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COXSACKIEVIRUS B
ANTIGEN SUBSTRATE SLIDE

CE

NOTE: Removed references to IgM testing.

PRODUCT AVAILABILITY

The following Coxsackievirus Group B Antigen Substrate Slides are available individually from Bion Enterprises, Ltd. (Bion):

Antigen Substrate Slide	Code No. REF	Code No. REF
Coxsackievirus B (1-6) Screen	CB-3312	CB-3306
Σ Number of Tests	12-Well	6-Well

INTENDED USE

The Bion COXSACKIEVIRUS B ANTIGEN SUBSTRATE SLIDES may be used as the antigenic substrate in indirect fluorescent antibody assays for the qualitative and/or semi-quantitative determination of Coxsackievirus B antibodies in human serum. Bion COXSACKIEVIRUS B ANTIGEN SUBSTRATE SLIDES are intended for use as an aid in the diagnosis of active infection and as a determination of immunological experience with Coxsackievirus B.

SUMMARY AND EXPLANATION

The Coxsackieviruses are any of 30 serologically different enteroviruses associated with a variety of symptoms and primarily affecting children during warm weather. Among the diseases associated with Coxsackievirus infections are herpangina, hand-foot-and-mouth disease, epidemic pleurodynia, myocarditis, aseptic meningitis and several exanthems.[1,2]

Coxsackieviruses were first recovered in 1949 in suckling mice inoculated with the fecal extracts from two children with paralytic poliomyelitis-like syndrome.[3] Currently, there are six Coxsackievirus B and twenty-four Coxsackievirus A serotypes recognized.[2,3]

The virus is spread primarily by fecal-oral contamination, although respiratory transmission also occurs. The virus initially colonizes the alimentary tract, growing in the lymphoid tissues of the nasopharynx and intestinal tract. Viremia subsequently occurs allowing secondary localization in other viscera and lymphoid tissues. Infection is often asymptomatic (30-90%), but a wide variety of notable syndromes may also occur such as paralytic disease, aseptic meningitis, encephalitis, pleurodynia, pericarditis, herpangina and lymphonodular pharyngitis. The most common clinical picture is that of an undifferentiated febrile illness with malaise, headache, myalgia and sore throat.[2,3,4]

Coxsackieviruses B have been implicated in occasionally fatal, generalized infection of newborns manifested by fever, meningitis, rash, diarrhea or shock. This occurs primarily in the summer and fall and is often associated with a recent maternal infection with the virus.[3,5] Some studies have shown that neutralizing titers of antibody to Coxsackievirus B are sometimes associated with disease development in polymyositis patients.[4,6]

PRINCIPLE OF THE IFA PROCEDURE

The Bion COXSACKIEVIRUS B ANTIGEN SUBSTRATE SLIDES may be utilized in the indirect fluorescent antibody assay method first described by Weller and Coons[9] and further developed by Riggs, et al.[10] The procedure is carried out in two basic reaction steps:

Step 1 - Human serum is reacted with the antigen substrate. Antibodies, if present, will bind to the antigen forming stable antigen-antibody complexes. If no antibodies are present, the complexes will not be formed and serum components will be washed away.

Step 2 - Fluorescein labeled antihuman IgG antibody is added to the reaction site which binds with the complexes formed in step one. This results in a positive reaction of bright apple-green fluorescence when viewed with a properly equipped fluorescence microscope. If no complexes are formed in step one, the fluorescein labeled antibody will be washed away, exhibiting a negative result.

REAGENTS

Bion COXSACKIEVIRUS B ANTIGEN SUBSTRATE SLIDES are individually foil-wrapped slides of six or twelve wells with a mixture of Coxsackievirus B(1-6; NIH strains) infected and uninfected A549 cells fixed onto each well. Each well contains an average on 20-30% infected cells per 200X field.

STORAGE AND STABILITY

The Bion COXSACKIEVIRUS B ANTIGEN SUBSTRATE SLIDES are stable in sealed foil pouches at 8°C or lower until labeled expiration date.

WARNINGS AND PRECAUTIONS

1. For *in vitro* diagnostic use. Thus, only staff trained in methods of in vitro diagnostics may perform the test. IVD
2. Substrate slides are for single use only and must not be used more than once.
3. Use with non-Bion reagents could result in erroneous results.
4. Care should be taken when handling substrate slides due to sharp edges.
5. The antigenic substrates have been fixed and contain no detectable live Coxsackievirus B. However, they should be handled and disposed of as any potentially biohazardous laboratory material.
6. Do not remove slides from pouches until ready for testing. Do not use if pouch has been punctured, as indicated by a flat pouch.
7. Antigen substrate slides should be brought to room temperature (20-25°C) prior to use.
8. Abnormal test results may be seen if the antigen substrate slides are allowed to dry during the staining procedure.
9. Refrigeration (2-8°C) of antigen substrate slides immediately upon arrival will insure stability until labeled expiration date.
10. Antigen substrate slides should not be used beyond stated expiration date.
11. Avoid microbial contamination of all reagents involved in the testing procedure or incorrect results may occur.
12. Incubation times or temperatures other than those specified may give erroneous results.
13. Reusable glassware must be washed and thoroughly rinsed free of detergents.
14. Care should be taken to avoid splashing or generation of aerosols.
15. Previously frozen specimens after thawing should be thoroughly mixed prior to testing. It is recommended that sera is freeze thawed no more than one time. If repeated testing is required, it is suggested that specimen be aliquoted.
16. Patient samples, as well as all materials coming into contact with them, should be handled at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the CDC/NIH manual “Biosafety in Microbiological and Biomedical Laboratories”, 1984 Edition. Never pipette by mouth. Avoid contact with skin and mucous membranes.

SPECIMEN COLLECTION

Blood should be collected fasting or at least one hour after meals to avoid lipemic serum, as excess lipids may produce a “film” over the substrate. Aseptically collect 5-8 ml of blood by venipuncture. Allow the blood to clot at room temperature(20-25°C) before separating serum to avoid hemolysis which could interfere with test results. Specimens should be stored refrigerated at 2-8°C and tested within one week of collection. Long term storage should be at -20°C in aliquots to avoid repeated freezing and thawing. Do not store in self-defrosting freezer.

Avoid using contaminated sera as they may contain proteolytic enzymes which will digest the substrate. It is unnecessary to heat inactivate serum specimens prior to testing; however, sera that have been heat inactivated may be used.

When testing paired samples to look for evidence of recent infection, the acute specimen should be obtained as soon as possible after onset of illness and the convalescent specimen obtained 7-14 days later. Acute and convalescent specimens must be tested simultaneously, in the same assay, looking for a significant change in antibody titer between the paired sera. If the first specimen is obtained too late during the course of the infection, a significant rise in the antibody titer may not be detected.

PROCEDURE

Detailed descriptions of indirect immunofluorescence techniques may be found in the references listed in the bibliography.[11,12,13]

MATERIALS PROVIDED

Bion COXSACKIEVIRUS GROUP B (TYPES 1-6) SCREEN ANTIGEN SUBSTRATE SLIDES. Lot Number provided on label. LOT

MATERIALS AVAILABLE FROM Bion

1. Fluorescent Antibody Conjugate with 0.01% Evans Blue counterstain
2. Phosphate Buffered Saline (PBS)
3. Mounting Medium

MATERIALS REQUIRED BUT NOT PROVIDED

1. Disposable test tubes (12 x 75 mm or comparable) and rack
2. Disposable serological pipettes
3. Calibrated pipettes to deliver 50 µl, 100 µl and 200 µl with disposable pipette tips
4. Pasteur pipettes and bulbs
5. Moist chambers
6. Plastic squeeze wash bottle
7. Coplin jars or staining dishes with slide racks
8. 24 x 60 mm #1 coverslips
9. Felt tip marking pen
10. Fluorescence microscope equipped with a mercury or tungsten-halogen light source, a 390-490 nm excitation filter and 515-520 nm barrier filter, and optics to give a total magnification of 200X or 250X. The excitation wavelength of FITC is 490 nm and the emission wavelength is 520 nm.

TEST PROCEDURE

1. **SPECIMEN PREPARATION**

   Screening:

   Each laboratory should establish its own protocol for the preparation of serum screening dilutions. Most indirect fluorescent antibody staining procedures utilize a 1:10 dilution of each patient's serum which is prepared by adding 0.05 ml (50 µl) of the patient's serum to 0.45 ml of PBS.

   Semi-quantitation:

   Serum dilutions are utilized to measure antibody titer. Each laboratory should establish its own titering protocol. The selection of either twofold or fourfold dilution procedures depends upon the experience level and training of the individual(s) reading the fluorescent antibody assay.

   The following fourfold serial titration is suggested for IgG testing:

   a. Prepare a 1:10 dilution of each patient's serum by adding 0.05 ml (50 µl) of patient's serum to 0.45 ml of PBS in tube #1.

   b. Add 0.3 ml PBS to tubes #2, #3, #4, and #5.

   c. Using a 100 µl pipette, transfer 0.1 ml (100 µl) from tube #1 to tube #2. Mix. Using a new tip for each dilution, transfer 0.1 ml (100 µl) from the second tube to the third, from the third tube to the fourth, and from the fourth tube to the fifth, mixing after each transfer.

2. **SLIDE PREPARATION**

   Remove reagents and as many slides as are required from the refrigerator or freezer and allow to equilibrate to room temperature (20-25°C) for at least five minutes. Remove slides from sealed foil pouches being careful not to touch the antigen surface. Identify each slide using a felt tip marking pen.

3. **SPECIMEN APPLICATION**

   Using separate Pasteur pipettes, apply one drop (20-30 µl) of the positive control, one drop (20-30 µl) of the negative control and one drop (20-30 µl) of each patient serum dilution to individual wells of the slide. Do not touch the antigen surface with the pipette while dropping. Do not allow drops to mix, as cross contamination of samples between wells could cause erroneous results.

4. **INCUBATION 1**

   Incubate in a moist chamber at room temperature (20-25°C) for 30 minutes. THE ANTIGEN MUST NOT BE ALLOWED TO DRY DURING ANY OF THE FOLLOWING STEPS. Nonspecific binding may occur if the reagent is allowed to dry on the slide.

5. **RINSE 1**

   Remove slides from moist chamber and rinse GENTLY with PBS using a squeeze wash bottle. Do not focus the PBS stream directly onto the wells. To prevent cross contamination tilt slide first toward wells 1-6 and, running a PBS stream along the midline of the slide, allow the PBS to run off the top edge of the slide. Then, tilt the slide toward wells 7-12 and repeat this procedure, allowing the PBS to run off the bottom edge of the slide. For six well slides, tilt slide down and run the PBS stream across the slide above the wells, allowing the PBS to run off the bottom edge of the slide.

6. **WASH 1**

   Place slides in Coplin jars or staining dishes and wash in two changes of PBS for not less than five minutes or more than ten minutes each, agitating gently at entry and prior to removal.

7. **CONJUGATE APPLICATION**

   Remove slides from the wash one at a time, shake off excess PBS, dry around outside edges if necessary and return each slide to the moist chamber. Apply one drop of an appropriate fluorescent antibody conjugate with counterstain (diluted to its predetermined proper working dilution) to each well of each slide, making sure that each well is completely covered.

8. **INCUBATION 2**

   Incubate in a moist chamber at room temperature (20-25°C) for 30 minutes. Protect slides from excessive light.

9. **RINSE 2**

   Remove slides from moist chamber and rinse GENTLY with PBS using a squeeze wash bottle. As suggested in step 5., do not focus PBS stream directly onto the wells.

10. **WASH 2**

    Place slides in Coplin jars or staining dishes and wash in two changes of PBS for not less than five minutes or more than ten minutes each, agitating gently at entry and prior to removal.

11. **COVERSLIP**

    Remove slides one at a time from the last PBS wash, shake off excess PBS and immediately add two to four drops of mounting medium across the slide. Tilt slide and rest the edge of the coverslip against the bottom of the slide allowing the mounting medium to form a continuous bead between the coverslip and slide. Gently lower the coverslip from the bottom of the slide to the top, being careful to avoid air bubbles. Drain excess mounting medium by holding the edge of the slide against absorbent paper. Wipe off back of slide.

12. **READ**

Examine stained slides as soon as possible using a properly equipped fluorescence microscope. It is recommended that slides be examined on the same day they are stained. If any delay is anticipated, store slides in the refrigerator (2-8°C) away from direct light and read the following day. Do not allow mounting medium to dry between slide and coverslip. If drying should occur, add additional mounting medium or recoverslip slide.

FLUORESCENT INTENSITY GRADING

Fluorescent intensity may be semi-quantitated by following the guidelines established by the Centers for Disease Control, Atlanta, Georgia:[14]

4+ = Maximal fluorescence; brilliant yellow-green.
3+ = Less brilliant yellow-green fluorescence.
2+ = Definite but dull yellow-green fluorescence.
1+ = Very dim subdued fluorescence.

The degree of fluorescent intensity is not clinically relevant and has only limited value as an indicator of titer. Differences in fluorescence microscope optics, filters and light sources may result in differences of 1+ or more fluorescent intensity when observing the same slide using different microscopes.

QUALITY CONTROL

SPECIFICITY

Both a positive and negative antibody control must be included with each run. These controls must be examined prior to reading test samples and should demonstrate the following results:

Negative Control:
Using a negative control serum on Bion COXSACKIEVIRUS B SUBSTRATE SLIDES, the infected cells should exhibit less than 1+ fluorescence and appear reddish-orange due to the counterstain.

Positive Control:
Using a positive control serum on Bion COXSACKIEVIRUS B SUBSTRATE SLIDES, the infected cells should exhibit well defined specific fluorescent staining at an intensity of 3+ or greater. With Coxsackievirus B, 20-30% of the cell population displays a variety of patterns from solid staining of the entire cell, to speckly staining of some cells, to just the rim of the cell staining, with the uninfected cells staining reddish-orange due to the counterstain.

Each control must demonstrate the expected reaction in order to validate the test. If the controls fail to appear as described above, the test results should not be reported and the test should be repeated. If upon repeat testing the controls still fail to show the proper reaction, do not report test results.

SENSITIVITY CONTROL

A titered control included with each run tests substrate sensitivity, as well as, checks technique, conjugate quality and the microscope optical system. The endpoint titer of this control must be determined and there must not be more than a twofold difference (+/-) in titer from this determined endpoint. Each run should include the endpoint dilution, one twofold or fourfold dilution above and one twofold or fourfold dilution below the endpoint dilution. The more concentrated dilution should be positive and the less concentrated dilution negative. If the control does not behave as described, the test results are invalid and the tests should be repeated. If the control again fails to show the proper reaction upon repeat testing, do not report the test results.

READING OF TEST RESULTS

NEGATIVE

A serum dilution is considered to be negative for Coxsackievirus B antibodies if the cells exhibit less than 1+ fluorescence and appear reddish-orange due to the counterstain, or if the fluorescence observed is not the specific staining pattern of Coxsackievirus B.

A sample is considered negative for Coxsackievirus B antibodies if it exhibits less than 1+ fluorescence at a serum dilution of 1:10 and all greater dilutions, or if the fluorescence observed is not the specific staining pattern of Coxsackievirus B.

... Negative samples may exhibit fluorescent staining of the infected cells slightly greater than the negative control, but less than 1+.

... Nonspecific staining of all cells observed in some sera at low dilutions is most likely due to the presence of autoantibodies against cellular components in either the nucleus or cytoplasm.

... Staining of areas other than the viral infected cells should be interpreted as negative and attention should be directed to specific steps in the staining method (e.g., RINSE and WASH steps).

POSITIVE

A serum dilution is considered positive for Coxsackievirus B antibodies if well defined fluorescent staining is observed in the infected cells at an intensity of 1+ or greater. With Coxsackievirus B, 20-30% of the cell population displays a variety of patterns from solid staining of the entire cell, to speckly staining of some cells, to just the rim of the cell staining. The number of cells exhibiting a positive staining reaction and the type of fluorescent staining pattern should closely approximate that seen in the positive control.

A sample is considered positive for Coxsackievirus B antibodies if it exhibits the characteristic Coxsackievirus B staining pattern with a fluorescent intensity of 1+ or greater at a serum dilution of 1:10 or greater.

NOTE: Each field should contain cells that exhibit no apple-green fluorescence. Should most of the cells in the patient test wells fluoresce apple-green in the nucleus and/or cytoplasm, an autoimmune staining reaction due to the presence of autoantibodies should be considered.[15,16] It is recommended that such samples be diluted beyond the interference for better interpretation. It is possible that autoantibody staining may mask specific staining such that an interpretation cannot be made. Should this occur, test results should be reported as "Unable to interpret due to the presence of interfering antibodies."

TITRATION

If a semi-quantitative titration is performed, the result should be reported as the reciprocal of the last dilution in which 1+ apple-green fluorescent intensity of the specific staining pattern is detected. When reading fourfold serial dilutions, endpoints can be extrapolated where necessary.

EXAMPLE OF ENDPOINT EXTRAPOLATION:
1:10 = 4+
1:40 = 3+
1:160 = 2+
1:640 = +/-
The extrapolated endpoint is reported as 320.

TROUBLESHOOTING

Possible solutions to problems that may occur in immunofluorescent assays are discussed in an accompanying brochure entitled "TROUBLESHOOTING IN IMMUNOFLUORESCENCE".

INTERPRETATION OF RESULTS

Detection of the presence of Coxsackievirus B antibodies indicates a current or previous infection with Coxsackievirus B. A significant (fourfold or greater) increase in titer between acute and convalescent serum samples usually indicates evidence of a recent or active infection.

LIMITATIONS OF THE PROCEDURE

1. Coxsackievirus B antibody test results should be used in conjunction with information available from clinical evaluation and other diagnostic information.
2. A single serological IgG antibody titer to Coxsackievirus B should not be used as the only criterion for diagnosis. Paired serum samples (acute and convalescent) may provide more meaningful data.
3. A negative test result does not necessarily rule out current or recent infection. The specimen may have been collected too early in the disease before demonstrable antibody is present.
4. Lack of significant rise in titer does not exclude the possibility of recent infection but may indicate an acute phase specimen was obtained too late.
5. In some instances, high IgG antibody levels in the first of paired specimens may prevent the detection of increases in total antibody, resulting in apparently stationary total antibody titer.
6. Test results on specimens from immunosuppressed patients and pregnant women may be difficult to interpret.
7. Positive test results may not be valid in persons who have received blood transfusions or various blood products within the past several months.
8. Antinuclear antibodies (ANA) present in serum may interfere with the Coxsackievirus B IFA test. They can be differentiated from Coxsackievirus B staining in that ANAs stain the nuclei in all cells; whereas, Coxsackievirus B antibodies exhibit staining only in the 20-30% infected cells.[15]
9. Cytoplasmic fluorescence in the majority of the cells may be due to the presence of antimitochondrial antibodies (AMA) often seen in primary biliary cirrhosis.[16] They can be differentiated from the specific antigen staining in that AMA will stain the cytoplasm of all cells; whereas, Coxsackievirus B antibodies exhibit staining in only the 20-30% infected cells.
10. Positive test results from cord blood or neonates should be interpreted with caution. The presence of Coxsackievirus B IgG antibodies in cord blood is usually the result of passive transfer from mother to the fetus. A negative test, however, may be useful in excluding possible infection. The method of choice to diagnose Enterovirus infection is viral isolation from a normally sterile body site such as blood, CSF, brain or liver.[8]
11. Endpoint reactions may vary between laboratories due to differences in type or condition of fluorescence microscope employed, diluting apparatus, as well as the experience level of personnel performing the assay.
12. If both the positive and negative control substrate cells are not visible when viewed using the fluorescence microscope, it may be necessary to replace or realign the light source and check the specific filters.
13. Cell culture substrate slides may exhibit nonspecific fluorescence due to contamination of antibodies or PBS rinse-wash solutions with bacteria or fungi. It is very important that personnel reading the staining results have experience in fluorescence microscopy.

SPECIFIC LIMITATIONS OF THE COXSACKIEVIRUS B ASSAY

Homotypic IgG antibody persist for many years post infection. Additionally, it has been documented that IgG antibody titers can be boosted by reinfection with the same Coxsackievirus serotype or by heterotypic responses elicited by other Coxsackieviruses or related Enteroviruses.[7] In most cases, especially where paired sera are available, testing for IgG specific antibodies seems to be sufficient for the serological diagnosis of a Coxsackievirus B infection.

SPECIFIC PERFORMANCE CHARACTERISTICS

Bion COXSACKIEVIRUS B ANTIGEN SUBSTRATE SLIDES have been evaluated for the presence of specific Coxsackievirus B antigen using commercial monoclonal antisera. In each case, positive reactions were identified with the Coxsackievirus B infected cell cultures when stained with its corresponding antisera. Also, there was no cross-reactivity with other specific viral or chlamydial monoclonal antisera and the Coxsackievirus B antigen.[17]

Each laboratory should determine its own performance characteristics using all reagents assembled to perform the IFA test.

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