Optimization of Binding, Washing and Elution Buffer for Development of DNA Isolation Kit

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Optimization of Binding, Washing and Elution Buffer for Development of DNA Isolation Kit

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Abstract. DNA isolation is one of the most crucial part in DNA analysis and is reflected by the abundance of ready-to-use DNA isolation kits available in the market. However, the chaotropic salts used in conventional kits during the binding step has been known to inhibit the downstream process of PCR and deteriorate when exposed to air. This study aims to design a better and faster DNA isolation process with better DNA isolation performance to replace the conventional one. This study aims to replace the chaotropic salt in binding buffer with organic acids or salt and improve the buffer used during the wash step. Sodium perchlorate and several other salts and acids were chosen as candidates for the binding buffer. Simultaneously, 10Mm NaCl and 10Mm Tris-Cl with varying concentrations of organic solvents were selected as candidates for the wash buffer. The performance of the selected buffers was then compared to the readily available commercial kit. Organic acid B was among the best candidates for binding buffer with 81.91% and 83.20% recovery rates. For wash buffer, it was observed that the DNA recovery increases with an increasing organic solvent concentration in 10Mm NaCl and 10Mm Tris-Cl. Wash buffer with 90% organic solvent shows the best compromise of DNA yield and purity compared to70%, 80%, and 100% organic solvent concentration in 10Mm NaCl and 10Mm Tris-Cl. A combination of organic acid B in binding buffer and 90% organic solvent A in wash buffer were tested against a commercial DNA extraction kit. The combination of organic acid B and 90% organic solvent yielded 72.81 ng/ul compared to 28.46 ng/ul by the commercial kit. The combination of the binding buffer organic acid B and 90% organic solvent in 10Mm NaCl and 10Mm Tris-Cl can replace the current commercial kits without the problems posed by the presence of chaotropic salt.

1. Introduction

The world market for nucleic acid isolation kit was estimated to worth USD 3.2 billion in 2020 and is expected to expand at 8.9% annually to USD 4.8 billion by 2025 [1]. This amount is reflected in the

number of readily available DNA extraction kits in the market. Nucleic acid isolation is a process of extracting DNA from a sample using several physical and chemical methods. DNA extraction is also known as the removal of DNA from the desired cells [2]. As DNA extraction is the most crucial step in any study involving DNA analysis, many ready-to-use DNA extraction kits are readily available. Before the invention of the DNA extraction kit, the nucleic acid isolation was complicated, time-consuming, and minimal output [3, 4]. Hence, the invention of DNA extraction in a box is one of the most critical innovations in the science sector as it makes DNA isolation more effective and easy to use. Innovations surrounding the choice of buffers during DNA extraction have also increased yield obtained from DNA extraction. There are 3 necessary steps in DNA isolation, which are lysis, precipitation, and purification. Lysis is the step of breaking the cell and nucleus to release the DNA. Next, precipitation is a method of adding some chemicals to separate the DNA from cellular debris. At the same time, purification is the method of rinsing the DNA with alcohol to remove unwanted materials [3].

Despite the improvements in the DNA isolation processover the years, there are still some obstacles to improve the DNA isolation process further. Chaotropic salts used in conventional kits for binding have been reported to inhibit the downstream process of Polymerase Chain Reaction (PCR). It is also highly deteriorating when exposed to air [5]. This is due to the crystallization of the highly concentrated salt, and these changes in salt concentration have the potential to inhibit PCR after DNA Isolation. Conventional DNA isolation kits in the market are based on the principle of binding DNA to a layer of silica followed by a wash step with organic solvents to remove impurities from the column. DNA will then be eluted by removing the DNA from the silica. In the conventional DNA extraction method, chaotropic salts play the role during binding. A high salt concentration is required for DNA to bond with silica. The most common chaotropic salt used in solid-phase extraction is guanidinium thiocyanate; it is the most potent chaotropic which disturbs the structure and activity between water and macromolecules like protein and nucleic acids. At the same time, it also helps in binding of DNA to silica surfaces [6].

It has been observed that DNA will be adsorbed by silica even without the presence of chaotropic salts in buffer solution and yielding a similar amount of eluted DNA with a slight adjustment to the buffer conditions [7]. It has also been shown that spin columns work well in a slightly acidic condition [8]. Thus by decreasing the pH to 5 during binding, adsorption of DNA to the silica layer is possible even without the presence of chaotropic salt. Also, nucleic acids can bind with silica without the presence of chaotropic salts but under the slightly acidic condition with kosmotropic salts [6]. Kosmotropic salt is also known as co-solvent, which helps in exhibiting the stability between water. It also helps in stabilizing the intramolecular interaction in macromolecules for examples like protein and nucleic acids too.

This study aims to create a new DNA isolation workflow by optimizing and improving the current DNA isolation process by finding an alternative to chaotropic salt during the binding stage of the DNA isolation process. This will be done by measuring the recovery rate of bacterial DNA concentration by different binding buffer alternatives. The concentration of organic solvent A during the wash stage will also be optimized to find the most effective concentration. The selected binding buffer alternatives will also be tested against a commercial DNA isolation kit.

2. Methods

2.1. Optimization of DNA Binding by Using Selected binding buffers

Initially, *Escherichia coli* bacterial culture in log phase (10^{16} cfu/ml) was prepared and used for DNA isolation. First, the bacteria culture was centrifuged with 13,000 rpm, and the supernatant was then removed slowly. The pellet was then added with 1× STE buffer as a bacterial suspension and followed by adding 20 µl of proteinase-K (20mg/ml). The suspension was then incubated inside a 65 ºC water bath for 15mins to break down the bacterial cell wall and protein digestion. 1% SDS was added to denature the proteins inside the DNA mixture. Then, 250 µl of digested suspension was then pipetted

into 5 sets (3 tubes each) of microcentrifuge tubes, and each tube was added with the tested binding solution at a ratio of 1:1.

Five different types of binding buffer solutions were prepared to test with 5 sets of digested suspension. The binding solutions were:

1) 50mM Tris with 6M sodium perchlorate salt as a positive control as it has a high chaotropic condition;

2) ddH_2O as negative control;

3) the binding solution of 0.25M organic acid A;

4) the binding solution of 0.25M organic acid B and;

5) the binding solution of 0.25M organic acid C. Each binding buffer was then added into each set of digested microcentrifuge tubes with suspension respectively to test the best optimizing organic salt [7].

Afterward, each binding solution was then added with 400mM potassium ions to standardize to pH 5 for a fair comparison between buffers. Each mixture was transferred to silica columns and centrifuged for 15 minutes. The supernatant from each collection tube was removed. After that, 500µl of organic solvent A was added into each mixture for optimizing the binding step and centrifuged for 2mins. The supernatant in each collection tube was removed too. These were then washed with 700µl of washing buffer [10mM NaCl, 10mM Tris. Cl and of organic solvent A] repeatedly and eluted with ddH_2O.

1% TAE Agarose gel electrophoresis was performed for 40 minutes at 90V for all eluted DNA samples for visualization. The purity of eluted DNA was measured at A260/A280 using Nanodrop (Thermo Scientific, Waltham, MA). The DNA concentration wasmeasured in the bacterial suspension before the extraction process and in the eluted DNA samples after extraction using a Nanodrop. The recovery rate was calculated using the following equation:

$$Recovery\ Rate = \frac{Concentration\ of\ DNA\ After\ Extraction}{Concentration\ of\ DNA\ Before\ Extraction} \times 100$$

2.2. Optimization of Wash Step

After knowing the optimum binding solution from the procedure of optimizing of DNA binding buffer by using selected organic acids and salt, the DNA binding steps from previous part were repeated by using the optimum binding solution and the mixtures were prepared in 3 spin columns (4 sets) for optimization of washing buffer. Four different types of washing buffer were prepared: 70%, 80%, 90%, and 100% of organic solvent A with 10mM NaCl and 10 mM Tris.Cl (pH 8.0) respectively. Then, each washing buffer was used to wash the DNA bound to each spin column prepared earlier. DNA concentration and purity from the 4 sets of eluted solutions were measured using a Nanodrop reader and running in a 1% TAE agarose gel for 40 minutes at 90V. The recovery rate was measured using the same equation in 2.1.

2.3. Comparison with commercial DNA Extraction Kit

A major brand of bacterial DNA isolation kit was chosen and compared with the binding buffers of organic acid A, organic acid B, and organic acid C. An optimized washing buffer method developed from this research. DNA from *Escherichia coli* bacterial culture in log phase (10^{16} cfu/ml) was isolated based on the manufacturer's protocol, and the optimized DNA extraction methods developed in this study. The same procedure was repeated for gel electrophoresis, DNA concentration, purity, and recovery.

2.4. Data Analysis

A One-Way Analysis of Variance (ANOVA) was used in this study to analyse the differences of DNA concentration and purity in the comparisons between the binding buffers, wash buffers, and the commercial kit [9]. A post hoc test, Duncan Multiple Range Test (DMRT) were performed for

pairwise comparison [10]. Both tests were analysed using software SPSS (IBM Corp, Armonk, NY) with alpha value, $\alpha = 0.01$.

3. Results and Discussions

3.1. Optimization of DNA Binding by Using Selected Organic Acids and Salt

In this study, the nutrient broth was used to culture the *E. coli* to test the binding ability for the DNA of *E. coli* towards the silica column by different organic acids and salts. After culturing *E. coli* overnight in nutrient broth, the bacterial culture in log phase (10^{16} cfu/ml) was transferred into a test tube and continued with DNA extraction. The aim of carrying this experiment in this particular part is to determine which organic acids and salts are giving an optimum result in binding to the silica spin column. Hence, each bacteria sample was added with different binding buffer, sodium perchlorate (SP, positive control), organic acid A, organic acid B, organic acid C and distilled water (ddH_2O, negative control). At the same time, all the buffers were standardized to pH 5 for a fair comparison.

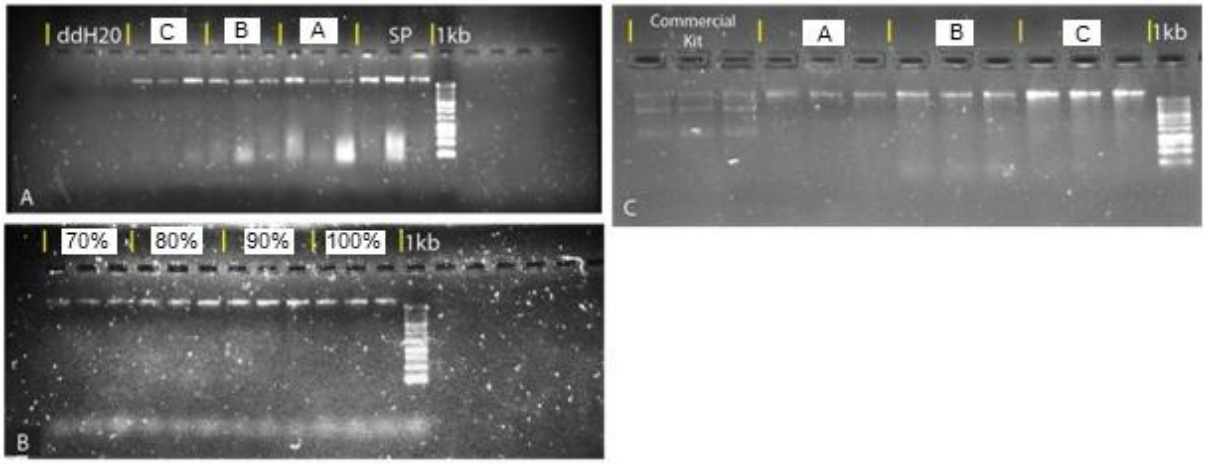

Figure 1. [A] 1% TAE Agarose gel electrophoresis of five binding buffers: ddH2O (Negative Control) = Deionized Water; C = 0.25M Organic acid C; B = 0.25M Organic acid B; A = 0.25M Organic acid A; SP (Positive Control) = 6M Sodium Perchlorate; Ladder = 1kb DNA Ladder; for 40 mins at 90V. [B] 1% TAE Agarose gel electrophoresis of four different organic solvent A concentration of washing buffers: 70%; 80%; 90%; 100%; Ladder = 1kb DNA Ladder; for 40 mins at 90V. [C] 1% TAE Agarose gel electrophoresis of three binding buffers with a commercial kit: Commercial Kit = Binding Buffer from Commercial Kit; A = 0.25M Organic acid A Binding Buffer; B = 0.25M Organic acid B Binding Buffer; C = 0.25M Organic acid C Binding Buffer; Ladder = 1kb DNA Ladder; for 40 mins at 90V.

Figure 1A shows gel electrophoresis for five extracted DNA samples by using different binding buffers, and each binding buffer was adjusted to pH5. The result in Figure 1A shows the bands that were formed indicating successful DNA isolation. Hence, all binding buffer, except ddH_2O (negative control), successfully bind DNA to the silica column. Due to PCR inhibition by chaotropic salt, replacing the chaotropic salt to organic acid B, organic acid A and organic acid C were tested as a new protocol in binding DNA to silica column as several methods have been reported to bind DNA to silica by either reducing the buffer to pH 5 without presence of any chaotropic salts [8] or by replacing chaotropic salt to kosmotrophic salt [6]. Our results show that sodium perchlorate (SP), organic acid B, organic acid A and organic acid C were also effective in DNA adsorption to silica.

Table 1 shows the mean concentration and ratio of 260/280 of DNA from different binding buffers by observing under Nanodrop meter. Each binding buffer is tested with the triplicate, and the mean value of concentration, purity, and recovery rates were selected for comparison. According to the result, without comparing with the positive and negative control, the highest DNA concentration

obtained among all the binding buffers is organic acid B, while the lowest concentration is organic acid C. The ANOVA result shows that the significant value for both concentration and ratio 260/280 of DNA extracted by five different buffers is 0.004, which is lower than the significance value of $\alpha = 0.01$, hence, the null hypothesis is rejected and significant difference does exist between the binding buffers. Table 1 also shows the DMRT result of DNA concentration of five dependent variables. There is only a significant difference between DNA concentration of the binding buffers and the negative control except for organic acid C, while no significant difference ($p > 0.01$) among the binding buffers. DMRT result of 260/280 DNA purity ratio shows a similar trend of the binding buffers being significantly different than the negative control while having no significant differences among all of the binding buffers tested.

Table 1. DNA concentration, purity, and recovery rates comparison of different binding buffers.

BUFFER	Conc., ng/ul (Mean ± SD)	260/280 (Mean ± SD)	Recovery Rate (Mean ± SD)
Sodium Perchlorate	37.48 (± 14.07) [a]	1.73 (± 0.16) [a]	83.20 (± 4.18) [a]
ddH_2O	13.47 (± 1.92) [b]	1.02 (± 0.04) [b]	25.86 (± 3.25) [c]
Organic acid A	40.20 (± 3.49) [a]	1.53 (± 0.20) [a]	73.32 (± 10.26) [ab]
Organic acid B	46.50 (± 8.19) [a]	1.59 (± 0.27) [a]	81.91 (± 1.61) [a]
Organic acid C	29.63 (± 6.07) [ab]	1.65 (± 0.07) [a]	67.06 (± 0.46) [b]
ANOVA Significant Value	0.004	0.004	0.000

[abc] Means with common superscripts are not significantly different ($P > 0.01$) using DMRT

Before extraction, the initial concentration of DNA was 49.723 ng/ul, as was measured using a nanodrop. Table 1 shows the recovery rate of each binding buffer calculated using the equation in 2.2 by substituting the value of '*Concentration of DNA before extraction*' with the measured value of 49.723 ng/ul. According to this result, without comparing with the positive and negative control, the highest recovery rate among all the binding buffers is organic acid B. At the same time, while the lowest is organic acid A. After analysing the data by ANOVA, the ANOVA result shows the significant value of recovery rate for five different binding buffers is lower than the $\alpha = 0.01$, hence, the null hypothesis is rejected and there is significant difference among variables. DMRT shows no significance different ($p > 0.01$) of recovery rate obtained among binding buffer of sodium perchlorate, organic acid A and organic acid B.

3.2. Optimization of Wash Step

The washing step in DNA extraction is a process to remove unwanted salts and proteins to produce a high quality of nucleic acid. Usually, rapid centrifugation is needed to wash away all these unwanted substances from DNA [3]. Other than organic solvent A, the 10mM of NaCl and 10mM of Tris-Cl were added in each washing buffer in this experiment. The role of NaCl is to help in removing proteins that are bound to DNA, too [11]. Besides, weak salting, like 10mM of NaCl, prevents protein aggregation, and keeps proteins dissolved in the aqueous layer [12]. After the DNA from sample bacteria was bound to silica column with the best binding buffer that was selected from the previous step, each spin column with sample DNA was then washed with four different concentration of organic solvent A buffers, which are 70%, 80%, 90% and 100% of organic solvent A with the presence of 10mM NaCl and 10mM Tris-Cl respectively. Figure 1B shows the agarose gel electrophoresis of four different organic solvents A concentration of washing buffer.

Table 2 shows the result of the concentration and ratio of 260/280 of DNA obtained from Nanodrop meter. In Nanodrop meter, pure nucleic acids will show a yield of 260/280 with a ratio of ~1.8 for DNA. This ratio is indicated and affected by the presence of protein, phenol or any contaminants absorbs near 280nm [13]. Based on the result, the washing buffer that produces the most

optimum ratio of of 260/280 is 90% organic solvent A of washing buffer as the value is the nearest to 1.8. In comparison,the lowest ratio value is 70% organic solvent A of wash buffer. Thus, the best washing buffer among all is 90% organic solvent A of wash buffer. The ANOVA significant value for both concentration and ratio 260/280 of DNA for four different washing buffers is less than the alpha value of 0.01. The null hypothesis is rejected, and there is a significant difference among the washing buffers. However, based on the homogenous subsets result in table 2 for four different washing buffers by DMRT, DNA concentration shows no significant difference ($p > 0.01$) between 90% and 80% of organic solvent A washing buffer. Simultaneously, the homogenous subset result shows no significance ($p > 0.01$) for the ratio of 260/280 of DNA obtained from 100% and 90% of organic solvent A washing buffer.

Table 2. DNA concentration, purity, and recovery rates comparison of different binding buffers.

BUFFER	Conc., ng/ul (Mean ± SD)	260/280 (Mean ± SD)	Recovery (Mean ± SD)
100% organic solvent A	35.00 (± 0.48) [a]	2.00 (± 0.07) [a]	96.77 (± 3.81) [a]
10Mm NaCl, 10Mm Tris-Cl, 90% organic solvent A	24.08 (± 0.08) [b]	1.97 (± 0.08) [a]	93.58 (± 2.19) [a]
10Mm NaCl, 10Mm Tris-Cl, 80% organic solvent A	24.34 (± 0.11) [b]	1.59 (± 0.02) [b]	66.77 (± 0.83) [b]
10Mm NaCl, 10Mm Tris-Cl, 70% organic solvent A	17.66 (± 0.39) [c]	1.38 (± 0.08) [c]	54.23 (± 0.12) [c]
ANOVA Significant Value	0	0	0

[abc] Means with common superscripts are not significantly different ($P > 0.01$) using DMRT

The recovery rates were calculated with the measured initial DNA concentration of 26.049ng/ul. Table 2 shows the recovery rate for each washing buffer other than the positive control, 90% organic solvent A of wash buffer is having the highest rate of recovery while 70% organic solvent A of wash buffer is the lowest. Thus, the best washing buffer among all is 90% organic solvent A. Moreover, the ANOVA significant value is less than the alpha value of 0.01, where the null hypothesis is rejected, and there is a significant difference among the washing buffers. DMRT recovery rate for four dependent variables shows no significant difference ($p > 0.01$) on the recovery rate obtained between organic solvent A wash buffer of 100% and 90% organic solvent A wash buffer.

3.3. Comparison with commercial DNA Extraction Kit

In this step, organic acid B was chosen as the optimal binding buffer while, 90% organic solvent A was chosen as the wash buffer based on the results in 3.1 and 3.2. However, organic acid A buffer and organic acid C buffer were also used to compare with a commercial kit. Figure 1C shows the result of gel electrophoresis for three different binding buffers and commercial kits. According to the result in Table 3, DNA concentration of organic acid B buffer is higher than that of the commercial kit while also having a ratio of 260/280 close to the optimal value of 1.8. ANOVA shows a significant difference ($P < 0.01$) for both the concentration and ratio of 260/280 of DNA, among all tested samples. While there is no significant difference between organic acid B and the commercial kits for both DNA concentration and 260/280 purity DNA ratio by DMRT, both methods were grouped in the same subset for 260/280 DNA purity ratio. They were grouped in a different subset for DNA concentration with organic acid B having a higher extracted DNA concentration than the commercial kit.

Table 3. DNA concentration and purity comparison of different binding buffers with a commercial DNA extraction kit.

Buffers	Conc., ng/ul (Mean ± SD)	260/280 (Mean ± SD)
Organic acid B	72.81 (± 2.14) [a]	1.59 (± 0.02) [a]
Organic acid A	31.24 (± 3.05) [b]	1.28 (± 0.04) [b]
Organic acid C	21.78 (± 0.53) [c]	1.30 (± 0.04) [b]
Commercial Kit	28.46 (± 0.13) [b]	1.67 (± 0.03) [a]
ANOVA Significant Value	0	0

[abc] Means with common superscripts are not significantly different (P > 0.01) using DMRT

4. Conclusions

The optimum binding buffer that produces the highest concentration of DNA and brightest band in gel electrophoresis among the entire buffers compared was organic acid B. Next, according to the experiment in optimization of washing step, the result shows that among all the washing buffer, the 90% organic solvent A washing buffer shows the best result for ratio 260/280 of DNA, which indicates 90% organic solvent A washing buffer is giving the purest DNA compare to the other washing buffers as it lies in the value of 1.8~2.0. A comparison between organic acid B and a commercial DNA extraction kit shows that organic acid B extracted 2.6 times more DNA than the commercial kit while having a similar 260/280 purity ratio. It is concluded in this study that substituting chaotropic salt with organic acid B in the binding step and using 90% organic solvent A during washing step have the potential to produce a DNA extraction kit with high DNA concentration and purity without inhibiting downstream PCR process and deteriorating risk when exposed to air.

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