Direct qPCR is a sensitive approach to detect Mycoplasma contamination in U937 cell cultures

Zain Baaity
Department of Medical Microbiology and Immunobiology, Faculty of Medicine, University of Szeged, Dom sq 10, H-6720 Szeged, Hungary

Sven Breunig
Procomcure Biotech Gmbh, Breitwies 1, A-5303 Thalgau, Austria

Kamil Önder
Procomcure Biotech Gmbh, Breitwies1, A-5303 Thalgau, Austria

Ferenc Somoyvári (somogyvari.ferenc@med.u-szeged.hu)
Szegedi Tudomanyegyetem Altalanos Orvostudomanyi Kar https://orcid.org/0000-0001-8409-892X

Research note

Keywords: Mycoplasma, qPCR, PCR, direct, elimination

Posted Date: September 26th, 2019

DOI: https://doi.org/10.21203/rs.2.15221/v1

Version of Record: A version of this preprint was published on November 1st, 2019. See the published version at https://doi.org/10.1186/s13104-019-4763-5.

Abstract

Objective In order to make the Mycoplasma contamination monitoring task easier, we optimized a commercially available quantitative PCR (qPCR)-based detection kit to detect Mycoplasma DNA without DNA purification in a U937 suspension cell culture. We compared the sensitivities of the direct qPCR and the qPCR with the purified DNA template. Results Our findings indicate that qPCR worked optimally with a 6 ml sample volume and a 52 o C annealing-extension temperature. We were able to decrease the annealing-extension step time from 60 sec to 20 sec without any major decrease in the reaction sensitivity. The total cycle time of the optimized direct qPCR was 65 minutes. The optimized qPCR protocol was used to detect Mycoplasma DNA directly and after DNA purification. Our findings indicate that the direct qPCR had a higher sensitivity compared to the regular qPCR. The Ct levels produced by the direct qPCR with 6 ml templates were almost identical to the Ct levels produced by the regular qPCR with DNA purified from a 60 ml cell culture sample (23.42 vs 23.49 average Ct levels respectively). The optimized direct qPCR protocol was successfully applied to monitor the elimination of Mycoplasma contamination from the U937 cell cultures.

Introduction

Mycoplasma is a small cell-wall free prokaryotic bacterium with a remarkable diversity at the species level. Besides human respiratory and urogenital tract infections, the Mycoplasma contamination of cell cultures is a frequent phenomenon. According to the DSMZ-German Collection of Microorganisms and Cell Cultures survey, the prevalence of Mycoplasma contamination of cell lines was 28% including Mycoplasma species M. orale, M. hyorhinis, M. arginini, M. fermentans, M. hominis and Acholeplasma laidlawii [1]. Mycoplasma contamination may be introduced by cross-infection with a Mycoplasma positive cell line, the laboratory personnel (e.g. M. orale) or by contaminated cell-culture reagents such as fetal bovine serum. Indeed, bovine Mycoplasma species M. arginini and A. laidlawii are frequent contaminating agents. Mycoplasma contamination is hard to prevent/eradicate since the bacterium is less sensitive to the antibiotics commonly applied in cell cultures, and due to its small size (0.3-1 μm) and non-rigid cell wall it is hard to remove by filtration. Mycoplasma infection has a pleiotropic effect on cellular physiology including altered metabolism, DNA, RNA and protein synthesis, pro- and anti-inflammatory effects [1–3]. U937 human monocytic cells, the cell-type used in this study, respond to the Mycoplasma infection by producing monocyte chemotactic protein-1, matrix metalloproteinase-12 [4] and interleukin-1[5]. Because of the high probability of introducing novel Mycoplasma infection into cell cultures, it is necessary to monitor cell culture ingredients and cell lines for Mycoplasma contamination. There is a wide variety of detection methods available including metabolism detection and Mycoplasma genome detection by PCR and qPCR. Regular PCR has a high sensitivity and specificity, but in the majority of cases it requires nucleic acid purification as a proxy, and gel electrophoresis as an endpoint detection of the PCR product. qPCR eliminates the gel electrophoresis step, but regular qPCR protocols also include nucleic acid purification. DNA purification can be a long and laborious procedure, especially if there are several samples to be purified. Direct PCR and direct qPCR eliminate the purification step,

significantly shorten the protocol, but the inhibitory effect of the direct sample can be present. Previously direct qPCR methods were successfully applied to monitor Chlamydia and herpes simplex virus-2 growth and the antimicrobial effects of various compounds [6–11]. In this study, we also wanted to leave out the DNA purification step and develop a short direct qPCR detection method that is suitable to detect Mycoplasma contamination directly from contaminated U937 cell cultures.

Materials And Methods

Cell culture

Mycoplasma infected U937 human monocytic cells were grown in a 25 cm^2 cell culture flask (Greiner Bio-One Hungary, Mosonmagyaróvár, Hungary) in an RPMI 1640 medium containing 10% heat-inactivated FBS (Sigma, St.Louis, MO, USA), and 50 mg/mL gentamicin at 37 ^oC in 5% CO_2.

Mycoplasma elimination

Mycoplasma elimination was performed using the *Mycoplasma* Elimination Reagent (Bio-Rad, Hercules, CA, USA). *Mycoplasma* Elimination Reagent was added to the RPMI 1640 medium at 0.5 mg/ml final concentration and the U937 cells were then cultured in this medium for 7 days.

DNA extraction and qPCR

DNA was extracted from *Mycoplasma* infected U937 cell supernatants via the Qiagen QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PhoenixDx® Mycoplasma Mix (Procomcure Biotech, Thalgau, Austria) was used in the qPCR experiments. qPCRs with 20 ml final volume were performed using the Bio-Rad CFX Connect qPCR real-time system. A statistical comparison of qPCR cycle threshold (Ct) values was performed with Student's t-test, as described previously [12].

Results

Figure 1.

Optimization of the *Mycoplasma* genus-specific direct qPCR and comparison of its performance with regular qPCR with purified DNA templates. A, effect of the qPCR annealing/extension temperature on the direct qPCR performance. Student's t-test was applied to compare the Ct values of the samples with various annealing/extension temperatures to those samples with a 50^oC annealing/extension temperature (n=3). B, effect of the annealing/extension time on the direct qPCR performance. Student's t-

test was applied to compare the Ct values of the samples with various annealing/extension times to those samples with a 60 sec annealing/extension time (n=4). C, effect of the sample volume on the direct qPCR performance. Student's t-test was applied to compare the Ct values of the samples with various template volumes with samples having a 10 ml template volume (n=3). D, comparison of the direct qPCR performance with the regular qPCR with a purified DNA template (n=3). The DNA was purified from a 6, 60 and 120 ml cell culture supernatant via the QIAamp protocol and eluted in a 100 ml elution buffer. 6 ml of the eluted DNA was used in the qPCR procedure. As a comparison, 6 ml of the cell culture supernatant was used in the direct qPCR. NA: no amplification was detected. *: $P<0.05$, **: $P<0.01$

To achieve an optimal sensitivity and the shortest possible reaction time of the direct qPCR, we followed a step-wise optimization protocol of the PhoenixDx *Mycoplasma* Mix (Procomcure Biotech, Thalgau, Austria) that was originally designed to amplify purified DNA samples. First, we tested the optimal annealing/extension temperature for detecting of unpurified *Mycoplasma* DNA in *Mycoplasma*-infected U937 cell culture supernatants (Figure 1A). The results indicated that the reactions with 50^{o}C-52^{o}C annealing/extension temperature produced the lowest Ct values (26.84+/-0.14 - 27.06+/-0.26). We chose the 52^{o}C annealing/extension temperature for further tests. Next, we tested to see whether reducing the annealing/extension time might influence the qPCR performance (Figure 1B). Our findings showed that the 60 sec annealing/extension time provided the lowest Ct values (23.56+/-0.47), but the 20 and 40 sec annealing/extension times led to only slightly higher Ct values (24.20+/-0.23, 24.11+/-0.27 respectively), which suggested that reducing the annealing/extension time from 60 sec to 20 sec had a minimal influence on qPCR sensitivity. 20 sec annealing/extension time was used for further qPCRs. Next, we tested the effect of sample volume on the qPCR performance (Figure 1C). The Ct levels of samples with 6 ml, 8 ml and 10 ml volumes of supernatants were similar (21.92-22.13 Ct value range), indicating that the qPCR sensitivity is influenced by the higher *Mycoplasma* DNA content and also by a higher level of qPCR inhibition in the 8 and 10 ml samples. In further experiments, we opted for the 6 ml sample volume. Next, we compared the performance of the direct qPCR and the regular qPCR with purified DNA samples (Figure 1D). The QIAamp DNA purification kit was used to isolate *Mycoplasma* DNA from the U937 cell cultures (medium+cells). The elution volume was 100 ml. A comparison of the 6 ml direct sample volume and 6 ml purified sample was not possible as just 6 ml of the 100 ml total elution volume could be used in the regular qPCR. Therefore, we also decreased the 6 ml direct sample volume by a factor of 6/100 (0.36 ml). In a comparison of these samples we found that the 6 ml purified sample produced lower Ct values (~ 2 cycles) than the 0.36 ml direct sample, suggesting a low level of qPCR inhibition of the supernatant. However, when we compared the Ct levels of the samples with 6 ml supernatant to the Ct levels of samples with purified DNAs we noticed that the Ct values produced with 6 ml supernatants were almost identical to those of the purified 60 ml supernatant (23.42+/-0.26, 23.49+/-0.30, respectively) indicating an altogether higher sensitivity of the direct qPCR.

Figure 2. Monitoring *Mycoplasma* elimination by direct qPCR. *Mycoplasma* contaminated U937 cells were treated with the Bio-Rad *Mycoplasma* Removal Agent at 0.5 mg/ml concentration. A, A comparison of qPCR Ct values in the absence and presence of *Mycoplasma* Removal Agent in the medium of contaminated U937 cells. Student's t-test was applied to compare the Ct values of the removal agent containing samples with the removal agent free samples (n=3). B, The first four days of the treatment monitored by direct qPCR is shown (n=4 at each time point). The *Mycoplasma* genome concentration on day 0 was defined as 100%.

As an application of the optimized direct qPCR we monitored the *Mycoplasma* elimination from the infected U937 cell culture. Our results showed that the removal agent containing and agent-free supernatants (n=4) resulted in nearly the same Ct levels (27.04 +/- 0.24 and 26.94 +/- 0.45, respectively) (Figure 2A), indicating that the presence of the removal agent did not influence the qPCR performance. The *Mycoplasma* DNA dropped rapidly (by ~80%) after a 24-hour treatment (Figure 2B). On the fourth day the *Mycoplasma* concentration was 2.3% of the original concentration. By the sixth day of the treatment, *Mycoplasma* DNA was no longer detectable (data not shown). Overall, the direct qPCR method was proved to be a quick and effective method for monitoring the decrease in *Mycoplasma* DNA during the elimination process.

Discussion

While various methods exist for the detection of Mycoplasma contamination [13, 14], probably the most frequently used ones are the biochemical detection of Mycoplasma metabolism and PCR-based detection of Mycoplasma DNA. Though the biochemical detection of mycoplasmal ATP generation (Mycoalert (Lonza, Basel, Switzerland)) is a quick protocol, it has certain disadvantages that should be mentioned, including the requirement of the reagents to be reconstituted and be brought to 22oC before each measurement, and the availability of a luminometer for ATP detection. Aspecificity due to ATP generated by other cells may lead to a high background and eventually false negative measurements. The Ureaplasma species which are also a common contaminant in a cell culture [15] cannot be detected by Mycoalert as their own ATP production relies on the hydrolysis of urea [16]. Lastly, the sensitivity of the biochemical detection was shown to be lower than that for PCR or qPCR methods [17, 18]. There is a variety of kits on offer based on regular PCR, followed by gel electrophoresis. The major advantage of these kits is the wide availability of regular PCR and electrophoresis equipments. However, the decreased specificity compared to a probe-based qPCR, the additional electrophoresis step, and the lack of quantitative monitoring of the decrease in the Mycoplasma genome concentration during treatment are clear drawbacks. The intercalation-based (e.g. SYBR Green) qPCR kits such as MycoSEQ Mycoplasma Detection Assay (Thermo Fisher, Waltham, MA, USA) eliminate the electrophoresis step and provide

quantitative information about the Mycoplasma genome concentration. The disadvantages of intercalation-based qPCR kits compared with the probe-based kits are the lower specificity, the lack of internal control and the potential effect of the cell culture composition, ionic composition and ionic strength on the melting temperature of the qPCR product [19–21]. Since the melting temperature is the basis for evaluating specificity in the intercalation based qPCRs, shifting the melting temperature in a direct qPCR can be a problem. The probe-based qPCRs such as PhoenixDx (Procomcure Biotech, Thalgau, Austria), Microsart RESEARCH Mycoplasma (Sartorius, Goettingen, Germany) and the qPCR Detection Kit (XpressBio, Frederick, MD, USA) avoid these problems and due the additional requirement of the binding of the probe sequence, these kits provide a higher specificity than the regular PCRs and intercalation-based qPCRs. Noting the above-mentioned advantages of probe-based qPCRs, we optimized the Procomcure PhoenixDx kit to perform a direct qPCR with a Mycoplasma infected U937 cell culture. Our results indicates that the optimal temperature was the same as that in the original protocol, so the primer+probe binding was not affected by the presence of the direct template. The fact that the optimal template volume was 6 µl (30% of the total qPCR volume) meant that the direct sample did not have a significant inhibitory effect on the qPCR. A major optimization step that we performed decreased the annealing/extension time from 60 sec to 20 sec, thus saving 40 seconds in each cycles. Interestingly, this decrease led to only a minor decrease in the sensitivity (~0.6 Ct level increase). In addition to decreasing the cycle number from 50 to 40, the total qPCR time required was reduced to 65 minutes. When we used the optimized qPCR protocol with direct and purified cell culture templates, we found that the Ct levels of a 6 µl direct template was almost identical with that of the purified DNA from a 60 µl cell culture. The reason for this is mainly due to a dilution of the original DNA content during the elution step at the end of the DNA purification. Overall in our case the direct qPCR sensitivity was higher than the qPCR with a purified template, with a saving in the cost/time of DNA purification. Then we followed the elimination of Mycoplasma contamination from the U937 cell culture using the optimized direct qPCR protocol. One of the concerns using pathogen DNA detection is that the non-viable pathogen's DNA can also be detected and lead to a false positive signal. In our case, however, the Mycoplasma DNA content dropped to ~20% of the original concentration after 1 day of treatment, and though days 1 and 2 contained a similar level of DNA, this decrease continued on day 3. In summary, with direct qPCR we were able to monitor the elimination of Mycoplasma over the treatment period. In conclusion, we optimized a probe-based qPCR to detect Mycoplasma contamination in a user-friendly manner. This direct qPCR method does not require a purification step, it maintains sensitivity and it offers a shorter 65-minutes protocol. Limitations While we did not observe a major qPCR inhibitory effect of U937 cell culture, it cannot be ruled out that components of other cell cultures may have an inhibitory effect. Most probe based qPCR kits, including the kit used here, contains an internal control (e.g. HEX-labeled probe), therefore the detection of qPCR inhibition (no FAM, no HEX signals) is straightforward. In the case of qPCR inhibition, dilution of the direct sample may be a solution for decreasing/eliminating qPCR inhibition.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare the following competing interests: SB and KÖ are employees of Procomcure Biotech GmbH, the manufacturer of the PhoenixDx® Mycoplasma Mix.

Funding

This project was supported by the University of Szeged, Faculty of Medicine (grant number: Hetenyi-5S 575 A202).

Authors' contributions

ZB: performed optimization experiments, was involved in preparing the manuscript and figures, FS: study design, performed optimization experiments, was involved in preparing the manuscript,

SB: performed optimization experiments, was involved in preparing the manuscript,

KÖ: involved in the study design, and preparing the manuscript.

Acknowledgements

Not applicable.

References

1. Drexler HG, Uphoff CC. Mycoplasma contamination of cell cultures: Incidence, sources, effects, detection, elimination, prevention. Cytotechnology. 2002;39:75–90.
2. Feng N, Huang X, Jia Y. Mycoplasma contamination affects cell characteristics and decreases the sensitivity of BV2 microglia to LPS stimulation. Cytotechnology. 2019;71:623–34.
3. Hoff FW, Hu CW, Qutub AA, Qiu Y, Graver E, Hoang G, et al. Mycoplasma contamination of leukemic cell lines alters protein expression determined by reverse phase protein arrays. Cytotechnology. 2018;70:1529–35.
4. Benedetti F, Davinelli S, Krishnan S, Gallo RC, Scapagnini G, Zella D, et al. Sulfur compounds block MCP-1 production by Mycoplasma fermentans-infected macrophages through NF-κB inhibition. J Transl Med. 2014;12:145.
5. Yang J, Hooper WC, Phillips DJ, Talkington DF. Interleukin-1beta responses to Mycoplasma pneumoniae infection are cell-type specific. Microb Pathog. 2003;34:17–25.
6. Eszik I, Lantos I, Önder K, Somogyvári F, Burián K, Endrész V, et al. High dynamic range detection of Chlamydia trachomatis growth by direct quantitative PCR of the infected cells. J Microbiol Methods. 2016;120:15–22.
7. Bogdanov A, Janovák L, Lantos I, Endrész V, Sebők D, Szabó T, et al. Nonactivated titanium-dioxide nanoparticles promote the growth of Chlamydia trachomatis and decrease the antimicrobial activity of silver nanoparticles. J Appl Microbiol. 2017;123:1335–45.
8. Lantos I, Virok DP, Mosolygó T, Rázga Z, Burián K, Endrész V. Growth characteristics of Chlamydia trachomatis in human intestinal epithelial Caco-2 cells. Pathog Dis. 2018;76.
9. Párducz L, Eszik I, Wagner G, Burián K, Endrész V, Virok DP. Impact of antiseptics on Chlamydia trachomatis growth. Lett Appl Microbiol. 2016;63:260–7.
10. Raffai T, Burián K, Janovák L, Bogdanov A, Hegemann JH, Endrész V, et al. Vaginal Gel Component Hydroxyethyl Cellulose Significantly Enhances the Infectivity of Chlamydia trachomatis Serovars D and E. Antimicrob Agents Chemother. 2019;63.
11. Virók DP, Eszik I, Mosolygó T, Önder K, Endrész V, Burián K. A direct quantitative PCR-based measurement of herpes simplex virus susceptibility to antiviral drugs and neutralizing antibodies. J Virol Methods. 2017;242:46–52.
12. Yuan JS, Reed A, Chen F, Stewart CN. Statistical analysis of real-time PCR data. BMC Bioinformatics. 2006;7:85.
13. Wehbe K, Vezzalini M, Cinque G. Detection of mycoplasma in contaminated mammalian cell culture using FTIR microspectroscopy. Anal Bioanal Chem. 2018;410:3003–16.
14. Soheily Z, Soleimani M, Majidzadeh-Ardebili K. Detection of Mycoplasma Contamination of Cell Culture by A Loop-Mediated Isothermal Amplification Method. Cell J. 2019;21:43–8.
15. Kotani H, McGarrity GJ. Ureaplasma infection of cell cultures. Infect Immun. 1986;52:437–44.

16. Volokhov DV, Graham LJ, Brorson KA, Chizhikov VE. Mycoplasma testing of cell substrates and biologics: Review of alternative non-microbiological techniques. Mol Cell Probes. 2011;25:69–77.
17. Molla Kazemiha V, Amanzadeh A, Memarnejadian A, Azari S, Shokrgozar MA, Mahdian R, et al. Sensitivity of biochemical test in comparison with other methods for the detection of mycoplasma contamination in human and animal cell lines stored in the National Cell Bank of Iran. Cytotechnology. 2014;66:861–73.
18. Molla Kazemiha V, Bonakdar S, Amanzadeh A, Azari S, Memarnejadian A, Shahbazi S, et al. Real-time PCR assay is superior to other methods for the detection of mycoplasma contamination in the cell lines of the National Cell Bank of Iran. Cytotechnology. 2016;68:1063–80.
19. Duguid JG, Bloomfield VA, Benevides JM, Thomas GJ. Raman spectroscopy of DNA-metal complexes. II. The thermal denaturation of DNA in the presence of Sr2+, Ba2+, Mg2+, Ca2+, Mn2+, Co2+, Ni2+, and Cd2+. Biophys J. 1995;69:2623–41.
20. Wang J, Pan X, Liang X. Assessment for Melting Temperature Measurement of Nucleic Acid by HRM. J Anal Methods Chem. 2016;2016:5318935.
21. MacGillivray AD, McMullen AI. On the influence of ionic strength on the melting temperature of DNA. J Theor Biol. 1966;12:260–5.

Figures

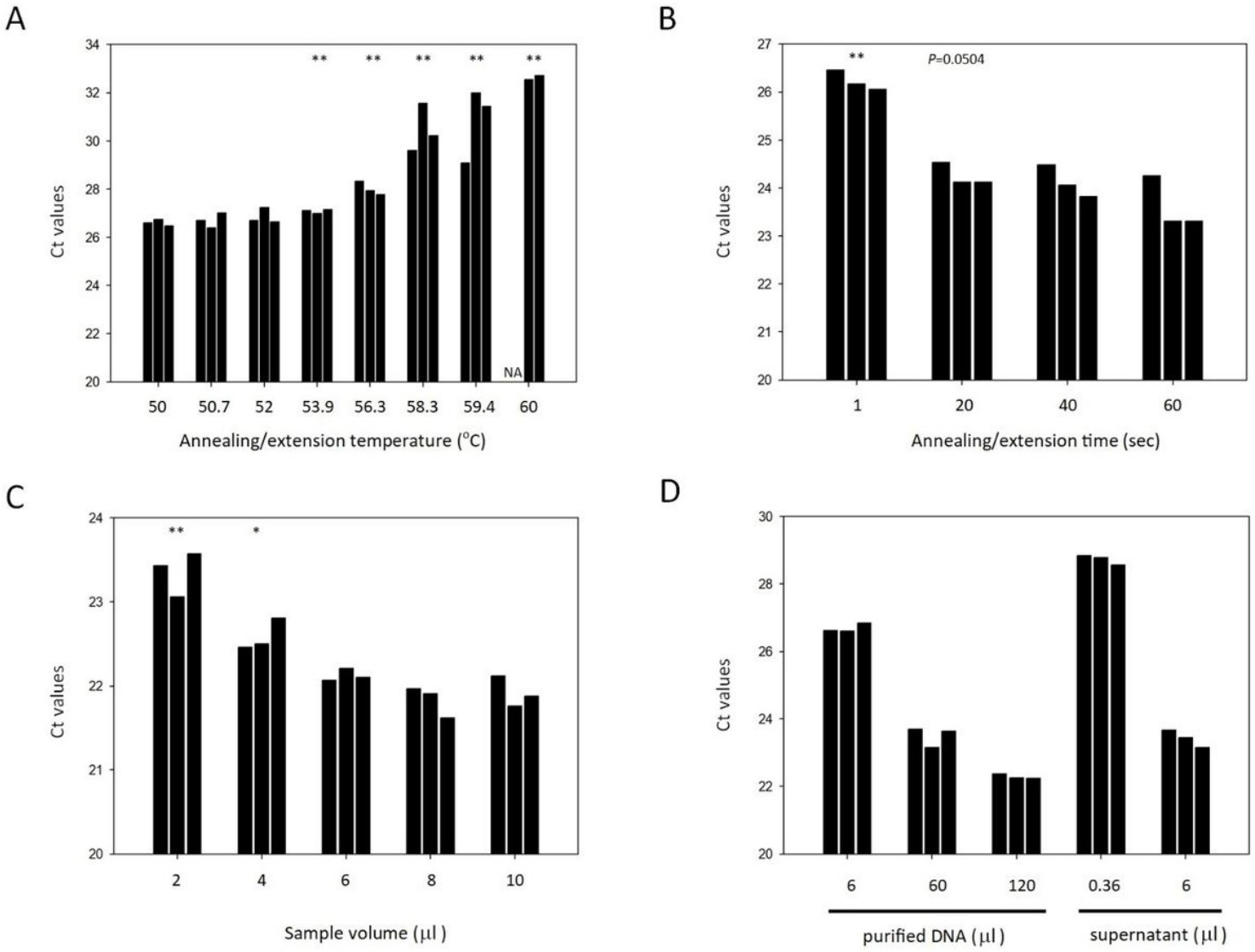

Figure 1

Optimization of the Mycoplasma genus-specific direct qPCR and comparison of its performance with regular qPCR with purified DNA templates. A, effect of the qPCR annealing/extension temperature on the direct qPCR performance. Student's t-test was applied to compare the Ct values of the samples with various annealing/extension temperatures to those samples with a 50oC annealing/extension temperature (n=3). B, effect of the annealing/extension time on the direct qPCR performance. Student's t-test was applied to compare the Ct values of the samples with various annealing/extension times to those samples with a 60 sec annealing/extension time (n=4). C, effect of the sample volume on the direct qPCR performance. Student's t-test was applied to compare the Ct values of the samples with various template volumes with samples having a 10 μl template volume (n=3). D, comparison of the direct qPCR performance with the regular qPCR with a purified DNA template (n=3). The DNA was purified from a 6, 60 and 120 μl cell culture supernatant via the QIAamp protocol and eluted in a 100 μl elution buffer. 6 μl of the eluted DNA was used in the qPCR procedure. As a comparison, 6 μl of the cell culture supernatant was used in the direct qPCR. NA: no amplification was detected. *: P<0.05, **: P<0.01

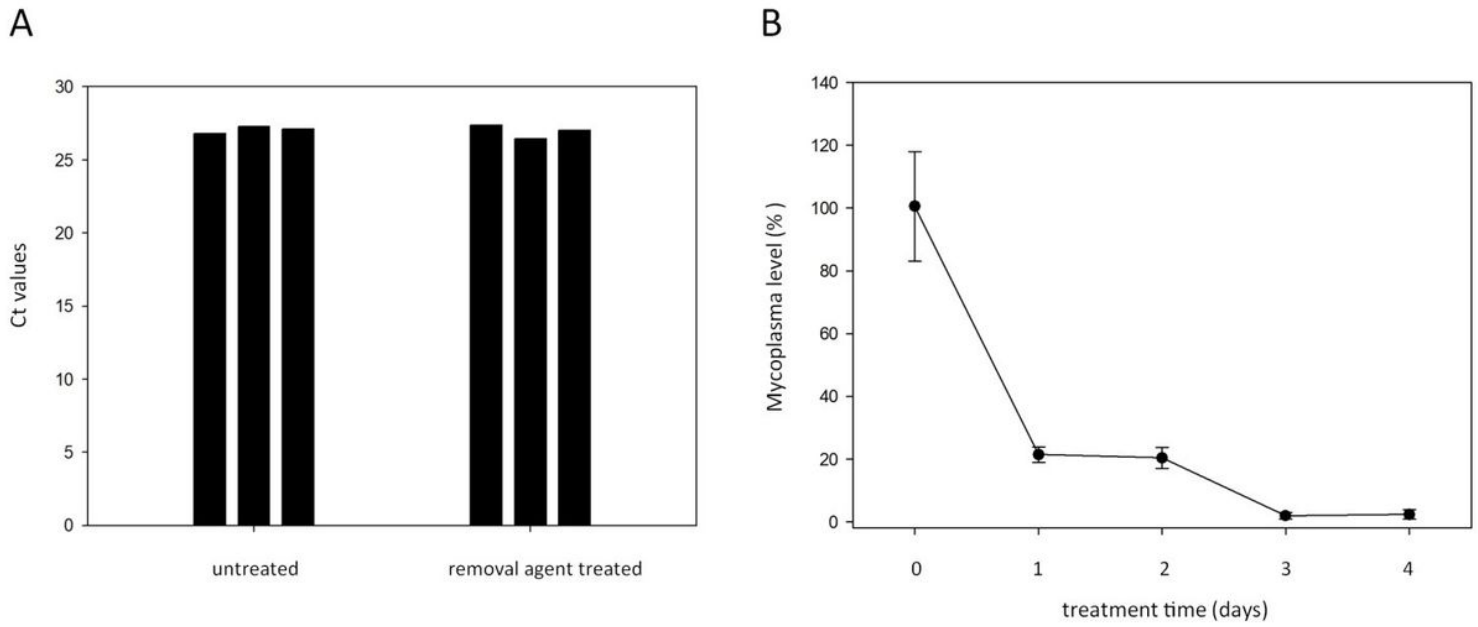

Figure 2

Monitoring Mycoplasma elimination by direct qPCR. Mycoplasma contaminated U937 cells were treated with the Bio-Rad Mycoplasma Removal Agent at 0.5 □g/ml concentration. A, A comparison of qPCR Ct values in the absence and presence of Mycoplasma Removal Agent in the medium of contaminated U937 cells. Student's t-test was applied to compare the Ct values of the removal agent containing samples with the removal agent free samples (n=3). B, The first four days of the treatment monitored by direct qPCR is shown (n=4 at each time point). The Mycoplasma genome concentration on day 0 was defined as 100%.