



Multiplexed, PCR Detection of *Mycoplasma pneumoniae*, *Chlamydia pneumoniae* and *Legionella pneumophila* in Broncho-alveolar Lavage (BAL) and Oropharyngeal (OP) Swab Specimens



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Introduction

Mycoplasma pneumoniae (Mpn), *Chlamydia pneumoniae* (Cpn) and *Legionella pneumophila* (Lpn) can cause both epidemic and endemic occurrences of acute respiratory disease and are responsible for 25-40% of cases of community acquired pneumonia. We developed and evaluated a multiplexed, real-time PCR assay for the detection of these agents in oropharyngeal swabs (OP) and broncho-alveolar lavage (BAL) specimens on the Luminex ARIES® instrument. This is a fully integrated, walk-away instrument for real-time PCR.

Methods

OP, collected in Universal Transport Medium (UTM), and BAL specimens were spiked with 10-fold dilutions of Mpn, Lpn or Cpn. Two hundred ul of each specimen plus primer-containing ReadyMix was added to the cassettes and then loaded onto the ARIES® instrument.

Primer pairs were obtained from Luminex. Internal (MHV 5) control primers were used to amplify the sample processing control (SPC) in each cassette. ReadyMix and cassettes were purchased from Luminex and contained all reagents for extraction, purification, amplification and detection.

The ARIES® instrument generates a Ct value, including one for the SPC, as well as a confirmatory melt temperature (Tm) value, for each reaction.

Instrument: ARIES® is an *in vitro* diagnostic medical device for detection of nucleic acids by fluorescence based PCR (Figure 1).



Figure 1. Luminex ARIES® instrument

Instrument Preparation: 8 µL of Primer mix was added to each ReadyMix tube, then attached to the cassette and loaded on the ARIES® instrument. After loading, the reaction then proceeded to completion in a hands-free manner. The Luminex ARIES® generates PCR amplification and melt curves for each target, with a resulting Ct value calculated for both the sample processing control and the targets (Figures 2 and 3).

Limit of detection (LOD) for Mpn, Cpn and Lpn, using data from both specimen types, were 200 cfu/mL, 200 cfu/mL and 20 cfu/mL, respectively. Twenty-three of 23 Mpn-positive samples tested positive (either OP or BAL specimens), giving a 100% **accuracy**. All Cpn-positive specimens (either OP or BAL specimens) were likewise positive using the ARIES® assay (100% **accuracy**) and 20 Lpn-positive samples (all BAL specimens) were confirmed positive using the ARIES® system (100% **accuracy**). All previously negative samples were confirmed negative using the ARIES® assay. **Reproducibility** of the resultant Ct values did not change over the course of the different testing period by different operator. No cross-reactivity was seen during any of the **specificity** testing. **Stability** of the collected specimen, in terms of reproducibility of the resultant Ct value, did not change over the course of the 8-day testing period. Since November 2016, a total of 836 patient specimens have been processed in our reference laboratory, with five positive Mpn and one positive Lpn. No specimens were positive for Cpn

Results

Cassette	Sample	Mycoplasma pneumoniae		Chlamydia pneumoniae		SPC		Legionella pneumophila		Ct on FOCUS Assay
		FAM CT	FAM TM	AP25 CT	AP25 TM	AP59 CT	AP59 TM	AP98 CT	AP98 TM	
1	PATIENT-1 (OP)	39.8	77.5			37.4	83.7			37.1
2	PATIENT-2 (OP)	38.7	77.6			33.2	84	39.4		35.1
3	PATIENT-3 (OP)	37.3	77.6			38.1	84	37.4		31.2
4	PATIENT-4 (OP)	39.7	77.6			36.6	83.8	38.5		35.5
5	PATIENT-5	37	77.6			33	83.6			27.6
6	PATIENT-6 (OP)	38.2	77.4			38.5	83.8	38.9		31.9
7	PATIENT-7 (OP)	38.9	77.3			34.9	84			30.4
8	PATIENT-8 (OP)	38.7	77.5	39.9		33.9	84	36.8		37.4
9	PATIENT-10 (OP)	32.5	77.4			32.1	83.8			31.1
10	PATIENT-11 (OP)	38.7	77.4			38.3	83.7			30.4
11	PATIENT-13	39.5	77.5			36.4	83.9	38		32.2
12	PATIENT-14	38.1	77.5			35.9	83.8			29.7
13	PATIENT-15	30.7	77.5			32.4	83.8			27.4
14	PATIENT-16	39.8	77.4			35	83.8			35.5
15	PATIENT-17	30	77.6			36.9	83.7			24.4
16	PATIENT-18	39.5	77.7			33.2	83.4			33.2
17	PATIENT-20	38.8	77.6			33.3	83.8			33.3
18	PATIENT-21	29.9	77.3			37.3	83.8	39.1		22.4
19	PATIENT-22	39.3	77.6			33.4	83.9			37.2
21	PATIENT-23 (OP)	34	77.6			32.6	83.8			34.6
22	PATIENT-24 (BAL)	29.7	77.5			39.9	83.9			22.4
23	PATIENT-25	38.9	77.6			33.3	83.7	39		36.9

Table 1: Mpn Accuracy : Twenty-three MPN positive samples were positive out of 23 samples with ARIES assay, giving a 100% accuracy.

Cassette	Sample	Mycoplasma pneumoniae		Chlamydia pneumoniae		SPC		Legionella pneumophila		Ct on FOCUS Assay
		FAM CT	FAM TM	AP25 CT	AP25 TM	AP59 CT	AP59 TM	AP98 CT	AP98 TM	
1	PATIENT-1 DNA OP			25.2	80.6	40	83.7			21.4
2	PATIENT-2 DNA OP			25.4	80.6	39.1	83.4	40		21.4
3	PATIENT-3 DNA OP			26.1	80.6	38.3	83.7			22
4	PATIENT-4 DNA OP			25.4	80.7	39.2	83.5			22.1
5	PATIENT-5 DNA OP			23.9	80.6	39.2	83.5	39.4		21.5
6	PATIENT-6 DNA OP			25.1	80.7	37.3	83.5	39		21.7
7	PATIENT-7 DNA OP			24.6	80.7	38.3	83.5	38.9		22.3
8	PATIENT-8 DNA OP			24.7	80.7	36.4	83.5	38.2		22.2
9	PATIENT-9 DNA OP			24.7	80.7	35.2	83.6	36.2		22.2
10	PATIENT-10 DNA OP			25.4	80.7	37.1	83.4			21.3
11	PATIENT-11 DNA OP			24.6	80.7	36.6	83.6			21.6
12	PATIENT-1 DNA BAL			25.3	80.8	39.3	83.6	39.7		21.7
13	PATIENT-3 DNA BAL			25.6	80.7	39.3	83.7	39.6		22.2
14	PATIENT-4 DNA BAL			25.2	80.6	39.8	83.7	40.1		22.2
15	PATIENT-5 DNA BAL			23.9	80.5	40	83.6			21.1
16	PATIENT-6 DNA BAL			24.5	80.6	37.4	83.5			21.6
17	PATIENT-7 DNA BAL			24.7	80.7	37.7	83.6			21.4
18	PATIENT-8 DNA BAL			24.6	80.5	36.8	83.7	37.6		21.4
19	PATIENT-9 DNA BAL			25.1	80.7	37.8	83.5			22
20	PATIENT-10 DNA BAL			25.5	80.7	37.2	83.7	39.2		22.1
21	PATIENT-11 DNA BAL			25.7	80.7	39.1	83.5			21.5
22	PATIENT-12 (BAL UTM)			38.2	80.7	34.2	84			34

Table 2: Cpn Accuracy - All Cpn-positive specimens (either OP or BAL specimens) were likewise positive using the ARIES® assay (100% accuracy).

Cassette	Sample	Mycoplasma pneumoniae		Chlamydia pneumoniae		SPC		Legionella pneumophila		Ct on FOCUS Assay
		FAM CT	FAM TM	AP25 CT	AP25 TM	AP59 CT	AP59 TM	AP98 CT	AP98 TM	
1	PATIENT-1 (small spot, mucus in BAL)					34.5	84.2	39	71	33.2
2	PATIENT-2 (Bloody and mucous BAL)					36.8	83.9	29.7	71.3	25
3	PATIENT-3 (Clear BAL)	26.1		26.3		33.8	83.8	34.6	71.1	30.7
4	PATIENT-4 (Clear BAL)					35.1	83.8	34.7	71.3	29.7
5	PATIENT-5 (Clear, slightly lumpy BAL)					32.9	83.8	31.9	71.5	27
6	PATIENT-6 (small spot of mucus in BAL)					34.9	84	38.6	71.2	34.5
7	PATIENT-7 (Clear BAL)					33.1	83.7	38.6	71.6	32
8	PATIENT-8 (Clear BAL)					35.6	84	38.7	71.3	34.5
9	PATIENT-9 (Clear BAL)	34.1				35.3	84.1	36.1	71.6	32.7
10	PATIENT-10 (Clear BAL)					32.4	83.8	35.5	71.5	32.2
11	PATIENT-11 (Clear BAL)					34.4	83.9	34.8	71.5	32.3
12	PATIENT-12 (Clear BAL)					35.2	83.9	34.7	71.3	30.7
13	PATIENT-13 (Clear BAL)					34.7	83.9	33.5	71.2	29.8
14	PATIENT-14 (Clear BAL)					33	83.8	38.1	71.4	31.3
15	PATIENT-15 (Bloody BAL)					33	83.8	35.2	71.4	30.8
16	PATIENT-16 (Clear BAL)					33.4	83.9	30.1	71.6	29
17	PATIENT-17 (Clear BAL)					32.9	83.8	36.8	71.5	31.7
18	PATIENT-18 (CLEAR BAL)					34.4	83.9	36.7	71.4	32
19	PATIENT-19 (CLEAR BAL)					36.1	83.7	36.9	71.4	31
20	PATIENT-20 (CLEAR BAL)					34.3	83.8	34	71.6	33.2

Table 3: Lpn Accuracy - 20 Lpn-positive samples (all BAL specimens) were confirmed positive using the ARIES® system (100% accuracy).

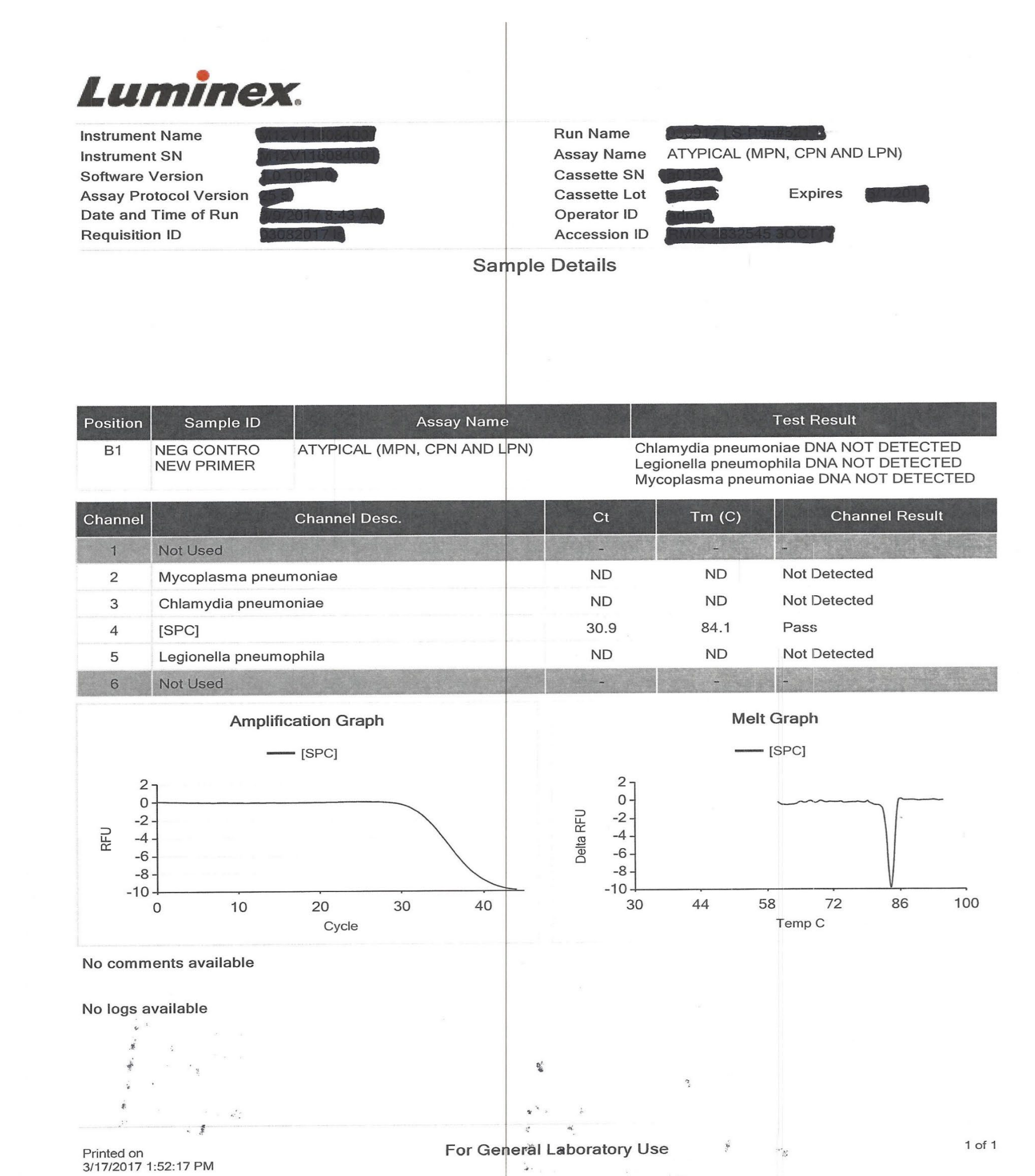


Figure 2: Negative control

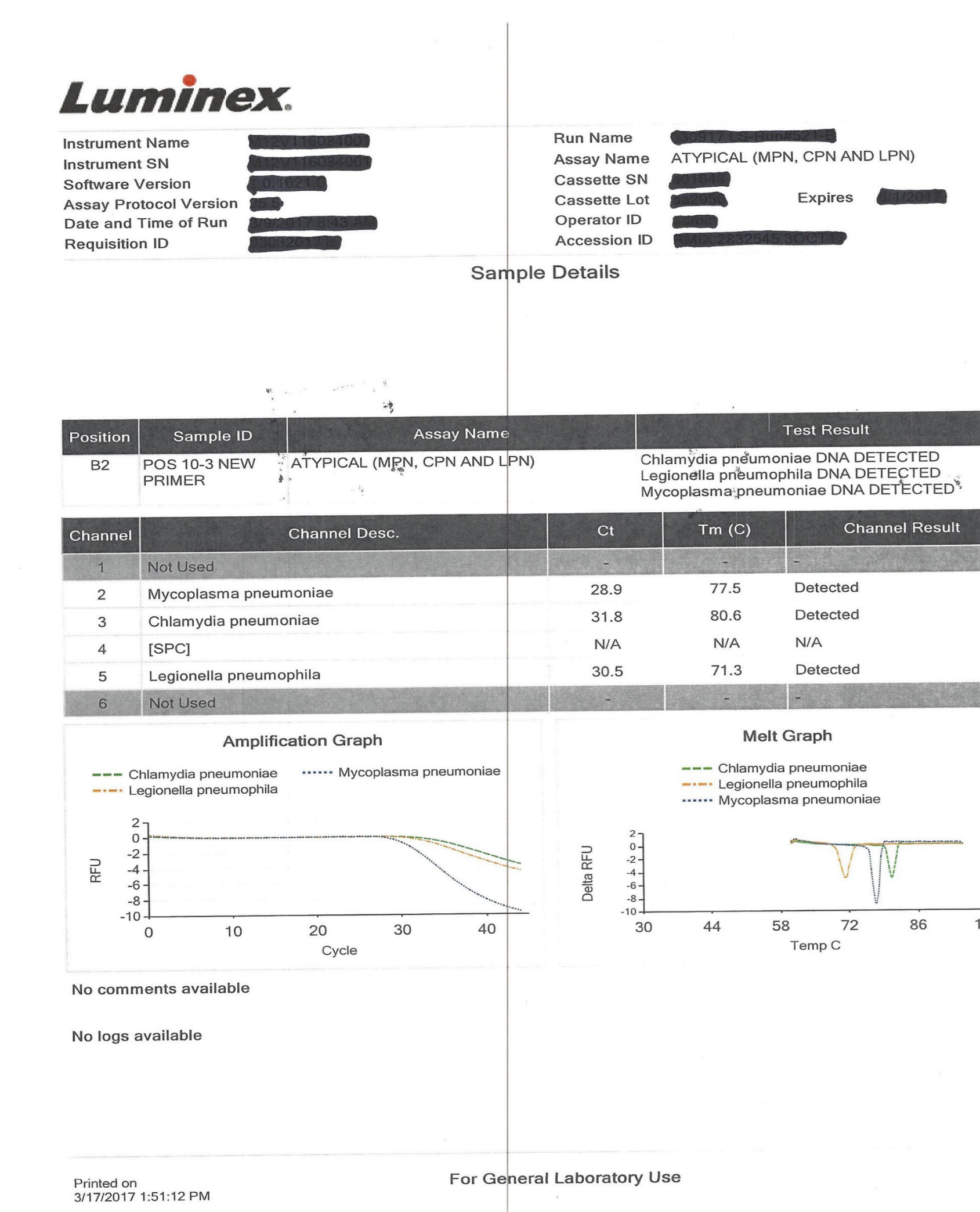


Figure 3: Positive control

Conclusion

The availability of a multiplexed, real-time PCR assay greatly enhances the ability to rapidly diagnose respiratory infections caused by these agents.

The Luminex ARIES® system involves minimal technologist time in sample preparation, and the cassettes contain all necessary reagents for extraction, amplification and detection.

The ARIES® software generates the curves, assigns the corresponding Ct values and confirmatory Tm values. This system is easily adapted to detect additional target sequences.

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