DNA extraction procedure based on magnetite nanoparticle: A Plackett-Burman design optimization

Tjoa Stanley Evander Emeltan[1,4], Mudasir Mudasir[2], Suharyadi Edi[3] and Daryono Budi Setiadi[1*]
1. Department of Biology, Universitas Gadjah Mada, Yogyakarta, 55281, INDONESIA
2. Department of Chemistry, Universitas Gadjah Mada, Yogyakarta, 55281, INDONESIA
3. Department of Physics, Universitas Gadjah Mada, Yogyakarta, 55281, INDONESIA
4. Konimex Diagnostic Center, PT Konimex, Solo, 57552, INDONESIA
*bs_daryono@mail.ugm.ac.id

Abstract
The MNPs-based DNA extraction method is a commonly used method, but research for its optimization has rarely been done using OFAT. Plackett-Burman is an experimental design used to analyze many factors. In this study, we used Plackett-Burman to screen several factors in the MNPs-based DNA extraction step such as MNPs type, MNPs amount, MNPs solvent, binding buffer, washing buffer, washing method, drying and elution buffer on the concentration and purity of the extracted DNA. Based on Plackett-Burman analysis, none of the factors had a significant effect on the concentration and purity of the extracted DNA.

The analysis also informed the interaction effect between the MNPs amount (in the range 2 – 5 mg) and type (the use of TEOS up to 0.25 w/v) on DNA concentration even though it was not significant. Model 9, one of the models given by the Plackett-Burman matrix was used to extract DNA from environmental bacteria and several types of specimens spiked with Mycobacterium tuberculosis. The extracted DNA has good integrity. It could also be amplified by conventional PCR and probe-based qPCR methods.

Keywords: DNA Extraction, Design of Experiment, Factorial Design, Magnetite Nanoparticle, Plackett-Burman

Introduction

DNA extraction using magnetite nanoparticles (MNPs) is a commonly used procedure[5,6]. The utilization of magnetite nanoparticles is favorable because the method is simple and easy to automate[3]. The preparation is simple and economical with similar function with various commercial magnetic nanoparticles[11]. The extraction procedure is also diverse with various options according to the needs of the type of sample to be extracted.

Despite its widespread use, the extraction procedure optimization is less common. Optimization of the extraction procedure has been carried out by a few studies. Research conducted by Bhati et al[1] optimized the lysis buffer. Research by Firoozeh et al[5] and Torres-Rodríguez et al[14] optimized the binding buffer.

**** Author for Correspondence***

Bhati et al[1] reported that RNAse and Proteinase K can help to increase the purity of extracted DNA in the A260/280 range of 1.72. Without RNAse, the A260/280 ratio increased, indicating RNA contamination. The addition of chaotropic salt is said to increase the concentration and purity of DNA. Chaotropic salt can also act as a binding buffer.

For binding buffer optimization, Torres-Rodríguez et al[14] reported that without binding buffer, the concentration and purity of DNA increased. The magnetic nanoparticles used were coated with silica and the binding buffer used was 20% PEG 8000 and 2.5 M NaCl. In contrary, Firoozeh et al[5] reported that binding buffer with a large amount of PEG will yield a high concentration and high purity of DNA. These studies used One Factor at a Time (OFAT) system. Simultaneous optimization is necessary to see the effect of each factor and possible interaction with each other.

Plackett-Burman is an experimental design used for factor screening. This design is useful to analyze multiple factors simultaneously. This analysis can also provide an overview of the interactions between the analyzed factors. This design is very efficient because multiple factors can be analyzed with a smaller number of experiments compared to OFAT which can save materials, time and energy[2,4].

The aim of this study is to determine the effect of the components that are involved in the MNPs-based DNA extraction such as MNPs type, MNPs amount, MNPs solvent, binding buffer, washing buffer, washing method, drying and elution buffer on concentration and purity of extracted DNA using Plackett-Burman design. Further optimization can be focused only on significant factors which will be identified in this study. In addition, we also want to apply the optimal model generated by this optimization to molecular tests such as conventional polymerase chain reaction (PCR) and probe-based quantitative PCR (qPCR).

Material and Methods

Material: In the synthesis of MNPs, the materials used were $FeSO_4$ and $FeCl_3$ (Glentham), tetraethyl orthosilicate (TEOS), NaOH and ammonia solution (Merck) and ethanol (Bratachem). For the DNA extraction procedure, the materials used were Tris, EDTA (Merck), Triton-X (Vivantis), SDS (BASF), PEG 6000 (Clariant), NaCl (Dominion Salt), Ethanol and Isopropanol (Bratachem).

Environmental bacteria from Konimex diagnostic center research laboratory were grown overnight using Tryptone soy broth medium. The *Mycobacterium tuberculosis* ATCC 25177 (Microbiologics) was grown using Lowenstein-Jensen (LJ) medium. All growth media used were from Himedia. For molecular testing application, Agarose (Himedia), MTB detection qPCR kit and MasterMix (KODC) were used. These materials would be used for electrophoresis, conventional PCR and qPCR testing.

Instrumentation: There were two categories of instruments. The first category was for MNPs synthesis. Magnetic stirrers (HSD180, Jisico), centrifuge (TGL16, Changsa Yingtai), oven (UN30, Memmert) and ultrasonicator (Powersonic 405, Nextgen Lab) were used. The second category was for DNA extraction and molecular applications. Magnetic table, waterbath (WNB7), nanophotometer (Implen-N60) were used for concentration and purity measurements based on A260/280. Thermocycler (BMC-Mic PCR) and horizontal electrophoresis set (Accuris) were used for DNA visualization.

Magnetite Nanoparticle Synthesis: MNPs or Fe_3O_4 synthesis was described as follows: $FeSO_4$ and $FeCl_3$ were diluted in 80 ml of water while stirring with a molarity ratio of 1:2. Then, 3 M NaOH solution was added dropwise with the help of a burette until the pH of the mixture reaches 8-10. The pH value was measured using pH indicator paper. The solution that had reached pH 8-10, was added by water until it reached a final volume of 100 ml. The solution was then allowed to settle and the supernatant was discarded. The precipitate was washed with water twice. The washed precipitate was dried in the oven at 60 - 100 ºC for two hours. Some of the dried precipitate was coated by TEOS according to Thangaraj et al[13].

DNA Extraction Procedure: The general DNA extraction procedure was as follows, the bacteria were lysed using 10 mM Tris, 1 mM EDTA, 0.6% SDS and 0.2% Triton X. The lysate was then mixed with MNPs prepared according to the design. Next, 200 µl of binding buffer was added to attach the DNA with the MNPs. The mixture was homogenized and allowed to stand at room temperature for 3 minutes. MNPs and supernatant were separated using a magnetic table for three minutes to ensure that all MNPs were bound. The supernatant was removed by decanting. After supernatant removal, the magnetic nanoparticles were washed with ethanol 70% in washing buffer twice and eluted by elution buffer.

Design and Analysis: The research design used Factorial design from Design of Experiment (DoE) with Plackett-Burman type from Minitab 19. The list of factors and factor levels used in this experiment is listed in table 1. All the experiments would be conducted using environmental bacteria. Experiments were run according to the matrix given by Plackett-Burman in table 2. Analysis was conducted using a Pareto chart of the effects to determine the factors that affect the concentration and purity of DNA based on A260/280. Model validation would also be monitored based on the observed and predictive value plots of the resulting model.

Application: The selected model would be used in the DNA extraction process from environmental bacteria and specimens (blood, serum, sputum and urine) spiked with *M. tuberculosis*. Environmental bacteria extraction results would be visualized by electrophoresis and amplified using primer pairs to ensure that the extraction procedure could be used for conventional PCR procedures[9]. In addition, extraction results of *M. tuberculosis* spiked specimens would be processed using the *M. tuberculosis* qPCR kit from KODC for CFP10 gene detection. It is a probe-based qPCR kit for *M. tuberculosis*. This step was to demonstrate the ability of this optimization to be used in the amplification of the probe-based qPCR method.

Results and Discussion

The amount and purity of DNA are commonly considered as quality parameters of the DNA extraction. Normally, the amount is associated with the concentration of the extraction. Purity is typically assessed by the A260/280 ratio.

Table 1
Factors and Factor Levels for Plackett-Burman Design

S.N.	Factor	Code	Level	
			Lower (0)	Upper (1)
1	MNPs	A	Without TEOS	With 0.25 w/v TEOS
2	MNPs amount (mg)	B	2	5
3	MNPs solvent	C	EDTA 0.5 M	Tris 10 mM; EDTA 1 mM
4	PEG (%) for binding	D	2	20
5	NaCl (mM) for binding	E	1.25	2500
6	Isopropanol (%) for binding	F	0	100
7	Tris (M) for washing	G	0	1
8	Washing	H	No re-suspension	Re-suspension
9	Drying	I	Without drying	With drying
10	Elution buffer	J	Sterile Water	Tris 10 mM; EDTA 1 mM; pH 8

The Plackett-Burman design was used to evaluate the effect of MNPs type, MNPs amount, MNPs solvent, concentration of PEG, NaCl and isopropanol in binding buffer, used of Tris in washing buffer, washing method with or without re-suspension, drying and elution buffer type on the quality of extracted DNA using magnetite nanoparticle in terms of concentration and A260/280. The experimental results of the 12 models given by Plackett-Burman are listed in table 2.

The factor selection was based on considerations related to concentration and purity. The MNPs type was based on the use of TEOS as a coating agent. The interaction of DNA with plain MNPs or Fe_3O_4 and MNPs coated with TEOS or with silica is different. This was analyzed together with its binding buffer and washing buffer. Similarly, the MNPs solvent is also related to the MNPs conditions before use. The amount of MNPs is related to the amount of DNA that can be bound.

Binding buffer analysis would be carried out for the amount of PEG. According to the research by Firoozeh et al[5], the higher is the level of PEG used, the better are the purity and concentration of DNA obtained. NaCl as a chaotropic salt would be used to help its interaction with the silica surface or to precipitate the DNA. Isopropanol was also analyzed because of its property to precipitate DNA, so it could affect the binding.

In washing, the use of Tris was carried out to determine its effect due to the washing stage. Many studies in the washing stage use salts[7,8,12,14], whereas this step is expected to clean the salts from the previous stage. Re-suspension was also analyzed. More is the contact area with the washing buffer, more effective the washing process would be. Before elution, the MNPs were dried. The aim was to avoid any remaining alcohol. This was conducted because alcohol will affect the A260/280 value[10]. The type of elution buffer was also analyzed because the difference in pH and ionic strength of the DNA solvent will affect the A260/280 value[15].

The analysis was continued by using a Pareto chart of the effects of each response. The results of the analysis could be seen in figure 1 (A) for the concentration response and (B) for A260/280 response. For both responses, none of the factors had a significant effect on the response. In the concentration response, the interaction between MNPs type (code: A) and MNPs amount (code: B) had the greatest effect although it had not crossed the reference line, perhaps in the range of 2 - 5 mg with the addition of TEOS up to 0.25 w/v ratio did not show significance on the extracted DNA concentration.

Looking at their interaction plot seen in figure 2 at a small MNPs amount (2 mg), the presence of TEOS produced a lower DNA concentration. Conversely, at a large amount of MNPs (5 mg), the presence of TEOS increased the DNA concentration. Research to determine the amount of MNPs and TEOS as a coating agent in a wider range is interesting to be carried out further to see its effect on DNA concentration. In the A260/280 response, none of the factors distinguished themselves. All of them were far from the reference line. The various factors tested were considered to be related to DNA purity as described earlier, but the results showed no influence at all as seen from the Pareto chart.

Table 2
Matrix of Plackett-Burman Design and Their Observed Data for Concentration (ng/µl) and Purity (A260/280)

Model	MNP	MNP amount (mg)	MNP solvent	PEG % (binding)	NaCl mM (Binding)	Isopropanol % (Binding)	Tris M (washing)	Washing (Resuspension or not)	Drying	Elution	Cons (ng/µl)	A260/280
1	0	5	0	2	2500	100	1	1	0	1	98.7	1.5
2	0	5	1	2	1.25	0	1	0	0	0	17.5	0.688
3	0	2	0	20	2500	100	0	0	0	0	44.75	1.441
4	0	5	1	20	1.25	100	0	1	1	1	11.05	1.188
5	0	2	1	20	2500	0	1	0	1	1	4.4	1.173
6	0	2	0	2	1.25	0	0	1	1	0	101.1	1.518
7	0.25	5	0	20	1.25	0	0	0	0	1	79.75	1.379
8	0.25	5	0	20	2500	0	1	1	1	0	14.7	1.642
9	0.25	5	1	2	2500	100	0	0	1	0	92.25	1.716
10	0.25	2	0	2	1.25	100	1	0	1	0	10.25	1.486
11	0.25	2	1	20	1.25	100	1	1	0	0	0	1.167
12	0.25	2	1	2	2500	0	0	1	0	1	5.75	1.494

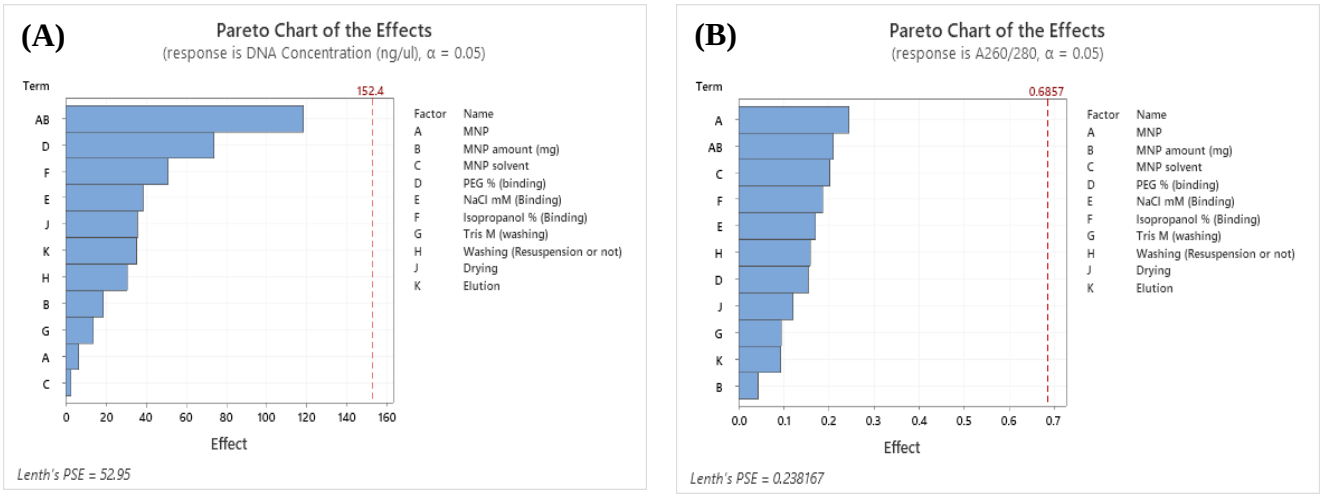


Figure 1: Pareto Chart of the Effects for Both DNA Quality Response. (A) Concentration (ng/µl). (B) A260/280

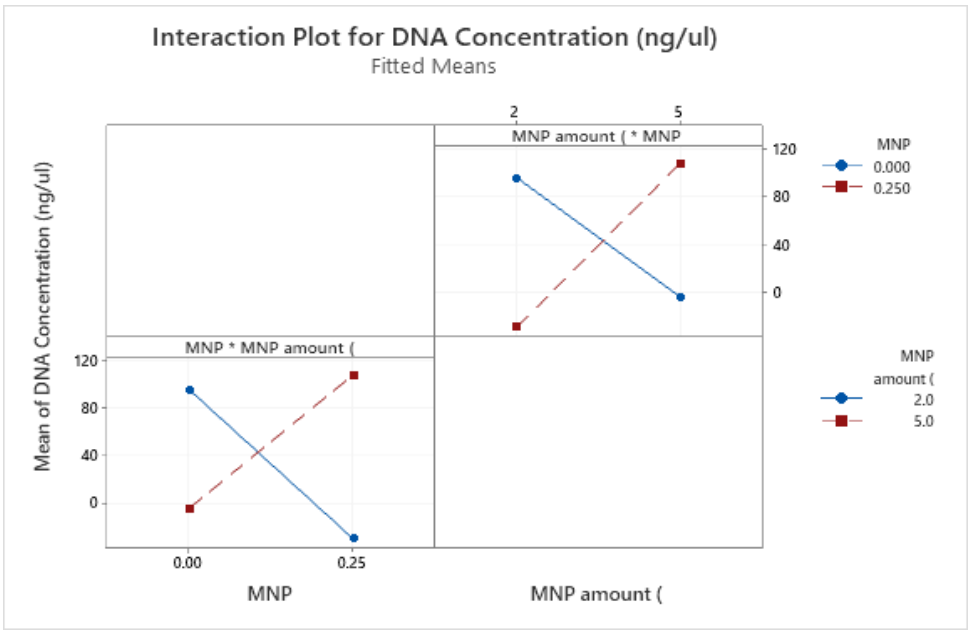
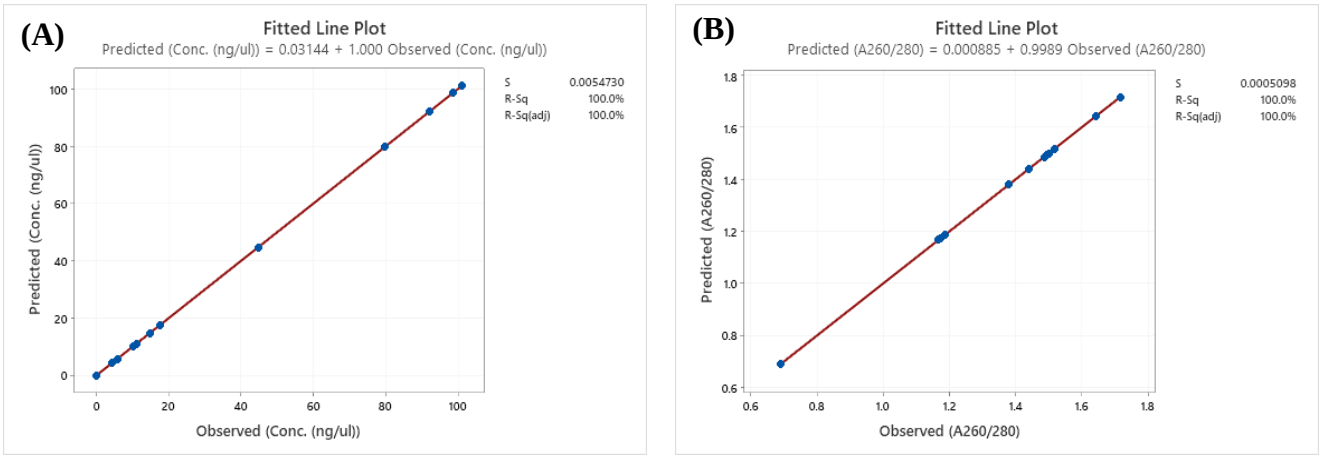


Figure 2: Interaction Plot for DNA Concentration Response of MNP and MNP Amount



Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	18426.0	18426.0	6.15143E+08	0.000
Error	10	0.0	0.0		
Total	11	18426.0			

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	0.842712	0.842712	3242193.70	0.000
Error	10	0.000003	0.000000		
Total	11	0.842714			

Figure 3: Validation Graph for Both DNA Quality Response. (A) Concentration (ng/µl). (B) A260/280

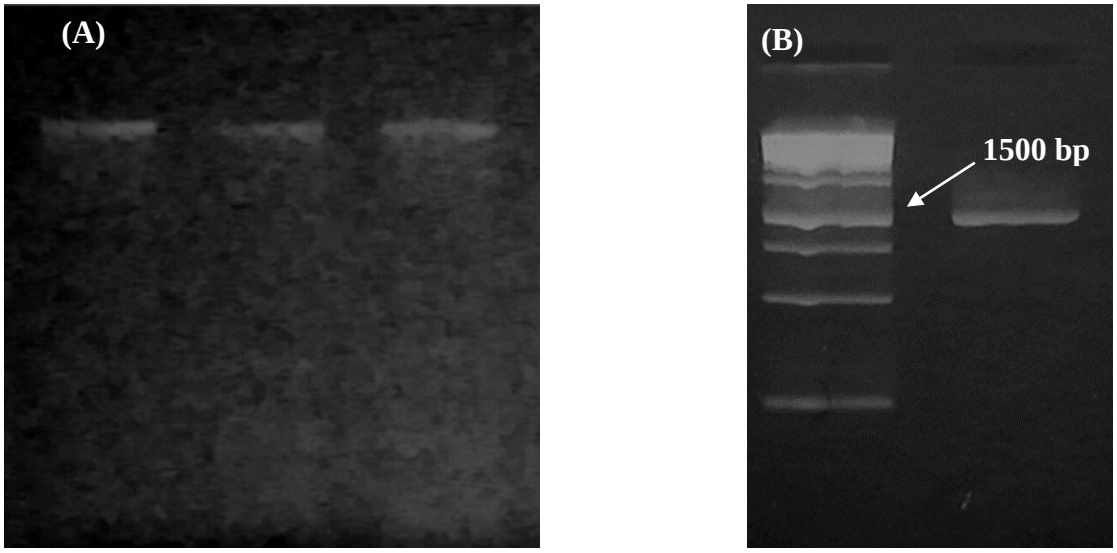


Figure 4: Electrophoresis Visualization of Extracted DNA Using Model 9. (A) Whole Genome. (B) Amplicon for 16S Gene, Size About 1500 bp

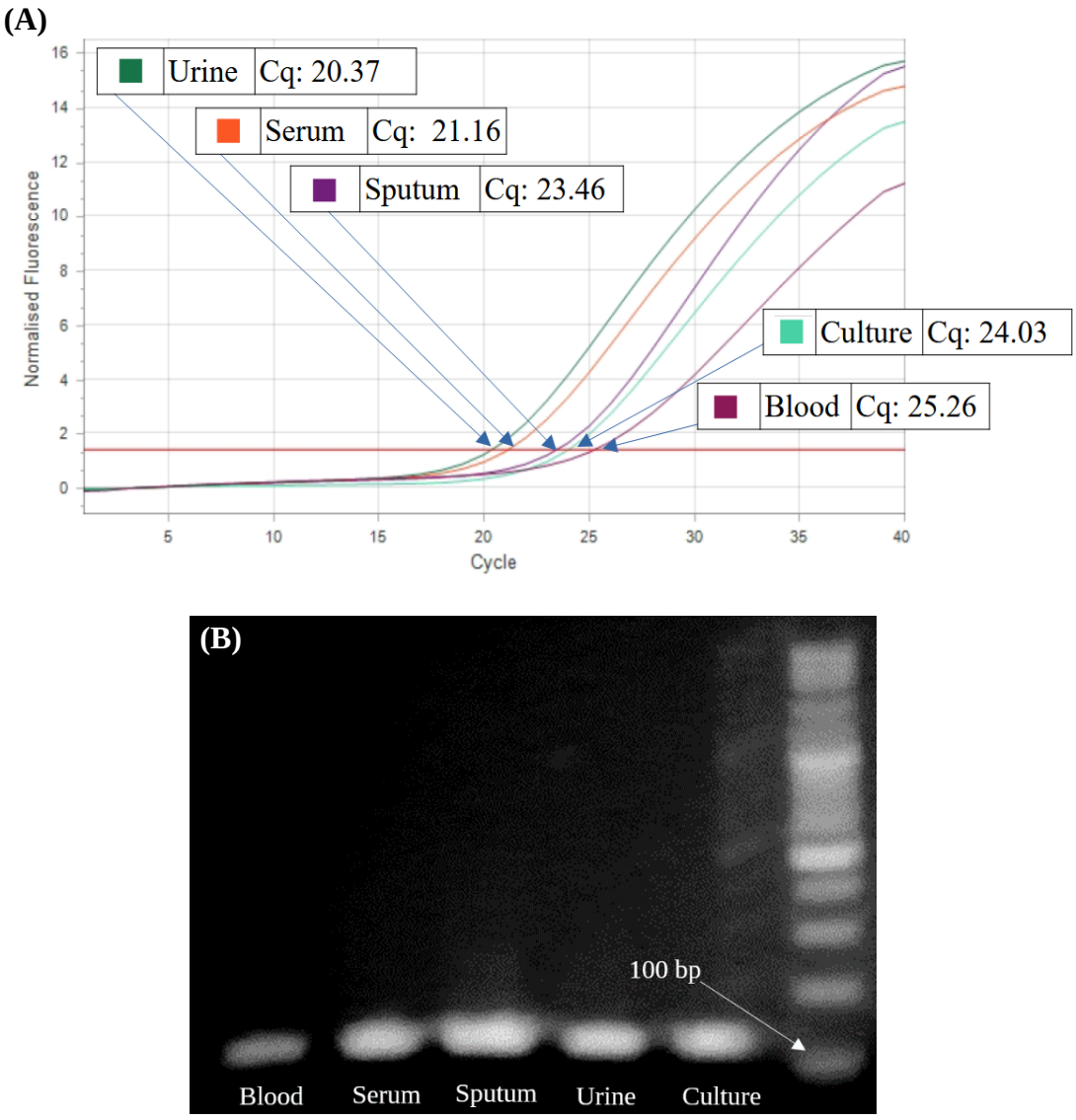


Figure 5: Amplification Result for Probe-Based qPCR for *M. tuberculosis* CFP 10 Gene from Blood, Serum, Sputum and Urine Compared with culture. (A) Graph of Normalised Fluorescence Signal and Cycle for CFP 10 Gene Amplification. (B) Electrophoresis Visualization for CFP 10 Gene Amplicon, Size About 120 bp

The statistical validation of the analysis was included in figure 3 fitting line plot and analysis of variance: (A) for concentration-response and (B) for A260/280 response. The analysis shows the relationship between the observed and predicted results with the coefficient of determination > 0.9 and the regression model's p-value < 0.05 which indicates statistical significance for both the responses. The equation used was the result of a previous factorial design analysis.

DNA concentration = 180.9 - 1132A - 33.24B - 2.783C - 4.103D - 0.01543E + 0.51F + 13.85G + 30.67H - 35.72I + 35.3J + 315.9AB

A260/280 = 1.424 - 0.9818A - 0.08448B - 0.2022C - 0.008574D + 0.000068E + 0.001863F - 0.09433G + 0.1588H + 0.1212I + 0.09367J + 0.5604AB

Fortunately, in this experiment, we got model 9 which produced a relatively high concentration with a purity of 1.716 close to 1.8. To confirm whether model 9 could be applied to a further test such as PCR, the experiment continued with electrophoresis visualization to see the integrity of the extracted DNA. The results of whole genome electrophoresis are shown in figure 4A. There was a DNA band without smear underneath which indicates that the extracted DNA was not fragmented or had good integrity. Subsequently, 16S gene amplification was also performed to ensure that the extraction results did not contain PCR inhibitors. The visualization results coul be seen in figure 4B. There was a 16S gene amplicon band that was about 1500 bp in size. Model 9 was also used to extract DNA from *M. tuberculosis* culture spiked into several types of specimens. The extracted DNA was amplified by probe-based qPCR method and visualized by electrophoresis. The results can be seen in figure 5. Model 9 could be used for DNA extraction from blood, serum, sputum and urine specimens and then can amplified by probe-based qPCR. DNA amplification results from various specimens gave a signal (Figure 5A). In comparison, the culture did not differ by more than 5 Cq. The visualization results could be seen in figure 5B. The size of the amplicons was about 120 bp.

Conclusion

We observed that factors such as MNPs type, MNPs amount, MNPs solvent, concentration of PEG, NaCl and isopropanol in binding buffer, used Tris in washing buffer. Washing method with or without re-suspension, drying and elution buffer type had no influence on the concentration and purity of extracted DNA in the Plackett-Burman design analysis. The regression model generated by the Plackett-Burman design showed the effect of the interaction between the amount and type of MNPs although the effect was insignificant. Future experiments may further explore the influence of these two factors.

In this study, we also applied model 9 for PCR amplification. The use of MNPs with 0.25 w/v TEOS, with an amount of 5 mg dissolved in TE buffer with binding buffer containing PEG 6000 2% and NaCl 2.5 M in isopropanol, without Tris in washing buffer, without re-suspension in washing process, with drying and sterile water as elution buffer could be used to extract DNA from environmental bacteria. *M. tuberculosis* was spiked into blood, serum, urine and sputum. The extracted DNA could be used in conventional PCR and probe-based qPCR procedures.

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