

The ZEUS IFA *Legionella pneumophila* Direct Fluorescent Antibody detection reagents are designed for the detection of *Legionella* (Groups 1 – 6) bacteria in various patient specimens, and are for *In Vitro* diagnostic use.

SIGNIFICANCE AND BACKGROUND

The direct fluorescent antibody (DFA) conjugates for Legionellaceae are recommended for the identification of the various *Legionella* bacteria in lung tissues, respiratory tract fluids, and cultures (1). They may be used on imprints of fresh lung tissue, on scrapings of formalin-fixed tissue, paraffin embedded tissue, and on lower respiratory tract fluids such as sputum, transtracheal aspirates, bronchial washings, and pleural fluids (1). They may also be used to stain culture smears. The direct FA test appears to be sensitive and highly specific, but should be used in conjunction with cultural isolation and biochemical characterization whenever possible (1 - 10).

PRINCIPLE OF THE ASSAY

The direct FA test is a simple technique. Antigen is fixed on a slide and is then overlaid with a small amount of FITC-labeled globulin directed against that antigen. The antigens bind the labeled immunoglobulins and the resulting antigen-antibody complexes are rendered visible upon excitation of the FITC by ultraviolet-blue light. When exposed to such light, FITC emits longer wavelengths of light in the yellow-green portion of the color spectrum, and the *Legionella* bacteria are observed as brilliantly fluorescent yellow-green rods.

TEST SYSTEM COMPONENTS

Materials Provided:

Each Test System contains the following components in sufficient quantities to perform the number of tests indicated on the packaging label. **NOTE: Conjugate and Controls contain a combination of Proclin (0.05% v/v) and Sodium Azide (<0.1% w/v) as preservatives. SAVe Diluent® contains Sodium Azide (<0.1% w/v) as a preservative.**

CONJ	+	1. Positive Conjugate: One, 2mL, amber bottle. Rabbit anti- <i>Legionella</i> (Groups 1 – 6) IgG labeled with fluorescein isothiocyanate (FITC). Contains phosphate buffer with BSA and counterstain. Ready to use.
CONTROL	+	2. Positive Antigen Control (<i>Legionella</i> bacteria): One, 1.0mL vial of <i>Legionella pneumophila</i> Group 1 Philadelphia strain, (formalin fixed). Bacteria are grown in artificial media and are formalin killed and suspended in formalinized <i>Legionella</i> PBS. The antigen suspensions are provided at the working dilution and must not be frozen.
CONJ	-	3. Negative Conjugate (Rabbit globulin): One, 2.0mL, amber bottle containing normal rabbit globulin labeled with FITC and diluted with Evans Blue in PBS, pH 7.6. Ready to use.
BUF	PBS	4. Phosphate-buffered-saline (PBS): pH 7.6 ± 0.2, 10g/packet. Empty contents of each buffer packet into one liter of deionized water. Mix until all salts are thoroughly dissolved. Two packets, sufficient to prepare 2 liters.
MNTMED		5. Mounting Media (Buffered Glycerol): Two, 3.0mL, white-capped, dripper tipped vials.

NOTE: Test System also contains a Component Label containing lot specific information inside the Test System box.

PRECAUTIONS

- For *In Vitro* diagnostic use.
- Follow normal precautions exercised in handling laboratory reagents. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing, gloves, and eye/face protection. Do not breathe vapor. Dispose of waste observing all local, state, and federal laws.
- These FA reagents should be used by properly trained individuals. As with all diagnostic specimens and their materials, precautions should be taken against danger of microbiological hazards. Specimens, containers and media should be sterilized after use.
- The *Legionella* positive control does not contain viable organisms. However, consider this material **potentially bio-hazardous** and handle accordingly.
- Adherence to the specified time and temperature of incubations is essential for accurate results. **All reagents must be allowed to reach room temperature (20 - 25°C) before starting the assay.** Return unused reagents to their original containers immediately and follow storage requirements.
- Improper washing could cause false positive or false negative results. Do not allow the wells to dry out between incubations.
- The Conjugates and Control contain Sodium Azide at a concentration of <0.2% (w/v). Sodium Azide has been reported to form lead or copper azides in laboratory plumbing which may cause explosions on hammering. To prevent, rinse sink thoroughly with water after disposing of solution containing Sodium Azide. This preservative may be toxic if ingested.
- Dilution or adulteration of these reagents may generate erroneous results.
- Never pipette by mouth. Avoid contact of reagents and patient specimens with skin and mucous membranes.
- Avoid microbial contamination of reagents. Incorrect results may occur.
- Cross contamination of reagents and/or samples could cause erroneous results.
- Reusable glassware must be washed and thoroughly rinsed free of all detergents.
- Avoid splashing or generation of aerosols.
- Do not expose reagents to strong light during storage or incubation.
- Allowing the slide packet to equilibrate to room temperature prior to opening the protective envelope will protect the wells and blotter from condensation.
- Collect the wash solution in a disposal basin. Treat the waste solution with disinfectant (i.e.: 10% household bleach - 0.5% Sodium Hypochlorite). Avoid exposure of reagents to bleach fumes.
- Do not expose any of the reactive reagents to bleach-containing solutions or to any strong odors from bleach-containing solutions. Trace amounts of bleach (Sodium Hypochlorite) may destroy the biological activity of many of the reactive reagents within this Test System.
- The components of this Test System are matched for optimum sensitivity and reproducibility. Reagents from other manufacturers should not be interchanged. Follow Package Insert carefully.
- All components are stable until the expiration date printed on the label, provided the recommended storage conditions are strictly followed. Do not use beyond the expiration date. Do not freeze.
- Evans Blue Counterstain is a potential carcinogen. If skin contact occurs, flush with water. Dispose of according to local regulations.
- Depending upon lab conditions, it may be necessary to place slides in a moist chamber during incubations.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Inoculation loop.
2. Bunsen or Meeker burner.
3. Incubator: CO₂ incubator or candle jar.
4. Coplin jars.
5. Clean FA slides.
6. Charcoal yeast extract medium.
7. Sterile petri dishes.
8. Sterile or clean scalpels.
9. Coverslips (#1).
10. Sterile or clean forceps.
11. Incubation chamber.
12. Deionized water.
13. 10% sterile neutral formalin.
14. For paraffin sections:
 - a. Oven at 58 - 60°C to fix sections.
 - b. Xylol to remove paraffin.
 - c. Absolute Ethanol.
 - d. 95% Ethanol.
15. 1.0% neutral formalin.
16. Test tubes (13 x 100mm).
17. Properly equipped fluorescence microscope assembly.

The following filter systems, or their equivalent, have been found to be satisfactory for routine use with transmitted or incident light darkfield assemblies:

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Transmitted Light		
Light Source: Mercury Vapor 200W or 50W		
Excitation Filter	Barrier Filter	Red Suppression Filter
KP490	K510 or K530	BG38
BG12	K510 or K530	BG38
FITC	K520	BG38
Light Source: Tungsten – Halogen 100W		
KP490	K510 or K530	BG38

Incident Light			
Light Source: Mercury Vapor 200, 100, 50 W			
Excitation Filter	Dichroic Mirror	Barrier Filter	Red Suppression Filter
KP500	TK510	K510 or K530	BG38
FITC	TK510	K530	BG38
Light Source: Tungsten – Halogen 50 and 100 W			
KP500	TK510	K510 or K530	BG38
FITC	TK510	K530	BG38

STORAGE CONDITIONS

 2°C - 8°C	Unopened Test System.
	Mounting Media, Conjugates, Positive Control.
	Rehydrated PBS (Stable for 30 days).
 2°C - 25°C	Phosphate-buffered-saline (PBS) Packets.

ASSAY PROCEDURE; SPECIMEN COLLECTION

Specimen Preparation:

During fixation and staining, each slide must be processed separately to ensure that organisms present in one specimen are not transferred to another specimen.

1. Preparation of tissue scrapings from formalin-fixed tissue:

- a. Select one or more areas of the lung or other tissue for testing. In the lung, areas of dense gray or reddish consolidation are selected.
- b. Transfer each tissue block to a sterile Petri dish.
- c. With a sharp scalpel, cut through these areas to produce new tissue faces for scraping.
- d. Grasp the tissue with forceps and holding the scalpel at a right angle to the tissue face, scrape it to produce a fine puree of tissue particles. (The lung tissue of victims of Legionellosis is usually quite friable). If the tissue is rubbery or spongy, a positive test is unlikely.
- e. Smear the particles of tissue and tissue fluids onto 1.5cm circles on a microscope slide using the scalpel blade to make the smears.
- f. Allow the smears to air dry. Gently heat fix.

2. Preparation of Fresh-Frozen Tissue (autopsy or biopsy):

NOTE: Process in a safety cabinet. If tissue is to be cultured, this should be done before making imprints.

- a. Using sterile instruments, cut a fresh face of tissue and, with the forceps, press and squeeze the tissue against clean slides. If the tissue is so moist that smears may be too thick, first blot on sterile gauze. Alternatively, grind the tissue with sterile distilled water and alundum in a sterile mortar, or homogenize the tissue in a Ten Broeck or comparable tissue grinder. Use sufficient sterile distilled water to give an approximate 10% tissue homogenate. Prepare smears in the same manner as described for formalin-fixed tissue. With larger pieces of tissue, this method produces a more representative sample and reduces sampling error.
- b. Air dry and heat fix.
- c. Fix smears for 10 minutes by covering with a solution of 10% neutral formalin. Place the slides in a moist chamber to prevent evaporation of the formalin.
- d. Drain off the formalin and rinse with a gentle stream of distilled water using a wash bottle; or briefly dip each slide into distilled water to remove the residual formalin.
- e. Air dry.

3. Preparation of Tissue Sections:

Legionella bacteria maintain their serologic integrity through histopathological processing and can be easily demonstrated in tissue sections if reasonably numerous. However, they are not as easily demonstrated in sections as in scrapings of formalin-fixed lung or imprints of fresh lung tissue. This is because many of the bacteria are intracellular; they lie at many different levels in the sections, and they are shrunk in size by the histological processing. Cut the tissue sections as thin as possible (4µ or less).

NOTE: For paraffin embedded tissue:

- a. Fix the sections for approximately 15 minutes at 58 - 60°C.
- b. De-paraffinize by two passages through xylol followed by two passages each through absolute ethanol, 95% ethanol and water.

4. **Preparation of Exudates from the Lungs** (process in the safety cabinet):

Sputum, transtracheal aspirates, bronchial washings, or other specimens from the lower respiratory tract are satisfactory materials for study. Legionellosis patients frequently do not produce much sputum and when produced, it may or may not be purulent; however, the secretions are extremely viscid and tenacious.

- a. Select a viscous portion of the specimen and prepare smears of moderate thickness within the 1.5cm diameter circles on the microscope slides.
- b. Air dry and heat fix.
- c. Fix in 10% neutral formalin as described above for fresh tissue.
- d. Drain off the formalin and rinse with a gentle stream of distilled water, or dip the slides briefly into distilled water.
- e. Air dry.

5. **Preparation of Pleural Fluids:**

NOTE: Process in safety cabinet.

Pleural fluids should be cultured for attempted isolation of the *Legionella* organism. Since the fluids are obtained aseptically, over growth of the *Legionella* organisms by contaminants is not a problem. Prepare thin smears, air dry, heat fix, and process as described for lung exudates above.

NOTE: Pleural fluids tend to form a fibrin clot on the slide and unless handled carefully, the entire film may be dislodged during processing.

6. **Preparation of Culture Smears:**

NOTE: Process in safety cabinet.

- a. Make suspensions of cultures of known or suspected *Legionella* bacteria in 1% neutral formalin to give a light turbidity (McFarland No. 1).
- b. Prepare smears on double ring or on multi-well slides.
- c. Air dry and heat fix.

FA Staining Procedure

Read each vial label carefully for proper selection. All liquid reagents are ready for use. Use appropriate Controls in each test. Run as described below:

1. Use the Positive and Negative Conjugates to screen specimens to identify if the specimens contain *Legionella* bacteria
 - a. Prepare test specimens as described above.
 - b. Prepare Positive Antigen Control as follows: Shake vigorously, make smears by applying antigen dropwise to multi-well slides, and aspirate any excess. Allow to air dry then heat fix.
 - c. Apply Positive and Negative Conjugates to the Positive Antigen Control preparation(s).
 - d. Apply Positive and Negative Conjugates to the test specimen. (See #1 above).
 - e. Incubate the Conjugates on the slide preparations for 20 minutes at room temperature (20 - 25°C).
 - f. Remove excess Conjugate by tapping the edge of the slide(s) against a paper towel. Quickly and gently rinse slide(s) with a stream of PBS. Do not aim directly at the specimens or smears. Avoid intermixing of Conjugates of different reactivities or specificities on the smears and specimens. Immerse slide(s) in PBS for 10 minutes. Gently rinse with a stream of distilled water or dip slide(s) in distilled water.
 - g. Air dry.
 - h. Add a small drop of buffered glycerol (pH 9.0) and cover slip.
2. **Stability of the Final Reaction:**
 - a. Stained slides are stable for 24 hours when held in the dark at 2 - 8°C. Read immediately or within 24 hours.
 - b. Examination of stained slides:
Examine first under 10X objective of the fluorescence microscope. Select areas of the smear where organisms are present and switch to the mid-range (40X - 63X) objective for rapid screening. The bacteria will be visible as single short rods or filaments showing strong peripheral staining with darker centers. Confirm observations with 100X oil immersion objective (see Interpretation of Test Results).

READING OF THE FINAL REACTION

Use the following numerical notations to describe the intensity of bacterial cell-wall fluorescence observed:

4+	=	brilliant yellow-green staining
3+	=	bright yellow-green staining
2+	=	definite but dull yellow-green staining
1+	=	dim yellow-green staining
Negative	=	absence of yellow-green staining

QUALITY CONTROL

1. The ZEUS Scientific Positive Antigen Control and Conjugates are used to verify proper performance. The Positive Conjugate must stain the Control antigen at 3+ to 4+ intensity, or the test must be considered invalid.
2. The Negative Conjugate must be used on each specimen to ensure that positive reactions obtained with the Positive Control are serologically specific (see Interpretation of Test Results).
3. Additional Controls may be tested according to guidelines or requirements of local, state, and/or federal regulations or accrediting organizations.

INTERPRETATION OF RESULTS

1. *Legionella* bacteria are pleomorphic bacilli. Organisms in culture are usually longer rods than those seen in tissues. In older cultures, long filaments, swollen rods, and other bizarre forms may be seen. The bacteria may produce flagella *in vivo* and in culture; however, flagellar antigens are commonly shared among the Legionellaceae; therefore, these antigens are not presently employed for diagnostic purposes.
2. In clinical specimens, the following criteria are used to evaluate and report test results:
 - a. ≥ 25 typical bacilli with 3+ to 4+ cell-wall fluorescence/smear: Report = FA Positive
 - b. < 25 typical bacilli with 3+ to 4+ cell-wall fluorescence/smear: Report Number of stained cells only.
 - c. No typical bacilli with 3+ to 4+ cell-wall fluorescence/smear (i.e., Bacteria of atypical morphology which stain brightly with either immune or negative control conjugates; or any bacteria which stain 2+ or less). Report FA Negative.
3. Cultural isolates that are morphologically typical and brightly fluorescent (3+ to 4+) are considered positive.
4. FA-positive results should be reported as positive for *Legionella*.
5. See the Limitations of the Assay section concerning cross-reactions among the *Legionella* cell isolates.

LIMITATIONS OF THE ASSAY

1. The direct FA test for *Legionella* is presumptively diagnostic. Whenever possible, a positive result should be confirmed by conventional isolation and biochemical techniques.
2. Direct FA staining of scrapings or sections of formalin-fixed lung, or fresh lung impressions, or of cultures and pleural fluids is rather straightforward.
3. Interpretation of the staining results for lower respiratory tract specimens is, however, more difficult. Tissue and white blood cells may be highly autofluorescent. Bacteria such as Staphylococci, Diplococcus, and Streptococci may fluoresce due to natural antibodies in the serum of the immunized rabbit, or to nonspecific reaction of the IgG molecule with certain bacterial cell-wall components, such as protein A. One strain of *Ps. fluorescence* has been found which is brightly and specifically stained by the working dilution of a *Legionella* conjugate (1). It is necessary to be familiar with the morphology and staining characteristics of the *Legionella* organisms if false-positive diagnoses are to be avoided. Also, relatively few *Legionella* bacteria are seen in lower respiratory tract specimens so that a smear should not be called negative until at least a 5 minute search has been made. If the lung tissue is rubbery or spongy, a positive test is unlikely.
4. A specimen with a negative result indicates only that the sample is negative for the *Legionella* species and serogroups represented by these ZEUS Conjugates. A negative result does not eliminate the possibility that a specimen may be positive for another *Legionella* isolate not represented in these reagents. Cross-reactions between heterologous *Legionella* may occur, and may or may not be reciprocal. These reactions may be either somatic or flagellar, and if somatic, usually will not exceed a 2+ staining intensity. With some heterologous antigens however, a small number of cells per microscopic field may be intensely staining (3+ to 4+). As new serogroups or species are identified, additional cross-reactions may be encountered.

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