PROCEDURE: PNEUMOCYSTIS JIROVECI IMMUNOFLUORESCENCE MONOFLUO ™ ASSAY

I. PRINCIPLE

The MONOFLUO™ Pneumocystis Immunofluorescent Antibody Test Kit is to be used for the detection of *Pneumocystis jiroveci (formerly P.carinii)* cysts and trophozoites in specimens collected from the respiratory tract.

The use of a direct, fluorescent-antibody procedure with induced sputum, bronchial wash or bronchoalveolar lavage samples provides a simple, highly specific procedure for detection of *Pneumocystis jiroveci.* The monoclonal antibodies in the MONOFLUO™ *Pneumocystis* Immunofluorescence Test Kit are chemically linked to fluorescein isothiocyanate (FITC) to produce a highly specific, direct, immunofluorescent reagent with a low level of background fluorescence. In addition to binding with *Pneumocystis jiroveci* cysts, the monoclonal antibodies also bind specifically to *Pneumocystis jiroveci* trophozoites, sporozoites, and the extracellular matrix. The test kit is used for the rapid identification of *Pneumocystis jiroveci* cysts and trophozoites directly in-patient specimens.

The MONOFLUO™ *Pneumocystis jiroveci* Staining Reagent contains murine monoclonal antibodies labeled with fluorescein isothiocyanate (FITC). These antibodies react with all *Pneumocystis jiroveci* forms (cysts, sporozoites and trophozoites) and also with the extracellular matrix present in infected specimens.

Slides are prepared for microscopy from clinical specimens as described in the "Preparation of Clinical Specimens" section. Following fixation, the slides are stained with the MONOFLUO™ *Pneumocystis jiroveci* Staining Reagent. The Staining Reagent binds to any *Pneumocystis jiroveci* organisms present in the specimen. A subsequent rinse step removes unbound Staining Reagent from the specimen. The slides are then mounted with the MONOFLUO™ Mounting Medium included in the kit. The Mounting Medium contains an anti-quencher, which increases the length of time slides may be examined before the fluorescence fades.

When viewed with a fluorescence microscope, infected specimens fluoresce bright apple-green. Stained cysts appear as large, round-to-elliptical structures found both individually and in clusters. Sporozoites and trophozoites may also be stained, appearing as relatively small, crescent-shaped or pleomorphic structures. In addition, the amorphous, extracellular matrix should also fluoresce brightly. Other organisms and cellular material from respiratory specimens are counterstained red to orange-red or gold without characteristic fluorescence of *Pneumocystis jiroveci* cysts.

II. AVAILABILITY

The test is available Monday-Friday, 7:30AM-2:30 PM.
Stat testing is available upon request if staffing allows.

III. TEST CODE

The test code is **DFPCP**

IV. SPECIMEN PREPARATION

NOTE: Clinical specimens should be freshly collected using standard methods for obtaining induced sputum, tracheal aspirate, bronchial wash or bronchoalveolar lavage. Specimens not processed immediately should be refrigerated for no longer than 72 hours. Expectorated sputum should not be used.

A. Induced Sputum Specimens including tracheal aspirates.

1. Put 2 to 3 mLs of sputum into a 15mL conical centrifuge tube. Add an equal amount of dithiothreitol reagent (DTT). Vortex the tube then incubate for 5-10 minutes at 37*C or 15 minutes at RT (15-30*C). If less than 2mls is received a disclaimer should be added indicating that results may be unreliable due to insufficient quantity of specimen.
2. After incubation, add an equal amount of 1X PBS pH 7.2 to sputum plus DTT (Sputolysin®), doubling the volume. Vortex.
3. Centrifuge at 1500 x G for 5 minutes
4. Carefully decant or aspirate the supernatant, leaving 0.5mL or less of the pellet and supernatant in the tube. Steps 2, 3, and 4 may be repeated, if necessary, to remove excess mucus.
5. Resuspend the pellet thoroughly. Using a pipettor, apply one drop (approximately 25-50ul) onto a microscope slide provided with the kit. Spread the drop evenly over the well, taking care not to scratch the slide. Place the slide on the slide warmer (35-37◦C) to dry.
6. Fix for 10 minutes in acetone. Slides may be processed immediately or frozen at -20*C for up to one month before staining.

B. Bronchial Wash and Bronchoalveolar Lavage Specimens
1. Non-mucoid samples such as bronchial washes or bronchoalveolar lavages will probably not require the mucolytic procedure.
2. For these samples, add up to 15 ml of sample into a clear conical centrifuge tube.
3. Proceed as in Steps 3-5 above.
4. Invasive, non-mucoid samples that are ≤1ml do not need to be spun, the slide can be inoculated directly. A disclaimer should be added indicating that results may be unreliable due to insufficient quantity of specimen.

V. MATERIALS AND EQUIPMENT

A. Materials
1. PNEUMOCYSTIS staining reagent-One 2.2mL dropper bottle containing FITC-labeled monoclonal antibodies (murine), Evans blue counterstain, 0.1% sodium azide and protein-stabilized buffer. Mix gently before use.
2. Mounting Medium- One 3.5mL dropper bottle containing buffered glycerol, 0.8% formaldehyde and anti-quencher. Ready to use
3. Fluorescence Microscopy Slides-24 (2 wells each)-Wipe with lint free tissue before use.

B. Equipment
1. Dithiothreitol (Sputolysin®) – dilute the contents of one vial with 100ml of sterile distilled water.
2. Phosphate Buffered Saline
3. Acetone, Reagent Grade, (store in a tightly closed container to prevent absorption of water)
4. Deionized water or tap water
5. Slide staining dishes
6. Humidified chamber
7. Coverslips 24x40mm, #1
8. Pasteur pipettes
9. Centrifuge
10. 15 mL conical centrifuge tubes
11. 37±ºC incubator
12. Biological safety cabinet
13. Fluorescent Microscope with FITC filter [wide band (420-490nm) or narrow band (470-490 nm) excitation wavelengths with a 515-nm dichromatic mirror and a 515 nm barrier filter

VI. STORAGE

A. Store staining reagent in the dark at 2-8ºC. Do not use the kit or any of its components beyond the expiration date.
B. Store reagents at 2-8ºC. Uninoculated slides should be stored at 2-28ºC. If stored at the specified temperatures, opened or unopened, reagents are stabile for the printed shelf life.
C. Diluted Sputolysin® is stable for 48 hours at 4ºC.
D. Unstained laboratory prepared control slides should be stored at -20ºC for up to one year.
E. Stained patient slides should be stored at -20ºC for up to 6 months.
F. Leftover patient sample is saved for 72 hours at 4°.

VII. QUALITY CONTROL

A. Positive and negative control slides are included each time the test is performed. Positive and negative control slides are prepared by the AFB/Mycology lab using previously tested patient specimens. Performance of the control slides must be acceptable for the test to be considered valid.
B. New shipments and/or new lots of the MONOFLUO™ Pneumocystis Immunofluorescent Antibody Test Kit are QC'd by using PCP Control Slides prepared by the AFB/ Mycology lab using previously positive and negative patient specimen. Performance of the positive and negative controls must be acceptable for the kit to be put into use.

VIII. TEST PROCEDURE

A. Allow any frozen slides to equilibrate to room temperature
B. Place the slide in a humidified chamber. A damp paper towel placed within the staining box can be used to provide moisture.
C. Mix staining reagent before each use by swirling gently.
D. Add sufficient amount of Pneumocystis jiroveci Staining Reagent to each well containing a specimen (2-3 drops) to cover the specimen, keeping within the boundary of the well.
E. Incubate for 30-35 minutes at 37±1°C. Do not allow the Pneumocystis jiroveci Staining Reagent to dry on the slide during the procedure.
F. Remove excess *Pneumocystis jiroveci* Staining Reagent by draining the slide onto clean paper toweling or by aspiration.
G. Wash the slide by placing into a Coplin jar filled with deionized water and soaking for 1 minute. Remove excess water by draining the slide onto clean paper towel.
H. Dry the slide thoroughly by air-drying or with a slide warmer at 37±1°C.
I. Apply one to two drops of Mounting Medium to the slide and apply a coverslip, being careful to avoid the formation of air bubbles.
J. Slides should be examined within 2-3 hours of staining.

IX. INTERPRETATION

A. Specimens infected with Pneumocystis jiroveci contain large round-to-elliptical shaped cysts, found both individually and in clusters, which will be stained bright apple-green
B. Two or more well-defined fluorescing cysts must be observed for the specimen to be considered positive.
C. Sporozoites and trophozoites may also be stained. They appear as small, crescent-shaped or pleomorphic structures. Brightly staining, amorphous, extracellular matrix may also be present. Specimens containing only brightly-staining, amorphous material or typical sporozoite and trophozoite forms should prompt active searching for cysts before a definite diagnosis can be made. In the absence of cysts, the specimen should be suspected as positive for Pneumocystis jiroveci, and another slide should be made for repeat staining. If, upon repeat staining, there are still no cysts present, the specimen should be suspected as positive for Pneumocystis jiroveci, and another specimen should be requested.

D. Other organisms and cellular material from respiratory specimens should be counterstained red to orange-red or gold.
E. A specimen is considered negative if characteristic fluorescence, as described above, is not observed.

X. REPORTING RESULTS

A. POSITIVE PCP**:** Report as: (Test Comment)
    1. *DFA POSITIVE for Pneumocystis jiroveci(carinii)*

B. NEGATIVE PCP: Report as: (Test Comment)
    1. *DFA negative for Pneumocystis jiroveci (carinii)*

C. INSUFFICIENT MATERIAL: Report as: (TC)
    1. Insufficient material on slide for interpretation. Notify the patient's nurse that the specimen was insufficient and suggest a repeat specimen.

D. If only trophozoites are seen, use:
    1. DFA is Suspected as positive for Pneumocystis jiroveci. Please submit another specimen for confirmation.

E. Rejected Specimen: Report as:
    1. Specimen quality suggests expectorated sputum, test not performed. BAL/bronch wash or induced sputum required for adequate PCP exam.

XI. PROCEDURE NOTES

A. FOR IN VITRO DIAGNOSTIC USE.
B. This test should only be performed by personnel who are properly trained in the handling of clinical specimens that may contain HIV-1. The slides should be examined by personnel who have experience in reading immunofluorescence assays.
C. Handle all clinical specimens as potentially infectious material. All procedures should be performed in a biological safety cabinet.
D. The staining reagent contains Evans Blue, which is an irritant. Avoid spilling or splashing the staining reagent on skin or clothing. Flush any areas, which have come in contact with staining reagent thoroughly with water.
E. The Mounting Medium contains formaldehyde, which, according to the United States National Toxicity Program (NTP), may be carcinogenic. However, existing data do not conclusively establish the carcinogenicity of formaldehyde at the concentrations used in this kit (<1.00%). Avoid spilling or splashing the Mounting Medium on skin or clothing. Flush any areas, which may have come in contact with the Mounting Medium thoroughly with water.
F. The Staining Reagent contains sodium azide. Sodium azide may react with lead or copper plumbing to form metal azides that are highly explosive. If the Staining Reagent is disposed of in the sink, flush plumbing with a large volume of water to prevent azide buildup.
G. Clean all reusable items thoroughly between uses.
H. Use a separate slide for each patient specimen to prevent any cross-contamination of Pneumocystis jiroveci organisms. It is also recommended that each slide be washed separately.
I. Unused Sputolysin® solution should be disposed of according to Rhode Island Hospital chemical waste policies. See hazardous waste disposal guidelines for appropriate disposal.

XII. LIMITATIONS

A. A negative result does not exclude the possibility of *Pneumocystis jiroveci* infection in the patient. Sample collection and preparation are critical steps in the testing procedure. Collecting the sample at an improper time during the course of disease, by an inappropriate method or with improper handling of the specimen, or misusing the reagents provided can result in failure to detect *Pneumocystis jiroveci*. Diagnosis should be made in conjunction with clinical symptoms.
B. Run only one specimen on each slide. Care must be taken to process, fix, stain, and wash each specimen separately to prevent any possible wash-over of *Pneumocystis jiroveci* 12organisms to other slides.
C. Excess mucus in specimens may prevent adequate staining. Certain thick sputum smears may prevent adequate staining. Care must be taken to make smears as thin as possible. Nonspecific trapping of the *Pneumocystis jiroveci* Staining Reagent may occur if the specimen is not adequately washed.
D. If the bronchial wash or bronchoalveolar lavage specimen is negative, but the patient continues to exhibit symptoms associated with respiratory illness, other etiologic agents should be suspected and appropriate diagnostic procedures (including culture) should be performed.

XIII. TECHNICAL SUPPORT

A. FOR TECHNICAL ASSISTANCE CONTACT BIO-RAD LABORATORIES AT 1-510-724-7000 OR WWW.BIO-RAD.COM

XIV. REFERENCES

A. Mills J: Pneumocystis carinii and Toxoplasma gondii infections in patients with AIDS. Rev Infect Dis 8(6):1001-1011, 1986.
B. Kovacs JA, Hiemenz JW, Macher AM, et al: *Pneumocystis carinii* pneumonia: A comparison between patients with the acquired immunodeficiency syndrome and patients with other immunodeficiencies. Ann Intern Med 100:663-671, 1984.
C. Posner D, Khan FA: Respiratory infections in AIDS: A comprehensive care approach. J Respir Dis pp 83-94, June 1987.
D. BIO-RAD package inserts, 2022

XV. REVISIONS

A. 02/05/2024 Updated testing availability under II. AVAILABILITY
B. 02/05/2024 Updated sample type to include tracheal aspirates and revised specimen collection and preparation requirements under IV. SPECIMEN PREPARATION.
C. 02/05/2024 Added patient and control slide storage requirements under VI STORAGE
D. 02/05/2024 Eliminated F. under Section X. REPORTING RESULTS - A negative result on an induced sputum should be verified on a subsequent specimen collected by bronchial wash or bronchoalveolar lavage.
E. 02/05/2024 Added guidance pertaining to disposal of unused Sputolysin® under Section XI. PROCEDURE NOTES