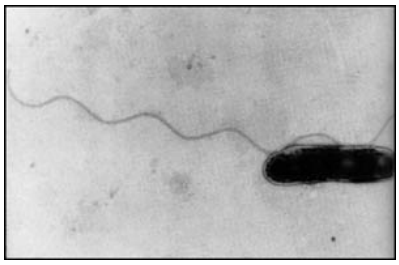
**Legionella species**
**(Legionnaires' disease)**
**Detection with real time PCR reagents**



**Overview:**



Legionella polar flagella
(1)

Legionella infection causes a spectrum of illness, from the brief self-limited Pontiac fever to the devastating, lethal Legionnaire's disease.

Legionnaires' disease is a life-threatening pneumonia with a low attack rate, about 5%, and an incubation period of 2 to 10 days. Non-specific symptoms initiate the illness with lethargy, headache, fever, chills, myalgia, and diarrhea. Initially respiratory symptoms are lacking, but later a dry, hacking cough starts and becomes productive after a few days. Shortness of breath and chest pain occurs in a third.

Pontiac fever is an acute, self-limited febrile illness with a high attack rate and short 36-hour incubation.

Legionella is thermophilic and grows well in water, accounting for its environmental source in cooling towers, hot water systems, showers, respiratory therapy devices, whirlpools, spas, and water misters in grocery stores. Person to person spread is not a problem. The organism is acquired by airborne transmission. Illness is more common in the over 50-age group and in the immune compromised. This does include children, newborns receiving respiratory therapy, surgical patients, and the immune suppressed child.

This gram-negative non-acid fast non-capsulated rod is aerobic and does not hydrolyze gelatin or produce urease and is non-fermentative. Legionella may be found ubiquitously in soils and aqueous environments. They require iron and cysteine for isolation. They are motile with 1 to 3 polar flagella.

Legionella genus contains over 30 species. L. pneumonphila is the most common pathogenic species; L. micdadei is the second most common pathogen. L. longbeachae and gormannii and dumoffii and bozemanii account for some of the other pathogenic strains. There are 14 different serovars of L. pneumonphila.

July 21, 1976 at the Bellevue Stratford Hotel, Philadelphia the American Legion convention started. The next day some became ill, and within four days some began to die of pneumonia. 221 were infected and 34 died. Dr Joseph McDade, a rickettsiologist at the CDC and his team discovered the cause.

…"McDade had been asked to rule out Q-fever as a causative agent. Thus, in his search for the Legionnaires' Disease bacteria, he used the same techniques for isolating rickettsial pathogens. Guinea pigs that were inoculated with material from victims all died of a febrile illness. Peering into a microscope, McDade observed several cocci and several small bacilli, none of which seemed significant at the time. He decided to innoculate embryonated eggs with suspensions of the guinea pig spleen tissue that had been treated with antibiotics to inhibit the growth of contaminating bacteria. The eggs grew nothing, causing McDade to suspect that the rickettsiae had been killed along with the rest of the microbes.

28 December, 1976, saw a year-end party that Joseph McDade did not particularly desire to attend. Uncomfortable with the crowd, he decided to return to the laboratory to wrap up some things before the end of the year. He took out his guinea pig slides again and put them under the microscope for review. This time he noticed something he had not noticed before: a cluster of the bacilli he'd seen previously were engulfed by a white cell.

Excited, McDade and his team of researchers plunged into the mystery afresh. New batches of innoculated eggs were prepared, this time without the antibiotics. Guinea pigs were then injected with yolk sac extracts, and developed the typical symptoms of Legionnaires' Disease. Blood samples taken from the survivors were mixed with the yolk sac isolates, and subsequently reacted. The Legionnaires' Disease bacteria had been found"…(2)

Since Legionella is found in water, care must be taken in processing specimens and DNA purification, such that they do not become contaminated with DNA from the environment.

Primers and probe are available for amplification and detection of Legionella species. The Legionella species primers are specific for a 212 bp portion of the 16S gene. The Borrellia detection probe is a molecular beacon. (3)

……

**Products**

| Products | Catalog No. | Quantity |
|---|---|---|
| **Legionella 16S FAM-BHQ1 Primers-probe** | **PP5400** | **0.055ml** |

*Store at –20ºC. 25-20 µl reactions*
*Typical use: Add 2X master mix and DNA, then thermal cycle.*
*Attostar reagent contains primers and probe. Detect at 510 nm.*

| | | |
|---|---|---|
| **AttoMaster 2X Mix for qPCR** | **AM10** | **1.25 ml** |

*Store at –20ºC. 125-20 µl reactions*
*Contains Taq polymerase (requires heat activation), dNTPs (0.4 mM) with optimal dUTP to dTTP ratio, heat labile UDG, Mg(6 mM), and buffer.*
*Typical use: Add Attostar Primer-probe, DNA, and then thermal cycle.*

| | | |
|---|---|---|
| **Legionella 16S Plasmid** | **PLAS5400** | **0.25ml** |

*Store at –20C.*
*Typical use: make serial 10 fold dilutions in TE for standard curve, diluting 5 µl into 45µl TE.*

……

**Detection of Legionella 16S DNA:**

……

**Thermal cycle conditions for PCR reactions on RotorGene***

*Similar cycle conditions and reaction volumes may be used on many other thermal cyclers.

25ºC 10 min (UDG treatment time)
95ºC 120 sec (activation for AttoMaster polymerase)
40 cycles
95ºC 15 seconds
60ºC 30 seconds RotorGene Channel Setup FAM/Sybr, Cy5; Gain 7
72ºC 30 seconds

FAM/Sybr has a source of 470nm and Detector 510nm (LightCycler use F1)
Cy5 has a source of 625nm and Detector 660hp nm
Quasar 670 has the same fluorescent absorption and emission as Cy5.

……

**Thermal cycle conditions for PCR reactions on LightCycler**

25ºC 10 min (UDG treatment time)
95ºC 120 sec (activation for AttoMaster polymerase)
40 cycles
95ºC 15 seconds
60ºC 30 seconds acquire fluorescent signal on F1 gain =1
72ºC 30 seconds
40ºC 30 seconds cool

……

**Use of the Legionella 16S plasmid:**

Dilute the plasmid in TE to prepare a standard curve. Common dilutions would be 10-fold from 200 to 0.002pg/ml. The 0.02 pg/ml plasmid dilution contains 12 copies of plasmid in 2 μl.

……

## Attostar Primers/probe PP5400 RotorGene-PCR 20µl reactions

| | RotorGene Reagents needed for 20 ul PCR final reaction tube volumes | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Reaction tube number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
| Attostar Primer-Probe FAM labeled (10X) | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 | µl |
| Master mix (2X) | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 | µl |
| Dispense 12 ul / reaction tube | | | | | | | | | | | |
| Add 8 ul DNA / reaction tube | | | | | | | | | | | |

## Attostar Primers/probe PP5400 LightCycler-PCR 20 µl reactions

| | LightCycler Reagents needed for 20 ul PCR final reaction tube volumes | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Reaction tube number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
| Attostar Primer-Probe (10X) | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 | µl |
| Master mix (2X) | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 | µl |
| BSA 1 mg/ml | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | µl |
| Dispense 13 ul / reaction tube | | | | | | | | | | | |
| Add 7 ul DNA / reaction tube | | | | | | | | | | | |

……

**Extraction / amplification control with T4 bacteriophage**

Adding T4 bacteriophage (BAC130) to the sample provides DNA for extraction, amplification, and reaction condition PCR controls.

When added to a sample, T4 adds a known amount of DNA. The T4 DNA can then be extracted, amplified, and detected as a control. T4 controls for the efficiency of DNA extraction, the presence of PCR amplification inhibitors, intact amplification reagents ( DNA polymerase, buffer, dNTPs), and instrument function (thermal cycling and fluorescent detection system).

The T4 DNA may be detected in a separate PCR reaction (using FAM labeled T4 probe in PP100). Or the T4 DNA and test organism DNA may be detected using a multiplex reaction (using Quasar 670 labeled T4 probe in PP160 and FAM labeled test organism probe).

**Brief procedure for use of T4 as extraction and amplification control:**

- Add 5µl T4 bacteriophage to the sample. Proceed with DNA extraction.
  Dilutions of the bacteriophage may be made to give a final PCR Ct value that is about 35. At this dilution, the phage is more sensitive, i.e. more likely, to detect a poor extraction or the presence of PCR inhibitors in the reaction.

  Please refer to the BAC130, PP100, and PP160 product literature.

**T4 multiplex reaction (PP160)**

**RotorGene Multiplex PCR 20µl reactions**

| | Multiplex Reagents needed for 20 ul PCR final reaction tube volumes | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Reaction tube number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
| Attostar Primer-Probe PP160 | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 | µl |
| Primer-Probe mix for TEST organism | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 | µl |
| 2 x master mix | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 | µl |
| Dispense 14 ul / reaction tube | | | | | | | | | | | |
| Add 6 ul DNA / reaction tube | | | | | | | | | | | |

| Reaction tube number | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Attostar Primer-Probe PP160 | 22 | 24 | 26 | 28 | 30 | 32 | 34 | 36 | 38 | 40 | µl |
| Primer-Probe mix for TEST organism | 22 | 24 | 26 | 28 | 30 | 32 | 34 | 36 | 38 | 40 | µl |
| 2 x master mix | 110 | 120 | 130 | 140 | 150 | 160 | 170 | 180 | 190 | 200 | µl |
| Dispense 14 ul / reaction tube | | | | | | | | | | | |
| Add 6 ul DNA / reaction tube | | | | | | | | | | | |

……

**Reference:**


1. http://www.diseaseworld.com/legion.htm
2. http://www.bbc.co.uk/dna/hub/A882371
3. http://www.molecular-beacons.org/Introduction.html




For technical support, contact Attostar@Attostar.com

10-19-07