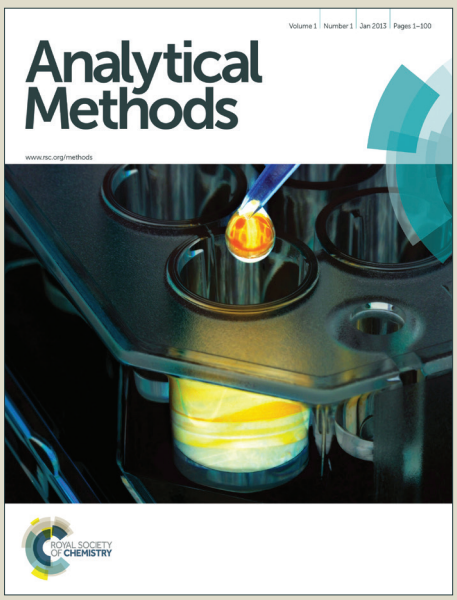
Analytical Methods

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Simultaneous Extraction, Separation and Purification of Microbial Genomic DNA and Total RNA from Acidic Habitat Samples

Jianping Xie[1,2,6*], Hui Yun[1,4,6*], Haigang Dong[3,5▵], Wenya Zhao[1,6], Guohua Wang[1,6], Guanzhou Qiu[1,6], Xinxing Liu[1,6]

1. School of mineral processing and bioengineering, Central South University, Changsha, Hunan, 410083, China
2. State Key Laboratory of Powder Metallurgy, Central South University, Changsha, Hunan, 410083, China
3. Kunming Institute of Precious metals, Kunming, Yunnan, 650106, China
4. Research Centre for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, 100085, China
5. State Key Laboratory of Advanced Technologies for Comprehensive Utilization of Platinum Metals, Kunming, Yunnan, 650106, China
6. Key Laboratory of Biometallurgy, Ministry of Education, Changsha, Hunan 410083, China

*These two author contribute equally to this study

▵Corresponding authors:

Haigang Dong
Tel: +86-13755128216
E-mail: donghaigang0404@126.com

Jianping Xie
Tel: 86-731-88836943
Email: whitewolf1101@gmail.com

Running title: Simultaneous Recovery of Microbial DNA and RNA

ABSTRACT. Although there were lots of methods focused on DNA and RNA extraction, only few studies focused on simultaneous extraction of high quality DNA and RNA from environmental samples, especially for acidic habitat samples. In this study, a widely used DNA extraction method (Zhou, J. Z., M. A. Bruns, and J. M. Tiedje. 1996) was optimized for simultaneous extraction of crude nucleic acid (including DNA and RNA), and LiCl salt based step-by-step precipitation method was also optimized for separation of DNA and RNA. The results showed that nucleic acid extraction buffer (pH 7.0) containing piperazine-1,4-bis(2-ethanesulfonic acid)(PIPES) salt could extract crude nucleic acid from acidic habitat samples effectively and optimized LiCl salt based step-by-step precipitation method could separate nucleic acid to high quality DNA and RNA. The results also showed that obtained DNA and RNA could be used for the downstream molecular analysis. This study developed a useful method for simultaneous extraction and separation of DNA and RNA from acidic habitat samples, which has a great potential as a standard method to obtain high quality nucleic acid from biohydrometallurgy process and extreme environmental samples.

Keywords: Metagenomics, metatranscriptomics, simultaneous extraction DNA and RNA, biohydrometallurgy, bioleaching

1. Introduction

Acid mine drainage (AMD) usually has low pH (<2) and high concentrations of sulfate and metal ions, is a prevalent, international environmental problem since it is harmful for diatoms[1], protozoans[2], aquatic invertebrates[3], piscivorous birds in freshwater lakes[4], groundwater, rivers, streams[5] and surrounding vegetation[6]. However, due to the relatively simple microbial community and extreme characteristic, microbial community in AMD was regarded as a model community to investigate linkages between microbial communities and geochemistry and even to reveal the mechanism of extreme life[7-9]. Better understanding of the microbial ecology and dynamic shift could provide a foundation for bioremediation of AMD contaminated environment. Moreover, it could be also accelerate the development of biohydrometallurgy (also known as bioleaching or biomining) technology, which is an energy-efficient valuable metal recovery technology from mine waste[7, 10]. Therefore, it is important to study the microbial community in the acid mine drainage, and there were lots of studies focused on microbial diversity in AMD during the past decades[11-17].

Nowadays, culture-independent nucleic acid techniques, such as restriction fragment length polymorphism (RFLP), quantitative reverse transcription polymerase chain reaction (qRT-PCR), Phylochip, GeoChip, pyrosequencing and metagenomic sequencing have greatly advanced environmental microbiology and were also gradually applied to analyze the microbial community structure and dynamics in the AMD[17-24]. However, the quality and quantity of the nucleic acid significantly affected the downstream molecular analysis, data processing and even the final conclusion[25, 26]. Therefore, how to extract high quality nucleic acid from AMD is still a key issue.

Although several methods were applied to extract genomic DNA and total RNA from extreme acidic habitat, such as AMD and bioleaching pool[15, 17, 27]. However, the simultaneous extraction of DNA and RNA from extreme acidic habitat samples has not yet been well investigated. There were only several methods focused on the simultaneous extraction of DNA and RNA from soil, tissue, et al[28-33]. There were two aspects advantages for simultaneous extraction of DNA and RNA, 1) demanding less sample amount, since DNA and RNA could be extracted simultaneously from one sample and therefore total sample assumption was less than extracting DNA and RNA separately. This point is important especially for precious samples; 2) guarantee the genomics and transcriptomics analysis results, the data is more reliable for DNA and RNA extracted simultaneous than extracted separately from one sample. Due to these advantages, several simultaneous DNA and RNA extraction methods

were developed for different samples, especially for those precious samples[28-31, 34, 35]. However, it is hard to apply these methods to the acidic samples due to low pH and high concentration of sulfide and heavy metal ion. Zammit 36 compared several nucleic acid extraction methods and developed an enzyme based method to recovery nucleic acid from biomining and acid mine drainage microorganisms by adapting Bond's method[16]. However, in Zammit's protocol, RNase or DNase was used for removing the residual RNA or DNA within total DNA or RNA. That operation causes nucleic acid loss. On the other hand, there is no standard method used for the extraction of nucleic acid from the biohydrometallurgy microbial community. Therefore, it's hard to compare the results among different studies, because different extraction methods might cause different results[36, 37]. In one word, standard, feasible, affordable and efficient simultaneous DNA and RNA extraction, separation and purification methods are necessary to be developed for recovery of genomic DNA and total RNA from acidic habitat samples.

The objective of this study was to develop a robust, effective, economic, simple and rapid method for the simultaneous extraction, separation and purification of intact genomic DNA and total RNA from the acidic habitat samples. A high salt Piperazine-1,4-bis(2-ethanesulfonic acid)(PIPES) buffer containing 0.1 M PIPES salt, 0.1 M EDTA, 1.5 M NaCl, 1% CTAB was used for total nucleic acid extraction. Nucleic acid extraction procedure was optimized based on a widely used DNA extraction method which showed excellent recovery of high-molecular-weight DNA from soil with diverse composition[28, 38, 39]. DNA and RNA separation procedure was optimized using different kinds of salt and separation conditions. Five bioleaching strains, including, *Acidithiobacillus ferrooxidans* (*A. f*), *Acidithiobacillus caldus* (*A. c*), *Acidithiobacillus albertensis* (*A. a*), *Leptospirillum ferrooxidans* (*L. f*), *Ferroplasma Thermophilium* (*F. t*), and several AMD samples from three different locations: Daye copper mine (Daye), Xiangxi gold mine (Xxi) and Axi gold mine (Axi) were used for confirming the optimized extraction and separation methods. Our results showed that genomic DNA (gDNA) and total RNA could be simultaneously extracted and separated from same sample and it will contribute to quantitative analysis for biohydrometallurgy microbial community structure and finally accelerate the efficiency of biohydrometallurgy.

2. Materials and Methods

2.1 Preparation of reagents and materials

All solutions, water, glassware and utensils used for nucleic acid extraction and separation were treated with 0.1 % diethylpyrocarbonate (DEPC) overnight at 37 ^{o}C and autoclaved at 121 ^{o}C for 30 min. Glassware and utensils used for nucleic acid extraction were then baked at 105 ^{o}C for 8 h. Disposable plastic ware was RNase free and working bench was treated with RNase removal reagent (Sigma, Shanghai, China).

2.2 Preparation of type strains and acidic environmental samples

Five strains, including *A. f*, *A. c*, *A. a*, *L. f* and *F. t* were used for optimizing the nucleic acid extraction and separation conditions. All strains were obtained from the Ministry of Education Key Laboratory of Biometallurgy Strain Library and grown in 9K liquid medium containing $(NH_4)_2SO_4$ 3.0 g/L, KCl 0.1 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, $Ca(NO_3)_2$ 0.01 g/L, K_2HPO_4 0.5 g/L, $Fe_2SO_4 \cdot 7H_2O$ 44.2 g/L or elemental S 10 g/L. The pH of culture medium was adjusted to 2.0 and the volume of medium was 200 mL for each flask. To obtain enough biomass, total 2 L liquid medium was cultured in 10 flasks for each time. Cells were harvested by centrifugation at 10,000×g for 10 min when cells density was around 10^7~10^8 cell/mL and cells pellet was immediately kept at -20 ^{o}C prior to total nucleic acid extraction. Several AMD samples (25 liters) were obtained from Hubei Daye copper mine (Daye I

and Daye II), Hunan Xiangxi gold mine (Xxi), and Xinjiang Axi gold mine (Axi I and Axi II), respectively. Each AMD sample was filtered (0.22-μm nylon filters), and the filters were stored at -20 °C until used for total nucleic acid extraction.

2.3 Nucleic acid extraction and separation protocol

The collected cells/sediments samples were mixed with 2 g sterilized silica sand and then ground from freezing to thawing for three times by using liquid nitrogen. The mixture was transferred into a RNase-free tube containing 16.5 mL PIPES extraction buffer (containing 0.1 M PIPES sodium salt, 0.1 M EDTA, 1.5 M NaCl and 1% CTAB) and mixed gently for several times. The pH of mixture was checked and adjusted to 7. Then 1.83 mL 20 % Sodium Dodecyl Sulfate (SDS) was added to the above mixture and incubated at 65 ^{o}C for 1 h with gentle inversion every 15 min. When 61 μL proteinase K was used, the mixture was incubated at 37 ^{o}C for 30 min first before SDS was added. After incubation, the mixture was centrifuged at 6,000×g for 10 min, and supernatant liquid was transferred to a new centrifuge tube and extracted with chloroform: isoamylol (V:V=24:1) for 10 min. The mixture was centrifuged at 6,000×g for 10 min, and supernatant liquid containing DNA and RNA was transferred to a new centrifuge tube. Nucleic acid was then precipitated with 0.6 volume isopropanol and then collected by centrifugation at 12,000×g for 30 min at room temperature. The nucleic acid pellet was air dried for around 10 min and resuspended with 100-200 μL RNase-free water.

Nucleic acid concentration was adjusted to optimized concentration and optimized salt was added to the nucleic acid and mixed well. RNA was first collected by centrifugation at 12,000×g for 30 min at 4 ^{o}C. After removal of RNA, DNA in the supernatant was then precipitated with 0.6 volume isopropanol overnight and then collected by centrifugation at 12,000×g for 30 min at room temperature. Both isolated DNA and RNA were purified with 70 % ethanol and collected by centrifugation at 12,000×g for 30 min at room temperature. The DNA and RNA pellet were air dried for 10~20 min and resuspended with 100-200 μL RNase-free water.

2.4 Optimization of nucleic acid extraction and separation protocol

2.4.1 Optimization of PIPES extraction buffer composition and conditions

PIPES extraction buffer was used to extract total nucleic acid. The conditions for extraction, such as incubation time, extraction buffer pH, and other controllable factors that could influence the extent of RNA damage, were optimized with a pure culture *A.f* cells based on the previously described DNA extraction and purification methods [38, 39]. The concentration of *A.f* cells is approximately 10^7 cells/mL, and we combine all cultured cell together and separate equally to make sure the biomass is same in each condition optimization experiment. The pH of PIPES extraction buffer was changed from 6.0 to 7.5 with the 0.5 increments. The effect of proteinase K on the extraction was examined using varied concentration of 0, 10, 50 and 100 mg/mL. The sensitivity of this method was tested using different *A. f* biomass differ from 1×10^9 cells to 4×10^9 cells.

2.4.2 Optimization of separation conditions

The nucleic acid of pure culture *A.f* was used to optimize the separation procedure, such as, different kinds of salt, different concentrations of selected salt, different initial concentrations of nucleic acid, incubation temperature and time, and other controllable factors that maybe influence the efficiency of the separation of RNA from DNA. Total three kinds of salt solutions, including one third volume of NaCl [Concentration was 1, 2, 3, 4, 5 M and saturated, respectively], equal volume of $CaCl_2$ [Concentration was 0.5, 2, 4, 6, 8 and 10 M, respectively]

and quarter volume of LiCl [Concentration was 10, 12.5, 15, 17.5 M and saturated, respectively], were selected for evaluating the RNA precipitation. These salt solutions were added to nucleic acid and mixed well, then incubated 0.5-1.5 h with 0.5 h interval at -20 ^{o}C. Precipitated RNA was collected by centrifugation at 12,000×g for 30 min at 4 ^{o}C. DNA in the supernatant was precipitated with 0.6 volume isopropanol and then collected by centrifugation at 12,000×g for 20 min at room temperature. Both DNA and RNA were purified with 70 % ethanol twice, centrifuged, air dried and resuspended with 100-200 μL RNase-free water. The universality of extraction and separation method was tested using another four pure cultures and three acidic environmental samples.

2.5 Separation and purification of RNA and DNA from total nucleic acid by using QIAGEN All Prep DNA/RNA Mini Kit

Total crude nucleic acid containing DNA and RNA can be also separated using QIAGEN All Prep DNA/RNA Mini Kit (Gene Company Limited, Shanghai, China) according to the manufacturers' protocols. Due to the low value of the $A_{260/230}$ of DNA and RNA separated by QIAGEN kit, the further purification process is needed. Firstly, add 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of cold absolute ethanol one by one to precipitate the resulted RNA and DNA, and then centrifuge the mixture at 12,000×g for 30 min at room temperature to get the nucleic acid pellet. Thirdly, the nucleic acid pellet was washed with 70 % ethanol twice, centrifuged, air dried and resuspended with 100-200 μL RNase-free water.

2.6 Quantification of DNA quality and quantity

Generally, two methods were used to measure the quality and quantity of extracted or/and purified DNA samples: (i) gel electrophoresis was proceeded with 1 % agarose gel stained with ethidium bromide and (ii) ratios of $A_{260/280}$ and $A_{260/230}$ absorbance were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). DNA with $A_{260/A280}$ and $A_{260/A230}$ ratios of >1.7 and >1.8, respectively, was thought good quality for further analysis. DNA and RNA quality was evaluated by further subsequent molecular analysis, such as polymerase chain reaction (PCR) and reverse transcription PCR.

2.7 PCR and reverse transcription PCR (RT-PCR)

RNA was reverse transcribed using primer *gln* A-F (5'-CATCCGCATTCCCTTCGTCAAC-3') for the glutamine synthetase gene with RT reagent kit (TAKARA BIOTECHNOLOGY CO., LTD, Dalian, China). 1 μL aliquots of the reverse transcription products from a total volume of 20 μL were used for PCR amplification in PCR buffer (12.5 μL 2×PCR Mix (TIANGEN Biotech Co., Ltd., Beijing, China), 1 μL of forward/reverse primer, 9.5 μL ddH_2O), in which the forward primer was *gln* A-F, the reverse primer was *gln* A-R (5'-GGCAGGTCGTAAAGATTCTTGTCC-3') and generated a 174-bp fragment. PCR amplification conditions included denaturation at 94 ^{o}C for 45 s, annealing at 55 ^{o}C for 45 s, and extension at 72 ^{o}C for 1.5 min for 30 cycles, followed by a final extension at 72 ^{o}C for 7 min (Bio-Rad Laboratories, Inc., Shanghai, China). The quality of DNA was also examined by PCR amplification of 16S rRNA gene using bacterial universal primers: 27F：5'-AGAGTTTGATCMTGGCTCAG-3', 1492R：5'-CGGTTACCTTGTTAC GACTT-3' [40]. PCR amplification was conducted in reaction mixtures containing 100 ng of DNA template, 1×PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl and 2 mM $MgCl_2$], 2 mM dNTPs, 5 pM each of the forward and reverse primers, 2.5 U Taq polymerase (Invitrogen, Shanghai, China), and deionized water to a final volume of 50 μL. The used thermal cycling protocol included an initial denaturation at 94 ^{o}C for 5 min, followed by 35 cycles of 94 ^{o}C for 45

s, 55 °C for 45 s, and 72 °C for 90 s. A final extension step of 72 °C for 7 min was also used. Negative controls with ddH_2O as the template were performed for all PCR reactions.

2.8 Statistical analysis

All experiments were performed in triplicate and repeated at least three times to check reproducibility of results. Statistical analysis was carried out using the Microsoft EXCEL 5.0 statistical package calculating mean and standard error.

3. Results

3.1 Optimization of PIPES extraction buffer pH and extraction conditions

PIPES buffer could be used as total nucleic acid extraction buffer at suitable pH [38]. Therefore, DNA and RNA extraction conditions were optimized with *A.f* in different conditions. The results showed that different amount of DNA and RNA was recovered from same amount of biomass with different pH of the extraction buffer (Tab.1; Fig. S1), which indicated that the PIPES extraction buffer pH affected the recovered amount of DNA and RNA. Total 42±2 μg DNA and 54±8 μg RNA could be extracted from 400 mL *A.f* with the concentration of 10^7 cells/mL when using PIPES buffer at pH 7.0. The amount of DNA and RNA extracted by pH 7.0 PIPES buffer was significant higher than the other three pH conditions (n=3; P<0.1). There's no difference for 1 h and 2 h incubation at 65 °C (data not shown). Total 42 μg DNA and 63 μg RNA were extracted when no proteinase K was added, however, the DNA and RNA amount decreased with adding increase amount of proteinase K (Tab.2; Fig.S2). It seemed proteinase K might cause DNA and RNA lost. These results indicated that pH 7.0, incubate at 65 °C for 1 h, and no proteinase K was the best extraction condition for the simultaneous extraction DNA and RNA from *A.f* (Fig. 1).

Table 1 DNA and RNA recovered by using different pH PIPES buffer

	DNA			RNA		
Buffer pH	Amount/μg	$A_{260/280}$	$A_{260/230}$	Amount/μg	$A_{260/280}$	$A_{260/230}$
pH 6	19±4	1.90±0.01	2.15±0.03	22±5	1.94±0.01	2.48±0.01
pH 6.5	16±0.9	1.89±0.01	2.17±0.02	22±3	2.02±0.01	2.43±0.04
pH 7	42±2	1.89±0.01	2.21±0.01	54±8	2.02±0.03	2.36±0.02
pH 7.5	27±18	1.84±0.07	2.1±0.1	20±13	1.9±0.1	2.38±0.07

Table 2 The effects of proteinase K for the DNA and RNA recovery

proteinase K mg/mL	DNA			RNA		
	Amount/μg	$A_{260/280}$	$A_{260/230}$	Amount/μg	$A_{260/280}$	$A_{260/230}$
0	42±8	1.62±0.01	2.24±0.02	63±20	1.81±0.02	2.30±0.02
10	14±13	1.7±0.2	1.5±0.4	49±34	1.74±0.04	1.8±0.4
50	31±14	1.62±0.05	1.9±0.3	66±32	1.80±0.02	2.1±0.3
100	24±11	1.65±0.06	2.0±0.2	51±24	1.82±0.03	2.0±0.3

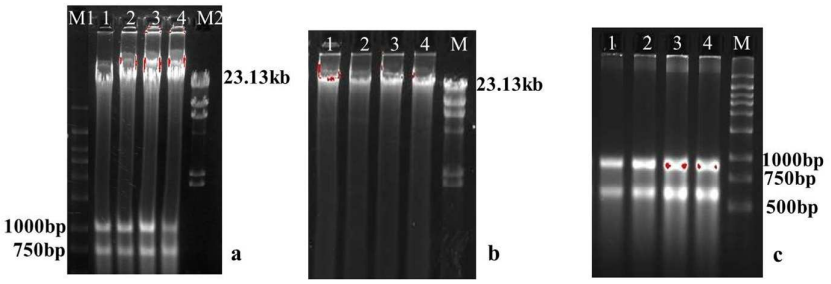


Figure 1 DNA and RNA simultaneously extracted from *A.f* by using the optimized method. (a) total nucleic acid extracted from *A.f*, lane M1: 1 kb ladder, lane M2: *Hind* III-cut lambda molecular size marker, lane 1-4 were the biological replicates; (b) DNA precipitated by isopropanol; (c) RNA precipitated by LiCl.

3.2 The sensitivity of the developed extraction method

To test the sensitivity of the PIPES extraction buffer, different amount of *A. f* biomass samples were used to extract DNA and RNA. The results showed that 6±2 μg DNA and 14±2 μg RNA could be simultaneously extracted from approximately 1×10^9 cells (Tab.3). Obtained DNA amount was significant linearly dependent on the biomass (R=0.99, *P*=0.0074). However, there was no linear relationship between RNA amount and biomass (Fig.S3). These results indicated that this method could extract large amount of DNA and RNA from limited cells, and extracted DNA is significant correlated with the amount of biomass.

Table 3 DNA and RNA extracted from different amount of biomass

Biomass/cells	DNA			RNA		
	Amount/μg	$A_{260/280}$	$A_{260/230}$	Amount/μg	$A_{260/280}$	$A_{260/230}$
1×10^9	6±2	1.7±0.1	1.8±0.1	14±2	1.97±0.03	2.29±0.03
2×10^9	21±6	1.85±0.02	2.13±0.04	17±2	1.92±0.03	2.31±0.02
3×10^9	34±8	1.87±0.01	2.20±0.01	20±3	2.07±0.07	2.28±0.09
4×10^9	56±23	1.83±0.05	2.0±0.2	90±2	2.1±0.1	1.9±0.5

3.3 Optimization of DNA and RNA separation conditions

Considering the cost and treatment capacity, a chemical way instead of QIAGEN All Prep DNA/RNA Mini kit was developed to separate DNA and RNA. Several DNA and RNA separation conditions including different kinds of salt and concentration, initial nucleic acid concentration and precipitation temperature and time were optimized. The purity and intactness were considered as important factors when choosing separation conditions. The results showed that RNA could not be precipitated by NaCl and DNA was co-precipitated with RNA by $CaCl_2$ (data not show). For 100 μL operational volume, 400 ng/μL initial total nucleic acid concentration and saturated LiCl solution showed the best separation efficiency (Tab.S1). No significant difference was found for DNA and RNA precipitation among incubation temperature at 4 ^oC, -20 ^oC and -80 ^oC (Fig.S4). No significant difference was found for DNA precipitation using 0.6 volume or 1 volume isopropanol (data not show). In order to optimize the initial nucleic acid concentration for the downstream DNA and RNA separation, 100, 200, 400, 600, 800 and 1000 ng/μL initial nucleic acid was used for RNA precipitation by 1/4 volume saturated LiCl first, and then DNA precipitated by 0.6 volume isopropanol. The results showed that separation efficiency decreased and degradation of RNA occurred when the concentration of initial nucleic acid was higher than 400 ng/μL (Tab.S2). On the contrary, nucleic acid was easy to lose when the concentration of initial nucleic acid was less than 400 ng/μL. Therefore, initial 400 ng/μL nucleic acid was regarded as the best concentration for 100 μL operational volume.

In short, the nucleic acid could be separated (Fig.2a, 2b and 2c) following the optimized separation procedure listed as below, RNA could be precipitated by saturated LiCl firstly from total nucleic acid at 400 ng/μL initial nucleic acid concentration after incubation at -20 ^{o}C for 30 min, then DNA could be precipitated by 0.6 volume isopropanol after incubation at -20 ^{o}C for 30 min. It is better to incubate the mixture of LiCl and total nucleic acid at 4 ^{o}C for 6-8 h after incubation at -20 ^{o}C for 30 min.

3.4 DNA and RNA extracted from the pure culture and environment samples

To evaluate the versatility of our optimized methods, several pure cultures (*A. c*, *A. a*, *L. f*, *F. t*) and environmental samples (Daye I, Daye II, Xxi, Axi I and Axi II) were used for the simultaneous extraction of total nucleic acid, and then RNA and DNA were separated from total nucleic acid. The results showed that total nucleic acid could be simultaneous extracted from all the tested samples (Fig.2 and Fig.3). Total 2-19 μg DNA and 1-116 μg RNA could be obtained from these samples with good quality except Xxi samples (Tab.4). The reason for poor quality nucleic acid of Xxi is mainly because the biomass in this sample was pretty low. All these results indicated that our optimized methods was also suitable for the simultaneous recovery of DNA and RNA from pure culture and environmental samples.

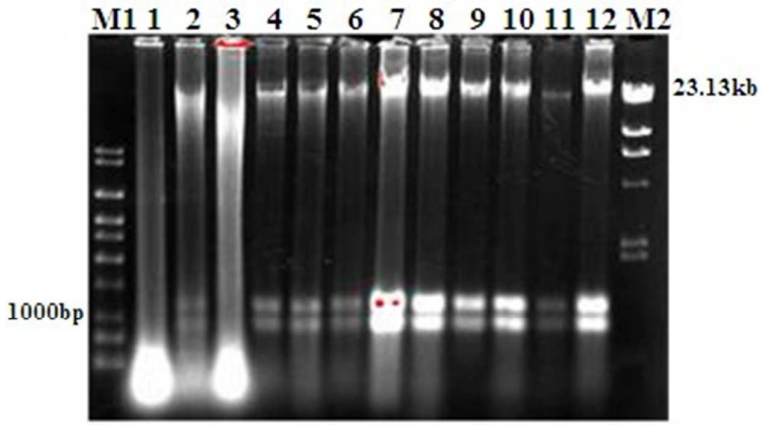


Figure 2 Simultaneous extraction of DNA and RNA from four pure cultures

Note: Lane M1 was 1 kb marker, lane 1-3 were *A. c* biological replicates, lane 4-6 were *A. a* biological replicates, lane 7-9 were *F. t* biological replicates, lane 10-12 were *L. f* biological replicates the lane M2 was *Hind* III-cut bacteriophage λ marker

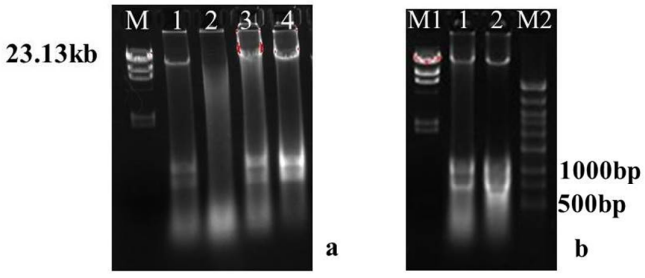


Figure 3 Simultaneous extraction of DNA and RNA from acidic environmental samples

(a) Extracted DNA and RNA of Xxi water sample (lane1), Daye sediment sample (lane 2) and Daye water samples (lane 3 and 4); (b) Extracted DNA and RNA of Axi water sample (lane 1) and sediment sample (lane 2). Lane M and M1 was *Hind* III-cut bacteriophage λ marker; Lane M2 was 1 kb marker.

Table 4 DNA and RNA extracted from different pure cultures and diverse environmental samples

Sample	DNA			RNA		
	Amount (μg)	$A_{260/280}$	$A_{260/230}$	Amount (μg)	$A_{260/280}$	$A_{260/230}$
A. c	7±3	1.94 ±0.03	1.9± 0.2	24 ±13	1.85±0.03	1.9 ±0.2
A. a	2 ±1	1.8 ±0.1	1.2 ±0.1	5 ±3	1.75 ±0.04	2.00 ±0.07
F. t	19 ±1	1.98 ±0.01	2.29 ±0.02	116 ±6	1.98±0.03	2.48 ±0.06
L. f	7±0.4	1.88 ±0.03	1.88 ±0.04	24 ±2	1.93± 0.02	2.35 ±0.02
Daye I	2±0.2	1.84±0.04	2.18±0.2	1±0.3	1.66±0.03	2.58±0.5
Daye II	16±2	1.94±0.00	2.37±0.03	24±2	2.00±0.01	2.45±0.03
Axi I	12±4	1.72±0.01	2.51±0.01	11±0.3	1.77±0.01	2.58±0.01
Axi II	18±1	1.59±0.01	1.72±0.01	10±0.2	1.67±0.01	2.03±0.01
Xxi	5±0.1	1.19±0.01	1.46±0.01	/	/	/

3.5 PCR and RT-PCR of DNA and RNA isolated from pure culture and environmental samples

To evaluate the quality of DNA and RNA isolated from pure culture and environmental samples, PCR and RT-PCR were performed. The results showed that DNA and RNA separated by LiCl were of high quality and suitable for the downstream analysis such as PCR and RT-PCR (Fig.4, Fig.5), no DNA contamination was found in RNA (Fig.5b).

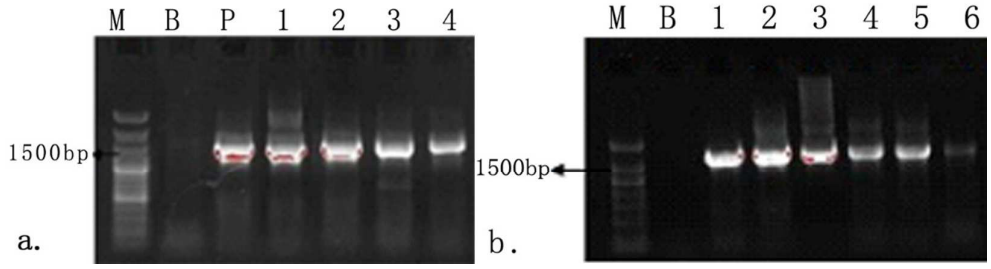


Figure 4 16S rRNA gene PCR for pure culture and environmental samples. a.) Lane M, 100 bp marker; Lane B, negative control for amplification; Lane P, positive control for amplification(*A. f*); Lanes 1-4, DNA separated from pure culture *A. c, A. t, F. t, L. f*; b.) Lane 1-6, DNA separated from Daye I, Daye II, Daye slurry I, Xxi, Axi and Axi slurry samples.

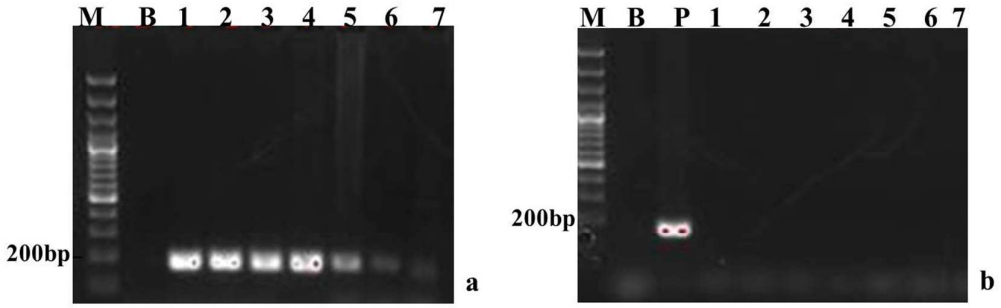


Figure 5 The RT-PCR for pure culture and environmental samples. Lane M, 100 bp marker; Lane B, negative

control for amplification; Lane P, positive control for amplification (*A. f*); a.) Lanes 1-7 RT-PCR for *A. f, A. c, A. t* DayeI, DayeII, Xxi and Axi; b.) Lanes 1-7 PCR amplification using RNA template extracted from *A. f, A. c, A. t* DayeI, DayeII, Xxi and Axi;
Note: B means negative control; P means positive control;

3.6 Evaluation of QIAGEN All Prep DNA/RNA Mini kit and LiCl separation methods

To evaluate the efficacy of QIAGEN All Prep DNA/RNA Mini kit and LiCl separation methods, four pure cultures mentioned above were tested using both methods. The results showed that both methods could successfully separate DNA and RNA from four strains, however, the amount of DNA and RNA separated by QIAGEN kit was less than the LiCl separation method (Tab.5). There is no significant difference for the ratio of $A_{260/280}$, however, the $A_{260/230}$ ratio of DNA and RNA separated by QIAGEN kit was lower than by LiCl separation method. Therefore, further purification using sodium acetate precipitation was necessary.

Table 5 Comparison of DNA and RNA separated using QIAGEN kit and LiCl separation methods

		A. c	*A. a*	*F. t*	*L. f*
Total nucleic acid[a]	Amount(μg)	101±55	37±13	56±9	33±4
	$A_{260/280}$	2.04±0.02	2.06±0.01	2.01±0.01	2.08±0.01
	$A_{260/230}$	1.49±0.04	2.07±0.01	2.19±0.01	1.78±0.01
DNA[b]	Amount(μg)	10±0.6	5±0.5	8±2	4±0.8
	$A_{260/280}$	1.86±0.01	1.79±0.03	1.79±0.03	1.75±0.06
	$A_{260/230}$	0.65±0.01	0.5±0.1	0. 6±0.2	0.26±0.04
RNA[b]	Amount(μg)	36±13	20±2	38±8	18±4
	$A_{260/280}$	2.04±0.01	2.08±0.01	2.09±0.02	2.09±0.01
	$A_{260/230}$	2.00±0.01	1.1±0.2	1.9±0.1	1.2±0.3
DNA[c]	Amount(μg)	14±0.4	16±0.2	29±2	7±0.5
	$A_{260/280}$	1.73±0.01	1.75±0.01	1.7±0.01	1.73±0.01
	$A_{260/230}$	2.81±0.05	2.43±0.01	2.56±0.02	2.32±0.02
RNA[c]	Amount(μg)	39±2	22±0.1	28±0.6	28±2
	$A_{260/280}$	1.84±0.02	1.82±0.01	1.81±0.02	1.78±0.02
	$A_{260/230}$	2.30±0.04	2.39±0.01	2.56±0.01	2.35±0.01

Note: [a.] Original nucleic acid extracted by optimized protocol; [b.] DNA and RNA separated by QIAGEN All Prep DNA/RNA Mini kit; [c.] DNA and RNA separated by LiCl separation methods.

4. Discussion

Nucleic acid extraction is a key issue for microbial community analysis. However, the process was often disturbed by the impurities, such as various metal ions and humic acid or other organic acid. For AMD, low pH and high concentration of heavy metal ions would not only shear the nucleic acid but also inhibit or influence enzyme's activity and stability during extraction and subsequent downstream molecular analysis. Much effort had been taken to improve the extraction methods, especially for soil samples. However, there are only a few reports focused on the extraction of DNA and RNA from AMD samples. Here we reported an optimized methods by adapting previously reported method[38, 39], which could simultaneously extract DNA and RNA from bioleaching pure cultures and AMD samples. The results showed that the quantity and quality of extracted DNA and RNA was

good enough for the following downstream molecular analysis.

In this study, a widely used efficient DNA extraction method [39] was optimized for the simultaneous extraction of crude nucleic acid (including DNA and RNA) from acidic habitat samples and a LiCl based DNA and RNA separation method was developed for DNA and RNA isolation from total nucleic acid. The pH of PIPES buffer ranged from 6.1 to 7.5. Based on our results, the pH 7.0 of PIPES extraction buffer was considered suitable for buffering the acidic sample and stabilizing the structure of nucleic acid. Higher buffer pH, such as pH 7.5, showed a negative influence on total nucleic acid recovery in our study. In general, the cell lysis is the first step of the nucleic acid extraction, of which alkali is commonly used. But once the release of cytoplasmic inclusion, the nucleic acid is easy to be degraded and it is difficult to conglomerate under such conditions due to their single strand existence form. On the contrary, the nucleic acid can be hydrolyzed under the acidic conditions. Thus, such buffer pH as 7.0 is best for the extraction of DNA and RNA. The main reason is supposed that buffer pH 7.0 could guarantee the integrity of the nucleic acid and avoid the influence from other ions.

To lyse the cells and minimize the DNA and RNA degradation from nuclease, we ground the cells with sterilized silica sand three times under -196 ^{o}C by using liquid nitrogen. The cells-sand mixture was then incubated at 65 ^{o}C for 1 h with 20 % SDS, so that the cells in the samples could lyse sufficiently. Meanwhile we tested Proteinase K by adding different quantity. A previous research showed that proteinase K could protect RNA to some extent [28]. However, our results showed that this kind of protection was not obvious. Actually, the high molecular weight bands of the nucleic acid extracted without proteinase K did not show less integrity than those extracted with proteinase K. On the contrary, proteinase K maybe lead to an excess release of protein as the observed thick and sticky middle layer between supernatant liquid layer and underlayer organic layer formed. As a results, these released proteins likely entangled with nucleic acid and caused loss.

The purity and intactness was considered important factors when selecting separation conditions. Halons or sulfate have been used to precipitate nucleic acid previously[35, 41]. On the one hand, alkali metal ions could compete bound-water with nucleic acid and improve its aggregation and sedimentation. On the other hand, the positive charge of ions can neutralize the negative charge of nucleic acid, thus changed the configuration of nucleic acid and finally weakened the repulsive interaction between nucleic acid molecules. Besides, the metal ions can combine with multiple nucleic acid as a bridge to precipitate the nucleic acid. By using different concentration of NaCl as a precipitator, DNA and RNA almost simultaneously disappeared from solution, which was not desired[42]. It is well known that 0.14 M NaCl is usually used for the DNA separation, however, in our study, there was no difference among different amounts of NaCl.

To purify plasmid, $CaCl_2$ was used to work on polysaccharide previously[43]. In addition, $CaCl_2$ was used for recovery of RNA[44]. Therefore, in this study we designed $CaCl_2$ concentration gradient to verify its function. Although $CaCl_2$[43] could sufficiently precipitate RNA, a lot of DNA also was co-precipitated. Our results confirmed above conclusion and we also found that low concentration of $CaCl_2$ may be helpful for DNA separation process. However, higher concentration of $CaCl_2$ might cause the serious loss of DNA.

Previous studies showed that LiCl could precipitate nucleic acid with different size of nucleic acid fragment under different extraction conditions[35, 45]. Therefore, it is necessary to test the LiCl for the separation of nucleic acid extracted from AMD samples simultaneously. Firstly, several low temperatures for RNA precipitation were compared based on the references. For RNA, the precipitation temperature lower than 4 ^{o}C might cause the trace DNA co-precipitation with RNA. In our results, RNA was precipitated at 4 ^{o}C for 30 min tended to dissolve in the solution again if RNA was collected using the centrifugation at room temperature. However, the results of both the gel electrophoresis and the amount from NanoDrop ND1000 showed that 4 ^{o}C precipitation and centrifugation is the best choice. It seems that at 4 ^{o}C, both DNA and RNA dynamically changed between dissolving and precipitating, which could be demonstrated by the reported 4 ^{o}C centrifugation temperature [46]. Therefore, it is better to keep the operation at 4 ^{o}C, not only for a higher recovery rate but also for minimizing the activity of nuclease. Our results also showed that the initial concentration of the nucleic acid could influence the separation

of DNA and RNA. When initial concentration of the nucleic acid was low (corresponding to the low total amount based on the equal volume), it was difficult to find the centrifugation pellet and the pellet was easy to loss. On the other hand, RNA and DNA will intertwine for their analogous structure and can be precipitated together when the tested nucleic acid solution concentration was high. Therefore, controlling the initial nucleic acid concentration is very important to separate DNA and RNA. In our experiment, we considered that 400 ng/μL initial concentration would be optimal for the 100 μL operational volume for separation of DNA and RNA.

In addition, the effect of the LiCl concentration was non-ignorable factors. Different initial concentrations with various doses were reported, of which one quarter volume with 10 M LiCl was commonly used[47-49]. However, in this study, adding 1/4 volume saturated LiCl solution was better than other concentrations. This was consistent with the reported 8 M LiCl with equal volume added[43, 50]. It was reported that the sedimentation was kept overnight that may be a challenge for the RNA integrity[48, 50]. Thus, we aimed to find a shorter time interval with the least influence simultaneously based on the RNA quality and quantity, and keeping 6 h at 4 ^{o}C was recommended according to our results. The main advantages of using kit are the time-saving and high efficacy when dealing with small amount. Compared with the QIAGEN All Prep DNA/RNA Mini Kit, the maximum load for each column was less than 100 μg, while using LiCl as precipitator did not had a "saturated load". DNA and RNA can be separated as efficaciously as even more than kit. Furthermore, repeated centrifugation was necessary when the volume was larger than 700 μL when using kit. Additionally, when the initial nucleic acid solution concentration was high, insufficient separation was commonly appeared and the recovery rate was pretty low when using the kit. Besides, it is interesting to find that LiCl was far efficient than the kit when recovery DNA from *F. t*, whose G+C % was about 34 % compared with others' 50-61.4 %.

The advantages of our optimized method as below: 1) simultaneously recover DNA and RNA as much as possible from one sample. Time consuming and complex processes for extraction of DNA and RNA separately is avoid, and DNA and RNA extraction time interval is also avoid. No RNase or DNase usage means no nucleic acid loss after extraction. 2) low cost. Saturated LiCl solution was used to separate DNA and RNA instead of high cost kit and special equipment, such as the equipment for CsCl-based ultracentrifugation procedures, decrease the cost of whole process. 3) high efficiency and quality. Our optimized method based on a widely used high efficient DNA extraction method and resulted in satisfied amount of DNA and RNA. The obtained DNA was greater than 23 kb. 16S rRNA and 23S rRNA were obviously observed in the gel electrophoresis. The DNA and RNA could be directly used for PCR and RT-PCR amplification, respectively, which means the downstream molecular analysis can be satisfied. 4) universal applied. This method can be effectively applied to Gram-negative bacteria (*A. f, A. c*, *A. a*, *L. f* and *E. coli*), Gram-positive bacteria (*S. t*) as well as archaea (*F. t*) and fungus in wastewater biofilm. This method could also be successfully applied to environmental samples.

All in all, our optimized protocol as below (Fig. S5):

1) The collected cells/sediments samples were mixed with 2 g sterilized sand and then ground from freezing to thawing for three times by using liquid nitrogen;

2) The mixture was transferred into a RNase-free tube containing 16.5 mL PIPES extraction buffer and 1.83 mL 20 % SDS, mixed well and incubated at 65 ^{o}C for 1 h with gentle inversion every 15 min;

3) After incubation, the mixture was centrifuged at 6,000×g for 10 min, and upper supernatant liquid layer was transferred to a new centrifuge tube and extracted with chloroform: isoamylol (V:V=24:1) for 10 min. The mixture was then centrifuged at 6,000×g for 10 min, and supernatant liquid containing DNA and RNA was transferred to a new centrifuge tube;

4) RNA in crude nucleic acid was first precipitated with 1/4 volume of saturated LiCl for 30 min at -20 ^{o}C and collected by centrifugation at 12,000×g for 30 min at 4 ^{o}C;

5) After remove RNA, DNA in the supernatant was then precipitated with 0.6 volume isopropanol for 30 min at -20 ^{o}C and then collected by centrifugation at 12,000×g for 30 min at room temperature;

6) Both isolated DNA and RNA were purified with 70 % ethanol and collected by centrifugation at 12,000×g

for 30 min at room temperature;

7) The DNA and RNA pellet were air dried for 10~20 min and resuspended with 100-200 μL RNase-free water.

5. Conclusion

Nucleic acid isolation is a key procedure for molecular analysis of environmental microbial community. Here we reported an optimized method, which could simultaneously extract total nucleic acid using high salt PIPES buffer (pH 7.0) and separated RNA and DNA by saturated LiCl solution from diverse bioleaching pure cultures (including *A. f*, *A. c*, *A. a*, *L. f* and *F. t*) and AMD samples (including AMD samples taken from Daye copper mine, Xiangxi gold mine and Axi gold mine). The obtained DNA and RNA could be amplified and also showed high quality ($A_{260/280}$ was around 1.8-2.0 and $A_{260/230}$ was around 1.8-2.2). This method could be widely used in the extraction and separation of DNA and RNA from bioleaching bacteria, which provided a solid foundation for the AMD microbial molecular analysis and environmental microbial ecology study.

ACKNOLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (No. 51104189), the 53rd China Postdoctoral Science Foundation (No. 2013M531814), the National Basic Research Program of China (No. 2010CB630901), and the opening projects of State Key Laboratory of Powder Metallurgy. We would like to thank Bin Liang who came from Harbin Institute of Technology for the improvement of English.

REFERENCES

1. A. Gerhardt, L. J. de Bisthoven, K. Guhr, A. M. V. M. Soares and M. J. Pereira, *Ecotoxicology*, 2008, 17, 47-58.
2. S. C. Tremaine and A. L. Mills, *Appl Environ Microb*, 1991, 57, 775-784.
3. J. V. Dsa, K. S. Johnson, D. Lopez, C. Kanuckel and J. Tumlinson, *Water Air Soil Poll*, 2008, 194, 185-197.
4. C. D. Levings, K. L. Barry, J. A. Grout, G. E. Piercey, A. D. Marsden, A. P. Coombs and B. Mossop, *Hydrobiologia*, 2004, 525, 185-202.
5. D. K. Nordstrom and C. N. Alpers, *Proceedings of the National Academy of Sciences of the United States of America*, 1999, 96, 3455-3462.
6. R. K. Tiwary, *Water Air Soil Poll*, 2001, 132, 185-199.
7. J. F. Banfield, N. C. Verberkmoes, R. L. Hettich and M. P. Thelen, *Omics*, 2005, 9, 301-333.
8. B. J. Baker and J. F. Banfield, *Fems Microbiology Ecology*, 2003, 44, 139-152.
9. P. L. Bond, G. K. Druschel and J. F. Banfield, *Applied and Environmental Microbiology*, 2000, 66, 4962-+.
10. W. Krebs, C. Brombacher, P. P. Bosshard, R. Bachofen and H. Brandl, *Fems Microbiol Rev*, 1997, 20, 605-617.
11. D. B. Johnson and K. B. Hallberg, *Sci Total Environ*, 2005, 338, 3-14.
12. L. Valenzuela, A. Chi, S. Beard, A. Orell, N. Guiliani, J. Shabanowitz, D. F. Hunt and C. A. Jerez, *Biotechnol Adv*, 2006, 24, 197-211.
13. C. Cid, L. Garcia-Descalzo, V. Casado-Lafuente, R. Amils and A. Aguilera, *Proteomics*, 2010, 10, 2026-2036.
14. A. Knob and E. C. Carmona, *Appl Biochem Biotech*, 2010, 162, 429-443.
15. P. L. Bond, G. K. Druschel and J. F. Banfield, *Appl Environ Microb*, 2000, 66, 4962-4971.
16. P. L. Bond, S. P. Smriga and J. F. Banfield, *Appl Environ Microb*, 2000, 66, 3842-3849.
17. J. P. Xie, Z. L. He, X. X. Liu, X. D. Liu, J. D. Van Nostrand, Y. Deng, L. Y. Wu, J. Z. Zhou and G. Z. Qiu, *Appl*

Environ Microb, 2011, 77, 991-999.

18. L. Y. Wu, D. K. Thompson, G. S. Li, R. A. Hurt, J. M. Tiedje and J. Z. Zhou, *Appl Environ Microb*, 2001, 67, 5780-5790.
19. M. Hamady, C. Lozupone and R. Knight, *Isme J*, 2010, 4, 17-27.
20. M. C. Schatz, A. M. Phillippy, P. Gajer, T. Z. DeSantis, G. L. Andersen and J. Ravel, *Appl Environ Microb*, 2010, 76, 5636-5638.
21. C. S. Riesenfeld, P. D. Schloss and J. Handelsman, *Annu Rev Genet*, 2004, 38, 525-552.
22. S. G. Tringe and E. M. Rubin, *Nat Rev Genet*, 2005, 6, 805-814.
23. Y. Zhang, J. Xie, M. Liu, Z. Tian, Z. He, J. D. van Nostrand, L. Ren, J. Zhou and M. Yang, *WATER RES*, 2013, 47, 6298-6308.
24. J. Z. Zhou, K. Xue, J. P. Xie, Y. Deng, L. Y. Wu, X. H. Cheng, S. F. Fei, S. P. Deng, Z. L. He, J. D. Van Nostrand and Y. Q. Luo, *Nat Clim Change*, 2012, 2, 106-110.
25. F. Martin-Laurent, L. Philippot, S. Hallet, R. Chaussod, J. C. Germon, G. Soulas and G. Catroux, *Appl Environ Microb*, 2001, 67, 2354-2359.
26. D. N. Miller, J. E. Bryant, E. L. Madsen and W. C. Ghiorse, *Appl Environ Microb*, 1999, 65, 4715-4724.
27. L. Zeng, J. Huang, Y. Zhang, G. Qiu, J. Tong, D. Chen, J. Zhou and X. Luo, *APPL MICROBIOL BIOT*, 2008, 79, 881-888.
28. R. A. Hurt, X. Qiu, L. Wu, Y. Roh, A. V. Palumbo, J. M. Tiedje and J. Zhou, *Appl Environ Microb*, 2001, 67, 4495-4503.
29. J. B. Xiong, Q. C. Yang, J. M. Kang, Y. Sun, T. J. Zhang, G. Margaret and W. Ding, *Electrophoresis*, 2011, 32, 321-330.
30. W. Mathieson and G. A. Thomas, *Anal Biochem*, 2013, 433, 10-18.
31. D. A. Triant and A. Whitehead, *J Hered*, 2009, 100, 246-250.
32. J. M. Tolosa, J. E. Schjenken, T. D. Civiti, V. L. Clifton and R. Smith, *Biotechniques*, 2007, 43, 799-804.
33. A. B. Hummon, S. R. Lim, M. J. Difilippantonio and T. Ried, *Biotechniques*, 2007, 42, 467-472.
34. M. Bauer and D. Patzelt, *Forensic Sci Int*, 2003, 136, 76-78.
35. S. Raha, F. Merante, G. Proteau and J. K. Reed, *GENE ANAL TECH*, 1990, 7, 173-177.
36. C. M. Zammit, L. A. Mutch, H. R. Watling and E. L. J. Watkin, *Hydrometallurgy*, 2011, 108, 87-92.
37. O. Inceoglu, E. F. Hoogwout, P. Hill and J. D. van Elsas, *Appl Environ Microb*, 2010, 76, 3378-3382.
38. J. P. Xie, L. Y. Wu, J. D. van Nostrand, Z. L. He, Z. M. Lu, H. Yu, J. B. Xiong, X. X. Liu and J. Z. Zhou, *J Cent South Univ*, 2012, 19, 3055-3063.
39. J. Zhou, M. A. Bruns and J. M. Tiedje, *Appl Environ Microb*, 1996, 62, 316-322.
40. T. Nunoura, H. Oida, M. Miyazaki and Y. Suzuki, *Int J Syst Evol Micr*, 2008, 58, 659-665.
41. R. D. Palmiter, *BIOCHEMISTRY*, 1974, 13, 3606-3615.
42. R. I. Griffiths, A. S. Whiteley, A. G. O'Donnell and M. J. Bailey, *Appl Environ Microb*, 2000, 66, 5488-5491.
43. V. Dal Cin, M. Danesin, F. M. Rizzini and A. Ramina, *Mol Biotechnol*, 2005, 31, 113-119.
44. J. M. Chirgwin, A. E. Przybyla, R. J. MacDonald and W. J. Rutter, *BIOCHEMISTRY*, 1979, 18, 5294-5299.
45. P. Krieg, E. Amtmann and G. Sauer, *Anal Biochem*, 1983, 134, 288-294.
46. D. W. Cullen and P. R. Hirsch, *SOIL BIOL BIOCHEM*, 1998, 30, 983-993.
47. T. Kondo, M. Mukai and Y. Kondo, *Anal Biochem*, 1991, 198, 30-35.
48. S. Chang, J. Puryear and J. Cairney, *PLANT MOL BIOL REP*, 1993, 11, 113-116.
49. Y.-K. Hong, S.-D. Kim, M. Polne-Fuller and A. Gibor, *J APPL PHYCOL*, 1995, 7, 101-107.
50. J. R. Diaz-Ruiz and J. M. Kaper, *PREP BIOCHEM*, 1978, 8, 1-17.