INTENDED USE
Focus Diagnostics’ Legionella IFA is intended for qualitatively detecting and semi-quantitating human serum antibodies to Legionella pneumophila.

SUMMARY AND EXPLANATION OF TEST
Legionella are gram negative, non-spore-forming bacilli. The Legionella genus includes at least 39 species and 61 serogroups. Legionella pneumophila causes over 90% of Legionnaires’ disease. 

Legionella cause a broad clinical spectrum of disease, ranging from asymptomatic infection to rapidly progressive pneumonia. Legionellosis is a CDC notifiable disease and is associated with 2 clinically and epidemiologically distinct illnesses: Legionnaires disease, which is characterized by fever, myalgia, cough, pneumonia, and Pontiac fever, a milder illness without pneumonia. Legionnaires’ disease cannot be distinguished clinically or radiographically from pneumonia caused by other agents, and evidence of infection with other respiratory pathogens does not rule out the possibility of concomitant Legionella spp. infection. About 5% to 30% of Legionnaires’ disease cases are fatal.

Inhaling aerosols of water contaminated with Legionella spp. is believed to be the primary mode of infection. Person-to-person transmission has not been observed. A person’s risk of acquiring legionellosis following exposure depends on a number of factors, including the type and intensity of exposure and the exposed person’s health status. Immunosuppression, advanced age, end-stage renal disease, cancer, and nosocomial acquisition of disease are each independently associated with a fatal outcome.

The incubation period for Legionnaires’ disease is generally 2 to 10 days. For U.S. Centers for Disease Control (CDC) epidemiologic purposes, laboratory-confirmed legionellosis that occurs in a patient who has spent > 10 days continuously in the hospital prior to onset of illness is considered definite nosocomial Legionnaires’ disease, and laboratory-confirmed infection that occurs 2 to 9 days after hospitalization is possible nosocomial infection.

Prevention of nosocomial Legionella infections include hospital staff education, surveillance and other measures. If there is evidence of continued transmission, then environmental investigation to determine the source(s) of Legionella and other infection control measures are warranted.

According to the CDC, cases can be confirmed using the following criteria:

Laboratory Confirmed (CDC) 
1. Isolating Legionella from respiratory secretions, lung tissue, pleural fluid, or other normally sterile fluids, or
2. Detecting > 4-fold rise in IFA antibody titer to > 1:128 against Legionella pneumophila serogroup 1 between paired acute- and convalescent-phase serum specimens, or
3. Detecting L. pneumophila serogroup 1 in respiratory secretions, lung tissue, or pleural fluid by direct fluorescent antibody testing, or
4. Demonstrating L. pneumophila serogroup 1 antigens in urine by radioimmunoassay or enzyme-linked immunosorbent assay.

According to the European Working Group for Legionella Infections, cases can be confirmed or presumed using the following criteria:

Microbiologically Confirmed (European Working Group) 
1. Isolating legionella from respiratory secretion, lung tissue or blood.
2. Detecting a > 4-fold rise in antibody titer to L. pneumophila serogroup 1 by IFA or microagglutination.
3. Detecting specific legionella antigen in urine using validated reagents.

Microbiologically Presumed (European Working Group) 
1. Detecting a > 4-fold rise in antibody titer to L. pneumophila or other serogroups or other legionella species by IFA or by microagglutination.
2. Detecting a single titre in specific serum antibody to L. pneumophila serogroup1 or other serogroups or other legionella species.
3. Detecting specific legionella antigen in respiratory secretion or direct fluorescent antibody (DFA) staining of the organism in respiratory secretion or lung tissue using evaluated monoclonal reagents.
4. Detecting legionella species DNA by polymerase chain reaction.

A single elevated antibody titer does not confirm a case of Legionnaires’ disease because IFA titers > 1:256 are found in 1% to 16% of healthy adults. IgA, IgG and IgM antibodies are produced to Legionella antigens. Overall, only 80% of culture positive persons develop a significant rise in titer. Of those 80%, a significant rise in titer may take up to 9 weeks. IgM antibody may be detectable for months.

The Focus Diagnostics Legionella IFA utilizes slides having 12 wells; and each well having 2 individual spots. 1 spot contains L. pneumophila serogroup 1, and the other spot contains a pool of L. pneumophila serogroups 2,3,4,5,6, and 8. The L. pneumophila are formalin fixed.

TEST PRINCIPLE
The Legionella IFA is a 2-stage “sandwich” procedure. In the first stage, the patient serum is diluted in PBS, added to slide wells in contact with the antigen, and incubated. After incubation, the slide is washed in buffered saline, which removes unbound antibodies. In the second stage, each antigen well is overlaid with fluorescein-labeled antibody to human immunoglobulin (IgG, IgM, IgA). The slide is incubated, allowing antigen–antibody complexes to react with the fluorescein-labeled anti-Ig. After the slide is washed, dried, and mounted, it is examined using fluorescence microscopy. Positive reactions appear as apple-green fluorescent rods. Semi-quantitative endpoint titers are obtained by testing serial dilutions of positive specimens.
MATERIALS SUPPLIED
Focus Diagnostics’ Test kit contains sufficient materials to perform 120 determinations.

Substrate Slides
10 slides of 12 wells each. Each well contains 2 spots: 1 spot contains L. pneumophila serogroup 1, and the other spot contains a pool of L. pneumophila serogroups 2,3,4,5,6, and 8. Store sealed slide packets at 2 to 8°C. The sealed slides are stable until the date stated on the slide packet labels. To avoid condensation, allow the slides to warm to room temperature before opening the sealed packets.

Polyvalent Conjugate, 3.5 mL
1 vial of fluorescein-labeled goat anti-human immunoglobulin (IgG, IgM, IgA). Contains Evan’s Blue counterstain, protein stabilizer and preservatives. Ready for use. Stable at 2 to 8°C until the date stated on the label. Do not use if cloudiness, discoloration or other indications of bacterial contamination are present. Allow to warm to room temperature before use.

Detectable Control, 0.30 mL
1 vial of human serum bottled at screening dilution. Contains preservatives. Stable at 2 to 8°C until the expiration date stated on the label. Do not use if cloudiness, discoloration or other indications of bacterial contamination are present. Allow to warm to room temperature before use. Do not pretreat or dilute.

Non-Detectable Control, 0.25 mL
1 vial of human serum bottled at screening dilution. Contains preservatives. Stable at 2 to 8°C until the expiration date stated on the label. Do not use if cloudiness, discoloration or other indications of bacterial contamination are present. Allow to warm to room temperature before use. Do not pretreat or dilute.

Mounting Medium, 2.5 mL
1 dropper bottle containing PBS-buffered glycerol at a pH of 7.2 ± 0.1. Contains preservatives. Stable at 2 to 8°C until the expiration date stated on the bottle label. Allow to warm to room temperature before use.

PBS
1 vial of phosphate buffered saline (PBS) powder. Reconstitute with 1 liter distilled (or purified) water. The reconstituted solution is a 0.01 M buffer at pH 7.2 ± 0.1. Before and after reconstitution, store PBS at 2 to 8°C. Allow to warm to room temperature before use. Do not use if cloudiness, discoloration, or other indications of bacterial contamination are present.

MATERIALS REQUIRED, BUT NOT SUPPLIED
1. 24 x 50 mm coverslips
2. Test tubes and rack, microcentrifuge tubes or microtiter plate for serum dilutions
3. Clinical centrifuge
4. 35 to 37°C incubator or water bath for slide incubation
5. 2 to 8°C refrigerator
6. Plastic wash bottle
7. Calibrated pipets or piston-type pipettors with disposable tips
8. Coplin jars or slide staining dish with slide holder
9. Clean volumetric flask or graduated cylinder, 1 liter
10. Humid chamber for incubation of slides
11. Distilled or purified water
12. Timer
13. Absorbent paper for blotting slides
14. Fluorescence microscope, recommended parameters
   Excitation Filter 470-490 nm
   Barrier Filter 520-560 nm
   Light Source HBO 100W, mercury
   Objective 20-40X, fluorescence quality, high dry

WARNINGS AND PRECAUTIONS
1. All blood products should be treated as potentially infectious. Source materials from which this product (including the non-detectable control) was derived have been screened for Hepatitis B surface antigen, Hepatitis C antibody and HIV-1/2 (AIDS) antibody by FDA-approved methods and found to be negative. However, as no known test methods can offer 100% assurance that products derived from human blood will not transmit these or other infectious agents, all controls, serum specimens and equipment coming into contact with these specimens should be considered potentially infectious and decontaminated or disposed of with proper biohazard precautions. CDC and the National Institutes of Health recommend that potentially infectious agents be handled at the Biosafety Level 2.6
2. Evan’s Blue is a carcinogen; however this product is below the reportable threshold (less than 0.1%).
3. Do not substitute reagents from different kit lots or manufacturers.
4. Use only protocols described in this insert.
5. Cross-contamination of patient specimens on a slide can cause erroneous results. Add patient specimens and handle slide carefully to avoid mixing of sera
from adjoining wells.
7. Bacterial contamination of serum specimens or reagents can produce erroneous results. Use aseptic techniques to avoid microbial contamination.
8. Mounting Medium contains 30 to 60 % glycerol which may cause irritation upon inhalation or skin contact. Upon inhalation or contact, first aid measures should be taken.

SHELF LIFE AND HANDLING
1. Kits are stable through the end of the month indicated in the expiration date when stored at 2 to 8°C.
2. Do not use test kit or reagents beyond their expiration dates.
3. Do not expose reagents to strong light during storage or incubation.

SPECIMEN COLLECTION AND PREPARATION
Serum is the preferred specimen source. No attempt has been made to assess the assay’s compatibility with other specimens. Hyperlipemic, hemolyzed, or contaminated sera may cause erroneous results; therefore, their use should be avoided.

Specimen Collection and Handling
Collect blood samples aseptically using approved venipuncture techniques by qualified personnel. Allow blood samples to clot at room temperature prior to centrifugation. Aseptically transfer serum to a tightly closing sterile container for storage at 2 to 8°C. If testing is to be delayed longer than 5 days, the sample should be frozen at −20°C or colder. Thaw and mix samples well prior to use.

Specimen Preparation
The serum screening dilution is 1:16 in PBS. To determine endpoint titers, use PBS to serially dilute beyond the screening dilution.

TEST PROCEDURE
1. Remove slides from cold storage. To avoid condensation, allow slides to reach room temperature before opening slide packets.
2. Apply 25 µL of Detectable Control, as bottled, to a slide well. Do not dilute.
3. Apply 25 µL of Non-Detectable Control, as bottled, to the slide well. Do not dilute.
4. For each patient sample to be tested, add approximately 25 µL of the diluted sample (see Specimen Preparation, above) to a slide well. Make notations to identify each well when reading results.
5. Incubate slide(s) in a humid chamber for 45 ± 2 minutes at 35 to 37°C.
6. Remove slides from the humid chamber and gently rinse each slide with a stream of PBS. Do not aim stream of PBS directly at the slide wells. Rinse 1 row at a time to avoid mixing of specimens. Wash slides by submersing the rinsed slides into Coplin or slide staining jars containing PBS for 20 minutes.
7. Dip the washed slides briefly in distilled or purified water, and allow the slides to air dry.
8. Add approximately 25 µL Conjugate to each slide well.
9. Incubate slides in a humid chamber for 30 ± 2 minutes at 35 to 37°C.
10. Repeat wash steps 6 and 7.
11. Place a few drops of Mounting Medium on the slide and cover with a 24 x 50 mm coverslip.
12. View wells at a final magnification of 400X on a properly equipped fluorescence microscope. For optimum fluorescence, read slides the same day the assay is performed. If this is not possible, store in the dark at 2 to 8°C until up to 24 hours.

QUALITY CONTROL
Each run (each time a slide, or group of slides, is processed) should include both Detectable and Non-Detectable Controls.
1. The Detectable Control should exhibit 1+ to 4+ fluorescence with both antigen spots.
2. If a 1+ Reading Control is desired, dilute the Detectable Control (see TEST PROCEDURE, above) 1:16 and read versus L. pneumophila Serogroup 1. Due to differing laboratory conditions, including equipment, the 1+ Reading Control may vary ± 1 two-fold dilution.
3. The Non-Detectable Control should exhibit negligible reactivity to all spots. Fluorescence that does not match the morphology and distribution of the detectable control is considered not detectable.

If controls do not exhibit these results, patient test results should be considered invalid and the assay repeated.

INTERPRETATION OF TEST RESULTS
Microscope optics, light source condition and type will determine overall fluorescent intensity and endpoint titers. Read control wells first during every run to ensure correct interpretation.

Reading the Slides
Read fluorescent intensity of the rods, and grade the fluorescence as follows:

<table>
<thead>
<tr>
<th>Fluorescence</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 to 4+</td>
<td>Moderate to intense apple-green fluorescence.</td>
</tr>
<tr>
<td>1+</td>
<td>Definite, but dim fluorescence.</td>
</tr>
<tr>
<td>Negative</td>
<td>No fluorescence or fluorescence corresponding to the Non-Detectable Control well.</td>
</tr>
</tbody>
</table>

Interpreting the Patient Specimens Results
The reciprocal of the highest serum dilution that gives definite (1+) apple-green fluorescence is termed the endpoint titer.

<table>
<thead>
<tr>
<th>Titer</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥1:16</td>
<td>A single specimen endpoint titer ≥ 1:16 should be considered evidence of exposure at an undetermined time. A second specimen drawn 10 to 21 days after the original draw should be tested in parallel with the first. If the second specimen exhibits a 4-fold increase over that of the initial specimen, recent exposure is indicated. Unchanging titers suggest past exposure.</td>
</tr>
<tr>
<td>&lt;1:16</td>
<td>Endpoint titers less than 1:16 suggest that the patient has not been recently exposed.</td>
</tr>
</tbody>
</table>

LIMITATIONS
1. The performance of this assay has not been established for the general population.
2. The performance of this assay has not been established for ruling out exposure to other organisms e.g., Streptococcus pneumoniae, Chlamydia pneumoniae, Mycoplasma pneumoniae, Hantavirus and other organisms. Alternative methods should be used for detecting these pathogens.
3. The performance of this assay has not been established for matrices other than serum, or monitoring antibiotic therapy.
4. All results from this and other serologies must be correlated with clinical history, epidemiological data, and other data available in evaluating the patient.
5. A single positive result only indicates previous immunologic exposure; level of antibody response may not be used to determine active infection or disease stage.

EXPECTED VALUES
IgA, IgG and IgM antibodies are produced to Legionella antigens. Overall, only 80 % of culture positive persons develop a significant rise in titer. Of those 80%, a significant rise in titer may take up to 9 weeks. IgM antibody may be detectable for months.
In the United States, single IFA titers of ≥ 1:256 are found in 1% to 16% of healthy adults.

PERFORMANCE CHARACTERISTICS

Reactivity with Legionella U.S. Sero-conversion Panel (n = 31)

An internal investigator evaluated the sensitivity of the Focus assay with 31 sero-conversion pairs supplied by a U.S. federal public health laboratory. Each of the 31 pairs was archived, and showed a 4-fold or greater increase in endpoint between acute and convalescent sera to *Legionella pneumophila* by a reference IFA. The Reference IFA used serogroup 1 antigen and polyclonal conjugate (IgA, IgG and IgM). Of the 31 patients, the Focus Legionella IFA showed a 4-fold increase in reactivity with 93.5% (29/31) with one or both of the antigen spots, one patient showed a low endpoint (1:64) to the 2-8 serogroup antigen, and one patient was negative. Of the 29 Focus Legionella IFA positives, 13 patients were positive for the serogroup 1 antigen, 1 patient was positive for the serogroup 2-8 antigen, and 15 patients were positive to both antigens (undifferentiated legionella positive).

<table>
<thead>
<tr>
<th>L. pneumophila Result</th>
<th>Focus %</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 4-fold increase</td>
<td>93.5%</td>
<td>78.6-99.2%</td>
</tr>
<tr>
<td>Low reactivity (&lt;1:256)</td>
<td>3.2%</td>
<td>0.1-16.7%</td>
</tr>
<tr>
<td>Negative</td>
<td>3.2%</td>
<td>0.1-16.7%</td>
</tr>
</tbody>
</table>

* 13 patients were sgp1 positive, one was sgp2-8 positive, and 15 were undifferentiated legionella positive (sgp1 and 2-8 positive).

Reactivity with Legionella EU Sero-conversion Panel (n = 29)

An external investigator evaluated the sensitivity of the Focus assay with 29 sero-conversion pairs supplied. Each of the 29 pairs was archived, and showed a 4-fold or greater increase in endpoint between acute and convalescent sera to *Legionella pneumophila* by a Reference IFA for serogroups 1-10. The Reference IFA used separate antigen spots for serogroups 1-10, and the Reference IFA used polyclonal conjugate (IgA, IgG and IgM). Of the 29 presumed acute positive patients, the Reference IFA detected four serogroup 1 patients, 12 serogroup 6 patients, 1 serogroup 2 patient, 1 serogroup 8 patient, and 11 undifferentiated legionella patients. Of the four Reference IFA serogroup 1 patients, the Focus IFA detected one as a serogroup 2-10 presumed acute patient, and three as undifferentiated legionella presumed acute patients. Of the 12 Reference IFA serogroup 6 patients, the Focus IFA detected six as serogroup 1 presumed acute patients, two as serogroup 2-10 presumed acute patients, three as undifferentiated legionella presumed acute patients, and one negative. The one Reference IFA serogroup 2 patient was detected by the Focus IFA as a serogroup 1 presumed acute patient. The one Reference IFA serogroup 8 patient was detected by the Focus IFA as an undifferentiated legionella presumed acute patient. Of the 11 Reference IFA undifferentiated legionella patients, the Focus IFA detected one as serogroup 1 presumed acute patient, the Focus IFA detected three as serogroup 2-10 presumed acute patients, and seven as undifferentiated legionella presumed acute patients.

<table>
<thead>
<tr>
<th>L. pneumophila Result</th>
<th>Focus IFA %</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 4-fold increase</td>
<td>96.6%*(28/29)</td>
<td>82.2-99.9%</td>
</tr>
<tr>
<td>Low reactivity (&lt;1:256)</td>
<td>0.0%</td>
<td>0.0-11.9%</td>
</tr>
<tr>
<td>Negative</td>
<td>3.4% *(1/29)</td>
<td>0.1-17.8%</td>
</tr>
</tbody>
</table>

* By the Reference IFA, 4 patients were sgp1 positive, 12 were serogroup 6, 1 was serogroup 2, one was serogroup 8, and 11 were undifferentiated legionella positive (sgp1 and 2-10 positive).

Reactivity with Legionella EU High Reactives (n = 31)

An external investigator evaluated the sensitivity of the Focus assay with 31 sera with high reactivity to a Reference Legionella IFA. The Reference IFA used separate antigen spots for serogroups 1-10, and the Reference IFA used polyclonal conjugate (IgA, IgG and IgM). Each of the 31 sera was archived, and endpointed above 1:128 with the Reference Legionella IFA. Of the 31 sera, Reference IFA detected 20 that were reactive with one of the serogroup 2-10 antigen spots, Reference IFA detected 3 that were reactive with just the serogroup 1 antigen spot, and the Reference IFA detected 8 sera that were undifferentiated legionella patients (reactivity within one 2-fold dilution between serogroup 1 and another serogroup). The Focus Legionella IFA overall reactivity (serogroup 1 and/or serogroup 2-8 reactivity) was within one 2-fold dilution of the Reference IFA for 96.8% (30/31) of the sera. The Focus Legionella IFA endpointed at least 8-fold lower with one serum.

Reactivity with Legionella EU Intermediate Reactives (n = 30)

An external investigator evaluated the sensitivity of the Focus assay with 30 sera with intermediate reactivity to a Reference Legionella IFA. The Reference IFA used separate antigen spots for serogroups 1-10, and the Reference IFA used polyclonal conjugate (IgA, IgG and IgM). Each of the 30 sera was archived, and endpointed at 1:64 or 1:128 with the Reference Legionella IFA. Of the 30 sera, Reference IFA detected 26 that were reactive with one of the serogroup 2-10 antigen spots, and the Reference IFA detected 4 sera that were undifferentiated legionella patients (reactivity within one 2-fold dilution between serogroup 1 and another serogroup). The Focus Legionella IFA overall reactivity (serogroup 1 or serogroup 2-8 reactivity) was within one 2-fold dilution of the Reference IFA for 93.3% (28/30) of the sera. The Focus Legionella IFA was 4-fold lower with one serum, and negative with a second serum.

Prevalence in a Normal Population (n = 30)

An external investigator (a reference laboratory located in France), evaluated prevalence with 30 sera from a normal population with the Focus assay. The sera were sero-negative with the investigator's Reference Legionella IFA. Of the 30 sera, 20% (6/30) showed low reactivity (1:16 or 1:32) with the Focus sero-group 1 antigen, and 10% (3/30) showed low reactivity (1:16 or 1:32) with the Focus sero-group 2-8 antigen.

Cross-reactivity

An external investigator (a reference laboratory located in France), compared cross-reactivity of the Focus Legionella IFA with a Reference Legionella IFA, using 16 sera that were sero-positive to pathogens that cause pneumonia. Of the 16 sera, five were IgM positive for *Mycoplasma pneumoniae*, five were sero-positive for *Chlamydia pneumoniae* (one of the five was also IgM positive for *C. pneumoniae*), and six were IgG positive for *Streptococcus pneumoniae*. The Reference IFA used separate antigen spots for serogroups 1-10, and the Reference IFA used polyclonal conjugate (IgA, IgG and IgM). The Focus IFA and the Reference IFA had low reactivity (endpoint ≤ 64) with 33% (2/6) of the *S. pneumoniae* positives. The Focus IFA had low reactivity with 40% (2/5) of the *M. pneumoniae* positives (1 of the 2 reactives endpointed at 1:128) and the Reference IFA had low reactivity with 60% (3/5) of the *M. pneumoniae* positives. The Focus IFA had low reactivity (endpoint ≤ 64) with 80% (4/5) of the *C. pneumoniae* positives, and the Reference IFA had low reactivity with 60% (3/5) of the *C. pneumoniae* positives.

<table>
<thead>
<tr>
<th>Population</th>
<th>FocusLegionella IFA % Reactive</th>
<th>ReferenceLegionella IFA % Reactive</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. pneumonia</td>
<td>33% (2/6)</td>
<td>33% (2/6)</td>
</tr>
<tr>
<td>Sero-positives</td>
<td>95% CI 4.3-97.7%</td>
<td>95% CI 4.3-97.7%</td>
</tr>
<tr>
<td>M. pneumonia</td>
<td>40% (2/5)</td>
<td>60% (3/5)</td>
</tr>
<tr>
<td>Sero-positives</td>
<td>95% CI 5.3-85.3%</td>
<td>95% CI 14.7-94.7%</td>
</tr>
<tr>
<td>C. pneumonia</td>
<td>80% (4/5)</td>
<td>60% (3/5)</td>
</tr>
<tr>
<td>Sero-positives</td>
<td>95% CI 28.4-99.5%</td>
<td>95% CI 14.7-94.7%</td>
</tr>
</tbody>
</table>
Inter-assay Reproducibility
An external investigator evaluated inter-assay reproducibility by endpointing 3 positive sera on three different days. All endpoints were within one 2-fold dilution.

Stability
An internal investigator assessed stability of the Focus Legionella IFA. Components were accelerated to an equivalent of 2 years at 2 to 8°C. The accelerated components were compared to unaccelerated components using 10 archived sera. Of the ten sera, one was highly reactive (≥ 1:512), one had intermediate reactivity (1:128), six had low reactivity (within 1:16 to 1:64), and three had no reactivity. All accelerated endpoints were within one 2-fold dilution of the unaccelerated endpoints.

REFERENCES
1. CDC. Legionellosis: Legionnaires’ Disease (LD) and Pontiac Fever. (December 2003).

This package insert is available in French, German, Italian, and Spanish at www.focusdx.com, and may be available in other languages from your local distributor.