PRINCIPLE OF THE IFA PROCEDURE

The MBL Bion COXSACKIEVIRUS B ANTIGEN SUBSTRATE SLIDES may be utilized in the indirect fluorescent antibody assay method first described by Weller and Coombs and further developed by Riggs, et al. 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 (or IgM) 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

MBL 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 MBL 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.

2. The antigenic substrates have been fixed and contain no detectable live Coxsackievirus B. However, they should be handled and disposed of as any potentially biobehavioral hazard material.

3. Do not remove slides from pouches until ready for testing. Do not use if pouch has been punctured, as indicated by a flat pouch.

4. Antigen substrate slides should be brought to room temperature (20-25°C) prior to use.

5. Abnormal test results may be seen if the antigen substrate slides are allowed to dry during the staining procedure.

6. Refrigeration (2-8°C) of antigen substrate slides immediately upon arrival will insure stability until labeled expiration date.

7. Antigen substrate slides should not be used beyond stated expiration date.

8. Avoid microbial contamination of all reagents involved in the testing procedure or incorrect results may occur.

9. Incubation times or temperatures other than those specified may give erroneous results.

10. Reusable glassware must be washed and thoroughly rinsed free of detergents.

11. Care should be taken to avoid splashing or generation of aerosols.

12. Previously frozen specimens after thawing should be thoroughly mixed prior to testing. It is recommended that sera be freeze thawed no more than once per time. If repeated testing is required, it is suggested that specimen be aliquoted.

13. 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/NHS 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.\(^{13,14,15}\)

**MATERIALS PROVIDED**

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

<table>
<thead>
<tr>
<th>MATERIALS AVAILABLE FROM MBL Bion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Fluorescent Antibody Conjugate with 0.01% Evans Blue counterstain</td>
</tr>
<tr>
<td>2. Coxsackievirus Group B Types 1-6 Positive Human Control Serum</td>
</tr>
<tr>
<td>3. Coxsackievirus Group B Types 1-6 Negative Human Control Serum</td>
</tr>
<tr>
<td>4. Phosphate Buffered Saline (PBS)</td>
</tr>
<tr>
<td>5. Mounting Medium</td>
</tr>
</tbody>
</table>

**MATERIALS REQUIRED BUT NOT PROVIDED**

- Disposable test tubes (12 x 75 mm or comparable) and rack
- Disposable serological pipettes
- Calibrated pipettes to deliver 50 µl, 100 µl and 200 µl with disposable pipette tips
- Pasteur pipettes and bulbs
- Moist chambers
- Plastic squeeze wash bottle
- Coplin jars or staining dishes with slide racks
- 24 x 60 mm #1 coverslips
- Felt tip marking pen
- 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.

**NOTE:** If testing for IgM specific antibodies using an IgM specific fluorochrome conjugate, each patient serum specimen must be pre-treated to remove any IgG interference by separating the IgM from the IgG, and then running the screening test on the IgM eluate. Suggested methodologies are ion exchange chromatography\(^ {16}\) or IgG immunoprecipitation.\(^ {17,18}\)

**Semi-quantitation:**

Serum dilutions are utilized to measure antibody titer. Each laboratory should establish its own tiering 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:

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

The following twofold titration is suggested for IgM testing:

- Prepare a 1:10 dilution of each patient’s serum using one of the treatment methodologies mentioned in the “Screening NOTE” above. This will be designated as tube #1.
- Add 0.2 ml PBS to tubes #2, #3, #4, and #5.
- Using a 200 µl pipette, transfer 0.2 ml (200 µl) from tube #1 to tube #2. Mix. Using a new tip for each dilution, transfer 0.2 ml (200 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.

These titrations will have the following dilutions:

<table>
<thead>
<tr>
<th>Fourfold</th>
<th>Twofold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube #1: 1:10</td>
<td>Tube #1: 1:10</td>
</tr>
<tr>
<td>Tube #2: 1:20</td>
<td>Tube #2: 1:20</td>
</tr>
<tr>
<td>Tube #3: 1:160</td>
<td>Tube #3: 1:40</td>
</tr>
<tr>
<td>Tube #4: 1:640</td>
<td>Tube #4: 1:80</td>
</tr>
<tr>
<td>Tube #5: 1:2560</td>
<td>Tube #5: 1:160</td>
</tr>
</tbody>
</table>

**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.

**NOTE:** For IgM testing, incubate the substrate slides in a moist chamber at 35-37°C for 90 minutes.

**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 (IgG or IgM) 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.

**NOTE:** For IgM testing, incubate in a moist chamber at 35-37°C for 60 minutes.

**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 recover slip slide.

**FLUORESCENT INTENSITY GRADING**
Fluorescent intensity may be semi-quantitated by following the guidelines established by the Centers for Disease Control, Atlanta, Georgia:¹⁹

- 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 MBL 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 MBL 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. 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. 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:**

\[
\begin{align*}
1:10 & = 4+ \\
1:40 & = 3+ \\
1:160 & = 2+ \\
1:640 & = +/
\end{align*}
\]

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 and/or a positive test for IgM specific antibodies usually indicates evidence of a recent or active infection.
LIMITATIONS OF THE PROCEDURE

1. Coxackievirus 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) and testing for IgM specific Coxackievirus B antibodies may provide more meaningful results.

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 or IgM 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 a positive Coxsackievirus B staining in that ANA stains the nuclei in all cells; whereas, Coxsackievirus B antibodies exhibit staining only in the 20-30% infected cells.

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. 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 only in 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. Because of the possibility of contamination of cord blood with maternal IgM, it is prudent to confirm positive viral IgM antibody results on cord blood samples by testing a follow-up specimen from the infant, preferably within the first five days of life.

11. Endpoint reactions may vary between laboratories due to differences in type or concentration of fluorescent microscope employed, diluting apparatus, IgG/IgM separation methods, 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 associated with IgM testing to avoid the many pitfalls that can trap the most experienced workers. IgM results must always be interpreted with caution.

SPECIFIC LIMITATIONS OF THE COXACKIEVIRUS B ASSAY

Homotypic IgG antibody and IgM 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. In most cases, especially where paired sera are available, testing for IgG and IgM specific antibodies seems to be sufficient for the serological diagnosis of a Coxsackievirus B infection; however, the determination of specific IgG antibody may add some valuable information, especially in the cases of suspected persistent disease.

SPECIFIC PERFORMANCE CHARACTERISTICS

MLB Bion COXACKIEVIRUS B ANTIGEN SUBSTRATE SLIDES have been evaluated for the presence of specific Coxsackievirus B antigen using commercial monolocal 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 monolocal antisera and the Coxsackievirus B antigen.

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

BIBLIOGRAPHY


25. Data on file, MBL Bion, Des Plaines, IL.
