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Modified Methods for Quick and Safe Extraction of DNA from Microbiological Samples

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Abstract

Isolating and purifying DNA are very important steps in DNA molecular techniques used in microbiological studies for the identification of genotypes, traits associated with genes of interest, and genetic diversity. Plant and soil materials are among the most difficult for high-quality DNA extractions. While carrying out DNA extraction for a total of 2000 different sample types (including water, soil, plant, algae, fungi and bacteria) using two different kits (ZYMO extraction kit and E.Z.N.A.® soil DNA Kit) and the crude extraction cetyltrimethylammonium bromide (CTAB) method, several modifications were made to ensure extraction of good quality DNA. These modifications in the methods were documented, repeated to confirm their accuracy and were done without the use of liquid Nitrogen. The extracted DNA concentrations obtained from CTAB extraction were extremely high and were diluted for use in PCR using a ratio of 1:10 of DNA to water while those obtained from the kit were used directly (but in small quantities ~ 0.5µL) or diluted in a ratio of 50:50. The mean DNA yields obtained for CTAB extraction was between 500 - 4000 ng/µl while that obtained for the kits were between 70 - 400 ng/µl. The 260/ 280 nm absorbance ratio had a high level of purity between 1.8 and 2.0. These modified methods can be used for day to day extraction processes in the laboratory.

Keywords: *Dna; Recovery; Extraction; Microbial; Efficient*

Introduction

For over three decades microbiologists have done numerous analyses to extract deoxyribonucleic acid (DNA) from environmental samples (eDNA) to enable them to study the diverse microorganisms in their various communities [1]. The first isolation of DNA was done in 1869 by Friedrich Miesche [2]. High-quality DNA is a major necessity for all experiments that involve DNA manipulation [1]. Every DNA extraction protocols follow the same basic steps including the rupturing of the cell wall, cell membrane and nuclear membrane to let out the DNA into the solution after which the DNA is precipitated out contaminate biomolecules (such as the proteins, polysaccharides, lipids, phenols and other secondary metabolites) are removed [3,4]. In several recent types of microbiological research, the need for molecular studies has become of major importance, hence, the need for effective efficient, simple and reliable methods for extraction of DNA [5,6]. DNA can be extracted from various sample types such as Plant leaf, Plant root, Algae, Soil samples, water samples, food samples, bacteria and fungi samples etc. The sample type and the DNA concentration needed determine the methodology of DNA extraction that will be followed by the researcher.

The need for more efficient methods to extract DNA with high qualities and yields has led to the development of several protocols. However, the fundamentals of DNA extraction remain the same [7,8]. CTAB extraction protocol is one of the earliest and cheapest methods

that has been used in the extraction of DNA for several sample types [9,10]. CTAB is a surfactant that has cationic nature and is required to break down the cellular membrane to initiate cell lysis so that the genic materials can be extracted. It is therefore employed in DNA extraction/isolation and the manufacturing of novel Gene Therapies. It sometimes takes a long time and requires the use of a lot of harmful chemicals and may still give low yields of DNA with poor quality but it is very cost-friendly especially for researchers in developing parts of the world and for commercial purposes [11].

Over time and in more recent years, DNA isolation and extraction kits have been invented and they have several advantages over crude methods (CTAB etc.). They are faster and simpler to use, and also rarely contain harmful chemicals such as phenol or chloroform and contact with samples and chemicals is highly reduced. They use spin columns or filters, which have silica-gel-based membranes that can bind to the DNA. The DNA when trapped in the membrane can be washed and cleaned free of contaminants and then eluted or released from the column (membrane) using nuclease-free water. The DNA obtained using kits are normally purer and cleaner than DNA extracted using crude methods. However, kits are very expensive, with prices ranging between $250 and above for 50 reactions (samples) [12]. This makes them unattractive for researchers in developing countries.

Plant and soil samples are among the most difficult for high-quality DNA extractions (13,14). Proper preparation of the tissues before extraction is essential to achieve good results. In many cases, the liquid nitrogen flash-freezing method is adopted followed by grinding the frozen tissue with a mortar and pestle [9,15,16]. Liquid nitrogen is very difficult and dangerous and harmful to use and therefore needs to be handled with care. We have therefore modified the previous DNA extraction protocol for two commercial kits and the CTAB method to suit the facilities that we have to meet our target.

Materials

Zymo extraction kit (Bacterial/fungal, Soil, Plant, Water)(manufactured by Zymo research Irvine, California, United States. www.zymoresearch.com)

E.Z.N.A.® soil DNA kit (manufactured by Omega Bio-Tek, *Headquarters*: 400 Pinnacle Way, Ste 450, Norcross, Georgia, 30071, United States ; Phone Number: (770) 931-8400 ; Website: www.omegabiotek.com)

Zymo extraction kit protocols: (https://www.zymoresearch.com/pages/genomic-dna-purification).

E.Z.N.A.® soil DNA Kit extraction protocol: (https://www.omegabiotek.com/wp-content/uploads/2013/04/D5625-Soil-DNA-Kit-120216-Online.pdf)

CTAB buffer

2% CTAB (hexadecyltrimethylammonium bromide), 100 mM TrisHCl [pH=8],20 mM EDTA, 1.4 M NaCl, 0.2% β-mercaptoethanol and 0.1 mg/mL proteinase K

NB: Add 4.1 g NaCl in 80 mL of distilled water and add 10 g of CTAB slowly while heating (≈65°C) and stir. It may take more than 3 hours to dissolve CTAB completely. The final volume should be adjusted to 100 ml by adding distilled water and sterilized in an autoclave at 121°C for 15mins at 1 atm.

Methods

The methods for the kits were modified from the extraction protocols given by the manufacturer and the well-known cetyltrimethylammonium bromide (CTAB) method where CTAB is used for DNA extraction (Kalendar *et al*., 2021) was also modified. The protocol for

the ZYMO kit and the CTAB method are simple and faster compared to other methods and the use of liquid nitrogen was not employed. The CTAB method was used for extracting Bacteria, Fungi, Algae and plant samples, the EZNA was used for only soil while the Zymo kit was used for extraction of DNA from Water, Soil, Plant, Algae etc. Each extraction was done using five replicates each. Plant samples, soil samples and water samples were processed as follows before extraction:

Plant samples

It should be noted that the leave and plant samples were allowed to dry in the oven leaving them with little or no moisture in them. The use of Nitrogen was not required.

Soil samples

Soil samples were allowed to dry to remove moisture from the samples.

Water samples

Water samples of about 500mL are collected and each sample is passed through a sterilized Buchner funnel sterilized weighing paper (Fisherbrand weighing paper cat No.09-898-12B made by fisher scientific Pittsburgh USA cut into the size of filter paper fitting the funnel) was used to filter the water sample. The forced pressure from the pump connected to the funnel was used to pull the water through the weighing paper. This took a long time due to the small pore size of the weighing paper. The weighing paper is used because it is easier to fold into the Eppendorf tube and takes lesser space.

The modified protocols are as follows

E.Z.N.A.® Soil DNA kit (Omega Bio-Tek, Norcross, GA, United States) modification to manufacturer's instructions

Add 500 - 700 mg (instead of 100 – 250 mg) soil sample to a disruptor tube. Add 725 µL, SLX-MLUS Buffer. Bead beat for 2 minutes and then mix manually for 2 minutes. Mix using a vortex at maximum speed for 5 minutes to lyse sample. Spin at 500 xg for 5 seconds to remove drops of liquid from the lid. Add 72 µL DS buffer vortex at maximum speed for 4minutes to mix thoroughly. Incubate at 70°C for 10 mins. Briefly mix using a vortex the tube once during incubation.

Centrifuge at 10,000 x g for 5 minutes at room temperature. Transfer 400 µL supernatant into a new 1.5 mL micro-centrifuge tube. Add 135 µL chilled p2 Buffer. Mix thoroughly using a vortex for 1 minute. Let it sit on ice for 5mins. Centrifuge at 13,000 x g for 3minute. Carefully transfer the supernatant to a new 1.5 µL micro-centrifuge tube. Add 200 µL HTR Reagent and mix thoroughly using a vortex. (HTR should be properly mixed). Let it sit at room temperature for 3 minutes.

Centrifuge at 13,000 x g for 5minutes. Transfer cleared supernatant (~ 500 µL) to a new 1.5 Ml micro-centrifuge tube (step should be repeated if the solution is still dark). Add an equal volume of XP1 Buffer. Mix thoroughly using a vortex. Insert a Hi-Band DNA Mini column into a 2 ml collection tube. Transfer up to 700 µL sample from step 16 to the Hi-Bind DNA Mini Column. Centrifuge at 13,000 x g for 5 minutes at room temperature. Discard the filtrate and reuse the Collection Tube. Discard the filtrate and reuse the collection Tube. Repeat steps 18 - 20 until all the lysate from step 16 has passed through the Hi-Bind DNA Mini Column.

Add 500 µL HBC Buffer (dilute with 32 mL 100% isopropanol before use). Centrifuge at 13,000 x g for 3minute. Discard the filtrate and the collection tube. Centrifuge at 20,000 x g for 1 minute to get rid of excess HBC Buffer. Transfer the Hi-Band DNA Mini Column into a new 2 mL collection tube. Add 700 µL DNA Wash buffer (diluted with100 Ml 100% ethanol). Centrifuge at 13,000 x g for 5minute. Discard the filtrate and reuse the collection tube.

The previous two steps should be repeated Centrifuge at 13,000 x g for 8 minutes. Transfer Hi-Bind DNA Mini Column into a clean 1.5 mL micro-centrifuge tube. Add 100 µL 70°c pre heated Elution buffer into the Centre of the Hi-Bind matrix. It should be allowed to sit at room temperature for 1 - 2 minutes. Centrifuge at 13,000 x g for 6 minutes. Collect filtrate and place it into the Centre of the same Hi-Bind DNA Mini column used in the procedure. Let sit at room temperature for 30 mins.

Centrifuge at 13,000 x g for 5 minute. Store eluted DNA at -20°C. Load on gel or check quality using a Nano-drop machine. Use for PCR and other molecular genetics studies.

Dna extraction procedure (using zymo kit)

Add 250 (50 - 100) mg (wet weight) fungal or bacterial cells (can be centrifuged from broth repeatedly until ¼ of the tube is full of bacteria cells) that have been re-suspended in up to 100 µl of water or isotonic buffer (e.g., PBS) or up to 200mg of tissue to a ZR bashing ™ Lysis Tube. Add 750ul Lysis Solution to the tube. Secure in a bead fitted with 2 ml tube holder assembly and process at maximum speed for > 5 minutes. Centrifuge the ZR bashing bead ™ Lysis Tube in a micro-centrifuge at > 10,000 x g for 1 minute. Transfer all the supernatant to a Zymo-Spin ™ IV Spin Filter (orange top) in a Collection Tube and centrifuge at 7,000 x g for 1 minute.

Add 700 µl of Fungal/Bacterial DNA Binding Buffer to the filtrate in the collection Tube from Step 4. Transfer 800 µl of the mixture from Step 5 to a Zymo-Spin ™ IIC column in a collection Tube and centrifuge at 10,000 x g for 1 minute. Discard the flow-through from the collection Tube and repeat Step 6. Add 150 µl DNA Pre-Wash Buffer to the Zymo-Spin ™ IIC column in a new collection tube and centrifuge at 10,000 x g for 1 minute. Add 350 µl Fungal/Bacterial DNA Wash Buffer to the Zymo-Spin ™ IIC column and centrifuge at 10,000 x g for 1 minute.

Transfer the Zymo-Spin ™ IIC Column to a clean 1.5 ml micro-centrifuge tube and add 70 ul DNA Elution Buffer directly to the column matrix. Centrifuge at 10,000 x g for 30 seconds to elute the DNA. Load on gel or check quality using a Nano-drop machine. Use for PCR and other molecular genetics studies.

Ctab extraction

This method can be used for bacteria, algae, soil and plant samples (Figure 1). Add 500mg bacteria and 500- 700 mg soil or plant into an Eppendorf tube. Add bashing beads of almost equal amounts to tubes for Bacteria and Plant samples while silver balls can be used for soil. Add 1 mL pre-heated CTAB DNA extraction buffer. Add 1 µL Ribonuclease A Solution (10 mg/ml in glycerol, 10 Mm Tris-HCL pH 8.0). Mix in tissue lyser for 15 mins. Incubate at 65°C for 1hour. Centrifuge at 13,000 rpm for 8 mins. Transfer Clear supernatant to new 2 mL micro-centrifuge tube. Add an equal volume of CIA (24:1 chloroform: Iso-amyl alcohol mix). Mix for 5 minutes in a disruptor.

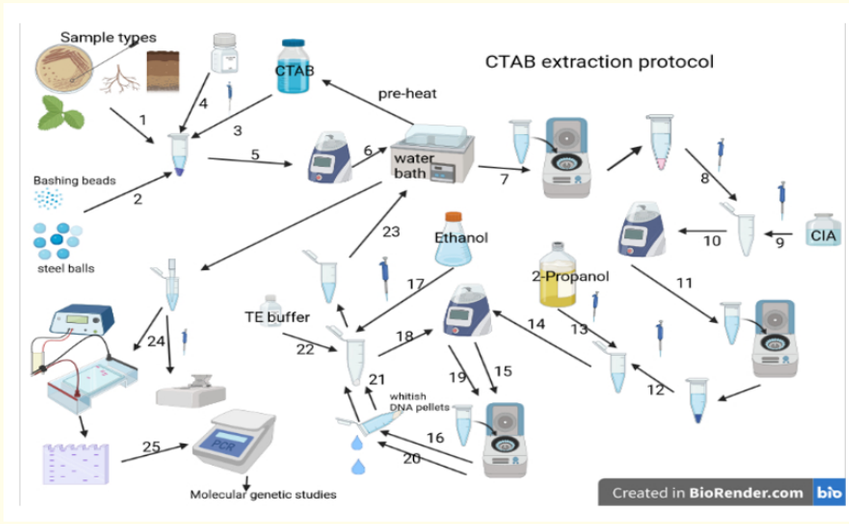

Figure 1: *Pictorial steps for DNA extraction using CTAB method.*

Centrifuge at 13,000 rpm for 2 mins. Transfer supernatant (clear) to new 2 mL Eppendorf tubes. Add equal volume of 2-propanol (100%). Vortex using a disruptor for 5mins. Centrifuge at 13,000 rpm for 5minutes. Discard supernatant. Wash pellet by adding 1.8 mL 70% ethanol. Place in a disruptor for 10 minutes. Centrifuge at 13,000 rpm for 5 minutes. Discard supernatant using micropipette or air dry by opening the tube till ethanol has completely evaporated. Check for whitish pellets of DNA at the base of the tube. Add 50 - 70 µL TE buffer (1 mM Na_3EDTA, 10 mM Tris-HCl pH 8.0). Place in a water bath at 65°C for 1 hour. Load on gel or check quality using a Nano-drop machine. Use for PCR and other molecular genetics studies.

Results

The modified CTAB DNA extraction method used in this study was observed to be the more efficient in extracting high DNA yields with better quality from the samples used and could be used in whole-genome sequencing, metagenomics, advanced sequencing technologies, and bioinformatics tools. Our results show that the concentrations of the DNA were highest for the CTAB extraction (172.47 ±23.37-4100.00 ± 655.74 ng/µl), but lower A_{260}/A_{280} ratio purity (0.78 ± 0.15- 1.74 ± 0.15) were observed with the CTAB method. Extraction using the ZYMO kit had higher concentrations in plant (137.9 ± 51.05 ng/µl) bacteria (146.1 ± 15.85 ng/µl), soil (84.77 ± 5.79 ng/µl) when modified method was used (table 1). Extraction using the ZYMO kit had higher concentrations in plant (137.9 ± 51.05 ng/µl) bacteria (146.1 ± 15.85 ng/µl), soil (84.77 ± 5.79 ng/µl) when the modified method was used (Table 1) but the A_{260}/A_{280} ratio purity were unaffected as they were similar. Extraction using the E.Z.N.A kit for soil also produced higher concentrations (172.46 ± 23.32 ng/µl) and A_{260}/A_{280} ratio purity (1.71 ± 0.04) when the modified method was used (Table 1).

Average DNA Concentration and Purity					
		Previous		Modified	
Extraction method	Sample type	Conc. (ng/µl)	Purity	Conc. (ng/µl)	Purity
ZYMO (kit)	Plant	27.27 ± 3.84	1.74 ± 0.15	137.9 ± 51.05	1.80 ± 0.08
	Soil	17.05 ± 0.95	1.89 ± 0.19	84.77 ± 5.79	1.87 ± 0.06
	Bacterial	33.16 ± 10.34	1.00 ± 0.10	146.1 ± 15.85	1.74 ± 0.09
	water	26.58 ± 7.68	1.35 ± 0.16	272.96 ± 30.66	1.91 ± 0.03
	Fungal	41.2 ± 11.43	1.71 ± 0.04	146.1 ± 15.85	1.89 ± 0.19
E.Z.N.A (kit)	Soil	27.47 ± 4.22	1.10± 0.12	172.46 ± 23.32	1.71 ± 0.04
CTAB	Plant	316.67 ± 74.08	1.60 ± 0.04	716.67± 104.08	1.8 ± 0.08
	Soil	616.67 ± 04.08	0.90 ± 0.15	616.67 ± 104.08	1.37 ± 0.28
	Bacterial	700.67 ± 00.08	0.92± 0.03	4100.00 ± 655.74	1.74 ± 0.15
	Fungal	172.47 ±23.37	0.78 ± 0.15	3505.35 ± 251.05	1.52±0.0

Table 1: *Comparism of the Concentration and Purity of extracted DNA using modified methods and previous methods.*

Discussion

In this study, three DNA extraction methods were used to extract high-quality DNA that can be efficiently amplified using PCR and for other molecular analyses. For all the modified methods liquid nitrogen was not used unlike in most methods of extraction where the use of liquid nitrogen to grind leaves, plant samples and soil samples is required as in the methods of Verma and Biswas, 2020; Kalendar *et al*., 2021 [17,18]. Mechanical beating using bashing beads enhances the disruption of the cells this was similar to the findings of Helmut *et al*., 2001 [19] where it was observed that the use of bead beating gave DNA of good qualities. It was observed that the mechanical grinding of cells directly in the DNA isolation buffer and the use of sufficient quantities of the sample were effective and efficient enough to avoid

the use of liquid nitrogen for the modified protocols. Weighing paper was used to filter water samples collecting more and tiny bacteria samples, differing from the method of Kawai *et al*., 2002; Djurhuus *et al*., 2017; Lee *et al*., 2019 [20-22] where filter paper was used as commonly practiced. Following the modified methods, the ZYMO kit chemicals were used for 100 reactions instead of the 50 reactions recommended by the manufacturers. DNA from CTAB extraction had higher yields while those from kits had higher purity this differs from the findings of Aboul-Maaty *et al*., 2019; Knüpfer *et al*., 2020 [23,24] where kit extraction had higher yields and yields obtain were about 500 µg/µl, but was similar to the findings of Edward *et al*., 1991 [25] where of extracted DNA were approximately 800 µg/µl although higher yields were obtained in this method. The purity of the DNA obtained were also similar to the A_{260}/A_{280} purity obtained in the work of Doyle and Doyle 1990; Wilson and Walker 2005; Aboul-Maaty *et al*., 2019 [23,26,27] where purity obtained were between 1.7 - 1.8.

The yield of DNA obtained was highly variable between the three different modified extraction methods and between samples and CTAB extracted DNA had the highest yield but the least quality this was similar to the findings of Minas *et al*., 2011; Tamari *et al*., 2013; Djurhuus *et al*., 2017; Wallinger *et al*., 2017 [28-30] and the extracted DNA were stable and applicable for marker-assisted selection, DNA fingerprinting, quantitative traits loci analysis, screening of transformants and enzymatic digestion.

Conclusion

We are able to describe efficient modified protocols using an established CTAB based extraction method and two commercial kits protocols for isolation and/or purification of high molecular weight genomic DNA from a range of fresh and difficult sources from a plant, animal, fungi, bacteria and soil material without the use of liquid Nitrogen.

CTAB-based extraction method can effectively extract DNA from most microbiological samples within a shorter time frame and that DNA extraction efficiency is dependent on the quantity of sample and the duration of beating the cells with bashing bead.

Conflict of Interest

The authors declare that there is no conflict of interest.

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