a maximum of 1:5 in wash buffer if necessary. Add positive control human serum (Ref) diluted in wash buffer according to a previous titration, and a negative human serum control (N) diluted 1:400 in wash buffer to a block of 6 wells each (see Fig. 1). Incubate plates for **1 hour at 37^0C** in a humidified chamber.

6. Wash 5X.

7. Dilute viral antigen in wash buffer according to a previous titration. Add 50 :l per well to the left three wells of each serum block (see Fig. 1). To the right three wells of each block, add 50 :l per well of normal antigen diluted in wash buffer to the same concentration as the viral antigen. **Incubate overnight at 4^0C** in a humidified chamber.

8. Wash 5X.

9. Add 50 :l per well of horseradish peroxidase-conjugated monoclonal antibody, broadly cross-reactive for the appropriate viral antigenic group, diluted in blocking buffer, according to a previous titration. **Incubate 1 hour at 37^0C** in a humid chamber.

10. Turn on plate reader to warm up, and remove TMB-ELISA from refrigerator.

11. Wash plates 5X **twice**. Turn the plates 180^o in the washer after the first series of 5 cycles. This promotes consistent results.

12. While the plate is at room temperature, add 75:l per well of TMB substrate to all wells. Immediately cover plates to block out light. Incubate at room temperature for 10 minutes. A blue color will develop in antibody-positive wells.

13. Add 50 :l per well of stop solution to all wells, including the outer rows of wells on the plate (the plate reader should be set to zero itself on some of these wells). The wells that were blue will now change to a yellow color. Allow plates to sit at room temperature for 1 minute. Read plates in microtiter plate reader by using a 450 nm filter.

Practical considerations

1. Plates can be coated and kept at 4^{o}C for up to a week.
2. Undiluted control sera can be stored at 4^{o}C for up to 2 weeks.
3. Reconstituted, undiluted viral and normal antigens can be stored at -20^{o}C for an undefined period of time.
4. Test and control sera can be diluted to the working dilutions and refrigerated one day prior to use. Antigens and conjugate *must be diluted to the working dilutions immediately prior to use.*

The MAC-ELISA should be restandardized periodically. This should occur when new lot numbers of reagents are introduced, and at the very least, once a year. It is recommended that the mean optical density of the positive control serum reacted on the viral antigen be set to approx. 1.0. The normal control serum reacted on the viral antigen should be around 0.2 (this varies). The standardization of reagents is normally achieved via titration, always comparing the optical densities of the reagents when reacted on viral and normal antigen.

Results

Before the results can be calculated for each clinical specimen, the test must be determined to be **valid**. For a valid test the following must be true:

$$\frac{\text{Mean OD of the positive control serum reacted on viral antigen (P)}}{\text{Mean OD of the negative control serum reacted on viral antigen (N)}}$$

must be greater than or equal to 2.0. **This is the P/N of the positive control.**

Test validity must be determined for each plate. Results for the clinical specimens may only be determined if the test is valid. If the test is not valid, then that plate must be repeated. If the test still fails after a repeat, then one or more of the reagent or test parameters was likely in error, and troubleshooting should be performed.

To determine whether the clinical specimens (S1-S8) contain IgM to the viral antigen (which would indicate recent infections with that virus) the following must be calculated:

$$\frac{\text{Mean OD of the test specimen reacted on viral antigen (P)}}{\text{Mean OD of the negative control serum reacted on viral antigen (N)}}$$

This is the P/N of the test specimen. For a specimen to be considered IgM-positive to the test virus, the P/N must be greater than or equal to 2.0.

In addition the value of P for the test specimen must be greater than or equal to twice the mean OD of the test specimen reacted on normal antigen. If this requirement is not met, non-specific background is being generated, and the result **MUST** be reported as uninterpretable.

Interpretation All patient P/N values greater than or equal to 2.0 should be reported as presumptive IgM-positive (see paragraph below), as long as they meet the requirements listed above. In the event that an early acute CSF or serum is negative by this test, a convalescent serum specimen must be requested and tested before that patient is reported as negative for serological evidence of recent viral infection. Without testing of a convalescent specimen, a negative result may reflect testing of an acute-phase specimen obtained before antibody has risen to detectable levels. In most patients, IgM is detectable 8 days post-onset of symptoms from an alpha-, flavi-, or California group virus infection. IgM persists for at least 45 days, and often for as long as 90 days.

The positive P/N cut-off value of 2.0 is empirical, based on experience and convention. P/N values that lie between 2.0 and 3.0 should be considered suspect false-positives. Further tests should be performed to determine the status of these specimens.

It should be stressed that the P/N value for a specimen at the screening dilution of 1:400 is not an indication of absolute antibody concentration, i.e., the P/N value is not

quantitative.

It is further recommended that for sera, all positive results should be confirmed by titration using 6, 2-fold dilutions of the serum specimens compared to a similar titration of the negative control serum. Linear curves indicate true seropositivity. Flat or undulating titration curves indicate false-positive results.

References

Tsai, TH: Arboviruses, In Rose NR, Marcario EC, Fahey JL, Friedman H, and Penn GM, (Eds): Manual of Clinical Laboratory Immunology, 4th Edition, American Society for Microbiology: 606-618, 1976.

Diagnosis of Infections caused by Viruses, Chlamydia, Rickettsia, In Koneman EW, Allen SD, Janda WM, Schreckenberger PC, and Winn Jr. WC , (Eds): Diagnostic Microbiology, 4th Edition, JB Lippicott Co: 956-1074, 1992.

Monath, TP, Nystrom, RR, Bailey, RE, Calisher, CH, and Muth, DJ:Immunoglobulin M antibody capture enzyme-linked immunosorbent assay for diagnosis of St. Louis encephalitis. Journal of Clinical Microbiology 20:784-790, 1984.

Martin, DA., Muth, DA., Brown, T., Karabatsos, N., and Roehrig, JT. Standardization of immunoglobulin M capture enzyme-linked immunosorbent assays (MAC-ELISA) for routine diagnosis of arboviral infections. J Clin Microbiol. 2000 May; 38(5): 1823-6.

IgG ENZYME-LINKED IMMUNOSORBENT ASSAY PROTOCOL

Introduction

Immunoglobulin G (IgG) is less virus-specific than IgM, appears in serum slightly later in the course of infection than IgM, and remains detectable until long after IgM ceases to be present. Using the IgG-ELISA in parallel with the IgM Antibody Capture Enzyme-linked Immunosorbent Assay (MAC-ELISA), one can observe the relative rises and falls in antibody levels in paired serum samples. The test is simple and sensitive. It is applicable to serum specimens but not generally to cerebrospinal fluid (CSF) samples. False-positive reactions due to rheumatoid factor are minimized.

Principle

The IgG-ELISA provides a useful alternative to immunofluorescence for identification of a viral isolate or documentation of a serologic response. IgG-ELISA is less subjective than immunofluorescence and large numbers of samples can be processed. In our laboratory, viral group-reactive monoclonal antibody is coated on a 96-well plate, followed sequentially by known viral antigen, patient serum, enzyme-conjugated human IgG, and lastly substrate for the conjugate used. This constitutes the IgG-ELISA.

Safety

The procedure should be performed under laboratory safety conditions that take into consideration the potential infectious nature of the serum specimens involved. Lab coat, gloves, and a laminar flow hood is recommended.

Materials and Reagents

Coating buffer: Carbonate/bicarbonate buffer pH 9.6
1.59g Na_2CO_3 + 2.93g $NaHCO_3$ diluted in 1L water.

Wash buffer: Phosphate buffered saline (PBS), 0.05% Tween 20, pH 7.2. PBS is available in powdered form from multiple commercial sources.

Blocking buffer: 3% goat serum, 1% Tween-20, in PBS.

Coating antibody: Group-specific monoclonal antibody, previously titrated.

Viral antigen: Sucrose-acetone extracted suckling mouse brain viral antigens, non-infectious, previously titrated

Normal antigen: Sucrose-acetone extracted suckling mouse brain antigen from mock-infected animals.

Detecting antibody conjugate: Alkaline phosphatase-conjugated goat anti-human IgG Fcγ portion, previously titrated (Jackson Immunoresearch cat# 109-055-098)

Substrate: 3 mg/ml p-nitrophenyl phosphate, disodium (Sigma 104, Sigma diagnostics cat# 104-105) in 1M Tris (base) pH 8.0 (note: the tris requires considerable conc. HCL for the pH adjustment)
Stop solution: 3M NaOH
Plates: Immulon II HB flat-bottomed 96 well plates
Dynatech Technologies cat# 3455
Microplate washer
Microplate reader
Incubator
Single and multi-channel pipettors
Reagent reservoirs
Ziploc bags, paper towels

Clinical specimens

Acute and convalescent human serum

NOTE: STORE ALL DIAGNOSTIC SPECIMENS AT 4°C PRIOR TO TESTING, AND -20°C AFTER ALL ANTICIPATED TESTING HAS BEEN COMPLETED. AVOID REPEATED FREEZE-THAW CYCLES.

Procedure

NOTE: THE FOLLOWING PROCEDURE INCLUDES INFORMATION ON QUALITY CONTROL AND INTERPRETATION. EACH SERUM SPECIMEN IS TESTED IN TRIPLICATE ON BOTH VIRAL AND NORMAL ANTIGENS. 8 TEST SPECIMENS CAN BE ANALYZED PER PLATE.

1. Using a fine-tipped permanent marker, number and label the plates (use Fig. 1. as a guide). Identify the location of each clinical specimen (S1-S8) by using the appropriate laboratory code number. *To keep timing of reagent addition consistent, process plates in the order that they are numbered during all steps of the procedure.* Plates should be kept in an enclosed, humidified environment during all incubation times with the exception of the coating step. A large ziploc bag containing a moist paper towel works well for this purpose.

2. Coat the inner 60 wells of 96 well plates with 75μl/well of the appropriate group-reactive monoclonal antibody diluted in coating buffer according to prior titration. **Incubate at 4^0 C overnight.**

3. Dump out the coating antibody and blot plates on paper towels. Block plates with 200μl blocking buffer per well. **Incubate at room temperature for 30 minutes.**

4. Wash wells 5X with wash buffer by using an automatic plate washer. Wells should be filled to the top each cycle.

5. Dilute viral antigen in wash buffer according to a previous titration. Add 50μl per well to the left three wells of each serum block (see Fig. 1). To the right three wells of each block, add 50μl per well of normal antigen diluted in wash buffer to the same concentration as the viral antigen. **Incubate overnight at 4^0C** in a humidified chamber.

6. Wash 5X.

7. Add 50μl per well of the patient's serum (S) diluted 1:400 in wash buffer to a block of 6 wells (see Fig. 1.).Add positive control human serum (Ref) diluted in wash buffer according to a previous titration, and a negative human serum control (N) diluted 1:400 in wash buffer to a block of 6 wells each. Incubate plates for **1 hour at 37^0C** in a humidified chamber.

8. Wash 5X.

9. Add 50μl per well of alkaline phosphatase-conjugated goat anti-human IgG diluted in blocking buffer, according to prior titration. **Incubate 1 hour at 37^0C** in a humid chamber.

10. Turn on plate reader to warm up and dissolve substrate tablets in tris buffer about 15 minutes prior to adding it to the plates.

11. Wash plates 5X **twice**. Turn the plates 180^o in the washer after the first series of 5 cycles. This promotes consistent results.

12. While the plate is at room temperature, add 75μl per well of Sigma 104 substrate to all wells. Immediately cover plates to block out light. Incubate at room temperature for 30 minutes. A yellow color will develop in antibody-positive wells.

12. Add 35μl per well of stop solution to all wells, including the outer rows of wells on the plate (the plate reader should be set to zero itself on some of these wells). Reactive wells

will remain a yellow color. Allow plates to sit at room temperature for 1 minute. Read plates in microtiter plate reader by using a 405 nm filter.

Practical considerations

1. Plates can be coated and kept at 4^{o}C for up to a week.
2. Undiluted control sera can be stored at 4^{o}C for up to 2 weeks.
3. Reconstituted, undiluted viral and normal antigens can be stored at -20^{o}C for an undefined period of time.
4. Test and control sera can be diluted to the working dilutions and refrigerated one day prior to use. Antigens and conjugate *must be diluted to the working dilutions immediately prior to use.*

The IgG-ELISA should be restandardized periodically. This should occur when new lot numbers of reagents are introduced, and at the very least, once a year. It is recommended that the mean optical density of the positive control serum reacted on the viral antigen be set to approx. 1.0. The normal control serum reacted on the viral antigen should be around 0.2 (this varies). The standardization of reagents is normally achieved via titration, always comparing the optical densities of the reagents when reacted on viral and normal antigen.

Results

Before the results can be calculated for each clinical specimen, the test must be determined to be **valid**. For a valid test the following must be true:

$$\frac{\text{Mean OD of the positive control serum reacted on viral antigen (P)}}{\text{Mean OD of the negative control serum reacted on viral antigen (N)}}$$

must be greater than or equal to 2.0. **This is the P/N of the positive control.**

Test validity must be determined for each plate. Results for the clinical specimens may only be determined if the test is valid. If the test is not valid, then that plate must be repeated. If the test still fails after a repeat, then one or more of the reagent or test parameters was likely in error, and troubleshooting should be performed.

To determine whether the clinical specimens (S1-S8) contain IgG to the viral antigen (which would indicate either recent or past infections with that virus) the following must be calculated:

Mean OD of the test specimen reacted on viral antigen (P)
Mean OD of the negative control serum reacted on viral antigen (N)

This is the P/N of the test specimen. For a specimen to be considered IgG-positive to the test virus, the P/N must be greater than or equal to 2.0.

In addition the value of P for the test specimen must be greater than or equal to twice the mean OD of the test specimen reacted on normal antigen. If this requirement is not met, non-specific background is being generated, and the result **MUST** be reported as uninterpretable.

Interpretation All patient P/N values greater than or equal to 2.0 should be reported as presumptive IgG-positive (see the explanatory paragraph on the following page), as long as they meet the requirements listed above. **Interpretations of IgG-ELISA's should always be made in the context of the corresponding MAC-ELISA, and the date of collection with respect to onset of symptoms.** A positive IgG-ELISA result on its own cannot distinguish a recent from a past infection due to the persistence of IgG from past infections. IgG is also more cross-reactive than IgM, which means that a positive result by the IgG-ELISA may in fact indicate the presence of antibody to a related virus. In most cases, IgG is detectable 12 days post-onset of symptoms from an alpha-, flavi-, or California group virus infection and persists for long periods of time, possibly for years.

Some examples of common scenarios are listed below:

1. A positive IgG-ELISA result with a positive MAC-ELISA result would indicate the presence of a recent infection.

2. A negative IgG-ELISA result with a positive MAC-ELISA result in an acute specimen would indicate a recent infection in which the IgG antibody had not yet risen to detectable levels.

3. A positive IgG-ELISA result and a negative MAC-ELISA result from a specimen timed between approx. 8 and 45 days post-onset of symptoms would suggest the occurrence of a past infection (remember that IgG to a virus is often cross-reactive with other viruses from the same genus).

4. For a single late specimen (obtained later than 45 days post-onset of symptoms) yielding a positive IgG-ELISA result

and a negative MAC-ELISA result, the distinction between the current infection and past infections cannot be made.

5. A negative IgG-ELISA result plus a negative MAC-ELISA result indicates the lack of any recent or past infections with the test virus.

The positive P/N cut-off value of 2.0 is empirical, based on experience and convention. P/N values that lie between 2.0 and 3.0 should be considered suspect false-positives. Further tests should be performed to determine the status of these specimens.

It should be stressed that the P/N value for a specimen at the screening dilution of 1:400 is not an indication of absolute antibody concentration, i.e., the P/N value is not quantitative.

It is further recommended that for sera, all positive results should be confirmed by titration using 6, 2-fold dilutions of the serum specimens compared to a similar titration of the negative control serum. Linear curves indicate true seropositivity. Flat or undulating titration curves indicate false-positive results.

References

Tsai, TH: Arboviruses, In Rose NR, Marcario EC, Fahey JL, Friedman H, and Penn GM, (Eds): Manual of Clinical Laboratory Immunology, 4th Edition, American Society for Microbiology: 606-618, 1976.

Diagnosis of Infections caused by Viruses, Chlamydia, Rickettsia, In Koneman EW, Allen SD, Janda WM, Schreckenberger PC, and Winn Jr. WC , (Eds): Diagnostic Microbiology, 4th Edition, JB Lippicott Co: 956-1074, 1992.

Johnson, AJ., Martin, DA., Karabatsos, N., and Roehrig, JT. Detection of anti-arboviral immunoglobulin G by using a monoclonal antibody-based capture enzyme-linked immunosorbent assay. J Clin Microbiol. 2000 May; 38(5): 1827-31.