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A systematic investigation of key factors of nucleic acid precipitation toward optimized DNA/RNA isolation

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ABSTRACT

Nucleic acid precipitation is important for virtually all molecular biology investigations. However, despite its crucial role, a systematic study of the influence factors of nucleic acid precipitation has not been reported. In the present work, via rational experimental design, key factors of nucleic acid precipitation, including the type of nucleic acid, temperature and time of incubation, speed and time of centrifugation, volume ratio of ethanol/isopropanol to nucleic acid solution, type of cation-containing salt solution and type of coprecipitator, were comprehensively evaluated in an attempt to maximize the efficiency of nucleic acid precipitation. Our results indicate that the optimal conditions of each influence factor of nucleic acid precipitation may vary in accordance with the chemistry, structure and length of nucleic acids.

METHOD SUMMARY

To maximize the efficiency of nucleic acid precipitation, the key factors, including incubation and centrifugation conditions, and the volume ratio of polar solvents, cation-containing salts and coprecipitators, were systematically evaluated. To gain more practical information, four types of commonly used nucleic acids were tested. The results indicate that the optimal conditions vary greatly in accordance with the type of nucleic acids under study.

KEYWORDS

ethanol • isopropanol • linear polyacrylamide • nucleic acid precipitation

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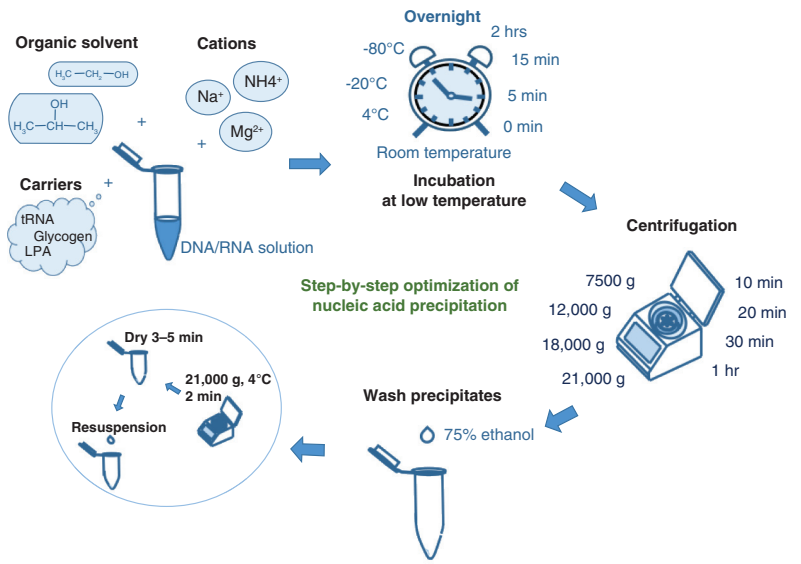
GRAPHICALABSTRACT



Nucleic acid precipitation is an elementary technique to de-salt and concentrate nucleic acids (DNA or RNA) from their aqueous solutions, and it is involved in virtually all molecular biology investigations, such as cloning [1], sequencing [2], hybridization [3], restriction enzyme analysis [4] and transformation [5]. To date, several different procedures for nucleic acid precipitation have been published [5–7]. The principle of this technique is straightforward. Nucleic acids are hydrophilic polar molecules because they carry negatively charged phosphate residues [8]. In aqueous solutions, the phosphate residues attract water molecules and form hydrated retia surrounding DNA/RNA. The large dielectric constant of water can hinder cations from binding to the phosphate residues [9]. However, the dielectric constant of some polar organic solvents, such as ethanol, is much lower than water, therefore allowing cations to neutralize anionic phosphate groups in ethanol [10,11]. Therefore, with the addition of ethanol and salts (e.g., sodium acetate [NaAc]) that contain cations to nucleic acid solution, the repulsive forces among the polynucleotide chains weaken, resulting in the disturbance of hydrated retia and polymerization of nucleic acids, eventually causing the precipitation of nucleic acids [12]. According to our test, negligible nucleic acids were recovered via adding either alcohol alone or NaAc alone (Supplementary Table 1), indicating both polar organic solvents and salts are essential for nucleic acid precipitation. In general, nucleic acid precipitation is composed of three steps. First, cations and polar organic solvents are added to the DNA/RNA solution, followed by incubation at low temperature to promote the precipitation of nucleic acids. Second, centrifugation is performed to pellet the insoluble DNA/RNA. Third, precipitates are washed with ethanol to remove the impurities (e.g., salts and proteins) [5,6,13–15].

Over the past decades, different types of nucleic acid precipitation protocol have been widely performed. Although each of them displays a different recovery rate for different types of nucleic acids, a systematic evaluation of the key factors of nucleic acid precipitation has not been performed. This is probably because for most of the ordinary molecular biological work, such as gene cloning, the harvest rate is not very important, especially when the resulting sequences could be amplified subsequently via PCR. However, it has been noticed that, for many of the recently developed techniques, such as SELEX (for aptamer development) [16] and next-generation sequencing [17], quantitative collection of nucleic acids from solutions is critically important. Here, via rational experimental design (Supplementary Figure 1 & Figure 1), key influential factors of nucleic acid precipitation, including the type of nucleic acids, temperature and time of incubation, force and time of centrifugation, volume ratio of ethanol/isopropanol to nucleic acid solution, type of cation-containing salts and type of coprecipitators, were comprehensively evaluated in an attempt to maximize the efficiency of nucleic acid precipitation for different types of nucleic acids.

MATERIALS & METHODS

Different types of nucleic acids of diverse chemistries (DNA/RNA), structures (single-stranded/double-stranded) and lengths (short/long) were comprehensively evaluated for the optimization of nucleic acid precipitation (Supplementary Figure 1). The concentration of nucleic acids was determined by an ultramicro-spectrophotometer (NanoDrop 2000; Thermo Scientific, MA, USA). Measurement of concentrations were performed twice (before and after precipitation process) in order to calculate the recovery rates of nucleic acids. All of the recovery rates were analyzed via least significant difference pairwise comparison methods using SPSS software version 13.0 (IBM Corporation, NY, USA). The recoveries which have no significant difference with each others were marked with same letter [18–20]. Besides, all RNA/DNA solutions were adjusted to a relatively low level (100 ng/μl) prior to precipitation procedures, owing to the fact that nucleic acids of high initial concentrations normally achieve a higher recovery rate compared with those of lower initial concentrations [5], which may obscure the distinctive effects of various conditions of each influence factor on nucleic acid recovery levels. Purity of nucleic acids met two standards: the A260/A280 ratios were between 1.8 and 2.0, whereas the A260/A230 ratios exceeded 2.0 [21,22].

Optimization of incubation conditions

To our knowledge, the incubation conditions of nucleic acid precipitation were first explored in this study. Prior to incubation, ethanol and NaAc were added into the nucleic acid solutions as polar organic solvent and source of cations, respectively. The volume ratio of ethanol to DNA/RNA solutions was 3:1, and the working concentration of NaAc was 0.3 M [23]. The incubation procedure was subsequently performed under a variety of conditions: -20°C overnight, 4°C overnight, -20°C for 2 h, -80°C for 5 min, room temperature (RT) for 0 min (immediate centrifugation), RT for 15 min and 4°C for 15 min, followed by centrifugation at 12,000 × *g*, at 4°C for 10 min. Average recovery rates of each type of nucleic acids were then calculated, and the optimal incubation conditions, which led to the maximum recovery rates, were applied in succeeding steps of the optimization.

Optimization of centrifugation force

Ethanol (v/v: 3:1) and NaAc (0.3 M) were added into each type of nucleic acid solution, followed by incubation of the nucleic acids under their optimized conditions, correspondingly. Next, nucleic acid suspensions were centrifuged at 7500, 12,000, 18,000 and 21,000 × *g* for 10 min, respectively. Average recovery rates of each type of nucleic acids were then calculated and the optimal centrifugation force resulting in maximum recovery rates was applied in succeeding steps of the optimization.

Optimization of centrifugation time

Ethanol (v/v: 3:1) and NaAc (0.3 M) were added into each type of nucleic acid solutions, followed by optimized incubation. Next, centrifugation was performed under optimized force for different lengths of time, which included 10, 20, 30 min and 1 h, respectively. Average recovery rates of each type of nucleic acids were then calculated, and the optimal centrifugation time leading to maximum recovery levels was applied in succeeding steps of the optimization.

Optimization of volume ratio of ethanol/isopropanol to nucleic acid solution

Ethanol or isopropanol of various volume ratios (ethanol: 2×, 3×, 4×; isopropanol: 0.5×, 0.75×, 1×) were added into each type of nucleic acid solution with NaAc (0.3 M), followed by incubation and centrifugation under optimal conditions. Average recovery rates of each type of nucleic acids were then calculated, and the optimal volume ratios of ethanol/isopropanol, which resulted in maximum recovery levels, were applied in succeeding steps of the optimization.

Optimization of cation types

Ethanol or isopropanol of optimized volume ratios was added into the nucleic acid solutions with different types of salts containing cations, which included NaAc, a mixture of NaAc and $MgCl_2$, $MgCl_2$, NH_4Ac and a mixture of NH_4Ac and $MgCl_2$. The working concentrations of NaAc, $MgCl_2$ and NH_4Ac were 0.3, 0.01 and 2.5 M, respectively. Optimized incubation and centrifugation were conducted afterwards. The average recovery rates of each type of nucleic acid were then calculated, and the optimal choices of cation containing salts resulting in maximum recovery rates were applied in the succeeding step of the optimization.

Optimization of coprecipitator (carrier)

Ethanol or isopropanol of optimized volume ratios and cation containing salts of optimal selections were added into nucleic acid solutions with different types of coprecipitators, which included yeast tRNA (Solarbio, Beijing, China), glycogen (Sangon, Shanghai, China) and linear polyacrylamide (LPA; Sigma-Aldrich, MO, USA). The working concentrations of yeast tRNA, glycogen and LPA were 20, 50 and 20 μg/ml [11], respectively. Optimized incubation and centrifugation were conducted afterward. The average recovery rates of each type of nucleic acids were then calculated.

RESULTS & DISCUSSION

In the present study, four types of the most commonly used nucleic acids with different chemistry and length, including short single-stranded RNA (e.g., microRNA:

Figure 1. Step-by-step optimization of nucleic acid precipitation.
RT: Room temperature.

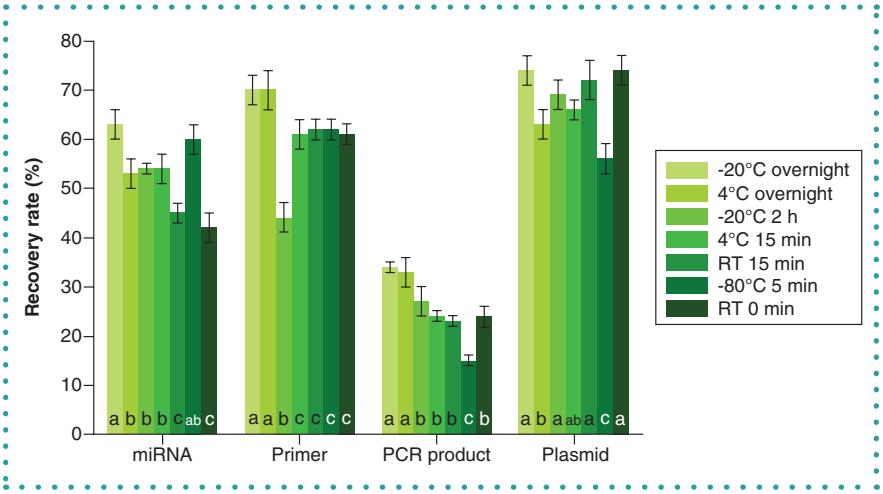


Figure 2. The recovery rates of different nucleic acids under different incubation conditions. Letters a, b, c and d represent statistical significance among different incubation conditions of each nucleic acid ($p < 0.05$). The recoveries with the same letter(s) indicate no significant difference. The letters 'ab' indicate that the recovery rates have no significant difference with the recoveries with letter 'a,' as well recoveries with letter 'b.' The original data of recovery rates are shown in Supplementary Table 2.

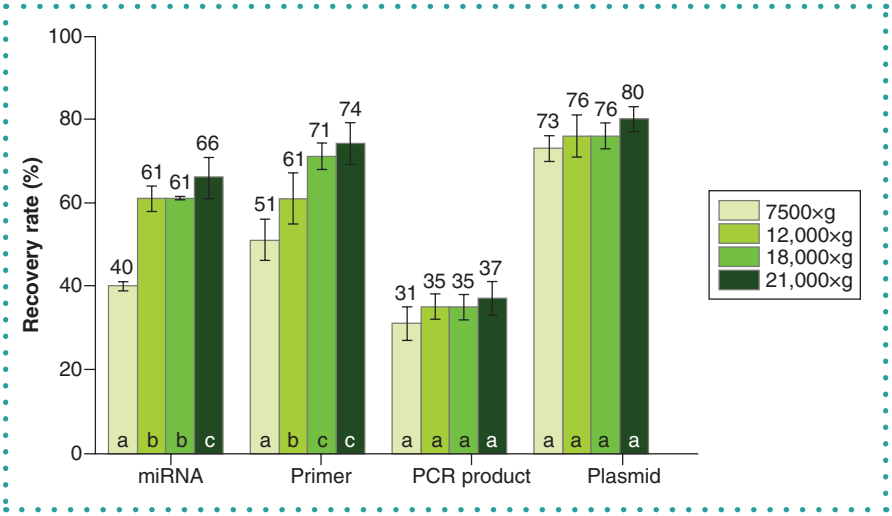


Figure 3. The recovery rates of miRNA, primer, PCR product and plasmid under different centrifugation force. Letters a, b and c represent statistical significance under different centrifugation force of each nucleic acid ($p < 0.05$). The recoveries with the same letter(s) indicate no significant difference. Statistically, a gradual increase with the increase of centrifugal force was observed on short RNA (miRNA) and DNA (primer sequences, although not for the medium-length PCR products and long plasmids. The original data of recovery rates are shown in Supplementary Table 3.

20 nt), short single-stranded DNA (e.g., primer: 20 nt), medium-length double-stranded DNA (PCR product: 150 bp) and long double-stranded DNA (plasmid: 10k bp) were tested. As shown in Figure 1, the evaluation was performed in a step-by-step manner starting with incubation condition optimization, then progressively followed by optimizations for centrifugation force, centrifugation time, volume ratio of organic solvent (ethanol/isopropanol), cation containing salts and type of coprecipitators. Optimum conditions leading to maximum recovery rates of their corresponding nucleic acids, obtained from each step of optimization, were subsequently applied in the succeeding steps. Therefore, the optimization work performed in this study was conducted on a systematic basis. Recovery rate of nucleic acids was represented by the ratio of the amount of nucleic acid after precipitation to its initial amount (before precipitation). All experiments were conducted in triplicates.

Incubation conditions

Commonly used incubation conditions for nucleic acid precipitation include -20°C overnight, 4°C overnight, -20°C for 2 h, -80°C for 5 min and RT for 0 min (without incubation). As shown in Figure 2, the highest recovery rates of miRNA (61%) and PCR products (33%) were achieved after overnight incubation at -20°C, whereas primer sequences achieved maximum recovery (72%) after overnight incubation at 4°C. Interestingly, for long DNA sequences (plasmid), the prolonged overnight incubation at -20°C did not display any type of a benefit compared with direct centrifugation (without incubation), with a 72 and 75% recovery rate for -20°C overnight incubation and without incubation, respectively (Figure 2 & Supplementary Table 2). Apparently, the recovery rates of nucleic acids generally displayed increase with the increase in incubation time. Under the same -20°C condition, after overnight incubation, the yields of miRNA (61%), primer (65%) and PCR product (33%) were significantly higher than that of the 2 h incubation groups (miRNA: 53%, $p = 0.009$; primer: 48%, $p = 0.000$; PCR product: 28%, $p = 0.001$), with the exception of plasmid, which did not display a significant difference under the tested conditions; $p = 0.055$ (-20°C overnight: 72%, -20°C for 2 h: 66%) (Figure 2 & Supplementary Table 2). Markedly, distinct reduction in recovery was observed at -80°C (5 min) in the cases of PCR product (15%) and plasmid (44%), in comparison with their 'adjacent' conditions, which were -20°C (2 h) (PCR product: 28%, plasmid: 66%) and without incubation (PCR product: 30%, plasmid: 75%) (Figure 2).

Centrifugal force

Evaluation of the effect of different centrifugal force, including 7500, 12,000, 18,000 and 21,000 × *g*, on the recovery rate was conducted by applying the optimum incubation conditions of each type of nucleic acid. As shown in Figure 3, the medium-length PCR products and long plasmids did not display statistical differences with a different centrifugation force, with the low spinning force (7500 × *g*) achieving a comparable recovery yield (31 and 73% for PCR product and plasmid) with the top force 21,000 × *g* (37 and 80% for PCR product and plasmid; $p = 0.079$, $p = 0.096$).

Although in the cases of the short RNA (miRNA) and DNA (primer) sequences, the yield demonstrated a gradual increase with the increase of centrifugal force. For example, the highest yield of 66% of miRNA with 21,000 × *g* is significantly higher than that of the 40% yield with 7500 × *g* ($p = 3.76$; E-07). A similar phenomenon was observed for primer sequences, with a 74% yield and a 51% yield for the 21,000 × *g* and 7500 × *g* groups, respectively ($p = 4.27$; E-04) (Figure 3).

Centrifugation time

Examination of the centrifugation time was performed based on the optimal conditions of incubation and centrifugation force (Figure 1). As shown in Figure 4, although increasing centrifugation time generally led to gradual rise of nucleic acid recovery rates, no significant difference was observed for different treatment groups. For all types of nucleic acids, even the lowest yields (63, 73, 37 and 77% for miRNA, primer, PCR products and plasmid) did not display prominent difference with the highest yield groups (69, 80, 44 and 83% for miRNA, $p = 0.081$, primer, $p = 0.079$, PCR products, $p = 0.053$, plasmid, $p = 0.21$).

Volume ratio of ethanol/isopropanol to nucleic acid solution

Based on the optimal conditions of incubation and centrifugation, optimization for volume ratio of polar organic solvent (ethanol or isopropanol) to nucleic acid solutions was conducted. Commonly used volume ratio of ethanol (2×, 3× and 4×) and isopropanol (0.5×, 0.75× and 1×) were tested. Interestingly, although for short sequences (miRNAs and primers), the ethanol group generally showed higher recovery rates than their counterparts with isopropanol (74 and 85% top yields for miRNA and primer in the ethanol groups vs 61 and 60% top yields for miRNA and primer in the isopropanol groups). However, similar results were observed between ethanol and isopropanol groups for the medium-length PCR products and the long plasmid sequences under the low volume ratio groups, whereas high volume ratio groups tend to the same level (Figure 5).

Specifically for the ethanol-mediated precipitation, the yields plateaued at 2× the ethanol amount for the medium-length PCR product and long plasmid, whereas the 2×, 3× and 4× groups showed no significant differences ($p > 0.05$). However, for short RNA (miRNA) and DNA (primer) sequences, it is apparent that the recovery rates display an increase with the increase of ethanol volume.

For example, whereas 4× of ethanol addition resulted in up to 85% recovery of primers, only 59% primers were precipitated when 2× ethanol were used. However, 1× isopropanol appears to have a comparable efficacy with the lower 0.75× groups, except in the primer group, in which the 1× isopropanol resulted in a notably higher recovery rate (60%) than the 0.75× group (42%). Clearly, the 0.5× isopropanol resulted in the lowest yields in all groups.

Cation types

Assessment of the optimized cation condition was performed on the basis of the optimum conditions of incubation, centrifugation and organic solvent volumes (Figure 1). Five different types of salts, which included NaAc only (0.3 M working concentration), combined NaAc and $MgCl_2$, $MgCl_2$ only (0.01 M working concentration), NH_4Ac only (2.5 M working concentration) and combined NH_4Ac and $MgCl_2$, were evaluated with either ethanol or isopropanol (Figure 6). Results indicated that nucleic acids with ethanol achieved their maximum recovery rates with the addition of $MgCl_2$ only (miRNA: 80%; PCR product: 63%) or NaAc only (primer: 88%; plasmid: 79%) (Figure 6). Similarly, nucleic acids with isopropanol also attained their highest recovery levels when incubated with $MgCl_2$ only (primer: 70%; PCR product: 59%) or NaAc only (miRNA: 61%; plasmid: 72%) (Figure 6).

Importantly, the addition of NaAc to all types of DNA/RNA solutions resulted in higher recovery rates compared with NH_4Ac. For example, in both the ethanol and isopropanol groups, higher recovery levels of primer were achieved after NaAc-only addition (88 and 68% for ethanol and isopropanol, respectively) than NH_4Ac-only (52 and 40% for ethanol and isopropanol, respectively) (Figure 6 & Supplementary Table 6). In addition, in most cases adding a mixture of NaAc and $MgCl_2$ into the nucleic acid solutions led to higher nucleic acid recovery levels than the addition of NH_4Ac and $MgCl_2$ mixtures, with miRNA as an exception when isopropanol was used, in which a higher recovery rate was achieved with the NH_4Ac and $MgCl_2$ mixture (46%) rather than the combined application of NaAc and $MgCl_2$ (43%) (Figure 6 & Supplementary Table 6).

Interestingly, all types of nucleic acids with ethanol demonstrated lower recovery rates when incubated with mixtures of salts compared with adding one type of salt. For instance, miRNA with ethanol attained less recovery levels on addition of the mixture ▶

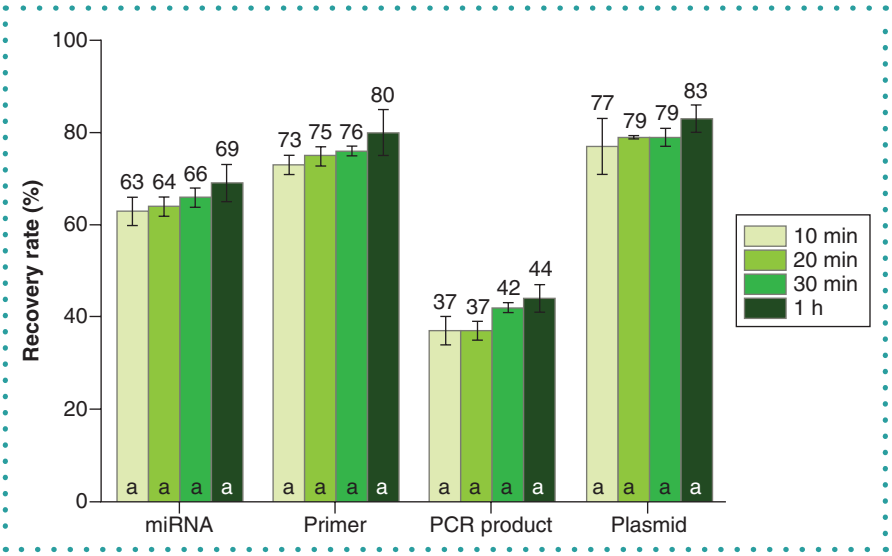


Figure 4. The recovery rates of different nucleic acids under different centrifugation time. The letter a represents statistical significance under different centrifugation times for each nucleic acid ($p < 0.05$). For all nucleic acid types, no significant difference was observed for different centrifugation time. The original data of the recovery rates are shown in Supplementary Table 4.

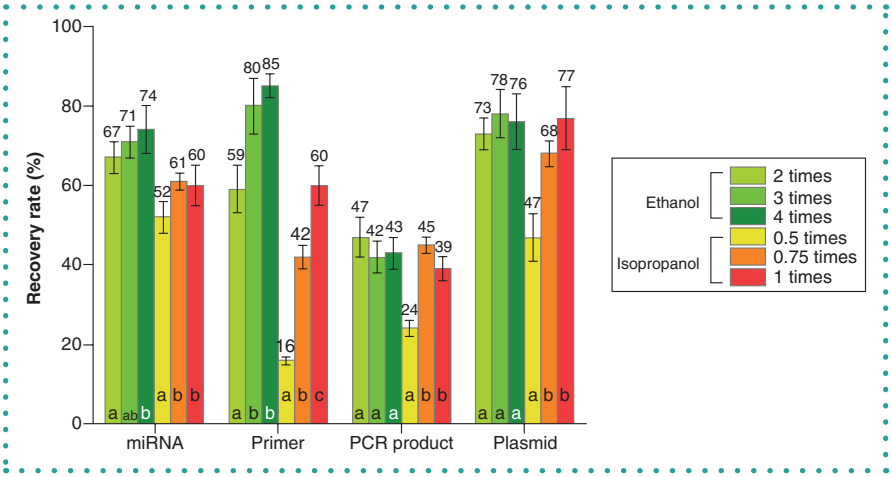


Figure 5. The recovery rates of different nucleic acids under different volume ratio of ethanol/isopropanol to nucleic acid solution. Letters a, b and c represent statistical significance under different volume ratio of ethanol/isopropanol ($p < 0.05$). The recoveries with the same letter(s) indicate no significant difference. The letters 'ab' indicate that the recovery rate has no significant difference with the recoveries with letter 'a,' as well as the recoveries with letter 'b.' The optimal polar solvents ethanol/isopropanol and their volume ratio vary greatly in accordance with the type of nucleic acids. The original data of the recovery rates are shown in Supplementary Table 5.

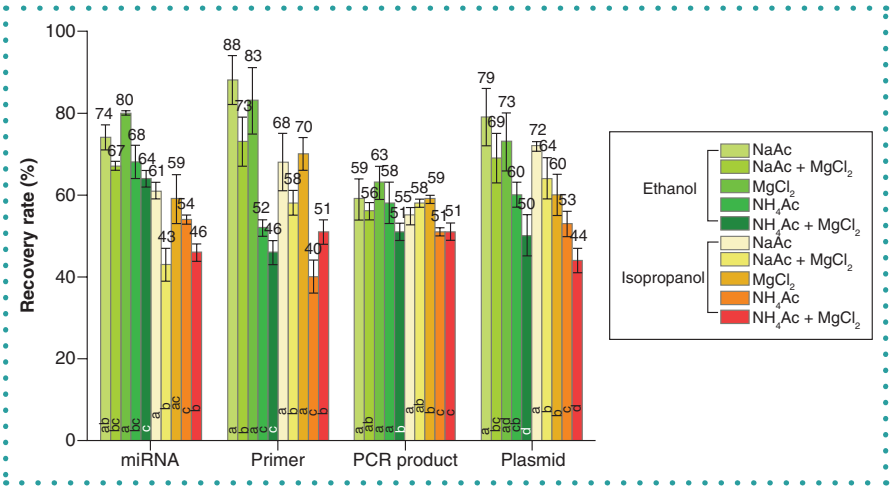


Figure 6. The recovery rates of different nucleic acids under different cation conditions. Letters a, b, c and d represent statistical significance under different cation conditions ($p < 0.05$). The recoveries with same letter(s) indicate no significant difference. The letters 'ab' indicate the recovery rate has no significant difference with the recoveries with letter 'a,' as well as the recoveries with letter 'b.' In most cases, adding one type of salt led to higher nucleic acid recovery levels than the addition of mixtures. The addition of NaAc was superior to NH_4Ac. The original data of the recovery rates are shown in Supplementary Table 6.
NaAc: Sodium acetate.

of NaAc and $MgCl_2$ (67%) compared with adding NaAc (74%) or $MgCl_2$ (80%) alone. Similarly, lower recovery was also attained for miRNA with ethanol when incubated with the NH_4Ac and $MgCl_2$ mixture (64%) in comparison with NH_4Ac (68%) or $MgCl_2$ (80%) alone (Figure 6). However, this phenomenon was not evident for the primer and the PCR product when isopropanol was employed, except for miRNA and plasmid (Figure 6).

Coprecipitator

Coprecipitators, which play the role as 'traps' or 'carriers' of DNA/RNA molecules, are added to nucleic acid solutions, together with cations and organic solvents, to improve the precipitation process, therefore maximizing the recovery level of nucleic acids precipitation [7,15]. This step is especially useful for the precipitation of a low amount of nucleic acid where a clear pellet after centrifugation is otherwise not visible. Applying the optimal conditions from the previous steps of optimization, three commonly used coprecipitators, including glycogen, LPA and yeast tRNA [7], were tested (Figure 7 & Supplementary Tables 7 & 8). However, it is important to note that the results of the yeast tRNA are only shown in Supplementary Table 7, because the recovery rates of nucleic acids with tRNA exceeded 100% owing to the fact that the absorption peak of tRNA (260 nm) overlaps with the tested DNA or RNA sequences. As a result, yeast tRNA is not suitable for experiments in which accurate quantification information of the resulted nucleic acids is required, such as reverse transcription and next-generation sequencing.

In general, with the addition of glycogen or LPA, all types of nucleic acids, with either ethanol or isopropanol, achieved higher recovery rates than their counterparts without using coprecipitators (Figure 7 & Supplementary Table 8). For example, PCR product with ethanol attained 72% of recovery with either glycogen or LPA, in contrast to the 63% recovery rate when carriers were not present. Similarly, PCR product with isopropanol achieved higher recovery levels when incubated with glycogen (65%) and LPA (67%), compared with the PCR products without using coprecipitators (54%) (Figure 7 & Supplementary Table 8).

Markedly, short single-stranded nucleic acids (miRNAs and primers) with ethanol achieved their highest recovery rates with the addition of glycogen (miRNA: 89%; primer: 90%) (Figure 7), medium-length PCR product (150 bp) with ethanol showed its peak recovery level when incubated with either glycogen (72%) or LPA (72%) (Figure 7), whereas longer nucleic acid (plasmid) with ethanol achieved its peak recovery level when incubated with LPA (93%) (Figure 7). However, most of the nucleic acids with isopropanol attained their highest recovery levels with the addition of LPA (miRNA: 73%, PCR product: 67%, plasmid: 80%), with the exception of primer, which achieved its highest recovery when incubated with glycogen (76%) (Figure 7).

In an attempt to maximize the efficiency of nucleic acid precipitation, key factors, including the type of nucleic acid, temperature and time of incubation, force and time of centrifugation, volume ratio of ethanol/isopropanol to nucleic acid solution,

type of cation-containing salts and type of coprecipitator, were systematically evaluated in this study. The results of the incubation conditions demonstrated that the maximum recovery rates of nucleic acids (except plasmid) were achieved under overnight incubation at -20°C/4°C except for plasmid. In addition, longer time (overnight) incubation under -20°C generally results in significantly higher yield than those incubated for 2 h ($p > 0.05$). These results were consistent with a previous report that observed improvements in DNA recovery along with the extension of incubation time [5]. Interestingly, in the case of plasmid, its prolonged incubation at low temperature (both -20 and 4°C) does not show benefit, which is evident by a comparable yield that was recorded with the group completely omitting the incubation step. The implication is that although a prolonged incubation at -20°C/4°C is generally suggested for short- (20 nt) and medium-length (150 bp) nucleic acids, this step is not necessary for long nucleic acids such as plasmid. This observation correlates with previous work conducted by Green and Sambrook [11]. According to their results, the minimum incubation time depends on the length and concentration of the DNA; the smaller the DNA fragments and the lower their concentration, the longer the time required for precipitation [11]. Of note, distinct drops in recovery rate were observed at -80°C (5 min) in the cases of PCR product and plasmid in comparison with -20°C (2 h) and even without incubation. This phenomenon agrees with the study performed by Zeugin and Hartley [5]. As reported, nucleic acids precipitate was suppressed at low temperatures and occurs more efficiently at RT rather than -20 or -80°C [5]. Although the investigation of the detailed mechanism of this phenomenon is over the range of this project, this information should be taken into consideration because it is increasingly common practice for researchers to shorten the incubation time by freezing samples at -80°C.

As expected, improvements in the recovery of nucleic acids of different types were observed with the increase in centrifugal force and extension in centrifugation time. Considering that the purpose of the centrifugation is to drive the DNA/RNA aggregate through the ethanol/isopropanol solution to the wall of the tube [5], it is not surprising that the faster centrifugation is more advantageous. However, although more powerful centrifugation is beneficial for the precipitation of short nucleic acids, such as primers and miRNAs (21,000 × *g*), in the cases of medium-length PCR products and longer nucleic acid plasmid, the yield reach plateau at 7500 × *g*, as a significant increase in the yield was not observed from 7500 × *g* to 21,000 × *g*, suggesting an ordinary centrifuge with relatively higher force capacity is sufficient for the precipitation of nucleic acids with length longer than 150 bp (Figure 2). It is generally accepted that a prolonged centrifugation time (e.g., up to 1 h) is necessary for enhanced nucleic acid recover during ethanol precipitation [10]. However, this opinion was not supported by our tests. As shown in Figure 3, for all types of nucleic acids, no significant difference was observed between different centrifugation time groups, with the 10-min centrifugation obtaining comparable yields with the prolonged 1 h centrifugation groups. This observation suggested that although extending centrifugation time could slightly increase the recovery rate, the yield of nucleic acid precipitation could be affected more by other factors. Generally, a 10-min centrifugation is sufficient for most downstream applications. As for the reason why no statistically significant difference on recoveries by extension of time, perhaps a 10-min centrifugation is underpowered to show statistical differences and requires less time centrifugation (e.g., 5 min or even 2 min).

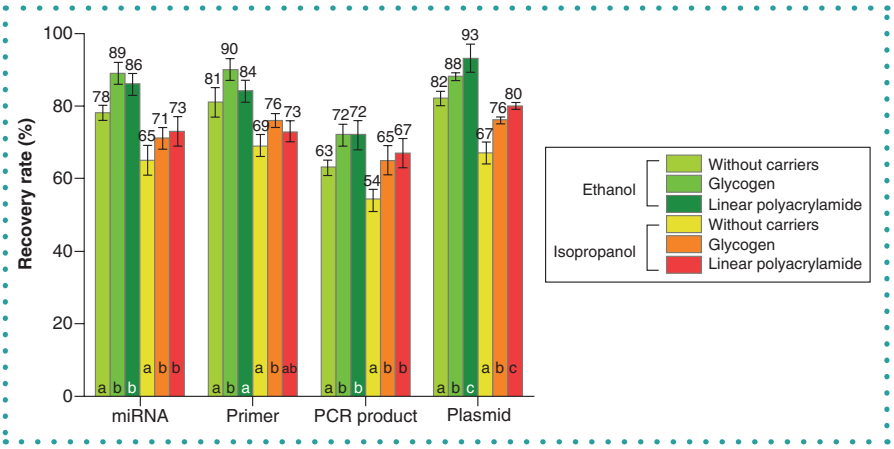


Figure 7. The recovery rates of different nucleic acids under different conditions of coprecipitators. Letters a, b and c represent statistical significance under different conditions of carriers of ethanol/isopropanol ($p < 0.05$). The recoveries with the same letter(s) indicate no significant difference. The letters 'ab' indicates the recovery rates no significant difference with the recoveries with letter 'a,' as well as the recoveries with letter 'b.' With the addition of coprecipitators, the recovery rates of all types of nucleic acids were increased, and in most cases, linear polyacrylamide is better than glycogen. The original data of the recovery rates are shown in Supplementary Table 8.

Both ethanol and isopropanol are organic solvents with strong polarity for nucleic acid precipitation [11]. Although ethanol is more commonly adopted for more purified nucleic acid preparation, the requirement of relatively higher volume ratio (more than 2× of the solution) makes it difficult to perform when a big initial solution volume is processed. In this case, isopropanol represents an alternative for the relatively small volume ratio required (1× for instance). In addition, as a better solvent of polysaccharide and proteins, isopropanol is especially favorable for processing solutions with high concentration of such pollutants [11,24]. Even so, it should be noticed that isopropanol is easy to cause the coprecipitation of salts, and resulting in higher salt contamination [11].

In this study, nucleic acids with different volumes of ethanol in most cases showed higher recovery rates than their counterparts with isopropanol, especially compared with the low volume ratio groups of isopropanol (0.5× and 0.75×) (Figure 5). Apparently, in ethanol groups, although the higher volume ratios of 3× and 4× generally resulted in higher recoveries, for medium-length PCR products and long plasmid, the lower volume ratio (2×) did not show a significant ▶

difference compared with the 3× and 4× volume ratios ($p > 0.05$). Similar trends were observed for isopropanol, a higher isopropanol volume rate (e.g., 1×) that generally saw higher recovery levels. However, it should be noted that a higher concentration of ethanol or isopropanol is prone to coprecipitate a higher concentration of contaminant [25], and extra washing steps using 75% ethanol is recommended to further purify the collected nucleic acids.

Nucleic acid precipitation only occurs when cations are available in sufficient quantity to neutralize the charge of the exposed phosphate residues of nucleic acids [11]. According to our results, the recovery rates of all of the four types of nucleic acids vary according to the presence of different cations. As shown in Figure 6, $MgCl_2$ or NaAc resulted in the highest recovery rates irrespective of the treatment of ethanol or isopropanol. The bivalent nature of $MgCl_2$ may contribute to this phenomenon. As reported previously by Zhou *et al.*, Mg^{2+} is more effective to precipitate small qualities of DNAs in case of ethanol-mediated nucleic acid precipitation [26]. In another case, Green and Sambrook recommended adding $MgCl_2$ to a final concentration of 0.01 M when the DNA molecule is short (<100 bp) or the amount of DNA is sufficiently low (<0.1 μg/ml) [11]. Although both NH_4Ac (2.5 M) and NaAc (0.3 M, pH 5.2) had been widely used for nucleic acid precipitation, the current study suggests that NaAc is preferential to NH_4Ac for different types of nucleic acids. However, as a volatile salt, NH_4Ac can effectively suppress the coprecipitation of contaminated components such as salts, dNTPs and oligosaccharides for more purified nucleic acid preparation [5,11]. Interestingly, specifically for ethanol-mediated precipitation, all types of nucleic acids demonstrated less recovery rates when incubated with combined cation-containing salts (e.g., NaAc + $MgCl_2$) compared with adding only one type of salt. Although this phenomenon cannot be explained with the current data, NaAc and $MgCl_2$ were recommended to add individually when using ethanol for precipitation.

Although optimized conditions could be explored for efficient nucleic acid precipitation, the efficacy of such methods could be detrimentally affected when the amount of nucleic acids in the solution is lower than 50 ng/ml [6]. This is because at such a low amount, it is difficult to pinpoint the faint pellets after even prolonged centrifugation. The black-box mode of process (no visible pellets to direct supernatant removal) definitely affects the yield of nucleic acid precipitation. The addition of coprecipitator presents a potent strategy to tackle this problem by providing a physically visible pellet after centrifugation [7]. Ideally, coprecipitators used for nucleic acid precipitation should be inert substances and not affect the quantification and downstream applications of the resulted nucleic acids [7]. According to this principle, yeast tRNAs, although widely used, decisively affect the quantification of the resulted nucleic acid products (tRNA itself is a kind of nucleic acid). As shown in Supplementary Tables 7 and 9, the application of tRNA results in obvious false results, with higher than 100% recovery rates recorded. Considering most of the downstream nucleic acid precipitations, such as reverse transcript, PCR, next-generation sequencing [27] and SELEX [28], rely on an accurate quantification of the initial input, yeast tRNA is not recommended unless quantification is not required. As a neutral carrier, LPA is an ideal choice in this aspect. First, LPA is not biologically derived and therefore does not contain potential nucleic acid contaminations to interfere with A260/A280 readings (Supplementary Table 9) [7,15]; secondly, as a type of inert material, LPA does not exhibit biological functions, and therefore is not compatible with various downstream molecular biological applications [7,15]. Similarly, glycogen, without absorption at 260 nm (Supplementary Table 9), represents another popular coprecipitator and is generally safe for subsequent biological reactions. As reported, unless with a high concentration (higher than 2 mg/ml), glycogen does not affect the activity of reverse transcriptase, and no T4 ligase activity was observed with up to 0.02 mg/ml glycogen concentration [29]. However, glycogen could potentially interfere with the interactions between DNA and proteins [11,15]. Therefore, caution needs to be taken when such a reaction is involved in downstream applications.

As expected, on the addition of glycogen or LPA, all types of nucleic acids with either ethanol or isopropanol treatments achieved significantly higher recovery rates than their counterparts without using coprecipitators. Markedly, short single-stranded nucleic acids (20 nt) with ethanol achieved their highest recovery rates on the addition of glycogen. This observation correlates with the investigation conducted by Gaillard and Strauss [15]. According to their results, very short DNA fragments (<20 nt) displayed lower level coprecipitate with LPA. Therefore, glycogen is more appropriate for precipitation of short DNA/RNA fragments. However, for both ethanol and isopropanol-mediated nucleic acid precipitation, LPA performed better for medium-length PCR product (150 bp) and the long plasmids (Figure 7), consistent with the previous studies that demonstrated that LPA displayed advantages over glycogen for normal DNA (e.g., bigger than 100 bp) precipitation [15]. Therefore, apart from short nucleic acids, LPA should be preferentially considered. This suggestion is further supported by a previous observation that application of glycogen potentially caused nucleic acid and biological enzyme contaminations (because of its biological origin) and negatively affected the downstream biological reactions [30].

CONCLUSION

Despite its critical importance in biological investigations, a systematic study of the influence factors of nucleic acid precipitation has not previously been reported. To our knowledge, this is the first time, via rational experimental design, that key factors of nucleic acid precipitation were systematically studied. Our results indicated that the optimal conditions vary greatly in accordance with the type of nucleic acids under study. Specifically, we suggest: whereas overnight incubation at -20°C is necessary for short- (20 nt) and medium-length (150 bp) nucleic acids, the whole incubation step is not compulsory for long nucleic acids (e.g., plasmid); -80°C incubation may result in reduced nucleic acid precipitation; an ordinary centrifuge with 7500 × *g* force is sufficient for isolation of nucleic acids longer than 150 bp, whereas centrifugal force up to 21,000 × *g* is required for short nucleic acids; although prolonged centrifugation generally

increase precipitation yields, a 10-min centrifugation is sufficient for most applications; ethanol is most preferred to isopropanol; whereas 2× volume ratio of ethanol is sufficient for medium-length PCR products and long plasmids, 3× volume ratio is preferred for short nucleic acids (e.g., miRNA); NaAc and $MgCl_2$ were recommended to add individually irrespective of ethanol or isopropanol selection; apart from short nucleic acid, linear polyacrylamide should be preferentially considered as coprecipitator.

FUTURE PERSPECTIVE

In this study, through conducting a carefully designed nucleic acid precipitation experiment, we provided invaluable references for researchers in a broad field of biological investigations, specifically in the fields in which quantitative nucleic acid recovery is crucial, such as SELEX (for aptamer development) and next-generation sequencing. With rapid progress in biotechnology areas, including material sciences, and separation techniques, we envision that the efficiency of the current nuclei acid precipitation could be further improved. Additionally, with the ongoing accumulation of knowledge in this area, commercial kits specifically designed for different types of nuclei acids may soon become available.

SUPPLEMENTARY DATA

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2019-0109

AUTHOR CONTRIBUTIONS

T Wang and F Zhang conceived the study concept and design. Y Li and S Chen performed the experiments, data acquisition and analysis, and drafted the manuscript. N Liu, L Ma, T Wang, RN Veedu, T Li and F Zhang revised the manuscript. H Zhou, X Cheng and X Jing assisted with the experiment.

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ETHICAL CONDUCT OF RESEARCH

We state that we have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

DATA SHARING STATEMENT

The authors certify that this manuscript reports original clinical trial data. Data reported in this manuscript are available within the article or posted publicly at www.clinicaltrials.gov, according to the required timelines. Additional data from the study are available upon reasonable request.

REFERENCES

1. Green MR, Sambrook J. Preparation of single-stranded bacteriophage M13 DNA by precipitation with polyethylene glycol. *Cold Spring Harb. Protoc.* 11, (2017).
2. Drmanac R, Peters BA, Kermani BG. US 00524369B2 (2016).
3. Chen H, Wu Y, Liu T, Chen Z, He N, Zhang L. Development of a micro-volume dynamic hybridizing system for nanoparticle-mediated nucleic acid hybridization. *J. Nanosci. Nanotechnol.* 16(12), 12244–12250 (2016).
4. Hahn MA, Li AX, Wu X, Pfeifer GP. Single base resolution analysis of 5-methylcytosine and 5-hydroxymethylcytosine by RRBS and TAB-RRBS. *Methods Mol. Biol.* 1238, 273–287 (2015).
5. Zeugin JA, Hartley JL. Ethanol precipitation of DNA. *Focus* 7(4), 1–2 (1985).
6. Shapiro DJ. Quantitative ethanol precipitation of nanogram quantities of DNA and RNA. *Anal. Biochem.* 110(1), 229–231 (1981).
7. Hengen PN. Carriers for precipitating nucleic acids. *Trends Biochem. Sci.* 21(6), 224 (1996).
8. Watson JD, Crick FH. Molecular structure of nucleic acids. *Nature* 171(4356), 737–738 (1953).
9. Arscott PG, Ma C, Wenner JR, Bloomfield VA. DNA condensation by cobalt hexaammine (III) in alcohol–water mixtures: dielectric constant and other solvent effects. *Biopolymers* 36(3), 345–364 (1995).
10. Hoopes BC, McClure WR. Studies on the selectivity of DNA precipitation by spermine. *Nucleic Acids Res.* 9(20), 5493–5504 (1981).
11. Green MR, Sambrook J. Precipitation of DNA with ethanol. *Cold Spring Harb. Proto.* 12, 1116–1120 (2016).
12. Bloomfield VA. DNA condensation. *Curr. Opin. Struct. Biol.* 6(3), 334–341 (1996).
13. Paithankar K, Prasad K. Precipitation of DNA by polyethylene glycol and ethanol. *Nucleic Acids Res.* 19(6), 1346 (1991).
14. Mamiatis T, Fritsch EF, Sambrook F, Engel J. Molecular cloning: a laboratory manual. *Acta Biotechnol.* 49(2), 411 (1982).
15. Gaillard C, Strauss F. Ethanol precipitation of DNA with linear polyacrylamide as carrier. *Nucleic Acids Res.* 18(2), 378 (1990).
16. Gopinath, SCB. Methods developed for SELEX. *Anal. Bioanal. Chem.* 387(1), 171–182 (2007).
17. Healey A, Furtado A, Cooper T, Henry RJ. Protocol: a simple method for extracting next-generation sequencing quality genomic DNA from recalcitrant plant species. *Plant Methods* 10(1), 21 (2014).
18. Zhao S, Huang Q, Yang P *et al.* Effects of ion beams pretreatment on damage of UV-B radiation on seedlings of winter wheat (***Triticum aestivum***). *Appl. Biochem. Biotechnol.* 168(8), 2123–2135 (2012).
19. Qi W, Zhang L, Feng W, Xu H, Wang L, Jiao Z. ROS and ABA signaling are involved in the growth stimulation induced by low-dose gamma irradiation in Arabidopsis seedling. *Appl. Biochem. Biotechnol.* 175(3), 1490–1506 (2015).
20. Wang L, Ma R, Yin Y, Jiao Z. Antioxidant response of ***Arabidopsis thaliana*** seedlings to oxidative stress induced by carbon ion beams irradiation. *J. Environ. Radioact.* 195, 1–8 (2018).
21. Olson ND, Morrow JB. DNA extract characterization process for microbial detection methods development and validation. *BMC Res. Notes* 5(1), 668 (2012).
22. Jing R, Wang H, Ju S, Cui M. Reference materials for molecular diagnostics: current achievements and future strategies. *Clin. Biochem.* 56, 11–17 (2018).
23. Friedmann T, Brown DM. Base-specific reactions useful for DNA sequencing: methylene blue-sensitized photooxidation of guanine and osmium tetraoxide modification of thymine. *Nucleic Acids Res.* 5(2), 615–622 (1978).
24. Zhang L, Qiu X, Xu J, Du Y, Qian B, Kennedy JF. Studies on fractionation and molecular weights of Chinese lacquer polysaccharide. *Carbohydr. Polym.* 19(3), 161–166 (1992).
25. Greco M, Sáez CA, Brown MT, Bitonti MB. A simple and effective method for high quality co-extraction of genomic DNA and total RNA from low biomass ***Ectocarpus siliculosus***, the model brown alga. *PLoS One.* 9(5), e96470 (2014).
26. Green MR, Sambrook J. Precipitation of DNA with ethanol. *Cold Spring Harb. Proto.* 12, (2016).
27. Wang T, Yin W, AlShamaileh H *et al.* A detailed protein-SELEX protocol allowing visual assessments of individual steps for a high success rate. *Hum. Gene Ther. Methods* 30(1), 1–16 (2019).
28. Wang T, Chen C, Larcher L, Barrero RA, Veedu RN. Three decades of nucleic acid aptamer technologies: lessons learned, progress and opportunities on aptamer development. *Biotechnol. Adv.* 37(1), 28–85 (2019).
29. Kershaw CJ, O'Keefe RT. Splint ligation of RNA with T4 DNA ligase. *Methods Mol. Biol.* 941, 257–269 (2013).
30. Bartram AK, Poon C, Neufeld JD. Nucleic acid contamination of glycogen used in nucleic acid precipitation and assessment of linear polyacrylamide as an alternative co-precipitant. *Biotechniques* 47(6), 1019–1022 (2009).