CHAPTER IV
RESULTS

1. Extraction of recombinant plasmid containing *C. trachomatis* (pCHL1) and *N. gonorrhoeae* (pJD1) DNA from the transformed *E. coli*

After extraction and purification, the DNA mixture containing recombinant plasmids of *N. gonorrhoeae* (pJD1) or *C. trachomatis* (pCHL1) were tested by conventional PCR. As expected, DNA fragments of 152 bps *from N. gonorrhoeae* and 108 bps from *C. trachomatis* were obtained (Figure 9).

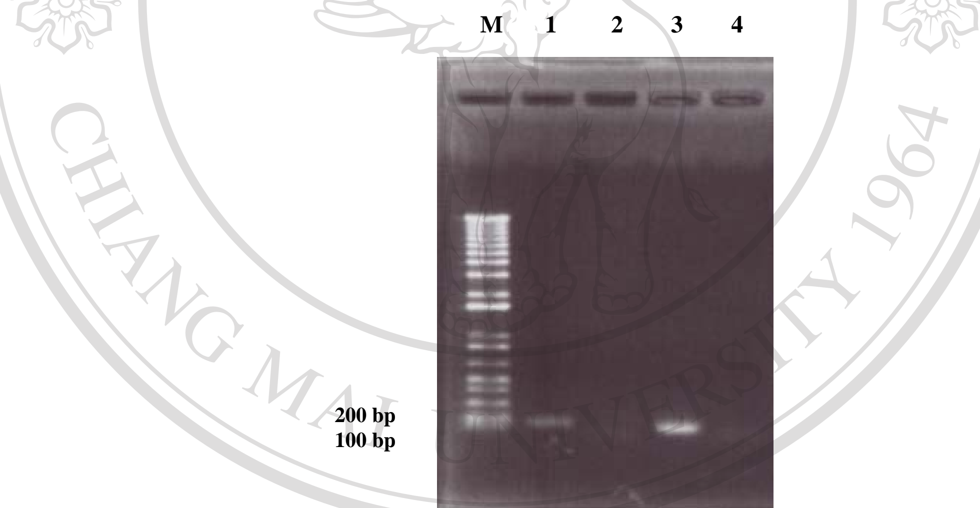

Figure 9. PCR amplified products of *N. gonorrhoeae* (pJD1) and *C. trachomatis* (pCHL1) plasmid DNA. Lane M demonstrates DNA marker, lane 1 shows PRC amplicons of pJD1 plasmid at 152 bps and lane 2 represents PRC amplicons of pCHL plasmid at 108 bps. Lanes 2 and 4 were the negative controls of each PCR reaction.

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The purified plasmid DNA was then quantified by spectrophotometry. The quality of DNA was determined by the ratio between the O.D. at 260 and 280. A ratio of greater than or equal to 1.8

indicates a high quality of DNA preparation. Performance of plasmid DNA from each preparation is shown in Table 3.

Table 3 The results of recombinant plasmid DNA preparation.

Plasmid DNA	O.D.260	O.D.280	O.D.260 / O.D.280 ratio	Dilution factor	DNA ng/ul
C. trachomatis	0.499	0.237	2.105	100	2,495
N. gonorrhoeae	0.760	0.398	1.910	50	1,900

2. Determination optimal concentration of probe and primer used in the assay.

2.1. In-house Taqman-based multiplex Real Time PCR for detection of *N. gonorrhoeae* and *C. trachomatis* plasmid DNA

In order to perform multiplex real time PCR, primer and probe should be adjusted to the optimal concentrations and ratio. Four concentrations of primer and probe were selected to determine the optimal concentration to be used in this test. The reaction was performed in duplicate; the average cycle threshold results are shown in Tables 4 and 5.

The optimal condition was defined as the lowest concentration ratio of primers and probe that could detect the same amount of target DNA at the lower cycle threshold (Ct). Primer and probe ratios of 200nM/100nM and 200nM/200nM gave slightly similar results with cycle threshold (Ct) values at 38.50 and 38.05, respectively (Table 4). When compared to the ratios 400nM/300nM and 400nM/200nM, which gave the Ct value at 36.42 and 34.40 respectively, although both concentration ratios yielded a better Ct value, the 400nM/300nM ratio employed 0.5 times as much probe compared to the 400nM/200nM ratio. However, there were relatively similar cycle thresholds in all concentrations of target DNA.

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Thus, the optimal concentration of primer and probe were considered to be 400nM and 200nM, respectively.

Table 4 Cycle thresholds (Ct) of Real Time PCR for detecting *N. gonorrhoeae* plasmid DNA under varying primer and probe concentrations.

Primer/Probe concentrations	Plasmid DNA		
	50 copies	5,000 copies	$5x10^5$ copies
200 nM/100nM	Ct 38.50	Ct 28.75	Ct 23.34
200nM/200nM	Ct 38.05	Ct 25.92	Ct 20.47
400nM/200nM	Ct 36.42	Ct 27.98	Ct 20.54
400nM/300nM	Ct 34.40	Ct 26.40	Ct 19.32

For detection of *C. trachomatis* (Table5), at a target DNA concentration of 50 copies per reaction, the primer and probe ratio of 200nM/100nM and 200nM/200nM yielded the best results of Ct value at 39.64 and 38.08, respectively, which is nearly at the limit of the PCR cycle (40 Ct). Concentration ratios of 400nM/200nM and 400nM/300nM yielded relatively lower Ct values of 37.12 and 21.97 respectively. However, , when performing a multiplex reaction the concentration of probe mixtures exceeded the upper limit concentration of 250 nM (133). Taken together, the optimal concentrations of primer and probe were 400nM and 200nM per reaction.

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Table 5 Results of Real Time PCR in detecting *C. trachomatis* plasmid DNA by varying primer and probe concentrations.

Primer/Probe concentration	Plasmid DNA		
	50 copies	5,000 copies	$5x10^5$ copies
200 nM/100nM	Ct 39.64	Ct 29.58	Ct 23.11
200nM/200nM	Ct 38.08	Ct 25.19	Ct 20.83
400nM/200nM	Ct 37.12	Ct 28.56	Ct 22.00
400nM/300nM	Ct 21.97	Ct 23.23	Ct 18.38

2.2. In-house Taqman-based Real Time PCR for detection of *N. gonorrhoeae* Porin A pseudogene DNA

Three concentrations of primers and probe were used to optimize the Real Time PCR reaction. The reaction was performed in duplicate at an *N. gonorrhoeae* genomic DNA concentration 500 pg.Table 6. As described earlier, the lowest concentrations of primer and probe (200nM/200nM) yielded a Ct value of 24.84, which was similar to those obtained from higher primer/probe concentrations. However, the amplification curve gave relatively low fluorescence intensity due to early exhaustion of primer and/or probe, making interpretation of the results difficult. When compared to Ct values for concentration ratios of 800nM/200nM and 400nM/200nM, the Cts values were 23.24 and 23.61 respectively. Thus, we decided to use the primer and probe concentration ratio at 400 nM /200nM per reaction in later experiments.

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Table 6 Demonstration the cycle threshold (Ct) results of Real Time PCR reaction in detecting *N. gonorrhoeae* Porin A pseudogene DNA by varying primer and probe concentrations.

concentrations	Probe 300 nM	Probe 250 nM	Probe 200 nM
Primer 200 nM	24.49	24.56	24.84
Primer 400 nM	23.26	23.52	23.61
Primer 800 nM	23.07	23.06	23.24

3. Determination the sensitivity of In-house Taqman-based Real Time PCR

3.1. In-house Taqman-based multiplex Real Time PCR for detection of *N.gonorrhoeae* and *C. trachomatis* plasmid DNA.

Serial 10-fold dilution of recombinant plasmid DNA from 1 pg/μl to10 ag/μl was used to determine the sensitivity of the assays. The assay was performed in duplicate and each experiment was repeated 3 times.

Five microliters of each plasmid DNA dilution were amplified by Taqman-based multiplex Real Time PCR that had been optimized as described above. The sensitivity of the assay was determined as the lowest concentration of plasmid DNA which can be detected at 100 percent within 40th cycle Tables 7 and 8).

For detection of *N. gonorrhoeae* the lowest concentration of plasmid DNA (1 fg/μl) could be detected in all experiments At a DNA concentration of 100 ag/μl, the assay could be detected in only 1 of 3 repeated experiments Thus, the sensitivity of the assay in detecting the *N. gonorrhoeae* plasmid DNA was defined as 1 fg/ul.

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Table 7 Results of In-house Taqman-based multiplex Real Time in detection of *N. gonorrhoeae* recombinant plasmid DNA

Plasmid mixture concentration	Cycle threshold (Ct)		
	1st	2nd	3 rd
1 pg/µl	27.92	26.85	29.22
100 fg/µl	30.73	29.46	32.15
10 fg/µl	36.12	33.98	35.49
1 fg/µl	40.00	38.00	38.13
100 ag/µl	35.25	Not detectable	Not detectable
10 ag/µl	Not detectable	Not detectable	Not detectable

For detection of *C. trachomatis* 8, the lowest concentration of plasmid DNA that could be detected at 100 % was at 1 fg/µl (equivalent to 308 copies of recombinant plasmid per µl). Thus, the 100 % sensitivity of the assay in detecting *C. trachomatis* recombinant plasmid DNA was determined to be 1 fg/µl.

Table 8 Results of In-house Taqman-based multiplex Real Time PCR in detection of *C. trachomatis* recombinant plasmid.

Plasmid mixture concentration	Cycle threshold (Ct)		
	1st	2nd	3 rd
1 pg/µl	27.18	24.58	26.15
100 fg/µl	30.41	26.61	29.73
10 fg/µl	34.71	32.92	34.41
1 fg/µl	36.45	35.14	36.41
100 ag/µl	Not detectable	36.40	35.66
10 ag/µl	Not detectable	Not detectable	Not detectable

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3.2 In-house Taqman-based Real Time PCR for detection of Porin A pseudogene DNA of *N. gonorrhoeae*

Serial 10-fold dilution of *N. gonorrhoeae* genomic DNA from100 pg/µl to 1 fg/µl was used to determine the sensitivity of the assays. The assay was performed in duplicate and each experiment was repeated 2 times.

Five microliters of each DNA concentration were amplified by Taqman-based Real Time PCR specific to the Porin A pseudogene DNA.. As described previously, the lowest concentration of target DNA that could be detected within 40 cycles was considered as the sensitivity of the assay. In this study, the lowest concentration of DNA 10 fg/µl that could be detected at Ct 37.08 and 37.58 (average Ct= 37.33), while at lower concentrations (1 fg/ul) the signal could not be detected. Thus, the sensitivity of the assay was determined as 10 fg/µl.

Table 9 Results of In-house Taqman-based Real Time PCR for Porin A pseudogene DNA of *N. gonorrhoeae*.

N. gonorrhoeae DNA concentration	Cycle threshold (Ct)	
	1st	2nd
100 pg/µl	24.31	24.13
10 pg/µl	28.23	28.26
1 pg/µl	31.25	31.38
0.2 pg/µl	33.46	34.04
100 fg/µl	34.71	34.21
10 fg/µl	37.08	37.58
1 fg/µl	Not detectable	Not detectable

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4. Determination specificity of In-house Taqman-based multiplex Real Time PCR for detection of *N. gonorrhoeae* and *C. trachomatis* and In-house Taqman-based Real Time PCR for detection of *N. gonorrhoeae* porin A pseudogene DNA.

To determine the specificity of above In-house Taqman-base Real Time PCR methods, primers and probe from each method were tested in two experiments.

4.1. Specificity testing by using computer software.

Several DNA sequences of related microorganisms, including commensal and pathogenic organism of the urogenital organs and the cryptic plasmid pJD1 (NC_001377), pCHL1 (NC_001372) and *N. gonorrhoeae* Porin A pseudogene (AJ223449), were downloaded from GenBank (http://www. genome.ou.edu) and used to align with each of the specific primer pairs and probes by using computer software such as Primer Premier 5, AmplifX version 1.37 and BioEdit. Moreover, other pathogenic and non-pathogenic microorganismal DNA sequences that ar efrequently found in the human upper respiratory tract were included for testing as well. The list of microorganisms tested is shown in Table 10.

Both primers and probes used in the multiplex real time PCR assay were highly specific to their expected sequences, for example, the primer RTCT-1-RTCT-2 and RTCT probe used for *C. trachomatis* detection aligned only with cryptic plasmid pCHL1 sequences of *C. trachomatis*, while the primer RTGC-1-RTGC-2 and RTGC probe were bound to cryptic plasmid pJD1sequence of *N. gonorrhoeae*. No cross binding with other DNA sequences from the microorganisms listed in Table 10 was observed. Primers and probe used for the *N. gonorrhoeae* Porin A pseudogene detection were also tested. These were shown to bind only to the Porin A pseudogene from *N. gonorrhoeae*. No cross binding was observed to other microorganisms, including the Porin A pseudogene from

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N. meningitides or other commensal species. These results indicate that all primers and probes used in both assays were highly specific to their sequences .

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Table 10 List of DNA sequences from microorganisms used as target DNA for specificity testing of primers and probes by using computer software.

No.	Micro-oranism	Accession No.
1.	*C. albicans* chromosome 7	AP006852
2.	*C. albicans* SC5314 mitochondrion	NC_002653
3.	*C.diphtheriae* NCTC 13129	NC_002935
4.	*C. psittaci* plasmid pCpA1	X62475
5.	*C. trachomatis* A/HAR-13	NC_007429
6.	*C. trachomatis* D/UW-3/CX	AE001273
7.	*E. coli* CFT073	AE014075
8.	*E. coli* CFT073	NC_004431
9.	*E. coli* E24377A	NC_009801
10.	*E. faecalis*	NC_004668
11.	*E. faecalis*	NC_008445
12.	*G. vaginalis*	EF194095
13.	*K. pneumoniae* strain NK29	EF382672
14.	*K. pneumoniae* subsp. *pneumoniae*	NC_009651
15.	*K. pneumoniae* subsp. *pneumoniae*	NC_009653
16.	*L. acidophilus* NCFM	NC_006814
17.	*L. lactis* subsp. *lactis*	NC_002662
18.	*L. lactis* subsp. *lactis*	NC_009751
19.	*L. rhamnosus*	DQ906101
20.	*M. catarrhalis* ATCC 25238	U10876
21.	*M. genitalium* G37	NC_000908
22.	*N. gonorrhoeae* porA pseudogene strain FA1090	AJ223447
23.	*N. gonorrhoeae* porA pseudogene,strain MS11	AJ223446
24.	*N. gonorrhoeae* porA pseudogene, strain NCTC8375	AJ223448
25.	*N. gonorrhoeae* PorB, strain 252	AY765457
26.	*N. gonorrhoeae* PorB, strain DGI 34	AY765460
27.	*N. gonorrhoeae* PorB, strain DGI 61	AY765461
28.	*N. gonorrhoeae* PorB, strain DGI 70	AY765458
29.	*N. lactamica* plasmid pNL18	DQ229165
30.	*N. meningitidis* plasmid pJS-B	NC_004758
31.	*N. meningitidis* sero-group C FAM18	AM421808
32.	*N. meningitidis* strain FAM18	NC_008767
33.	*N. meningitidis* strain MC58	NC_003112
34.	*N. meningitidis* strain Z2491	NC_003116
35.	*N. mucosa* porin precursor	AF121872
36.	*N. polysaccharea*	Y09309
37.	*N. polysaccharea* 16S ribosomal RNA	L06167
38.	*N. sicca* gene for porin	X65461
39.	*S.aureus* subsp. *aureus*	NC_002758
40.	*S. aureus* subsp. *aureus*	NC_002952
41.	*S. pyogenes* MGAS8232	NC_003485

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4.2.Specificity testing by performing the real time PCR amplification with related micro organisms.

Specific and non-specific target DNA extracted from the organisms in table 11 was used to test the specificity of the assays. *N. gonorrhoeae* (pJD1) and *C. trachomatis* (pCHL1) plasmid DNA extracted from the transformed *E. coli* were included as positive controls. The positive signal was observed only from the positive control DNA, and DNA extracted from both clinically isolated and standard strains of *N. gonorrhoeae.* All DNA samples extracted from either *Neisseria* species, including *Neisseria meningitidis* and other urogenitally related pathogenic and non- pathogenic micro organisms, gave negative results. It is interesting that our assays did not detect DNA samples extracted from *T. vaginalis, G. vaginalis* and *C. albicans,* which are mostly found in and often cause disease of the urogenital area. Moreover, the assay also did not detect some respiratory commensal and pathogenic bacteria such as alpha- and beta- streptococci, *S. pneumoniae*, *K. pneumoniae*, or *H. influenzae*.

Taken together, that the results indicate that the assay developed in this study was highly specific to the pathogenic *N. gonorrhoeae* and *C. trachomatis,* since cross reactivity to other related microorganisms was not observed.

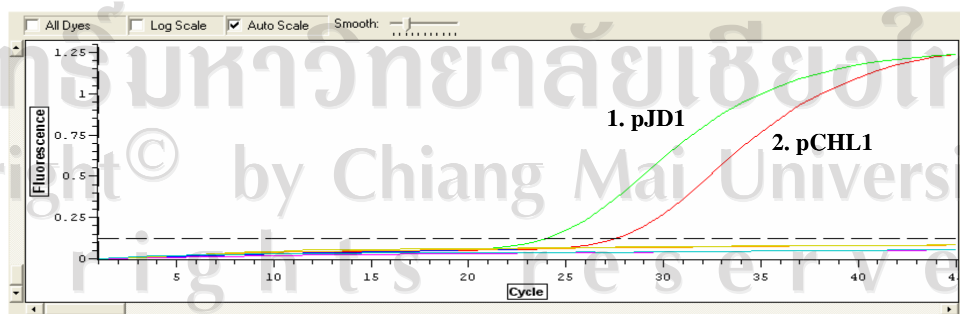

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Figure 10 Illustration of the results from specificity testing by using real time PCR assays. Positive results from pJD1and pHCL1 are demonstrated

as an exponential curve, while the negative results appear as a flat line below the threshold.

Table 11 Demonstration list of non-pathogenic and pathogenic micro-organisms used for specificity testing by real time PCR assay.

Bacterial	Source	Number of isolate
Neisseria species 1. *Neisseria lactamica*	ATCC 23970, 49142	2
2. *Neisseria subflava*	ATCC 14799	2
3. *Neisseria sicca*	TNCC 000871	2
4. *Neisseria cinera*	TNCC 000549	2
5. *Neisseria meningitidis*	ATCC 35561, 13090 and Clinical isolate	4
6. *Neisseria gonorrhoeae*	Clinical isolate	10
Urogenital related organisms 7. *Candida albicans*	Clinical isolate	4
8. *Escherichia coli*	Clinical isolate	4
9. *Tricomonas vaginalis*	Clinical isolate	4
10. *Gardnerella vaginalis*	Clinical isolate	2
11. *Proteus mirabilis*	Clinical isolate	2
12. *Proteus vulgaris*	Clinical isolate	2
13. *Lactobacillus*	Clinical isolate	2
Respiratory related organisms 14. *Staphylococcus epidermidis*	Clinical isolate	3
15. *Staphlycoccus aureus*	Clinical isolate	4
16. *Streptococcus pneumoniae*	Clinical isolate	3
17. *Haemophilus influenzae*	Clinical isolate	4
18. *Klebsiella pneumoniae*	Clinical isolate	3
19. *Pseudomonas aeruginosa*	Clinical isolate	3

5. Comparison of In-house Taqman-based multiplex Real Time PCR with Roche Multiplex AMPLICOR CT/NG PCR assay in detecting *N. gonorrhoeae* and *C. trachomatis* from urine samples.

To compare the performance of In-house Taqman-based multiplex Real Time PCR with Roche Multiplex AMPLICOR CT/NG PCR test, 191 first void urine samples were collected and tested for *N. gonorrhoeae* and *C. trachomatis* using the Roche Multiplex AMPLICOR CT/NG PCR at the Regional Medical Sciences Center, Chiang Rai. The left-over samples

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were sent to be tested for *N. gonorrhoeae* and *C. trachomatis* by using In-house Taqman-based multiplex Real Time PCR at the Division of Clinical Microbiology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University. Two assays were performed double blind in two separate laboratories. After all samples had been tested, the results were analyzed by comparing the positive and negative results obtained from both assays. Samples with concordant results were determined as positive or negative samples. However, samples with discordant positive results were confirmed by an assay that used different primer specificity. In the case of *N. gonorrhoeae*, we employed the Taqman-based Real Time PCR with primers and probe specific to the Porin A pseudogene. For confirmation of *C. trachomatis*, the conventional PCR with primers specific to the MOMP gene was used. The result of conventional PCR detection of the MOMP gene is shown in Figure 11. All negative samples were confirmed negative by amplification of the simulated ß–globin DNA using conventional PCR assay. The results are shown in Figure 12. The ß–globin gene positive samples were determined as negative samples as there were no PCR inhibitors, while the samples with ß–globin DNA negative amplification were determined as a false negative and were excluded from the analysis.

From 191 samples, 3 samples were negative for ß–globin DNA amplification and were excluded. Among 188 samples, 3 were positive and 185 were negative for *N. gonorrhoeae* by the Roche assay, while 11 samples were positive and 177 were negative by Real Time PCR (table 12).

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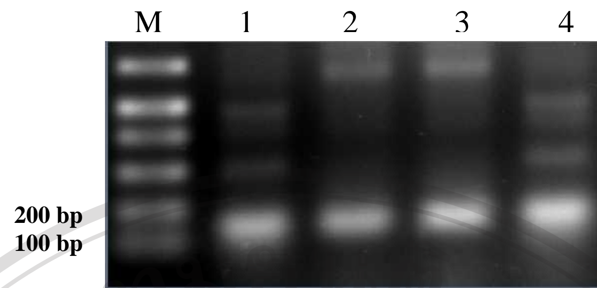

Figure 11 Illustration of conventional PCR detection of the MOMP gene in *C. trachomatis*. Lane M represents the marker and lanes 1-4 showed positive results of PCR to amplify products at 108 bp

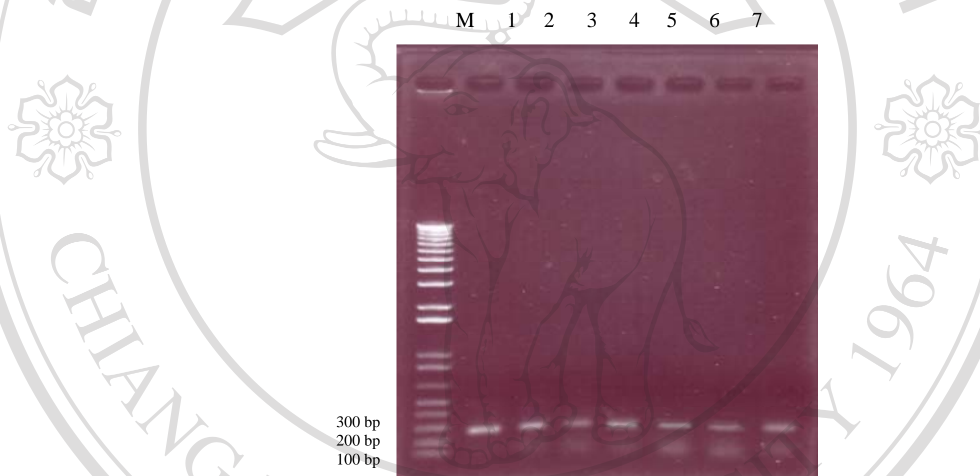

Figure 12 Detecting added human-beta globulin DNA by conventional PCR. Lane M represents the marker and lanes 1-7 showed positive results of PCR products at 265 bp

From all 191 samples, there were 3 samples that were negative by conventional PCR for human β-globin gene and were discounted.

Among 188 samples, 3 were positive for *N. gonorrhoeae* DNA by the Roche Multiplex AMPLICOR CT/NG PCR but only 2 samples were positive by In-house Taqman-based multiplex Real Time PCR for detection of *N. gonorrhoeae* and *C. trachomatis*. One sample that had been missed by our assay was later confirmed as negative by In-house Taqman-based Real Time PCR for Porin A pseudogene and was determined as a

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true negative. However, 176 negative samples for *N. gonorrhoeae* by Roche Multiplex AMPLICOR CT/NG PCR were determined as negative by In-house Taqman-based multiplex Real Time PCR for detection of *N. gonorrhoeae* and *C. trachomatis* as well. (Table 12). From 11 positive samples, only 2 were concordant and 9 were discordant with the Roche assay. However, all 11 Real Time PCR positive samples were later confirmed with porin A pseudogene DNA amplification. The results were confirmed positive in all samples. Among 3 positive samples with Roche assay, only 2 were positive by Real Time PCR. One sample that missed detected by our assay was also confirmed by porin A pseudogene DNA amplification and the result scored as negative, since no amplification signal was observed

Since a cryptic plasmid lacking strain of *N. gonorrhoeae* has been reported in the literature, all 176 samples negative by cryptic plasmid specific Real Time PCR were re-tested or confirmed by using Real Time PCR for the *N. gonorrhoeae* specific Porin A pseudogene DNA. There were 5 (2.84%) from 176 samples that were positive for Porin A pseudogene DNA. Thus, the cryptic plasmid lacking strain of *N. gonorrhoeae* present in our study subjects was at least 2.84%. Unfortunately, this finding might under represent the number of *N. gonorrhoeae* false negative results when screening with the cryptic plasmid specific assay.

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Table 12 Comparison between In-house Taqman-based multiplex Real Time PCR results and Roche Multiplex AMPLICOR CT/NG PCR results detection *N. gonorrhoeae* in urine samples

Assays		Roche Multiplex AMPLICOR CT/NG PCR		Total
		Positive	Negative	
In-house Taqman-based multiplex Real Time PCR for detection of *N. gonorrhoeae* and *C. trachomatis*	Positive	2	9	11
	Negative	1	176	177
Total		3	185	188

For *C. trachomatis* detection (Table 13), the Roche AMPLICOR assay could detect *C. trachomatis* in 19 (10.11%) from 188 samples while Real Time PCR detected more positive samples; 23 (12.23%) samples. In the comparison between these two assays, the concordant positive and negative results were observed in 184 (97.87%) of 188 samples. As for *N. gonorrhoeae* detection, the Real Time PCR assay detected more *C. trachomatis* positive samples than the Roche AMPLICOR assay; 23 vs.19 samples. Four samples that were positive only by Real Time PCR were later confirmed by using conventional PCR detecting the MOMP gene of *C. trachomatis*. All those 4 samples were confirmed positive for *C. trachomatis*. An example of a PCR positive result by agarose gel electrophoresis is shown in Figure 11. Moreover, 165 samples negative by Real Time PCR were all negative by Roche AMPLICOR assay, revealing the highly specific nature of the Real Time PCR assay.

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Table 13 Comparison between In-house Taqman-based multiplex Real Time PCR results and Roche Multiplex AMPLICOR CT/NG PCR results detection *C. trachomatis* in urine samples.

Comparable test		Roche Multiplex AMPLICOR CT/NG PCR		Total
		Positive	Negative	
In-house Taqman-based multiplex Real Time PCR for detection of *N. gonorrhoeae* and *C. trachomatis*	Positive	19	4	23
	Negative	0	165	165
Total		19	169	188

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