# Development of a Real-time PCR assay for *Pneumocystis jirovecii* on the Luminex ARIES® Platform

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## Introduction And Objective

*Pneumocystis* pneumonia is an opportunistic infection caused by *Pneumocystis jirovecii*. Infection with *P. jirovecii* can result in a serious illness in patients with weakened immune systems and can lead to death. Direct detection of *P. jirovecii* in bronchoalveolar lavage (BAL) is preferred for rapid diagnosis. We describe a diagnostic real-time PCR assay that targets the multi-copy gene mitochondrial large subunit ribosomal RNA gene (mtLSU rRNA) of *P. jirovecii* using BAL specimens.

## Methods & Materials

Specimens: The sensitivity studies were performed by testing BAL, collected from pooled negative BAL samples (previously tested by *P. jirovecii* PCR), and spiked with 10-fold dilutions of a *P. jirovecii* positive control (Zeptometrix). For the accuracy study, 24 previous positive specimens for *P. jirovecii* and 10 negative clinical samples were used (University of Michigan). Testing also included 17 positive and 14 negative blinded samples prepared for an additional accuracy study by spiking with known concentrations of a *P. jirovecii* control. Finally, specificity, precision and stability studies were conducted to validate the assay.

Reagents: Primer pairs were designed to include a fluorescent reporter dye labeled primer with a unique MultiCode® base pair isoC on the 5'end, and an unlabeled primer obtained from IDT. The primer sequences for PCP mtLSU are: forward 5'-CAG ACT ATG TGC GAT AAG GTA GAT AGT CG-3'; reverse 5'-/56-FAM/iMe-isodC/GGA GCT TTA ATT ACT GTT CTG GGC -3'. Each primer was used at a final concentration of 200 nM. MHV control primers 2 from Luminex were used to amplify the sample processing control in the cassette. The cassette contains the reagents needed to perform all of the steps including extraction, purification, amplification, and detection, plus sample processing control.

Control Materials: Recombinant PCP positive controls were purchased from Zeptometrix (NATtrol *P.jirovecii* Recombinant External Run Control, Catalog number 320679, Buffalo, NY) and EXACT Diagnostics (Custom *Pneumocystis jirovecii* Run control, Reference number PCPRC, Fort Worth, TX).

Organisms: Eleven different fungi (*Candida dubliniensis, Candida parapsilosis, Candida glabrata, Candida krusei, Candida tropicalis, Cryptococcus neoformans, Saccharomyces cerevisiae, Histoplasma capsulatum [yeast form], Aspergillus fumigatus, Aspergillus versicolor and Aspergillus niger)*, eight different bacteria (*Streptococcus pneumoniae, Chlamydophila pneumoniae* strain CWL 029, *Bordetella pertussis* A639, *Bordetella strain parapertussis* A747, *Mycoplasma pneumoniae* Strain M129, *Chlamydia pneumoniae, Legionella pneumophila, Mycoplasma pneumoniae*) and thirteen different viruses (Enterovirus strain Coxsackie A9, Influenza A strain California 07/2009 [H1N1 pdm], Influenza B Massachusetts 2/2012, Coronavirus NL63, Parainfluenza strain Type 2 and RSV strain Type B CH93 (18), Parainfluenza virus IV strain type 4, Rhinovirus/Enterovirus Echovirus 30 (E-30), Influenza B Strain Brisbane 60/2008 and Parainfluenza Strain Type 3, Adenovirus Strain type 21, Influenza A Strain New York 18/2009 (H1N1 pdm), Human metapneumovirus strain B2) were spiked into negative BAL and tested to assess cross-reactivity.

## *P. jirovecii* Life Cycle

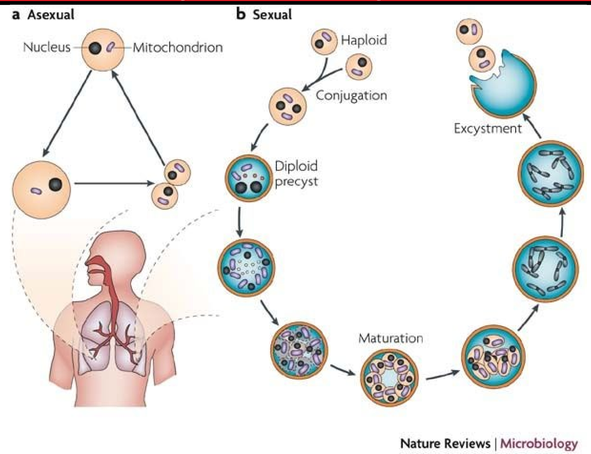



**Figure 1.** There are two predominant life-cycle forms of *Pneumocystis*, the trophic form and the cyst form. It has been hypothesized that the trophic form can conjugate by binary fission and therefore undergo asexual reproduction (**a**). In addition, there is a sexual cycle (**b**). Three intermediate cyst stages have been visualized by electron microscopy, and they contain complements of 2, 4 and 8 nuclei, respectively The mature cyst contains 8 intracystic nuclei. It has been suggested that trophic forms emanate from the intracystic nuclei of the mature cyst as it ruptures, and then undergo vegetative growth or conjugate to re-form the cyst. Modified, with permission of J. Ruffalo, from Ref. 155 © (1998) Arnold Publishing. Copied from Nature reviews/ Microbiology 5, 298-308, 2007.

## Results

**Analytical Sensitivity:**

- The assay could reliably detect 3000 copies of mtLSU that equated to 200 organisms/mL of *P. jirovecii* (Table 4) in respiratory specimens.

**Accuracy:**

- Analytical sensitivity for the *P. jirovecii* assay was 89.1% (95% CI 76.43-96.38%), while analytical specificity was 100% (95% CI 87.23 -100).

**Precision:**

- The results were highly reproducible, demonstrated by testing positive controls and negative controls with different technologists on different days.

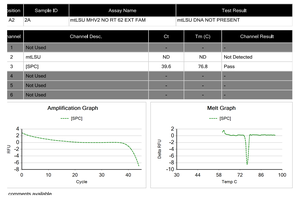
**Analytical stability:**

- Stability studies were performed using positive control material spiked in BAL and stored at 2-8°C for eight days. Results showed that DNA was detected by the PCR assay under these storage conditions.

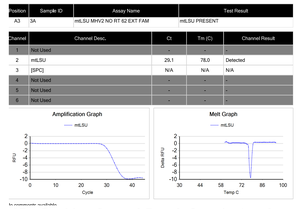
**Analytical specificity:**

- Finally, a variety of fungal, bacterial organisms and viruses were tested to establish specificity. There was no cross-reactivity observed with the *P. jirovecii mtLSU* primers using 11 different fungi, 8 different bacteria, and 13 viruses.

**Figure 2. Sample Data from ARIES Run**




**2A. Negative Control Result** Sample data from ARIES® instrument with a negative control illustrating the amplification curve and melt temperature for *P. jirovecii*.




**2B. Positive Control Result** Sample data from ARIES® instrument with a positive control illustrating the amplification curve and melt temperature for *P. jirovecii*.

## Instrument

Instrument: ARIES® is an *in vitro* diagnostic medical device for detection of nucleic acids by fluorescence based PCR (Figure 3).

Instrument Preparation: 6 µL of Primers mix were added to each ReadyMix tube, attached to the cassettes and loaded on the ARIES®. After loading, the reaction then proceeded to completion in a hands-free manner. The Luminex ARIES® generates RT-PCR amplification and melt curves for the target, with resulting Ct and Tm values calculated for both the sample processing control and the target.

**Figure 3. Luminex ARIES® instrument**

## Discussion and Conclusion

Sensitivity, specificity, accuracy, precision and stability studies were completed, indicating the utility for diagnostic testing in suspect patients.

The technology is based on the unique Multicode ® base pair, isoC: isoG. Multicode ® bases hydrogen bond only with each other and is site-specifically incorporated during amplification.

Automated extraction of nucleic acid and PCR is completed in 2 hours, involving minimal technologist time in sample preparation. The ARIES® system is easily adapted to additional target sequences.

The method allows for rapid and sensitive detection of *Pneumocystis* pneumonia infections with *P. jirovecii* using clinical specimens, while detecting potential sample inhibition.

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