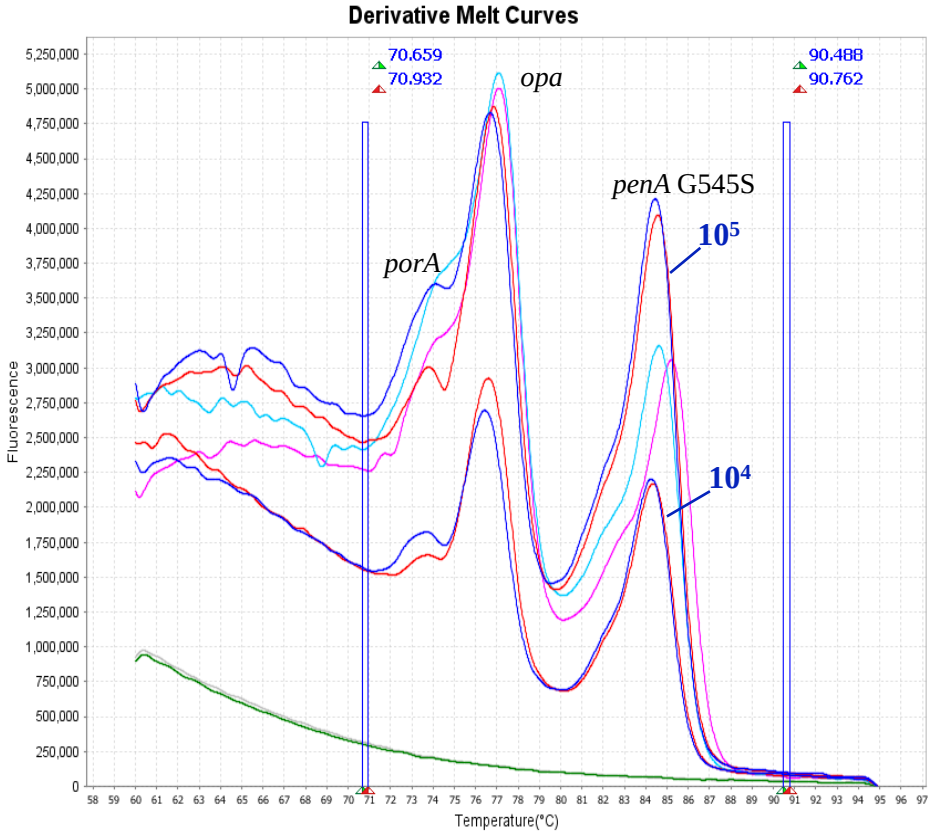
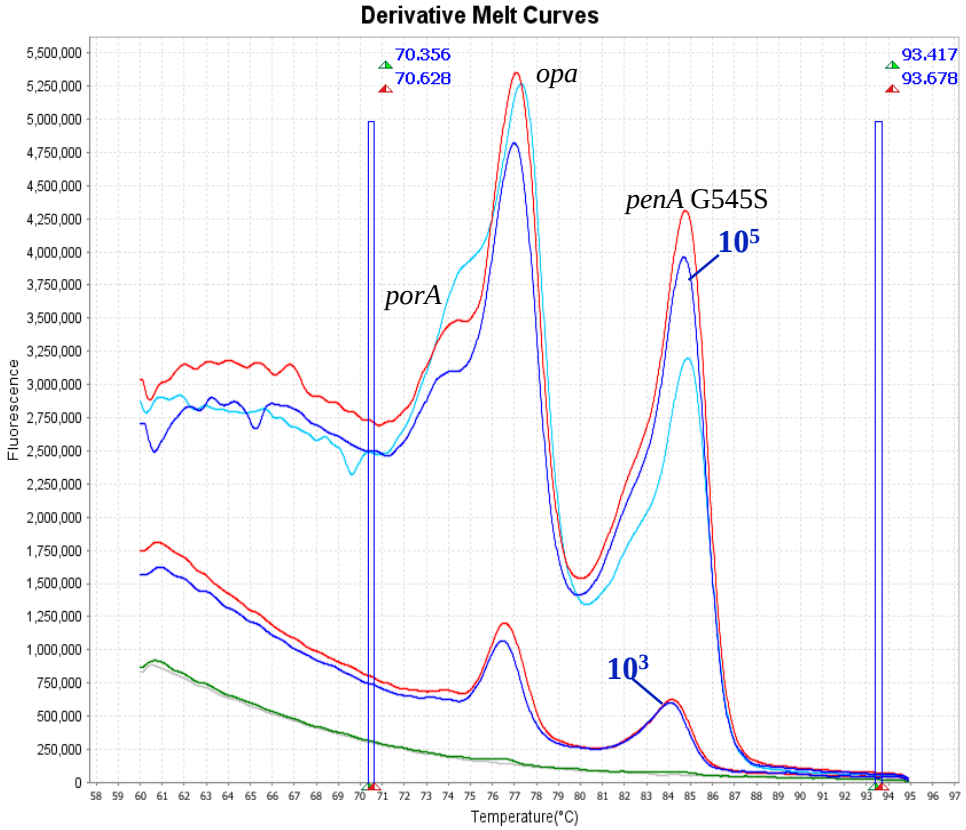
Figure S2-A: Spiked negative pharyngeal samples

Triplex *opa* + *porA* + *penA* Gly545Ser



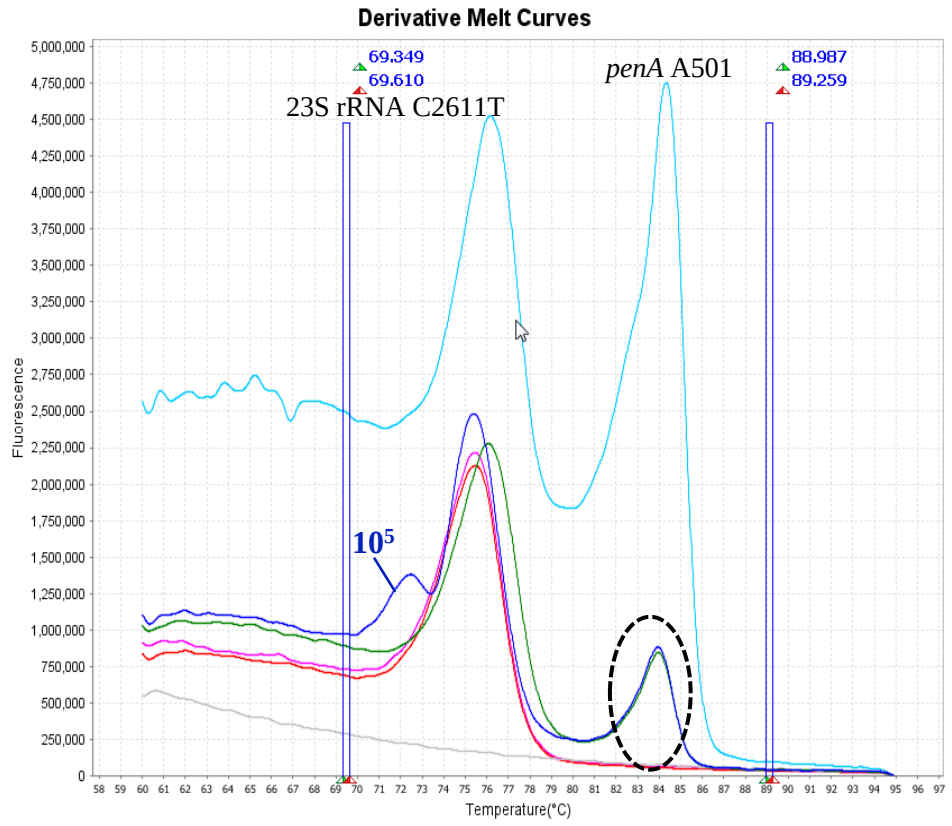


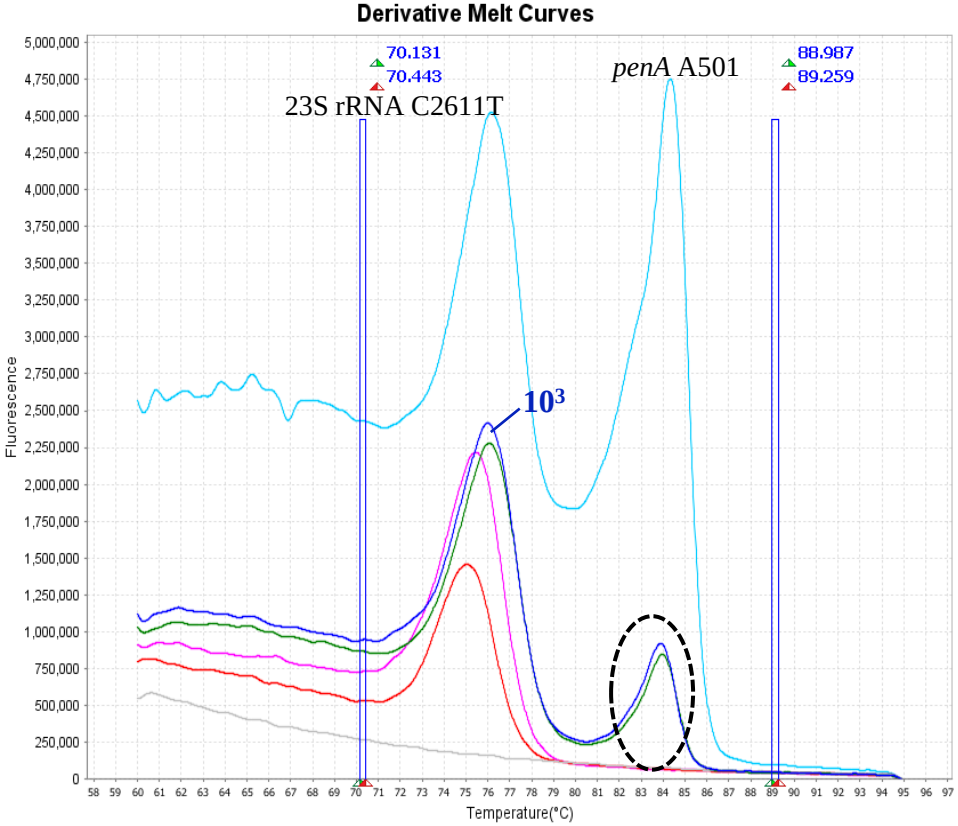
Dark blue: pharyngeal sample spiked with *Neisseria gonorrhoeae* (NG); mutated *penA* Gly545Ser
Red: isolated culture of NG; mutated *penA* Gly545Ser
Light blue: control NG; mutated *penA* Gly545Ser (10^7 gDNA copies/reaction)
Pink: control NG; WT *penA* Gly545 (10^7 gDNA copies/reaction)
Green: unspiked pharyngeal sample
Gray: negative control

Figure S2-A. Derivative melt curves of spiked pharyngeal specimens for the triplex reaction *opa* + *porA* + *penA* Gly545Ser. Dark blue: pharyngeal sample spiked with *Neisseria gonorrhoeae* (NG) with mutated *penA* Gly545Ser. **Red**: isolated culture of NG with mutated *penA* Gly545Ser. **Light blue**: control NG with mutated *penA* Gly545Ser (10^7 gDNA copies/reaction). **Pink**: control NG with wild-type (WT) *penA* Gly545 (10^7 gDNA copies/reaction). **Green**: unspiked pharyngeal sample. **Gray**: negative control.
No background amplification from commensals was observed in all 4 specimens tested. No difference between reactions containing spiked specimens (dark blue) or solely the gDNA of the corresponding NG isolate at the same concentration (red) was observed.

Figure S2-B: Spiked negative pharyngeal samples

Duplex 23S rRNA C2611T + *penA* Ala501



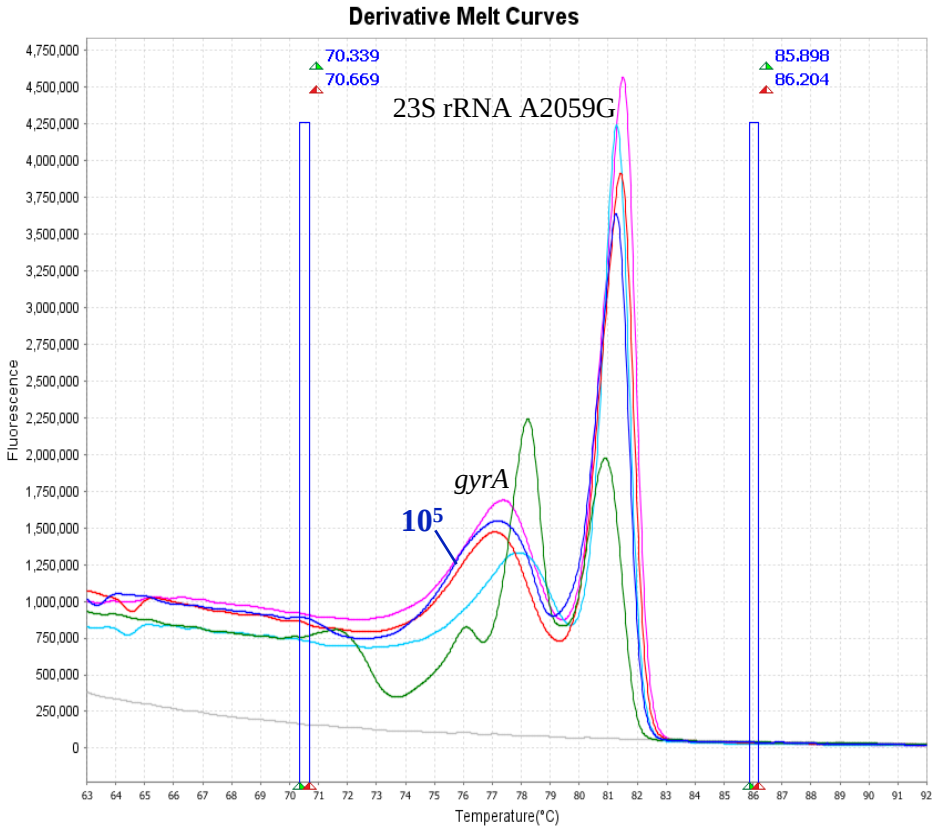


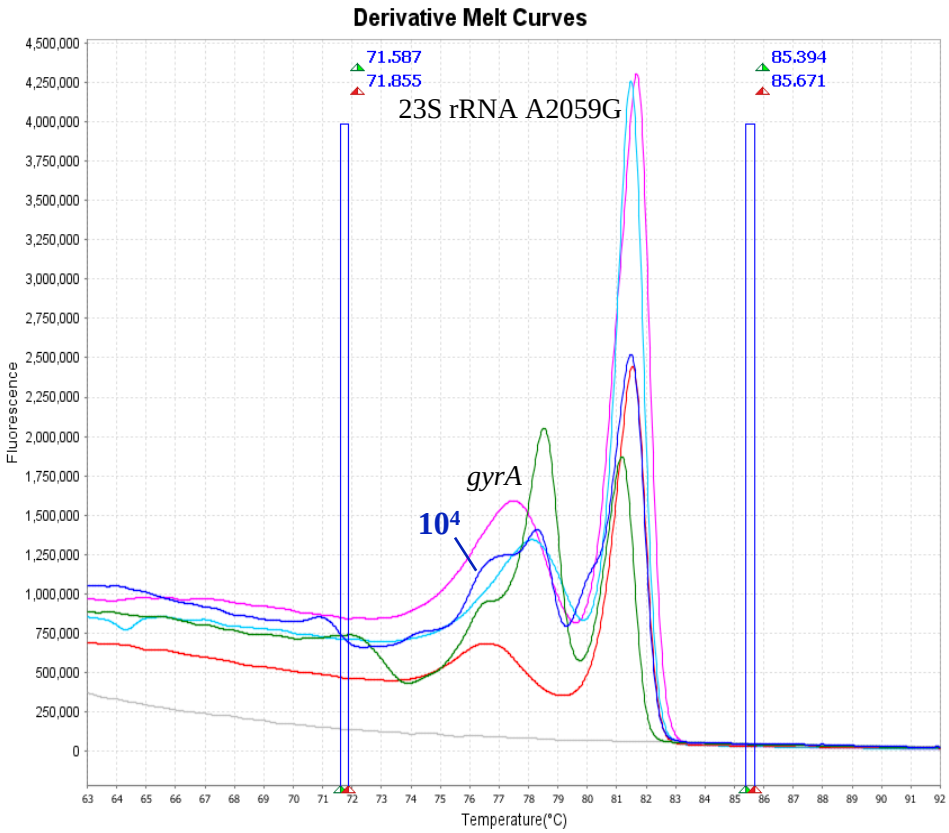
Dark blue: pharyngeal sample spiked with *Neisseria gonorrhoeae* (NG); mutated 23S rRNA C2611T, non-mosaic *penA*
Red: isolated culture with NG; mutated 23S rRNA C2611T, non-mosaic *penA*
Light blue: control NG; WT 23S rRNA C2611, mosaic *penA* (10^7 gDNA copies/reaction)
Pink: control NG; mutated 23S rRNA C2611T, non-mosaic *penA* (10^7 gDNA copies/reaction)
Green: unspiked pharyngeal sample
Gray: negative control

Figure S2-B. Derivative melt curves of spiked pharyngeal specimens for the duplex reaction 23S rRNA C2611T + *penA* Ala501. Dark blue: pharyngeal sample spiked with *Neisseria gonorrhoeae* (NG) with mutated 23S rRNA C2611T and non-mosaic *penA*. **Red**: isolated culture of NG with mutated 23S rRNA C2611T and non-mosaic *penA*. **Light blue**: control NG with wild-type (WT) 23S rRNA C2611 and mosaic *penA* (10^7 gDNA copies/reaction). **Pink**: control NG with mutated 23S rRNA C2611T and non-mosaic *penA* (10^7 gDNA copies/reaction). **Green**: unspiked pharyngeal sample. **Gray**: negative control.
There was strong background amplification of WT 23S rRNA C2611 due to commensals in all 4 tested specimens, which completely masked the presence of the mutation in gonococcus in samples spiked with 10^3 gDNA copies. In samples spiked with both 10^5 and 10^4 gDNA copies/reaction the 23S rRNA C2611T mutation was still detectable, but a shoulder peak indicated the formation of heteroduplexes between the gonococcal mutated and the commensal WT sequences. Positive background amplification of the *penA* Ala501 reaction due to commensals was observed in 2 pharyngeal samples (dashed circle).

Figure S2-C: Spiked negative pharyngeal samples

Duplex *gyrA* Ser91Phe + 23S rRNA A2059G



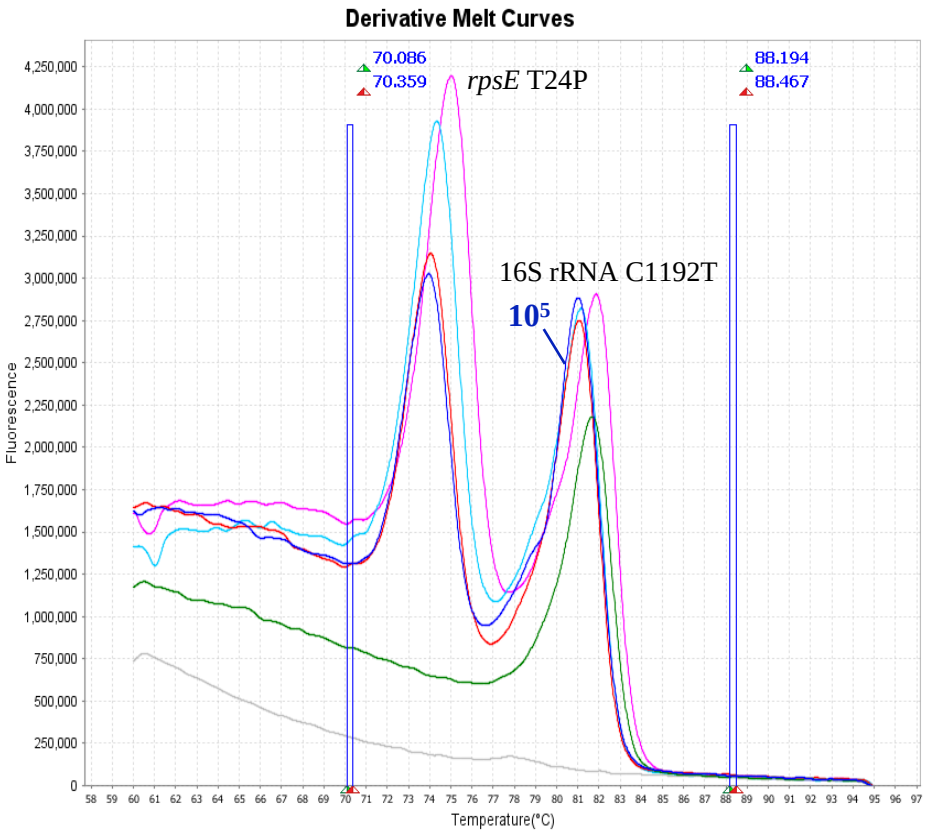


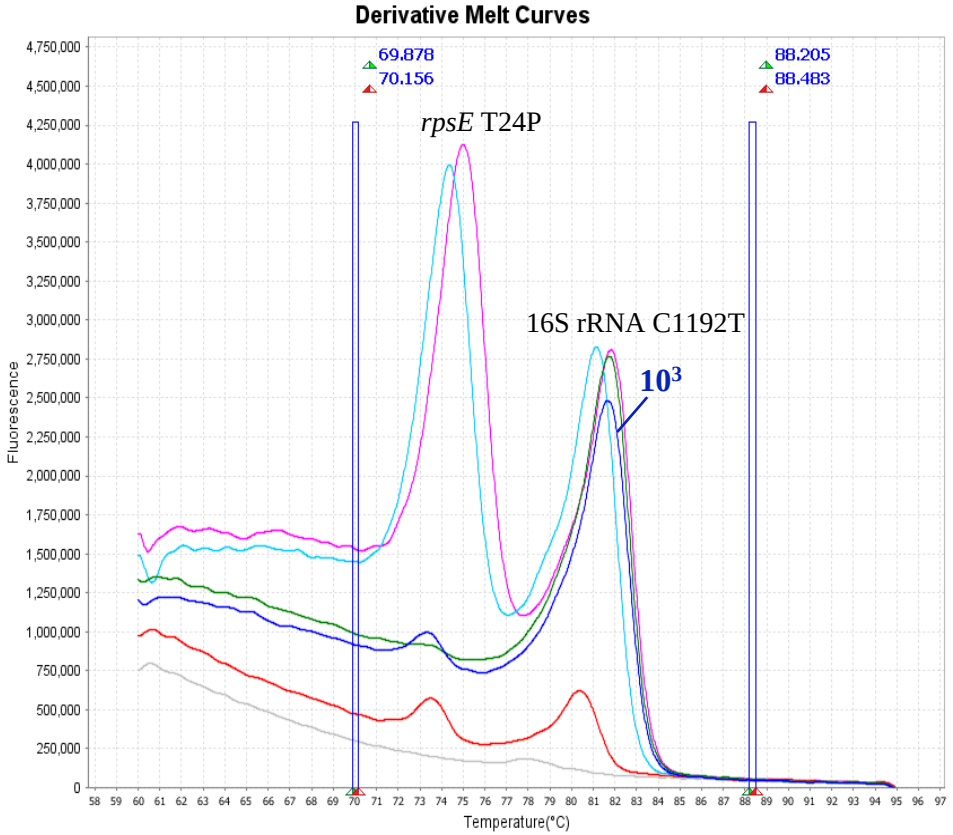
Dark blue: pharyngeal sample spiked with *Neisseria gonorrhoeae* (NG); mutated *gyrA* Ser91Phe, mutated 23S rRNA A2059G
Red: isolated culture with NG; mutated *gyrA* Ser91Phe, mutated 23S rRNA A2059G
Light blue: control NG; WT *gyrA* Ser91, WT 23S rRNA A2059 (10^7 gDNA copies/reaction)
Pink: control NG; mutated *gyrA* Ser91Phe, mutated 23S rRNA A2059G (10^7 gDNA copies/reaction)
Green: unspiked pharyngeal sample
Gray: negative control

Figure S2-C. Derivative melt curves of spiked pharyngeal specimens for the duplex reaction *gyrA* Ser91Phe + 23S rRNA A2059G. **Dark blue**: pharyngeal sample spiked with *Neisseria gonorrhoeae* (NG) with mutated *gyrA* Ser91Phe and mutated 23S rRNA A2059G. **Red**: isolated culture of NG with mutated *gyrA* Ser91Phe and mutated 23S rRNA A2059G. **Light blue**: control NG with mutated *gyrA* Ser91Phe and wild-type (WT) 23S rRNA A2059 (10^7 gDNA copies/reaction). **Pink**: control NG with mutated *gyrA* Ser91Phe and mutated 23S rRNA A2059G (10^7 gDNA copies/reaction). **Green**: unspiked pharyngeal sample. **Gray**: negative control.
There was strong aspecific amplification due to commensals in all 4 specimens (green). At 10^5 gDNA copies/reaction background amplification due to commensals masked the presence of the 23S rRNA A2059G mutation in gonococcus. Distinction of the *gyrA* Ser91Phe mutation by comparison with the appropriate controls (10^7 gDNA copies/reaction) was only possible in samples containing 10^5 gDNA copies/reaction.

Figure S2-D: Spiked negative pharyngeal samples

Duplex *rpsE* Thr24Pro + 16S rRNA C1192T



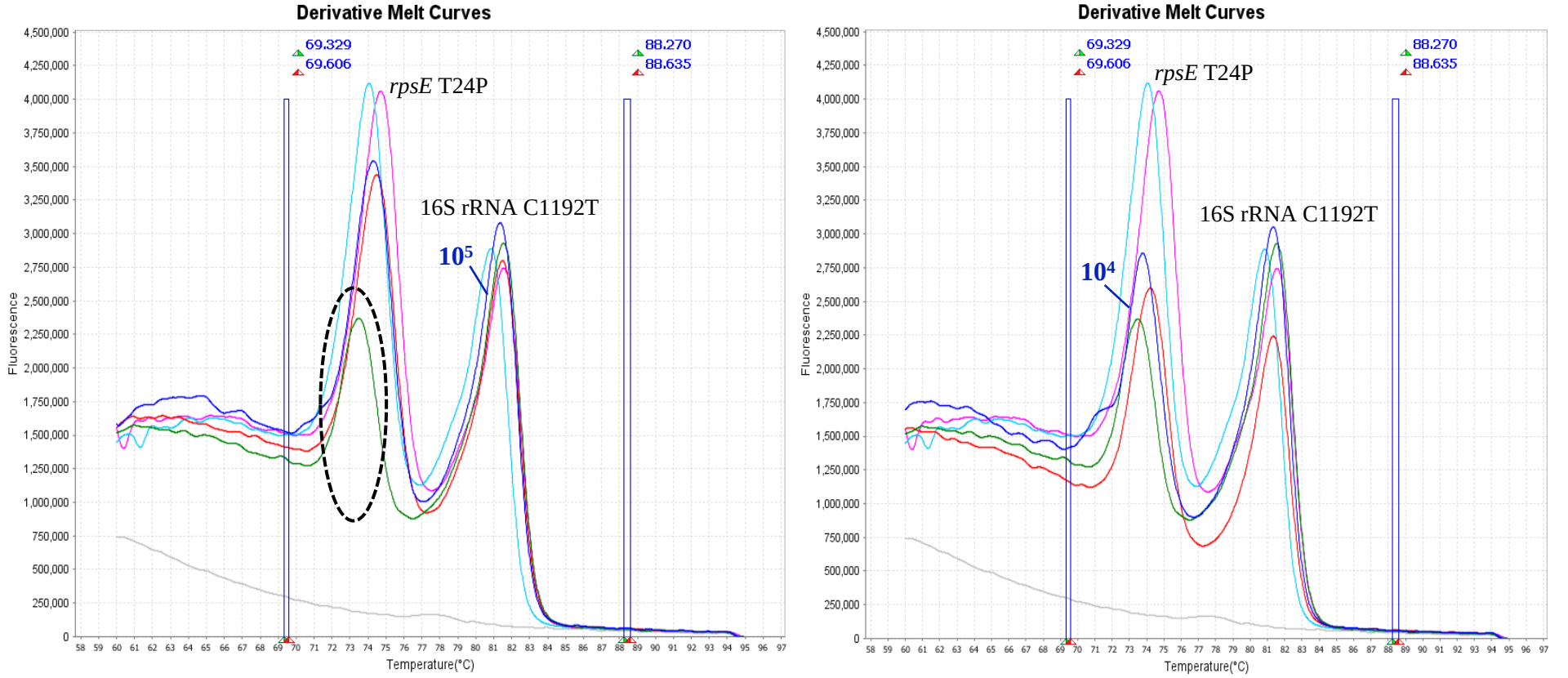


Dark blue: pharyngeal sample spiked with *Neisseria gonorrhoeae* (NG); WT *rpsE* Thr24, mutated 16S rRNA C1192T
Red: isolated culture with NG; WT *rpsE* Thr24, mutated 16S rRNA C1192T
Light blue: control NG; WT *rpsE* Thr24, mutated 16S rRNA C1192T (10^7 gDNA copies/reaction)
Pink: control NG; mutated *rpsE* Thr24Pro, WT 16S rRNA C1192 (10^7 gDNA copies/reaction)
Green: unspiked pharyngeal sample
Gray: negative control

Figure S2-D. Derivative melt curves of spiked pharyngeal specimens for the duplex reaction *rpsE* Thr24Pro + 16S rRNA C1192T. Dark blue: pharyngeal sample spiked with *Neisseria gonorrhoeae* (NG) with wild-type (WT) *rpsE* Thr24, mutated 16S rRNA C1192T. **Red**: isolated culture of NG with WT *rpsE* Thr24, mutated 16S rRNA C1192T. **Light blue**: control NG with WT *rpsE* Thr24, mutated 16S rRNA C1192T (10^7 gDNA copies/reaction). **Pink**: control NG with mutated *rpsE* Thr24Pro and WT 16S rRNA C1192 (10^7 gDNA copies/reaction). **Green**: unspiked pharyngeal sample. **Gray**: negative control.
There was strong background amplification due to commensals for the 16S rRNA C1192T reaction in all 4 tested specimens (green), which masked the presence of the 16S rRNA C1192T mutation in gonococcus in samples spiked with 10^3 gDNA copies.

Figure S2-E: Spiked negative pharyngeal samples

Duplex *rpsE* Thr24Pro + 16S rRNA C1192T

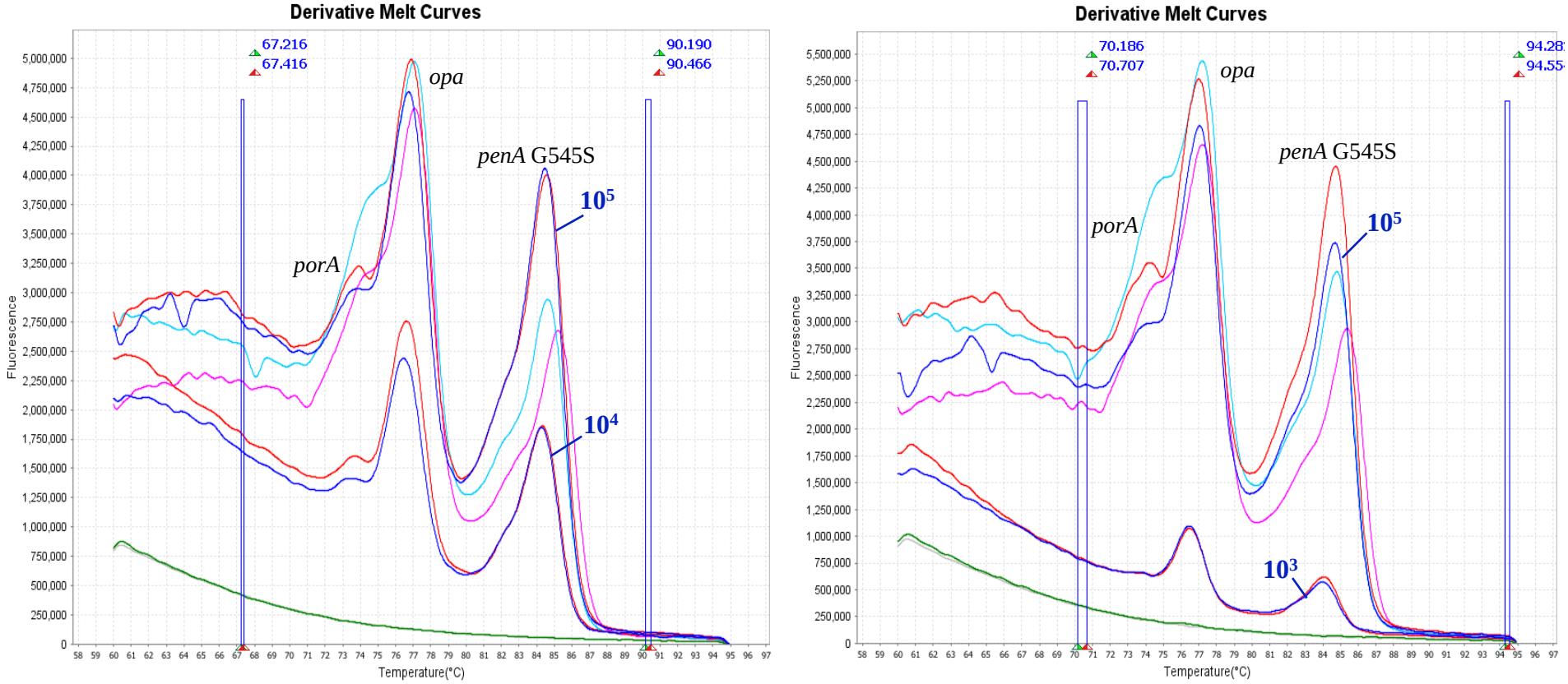


Dark blue: pharyngeal sample spiked with *Neisseria gonorrhoeae* (NG); mutated *rpsE* Thr24Pro, WT 16S rRNA C1192
Red: isolated culture with NG; mutated *rpsE* Thr24Pro, WT 16S rRNA C1192
Light blue: control NG; WT *rpsE* Thr24, mutated 16S rRNA C1192T (10^7 gDNA copies/reaction)
Pink: control NG; mutated *rpsE* Thr24Pro, WT 16S rRNA C1192 (10^7 gDNA copies/reaction)
Green: unspiked pharyngeal sample
Gray: negative control

Figure S2-E. Derivative melt curves of spiked pharyngeal specimens for the duplex reaction *rpsE* Thr24Pro + 16S rRNA C1192T. Dark blue: pharyngeal sample spiked with *Neisseria gonorrhoeae* (NG) with mutated *rpsE* Thr24Pro, wild-type (WT) 16S rRNA C1192. **Red**: isolated culture of NG with mutated *rpsE* Thr24Pro, WT 16S rRNA C1192. **Light blue**: control NG with WT *rpsE* Thr24, mutated 16S rRNA C1192T (10^7 gDNA copies/reaction). **Pink**: control NG with mutated *rpsE* Thr24Pro and WT 16S rRNA C1192 (10^7 gDNA copies/reaction). **Green**: unspiked pharyngeal sample. **Gray**: negative control.
Strong background amplification due to commensals for the *rpsE* Thr24Pro reaction was observed in one specimen (green, dashed circle), which masked the presence of the *rpsE* Thr24Pro mutation in gonococcus in samples spiked with 10^4 gDNA copies.

Figure S3-A: Spiked negative rectal samples

Triplex opa+*porA*+*penA* Gly545Ser

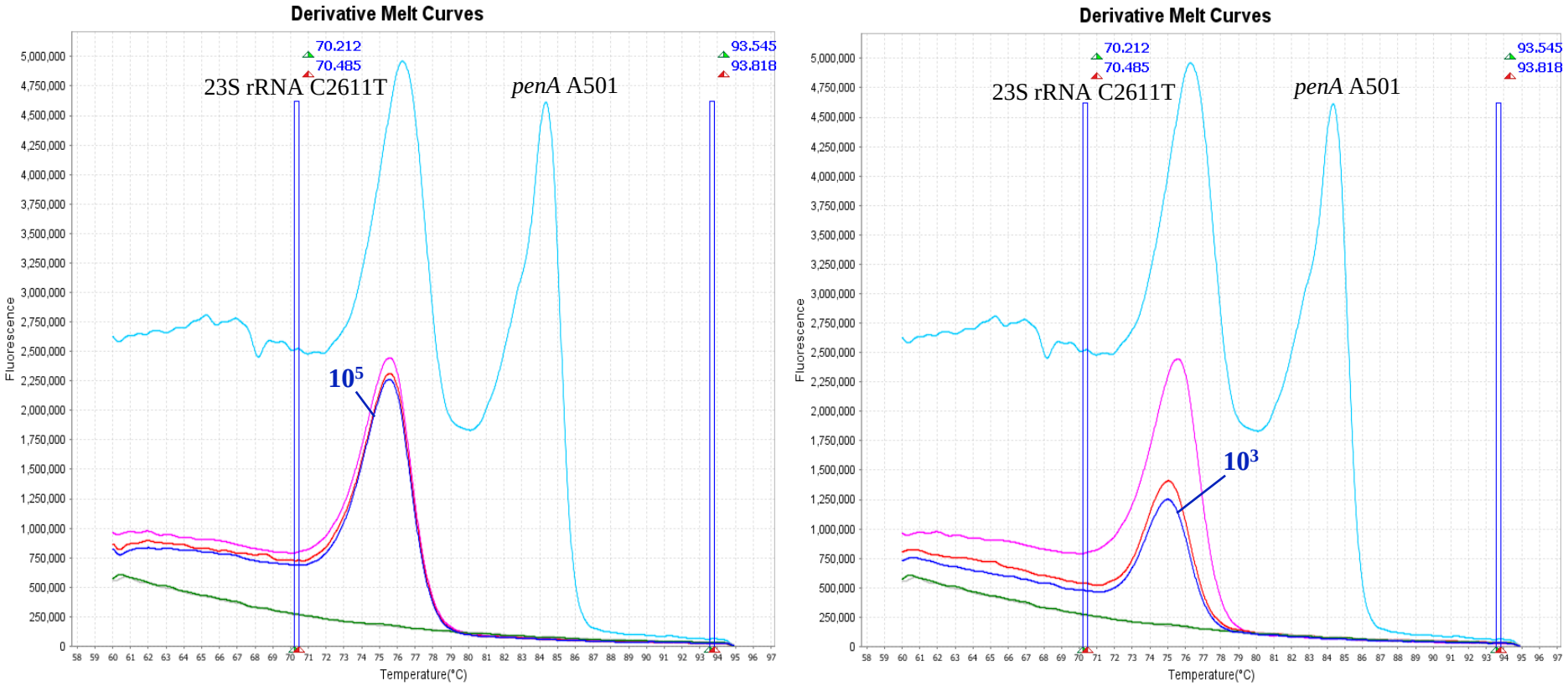


Dark blue: rectal sample spiked with *Neisseria gonorrhoeae* (NG); mutated *penA* Gly545Ser
Red: isolated culture with NG; mutated *penA* Gly545Ser
Light blue: control NG; mutated *penA* Gly545Ser (10^7 gDNA copies/reaction)
Pink: control NG; WT *penA* Gly545 (10^7 gDNA copies/reaction)
Green: unspiked rectal sample
Gray: negative control

Figure S3-A. Derivative melt curves of spiked rectal specimens for the triplex reaction *opa* + *porA* + *penA* Gly545Ser. Dark blue: rectal sample spiked with *Neisseria gonorrhoeae* (NG) with mutated *penA* Gly545Ser. **Red**: isolated culture of NG with mutated *penA* Gly545Ser. **Light blue**: control NG with mutated *penA* Gly545Ser (10^7 gDNA copies/reaction). **Pink**: control NG with wild-type (WT) *penA* Gly545 (10^7 gDNA copies/reaction). **Green**: unspiked rectal sample. **Gray**: negative control.
No background amplification from commensals was observed in all 4 specimens tested. No difference between reactions containing spiked specimens (dark blue) or solely the gDNA of the corresponding NG isolate at the same concentration (red) was observed.

Figure S3-B: Spiked negative rectal samples

Duplex 23S rRNA C2611T+*penA* Ala501

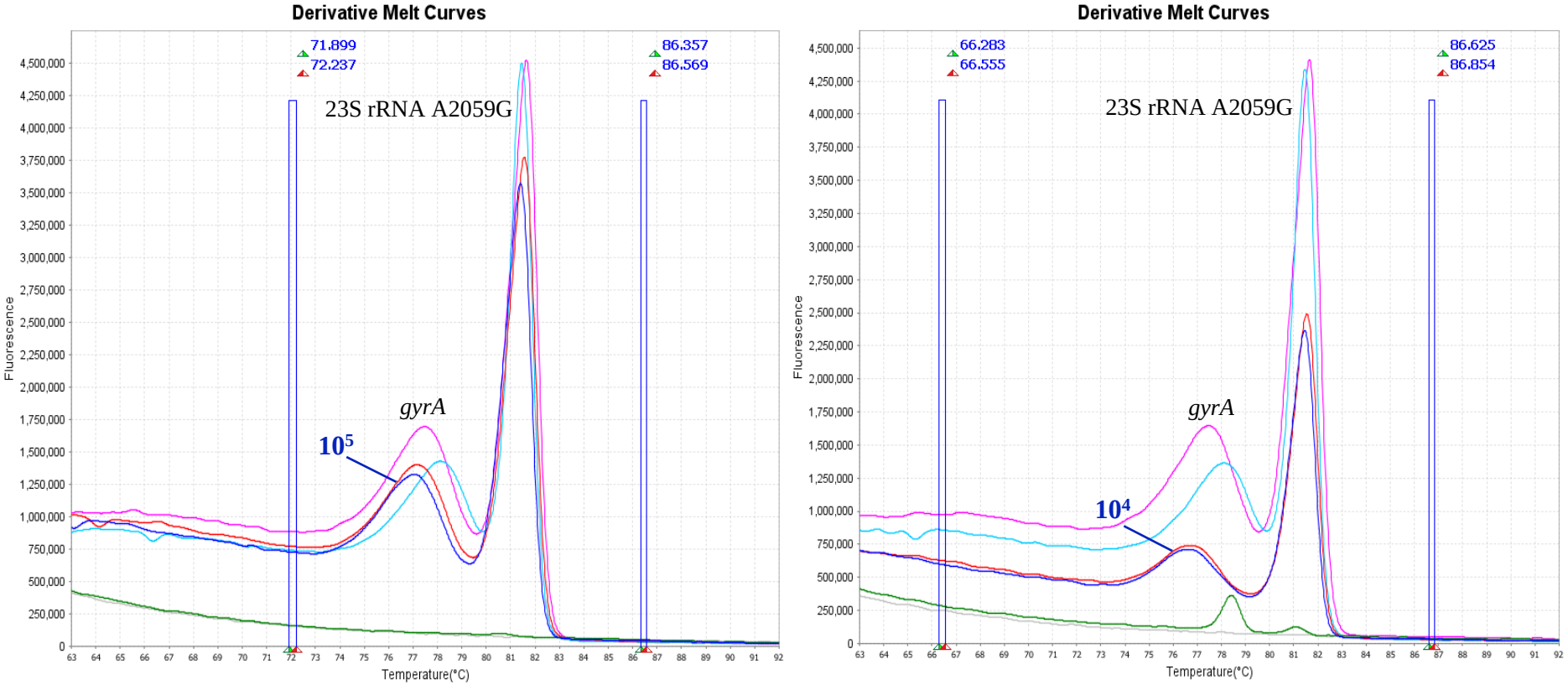


Dark blue: rectal sample spiked with *Neisseria gonorrhoeae* (NG); mutated 23S rRNA C2611T, non-mosaic *penA*
Red: isolated culture with NG; mutated 23S rRNA C2611T, non-mosaic *penA*
Light blue: control NG; WT 23S rRNA C2611, mosaic *penA* (10^7 gDNA copies/reaction)
Pink: control NG; mutated 23S rRNA C2611T, non-mosaic *penA* (10^7 gDNA copies/reaction)
Green: unspiked rectal sample
Gray: negative control

Figure S3-B. Derivative melt curves of spiked rectal specimens for the duplex reaction 23S rRNA C2611T + *penA* Ala501. Dark blue: rectal sample spiked with *Neisseria gonorrhoeae* (NG) with mutated 23S rRNA C2611T and non-mosaic *penA*. **Red**: isolated culture of NG with mutated 23S rRNA C2611T and non-mosaic *penA*. **Light blue**: control NG with wild-type (WT) 23S rRNA C2611 and mosaic *penA* (10^7 gDNA copies/reaction). **Pink**: control NG with mutated 23S rRNA C2611T and non-mosaic *penA* (10^7 gDNA copies/reaction). **Green**: unspiked rectal sample. **Gray**: negative control.
No background amplification from commensals was observed in all 4 specimens tested. No difference between reactions containing spiked specimens (dark blue) or solely the gDNA of the corresponding NG isolate at the same concentration (red) was observed.

Figure S3-C: Spiked negative rectal samples

Duplex *gyrA* Ser91Phe+ 23S rRNA A2059G

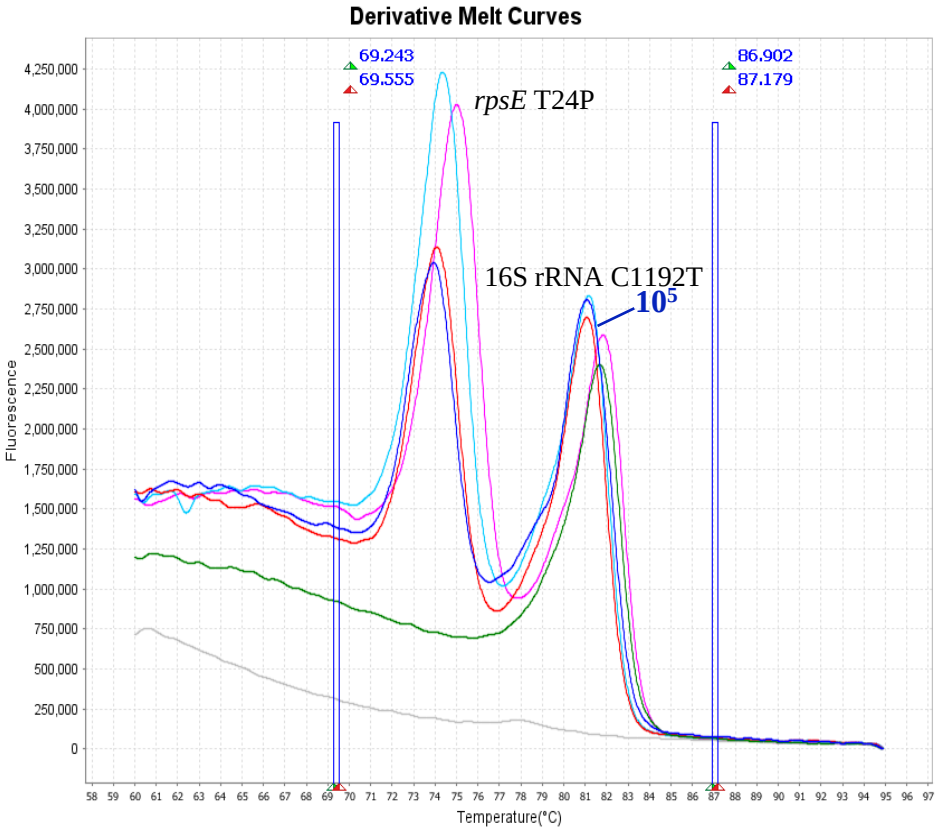


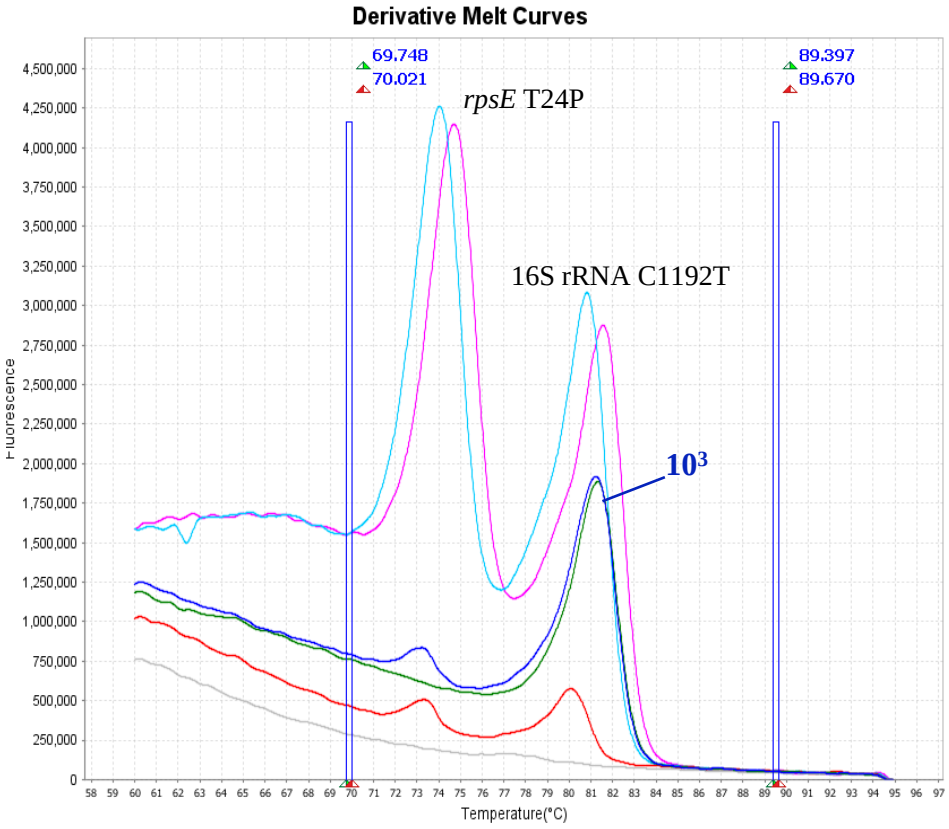
Dark blue: rectal sample spiked with *Neisseria gonorrhoeae* (NG); mutated *gyrA* Ser91Phe, mutated 23S rRNA A2059G
Red: isolated culture with NG; mutated *gyrA* Ser91Phe, mutated 23S rRNA A2059G
Light blue: control NG; WT *gyrA* Ser91, WT 23S rRNA A2059 (10^7 gDNA copies/reaction)
Pink: control NG; mutated *gyrA* Ser91Phe, mutated 23S rRNA A2059G (10^7 gDNA copies/reaction)
Green: unspiked rectal sample
Gray: negative control

Figure S3-C. Derivative melt curves of spiked rectal specimens for the duplex reaction *gyrA* Ser91Phe + 23S rRNA A2059G. **Dark blue**: rectal sample spiked with *Neisseria gonorrhoeae* (NG) with mutated *gyrA* Ser91Phe and mutated 23S rRNA A2059G. **Red**: isolated culture of NG with mutated *gyrA* Ser91Phe and mutated 23S rRNA A2059G. **Light blue**: control NG with mutated *gyrA* Ser91Phe and wild-type (WT) 23S rRNA A2059 (10^7 gDNA copies/reaction). **Pink**: control NG with mutated *gyrA* Ser91Phe and mutated 23S rRNA A2059G (10^7 gDNA copies/reaction). **Green**: unspiked rectal sample. **Gray**: negative control.
There was little or no background amplification due to commensals in all 4 rectal specimens. However, in all 4 samples the Tm of the mutated 23S rRNA A2059G amplicon (blue) was decreased in comparison to the Tm observed for the corresponding NG isolate (red) strongly affecting the interpretation of the melting curve.

Figure S3-D: Spiked negative rectal samples

Duplex *rpsE* Thr24Pro + 16S rRNA C1192T





Dark blue: rectal sample spiked with *Neisseria gonorrhoeae* (NG); WT *rpsE* Thr24, mutated 16S rRNA C1192T
Red: isolated culture with NG; WT *rpsE* Thr24, mutated 16S rRNA C1192T
Light blue: control NG; WT *rpsE* Thr24, mutated 16S rRNA C1192T (10^7 gDNA copies/reaction)
Pink: control NG; mutated *rpsE* Thr24Pro, WT 16S rRNA C1192 (10^7 gDNA copies/reaction)
Green: unspiked rectal sample
Gray: negative control

Figure S3-D. Derivative melt curves of spiked rectal specimens for the duplex reaction *rpsE* Thr24Pro + 16S rRNA C1192T. Dark blue: rectal sample spiked with *Neisseria gonorrhoeae* (NG) with wild-type (WT) *rpsE* Thr24 and mutated 16S rRNA C1192T. **Red**: isolated culture of NG with WT *rpsE* Thr24 and mutated 16S rRNA C1192T. **Light blue**: control NG with WT *rpsE* Thr24 and mutated 16S rRNA C1192T (10^7 gDNA copies/reaction). **Pink**: control NG with mutated *rpsE* Thr24Pro and WT 16S rRNA C1192 (10^7 gDNA copies/reaction). **Green**: unspiked rectal sample. **Gray**: negative control.
Background amplification due to commensals was observed in all 4 samples, which masked the presence of the 16S rRNA C1192T mutation in samples spiked with low amounts of gonococcal gDNA (10^3 gDNA copies/reaction).