Department of Microbiology
Legionella DFA Procedure

I. Introduction and Clinical Significance

Legionella is a gram negative bacillus that is found in the environment, including fresh water lakes, tap water, air conditioning systems, and recreational whirlpools. Although a number of species within this genus are associated with human disease, more than 80% of the organisms isolated from patients have been identified as *L. pneumophila*.

There are two distinct illnesses caused by *L. pneumophila*: pneumonia (Legionnaire's disease), an acute respiratory infection, and Pontiac fever, a non-pneumonia, self-limited respiratory illness.

Transmission of *Legionella* is related to exposure to aerosols such as those created by air conditioners, jet nebulizers, and room humidifiers. An increased risk of acquiring Legionnaire's disease is associated with cigarette smoking, alcohol consumption, and the presence of pre-existing disease resulting in reduced immunocompetence.

II. Principle

The MONOFLUO™ anti-*Legionella pneumophila* staining reagent contains a single monoclonal antibody that is labeled with fluorescein isothiocyanate. The antibody reacts with a protein present in the outer membrane of all known serogroups of *L. pneumophila.*

Smears are prepared on microscope slides, as described in the test procedure, either directly from patient specimens or from organisms isolated in culture. The smears are stained with the MONOFLUO™ anti-*Legionella pneumophila* staining reagent. The labeled monoclonal antibody binds to any *L. pneumophila* in the smear. A subsequent rinse step removes unbound antibody. When samples are viewed with a fluorescent microscope, *L. pneumophila* appear as brightly fluorescing apple-green bacilli or coccobacilli. Other organisms will appear dark red or dull gold due to counterstain.

III. Precautions

A. Do not use the kit after the stated expiration date.
B. Handle all specimens as potentially infectious. Procedures that could create aerosols should be conducted in a biological safety hood.
C. The Staining Reagent and Positive Control Antigen Suspension contain sodium azide. Sodium azide may react with lead and copper pipes to form metal azides that are highly explosive. To prevent azide buildup, flush plumbing with a large volume of water, if reagents are disposed of in the sink.
D. Clean Coplin dishes thoroughly between uses to prevent carryover of positive cells.
E. Do not allow the staining reagent to dry on the slides during the staining procedure. This will lead to staining artifact and the possibility of false-positive results.

IV. Specimen Preparation Prior to Staining

A. Sputum, transtracheal aspirate, bronchial washings or brushes, bronchoalveolar lavage, and induced sputum are acceptable specimens, as is virtually any type of lower respiratory tract specimen.
   1. Select portions of the specimen that are milky or bloody.
   2. Prepare smear by placing 2 drops of sample on the well closest to the etched edge of the slide.

B. For pleural fluid, centrifuge the specimen at 4000 X *g* for 30 min. Discard all but 0.5 mL of supernatant, and re-suspend the pellet. With a sterile pipette, prepare a thin smear on a two-well slide. Use the Cytospin as an alternative method of concentration for fluids.

C. Tissue (fresh or fresh-frozen) should be processed using sterile forceps and scalpels. Selecting dense areas of gray or reddish consolidation, cut a fresh surface on the tissue; with forceps, press and squeeze the tissue against the slide to fill the wells. If culture is to be performed, do so before making the smears.

D. For all of the above specimen types:
   1. Use a slide warmer set at 35-45 °C to dry the slides, and then heat fix the smears by rapidly passing the slide through a flame.
   2. Place the slides on a rack, and cover each smear with 10% formalin in saline. Do not allow slide to dry. Fix for 10 min at room temperature.
   3. Drain off the formalin, and briefly dip the slides in deionized water.
   4. Soak the slides in fresh deionized water for 2 min to remove the remainder of the formalin.
   5. Air dry, and proceed with the Fluorescence Staining Procedure.

V. Culture Confirmation

A. Work should be performed in a biological safety cabinet.

B. Clean and label a fluorescence microscopy slide.

C. Select an isolated colony of the potential *L. pneumophila*; suspend it in a test tube containing about 0.5 mL of 1% formalin in saline, bringing it to the turbidity of a McFarland No. 1 standard.

D. Using a Pasteur pipette, dispense 2 drops of the suspension into one of the slide wells; then remove it with the same pipette. A thin film of organism will remain on the well.

E. Use a slide warmer set at 35-45 °C to dry the smear under the hood, and then heat fix it by rapidly passing it through a flame.

F. Proceed with the Fluorescence Staining Procedure.

VI. Reagents

A. Anti-*Legionella pneumophila* staining reagent. Contains FITC-labeled monoclonal antibody, counterstain, sodium azide, and protein-stabilized buffer. Store at 2-8 °C.

B. Positive control antigen suspension. Contains killed *L. pneumophila*, buffered saline, and sodium azide. Shake before use. Store at 2-8 °C.

C. The mounting medium contains buffered glycerol and anti-quencher. The

anti-quencher increases the amount of time that the slides may be scanned before fluorescence fades. Store at 2-8 °C.

D. Fluorescence microscopy slides. Wipe with lint free tissue before use.

E. Micropipette bulbs

VII. Quality Control

A. Each day the test is performed, positive and negative control slides must be stained. **The positive control slide should be processed separately from the patient smears.**

B. Quality control slides are prepared from the *L. pneumophila* antigen (positive control) and a suspension of *E. coli* ATCC 25922 (negative control).

C. The positive control slides are prepared as follows:

1. Clean and label a two-well slide.
2. Shake the bottle containing the Positive Control Antigen suspension to re-suspend the cells.
3. Add 1 drop of the suspension on the well closest to the etched edge of the slide, and using a fresh pipette, **remove the liquid from the well and discard.**
4. Use a slide warmer set at 35-45 °C to dry the slides. Then heat-fix the sample by rapidly passing it through a flame.
5. Store controls at -20 °C.

D. The negative control smears are prepared using *E. coli*, according to the procedure for smear preparation for culture confirmation as outlined above in section V.

E. Enter QC results into the LIS. If QC slides do not produce expected results, do not report patient test results from batch and notify supervisor.

VIII. Fluorescence Staining Procedure

A. Dispense 1 drop (20 µL) of anti-*Legionella pneumophila* staining reagent. This should be sufficient to cover the specimen. Keep within the boundary of the well, or if it is a smear submitted by a client, keep the reagent within a circle etched on the smear prior to staining that is similar in size to a slide well.

B. Place the slides in a moisture chamber, and incubate for 30 min at 35 °C.

C. Remove excess staining reagent by tipping the edge of the slide against a paper towel to absorb the stain. Briefly dip the slide in deionized water, followed by two soaks for 5 min each in fresh deionized water. **The positive control slide and any culture confirmation slides should be dipped in separate containers from the direct smear patient slides**.

D. Air dry slides.

E. Add 1-2 drops of mounting medium to the slide. Apply a coverslip.

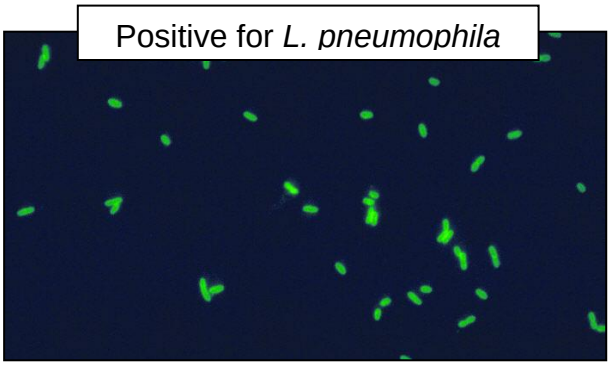
IX. Examination and Evaluation of Test Results

A. Interpret the slides within several hours of staining. The slides may be stored overnight in the dark at 2-8 °C or for longer periods at -20 °C.

Slides stored in the cold must be allowed to reach room temperature before reading to avoid condensation interfering with smear interpretation.

B. Scan each smear with the 20X objective. If fluorescent speckles are observed, use the 60X or 100X objective to confirm that the cellular morphology is consistent with *Legionella.*

C. Stained *L. pneumophila* appear as intra- or extracellular fluorescing apple green rods or coccobacilli. *L. pneumophila* are pleomorphic organisms that can range from very short coccobacillary forms to long filamentous rods. The staining characteristics and morphology of the organisms should resemble those in the positive control. Antibiotic therapy may result in organisms with uncharacteristic morphology.



1. If >5 fluorescing bacteria are seen per 2-well slide, report: **Legionella pneumophila seen by Direct Fluorescent Antibody stain. [LEGST]**
2. If 1 to 5 fluorescing bacteria are seen per 2-well slide, report: **Direct Fluorescent Antibody stain is indeterminate for Legionella pneumophila. Result should be confirmed by culture.**
3. Samples are negative when organisms appear dark red or dull gold. Report: **No Legionella pneumophila seen by Direct Fluorescent Antibody stain. [NLEGST]**
4. Attach the following comment to all reports: **The Legionella DFA test has a low sensitivity of 25-75% and a specificity of 95-99%. Legionella culture is a superior test with a sensitivity of 80-99% and a specificity of 100%. [LFAC]**

D. In a culture confirmation test, overcrowding of cells in the smear may significantly reduce staining intensity. If necessary, examine the outer edges of the well or portion of the smear containing fewer cells.

X. Limitations of the Procedure

A. A negative result does not exclude the possibility that the patient is infected with *L. pneumophila* or another *Legionella* species. Sample collection and preparation are critical variables in the procedure.

B. A limited number of organisms, *Staphylococcus aureus* and some *Lactobacillus* spp., may fluoresce due to non-specific binding of the antibody.

C. Use only fresh or frozen specimens. Histopathologically processed specimens and formalin preserved materials are inappropriate specimens for this test.

D. Certain purulent sputum specimens may exhibit increased background staining and non-specific binding of the antibody to cells. Any specimen exhibiting these characteristics should be confirmed by culture.

XI. References

A. BioRad MONOFLUO™ Legionella pneumophila IFA Test Kit. Product No. 32514. September 2009.

Document Control
Effective 01/1994
Microbiology Director Approval: Dr. Ann Robinson 04/12/2000
Microbiology Supervisor Reviews: Jerry Claridge 03/1994, 11/1995, 06/1996, 08/1997, 04/1998, 06/1999, 04/2000, 04/2001, 04/2004, 11/2005, 02/2006, 01/2007, 09/2007, 09/2008, 09/2009, 03/2011, 03/2013, Jason Ammons 05/2015
Revisions & Updates: 03/25/2005 AR revised. 05/27/2011 Updated for preparing only one smear for staining to conserve resources. Added test sensitivity comment.