

## Detection of nudibranch *Chromodoris quadricolor* associated microbiome using two DNA extraction methods

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### ABSTRACT

DNA extraction with high quality is critical to all molecular genetic analyses. However, obtaining DNA from microbes associated with animals is challenging. Despite the availability of various DNA extraction kits in the markets, no studies were conducted to date to evaluate their potential for the invertebrates such as nudibranch, one of Mollusca. This study compared the Quick—DNA Fungal/Bacterial Miniprep Kit and CTAB methods and tested them across four samples of *Chromodoris quadricolor* gut and skin tissues. The universal bacterial primers 331f and 797r and the animal-specific primers LCO1490-JJ and HCO2198-JJ were used to amplify the 16S rRNA gene and partial mitochondrial cytochrome oxidase I gene using extracted DNA as a template. The DNA and PCR products' quality and concentration were verified with agarose gel and Nanodrop, respectively. The two methods' quality assessed using the deep pyrosequencing of the 16S rRNA gene in capturing a more diverse microbiome. The highest yield and purity (over 1000 ng /  $\mu$ L) were obtained with the CTAB method, while it was not exceeded 260 ng/ $\mu$ L with Quick DNA kit and display high purity. Also, 16S rRNA community amplicon sequencing revealed that the CTAB way could catch more diverse bacterial groups. The most efficient method of DNA extraction was CTAB, as it achieved both high concentration and purity.

### 1. Introduction

Nudibranchs belong to Mollusca, Gastropoda. Their shell is disappeared after the larval stage and is predominantly brilliantly colored and slow-moving [1]. Even though several microorganisms associated with numerous marine organisms have been broadly studied, the microbial communities associated with nudibranchs are relatively unknown. As molecular biology techniques are developing into more valuable tools for understanding microbial communities and their structure from an ecological perspective, it is imperative to produce pure DNA and overcome the tissue problem that challenges the DNA extraction process [2-6]. In contrast, pure DNA is a base for further molecular genetic analyses [7, 8], like PCR and real-time PCR analysis, next-generation sequencing, cloning, and other genotyping procedures. There are several manual DNA extraction protocols and commercial DNA extraction kits. Manual DNA extraction protocols mainly depend on chloroform and phenol to ensure the complete separation for the organic phase, including the genetic material and the liquid phase, containing the cell debris and other contaminants [9].

Many kits are available nowadays. They differ in isolation technique, type and source of the sample, and quantity, along with the runtime required for each sample, elution volume, the yield of DNA, and possible final applications. These kits mostly rely on DNA purification in the solid phase [10] and are performed using a spin column (under centrifugal force). Compared to conventional methods, some kits result in a fast and efficient purification of DNA, such as the CTAB or Phenol chloroform method. Nudibranchs often possess chemical defenses such as producing lipid and protein-rich mucus to clean their surface shield their epidermis from pathogens [11], the main restriction of the DNA extraction process. It was previously reported that the commercial kits and DNA extraction methods give several results to different animals, cells, and animal tissues. Accordingly, for better results, the extraction methods should be optimized for each tissue [12]. Simultaneously, following the steps with slight modification in the lysis and purification processes will produce sufficient DNA for molecular genetics techniques. This study aimed to evaluate two different methods, the conventional methods CTAB based method and the commercially available kit (Quick—DNA Fungal/Bacterial Miniprep) to extract DNA of *C. quadricolor* tissue. The evaluation of the two methods was based on assessing DNA concentration and their convenience for further molecular applications.

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## 2. Materials and Methods

### 2.1 Nudibranch sample collection

A frozen sample of *C. quadricolor* was used for genomic DNA extraction (Figure 1). In contrast, the sample was collected by SCUBA diving in the Red Sea near El Tor in Suez, Egypt (28.2278 ° N, 33.6211 ° E) while being preserved in DNA/RNA stabilization buffer and then sent to the laboratory for further genomic studies. The amount of tissue used was 250 mg per sample, four samples for each DNA extraction method.



Figure 1. *C. quadricolor* used in this study

### 2.2 Samples preparation

*C. quadricolor* sample was stored in DNA/RNA after collecting, then transferred to the laboratory while kept at  $-20^{\circ}\text{C}$ . Then it was transported to  $4^{\circ}\text{C}$  overnight and washed with a new solution of RNA stabilizing solution. The specimen was dried with clean paper towels to remove the excess stabilizing solution. Next, the sample was longitudinally cut into two parts, and each one was separated into the skin and gut with a sterile razor blade. Then the tissues were homogenized in liquid nitrogen.

### 2.3 DNA extraction

#### 2.3.1 Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, CA, USA)

The DNA extraction of *C. quadricolor* was performed using Fungal/Bacterial Miniprep Kit following the manufacturer's instructions, designed to isolate DNA from challenging microorganisms cells, such as yeast, filamentous fungi, gram-positive and gram-negative bacteria that need to be lysis. Zymo kit combines a ZR BashingBead™ Lysis technology without using organic denaturants or proteinases with Zymo-Spin™ Technology. The time spent in this method was about 30 mins for four samples. The only modified step was performing tissue lysis with liquid nitrogen to improve the digestion process. Then DNA extraction carried out using 250 mg per animal part; following the manufacturer's instructions briefly, 250 mg was added to the lysis tube gather with 750  $\mu\text{L}$  of BashingBead™ Buffer, then vortex at maximum speed for 10 mins, the cell debris was removed with a centrifuge at 10000  $\times\text{g}$  for 1 min. Then purification and washing steps

using two types of spin tubes and washing buffers with several centrifugation steps followed by the final elution step.

#### 2.3.2 Cetyltrimethyl ammonium bromide (CTAB) based method

The C-TAB protocol isolated the *C. quadricolor* DNA with a slight modification [13]. The homogenized samples were mixed with a preheated digestion buffer that consisted of 20  $\mu\text{L}$  Proteinase K (20 mg/mL) and 500  $\mu\text{L}$  CTAB- $\beta$ -mercaptoethanol (1 g CTAB, 0.7 M NaCl, 10 mM EDTA, 50 mM Tris-HCl pH 8.0, and 100  $\mu\text{L}$   $\beta$ -mercaptoethanol) per 50 mL. The samples were incubated on a heat block for 1 hour at  $60^{\circ}\text{C}$  with inverting the tubes every 15 mins. ZR BashingBead was added to samples and vortexed for five min at maximum speed to increase the sample homogeneity. The mixtures were then incubated for 30 mins to allow the sample to settle down. Each mixture was then transferred to a clean tube with adding 5  $\mu\text{L}$  RNase A (20mg/mL) and incubated for 1 hour at  $60^{\circ}\text{C}$  while shaking. The samples were centrifuged at 14,000 $\times\text{g}$  for 90 sec. Subsequently, 500 $\mu\text{L}$  of chloroform: isoamyl alcohol (24:1) was combined with the sample and mixed by inversion then vortexed for 10 min at low speed. Again samples were centrifuged at 14,000 $\times\text{g}$  for five min, and the upper phase poured into a new tube, and 500  $\mu\text{L}$  isopropanol was combined and mixed by inverting the tubes ten times. After centrifugation for 20 min at 18,000 $\times\text{g}$  at four  $^{\circ}\text{C}$ , the liquid is pipetted out, and 1mL of 70% ethanol is added to wash the pellets and invert for 5 min. Then the pellet with ethanol transfers to a Zymo-Spin tube; this step improves the DNA purity. Finally, the sample was centrifuged for 1 min at 14,000 $\times\text{g}$ ; the ethanol was removed, and the DNA pellet air-dried was then resuspended in 50  $\mu\text{L}$  of nuclease-free water. A portion of the isolated DNA was visualized using gel electrophoresis and stored at  $-20^{\circ}\text{C}$ .

### 2.4 DNA spectrophotometric analyses

The DNA quantity and purity were determined by detecting the absorbance ratio at A260/A280 and A260/A230 using NanoDrop OneC Microvolume UV-Vis Spectrophotometer w/ Cuvette (Thermo Fisher Scientific Inc, Wilmington, DE, USA). Typically pure DNA has A260/A280 ratios of 1.8 to 2.0. One  $\mu\text{L}$  was poured onto the lower pedestal, then the sampling arm was closed, and spectrophotometric measurement was initiated using the operating software.

### 2.5 PCR amplification

The genomic DNA aliquots were used as a template to amplify two of the most frequently used DNA markers in prokaryotes (16S rRNA) and eukaryotes (Partial mitochondrial cytochrome oxidase I).

The genomic DNA aliquots were used as a template to amplify two of the most frequently used DNA markers in prokaryotes (16S rRNA) and eukaryotes (Partial mitochondrial cytochrome oxidase I). The LCO1490-JJ and HCO2198-JJ primers were used to amplify (*COI*) gene sequences [14]. The universal 331f and 797r primers were also utilized to amplify the 16S rRNA gene [15].

**Table 1:** List of existing primers used in this study.

Target gene	Primer	Sequence	Annealin g TM/Sec	Amplicon length/bP
16S rRNA	331f	TTCTACGGGAGGCAGCAGT	5	460
	797r	GGACTACCAGGGTATCTAATCCTGTT		
COI	LCO1490-JJ	GGTCAACAAATCATAAAGATATTGG	8	708
	HCO2198-JJ	TAAACTTCAGGGTGACCAAAAAATCA		

All PCRs were conducted in a 20  $\mu$ L using DNA Polymerase II (Fisher Scientific, Waltham, USA). Each PCR reaction consisted of 10 mM dNTPs (New England Biolabs, USA), DNA Polymerase II Buffer 5  $\mu$ L, 10 pmol of either primer, 20 mg/ml BSA, or 1u DNA polymerase II. All PCR reactions have an initial denaturation step at 98  $^{\circ}$ C for 30 sec followed by 34 cycles, that started with denaturation at 98  $^{\circ}$ C for 5 sec, then annealing temperature regarding primer sequence (Table1), then extension at 72 $^{\circ}$ C for 12 sec, and following with a final extension at 72  $^{\circ}$ C for 1 min.

### 2.6 16S rRNA community analysis of *C. quadricolor*

The quality of the extraction methods to access diversity in the microbiome associated with *C. quadricolor* was examined by 16S rRNA community analysis and next-generation sequencing, whereas bioinformatics analyses and ecological statistics were performed as described previously [16]. The V4 region of 16S rRNA was amplified to prepare Illumina MiSeq libraries using environmental DNA protocol generated by Kozich *et al.* [17]. Briefly, the Platinum<sup>®</sup> PCR SuperMix was used to generate V4 amplicons with primers 515F and 806R appended with Illumina-specific adapters [18].

### 2.7 Agarose gel electrophoresis

Verification of genomic DNA and amplified sequences was performed using agarose gel with 1 and 1.5% agarose (Biobasic inc., Canada), 1X TAE, DNA gel stain GreenView<sup>™</sup> 1 ml (GeneCopoeia TM, USA) and 5  $\mu$ l of 50 bp -10 Kb DNA Ladder (Hi-Lo DNA) (Minnesota Molecular, Minnesota, USA), or 5  $\mu$ l of 250 bp-10 Kb DNA Ladder (GoldBio, Missouri, USA).

## 3. Results and discussion

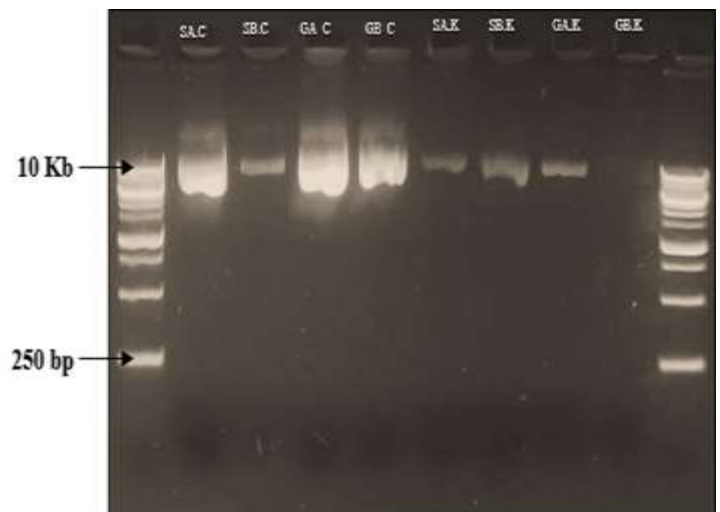
The present study aimed to evaluate and compare two available methods to extract the microbiome DNA associated with *C. quadricolor*, which may be difficult due to the high protein content in animal tissues and the diversity of the microbiome, which requires different methods of isolating the DNA that depending on the composition of the bacterial cell walls. We used the Fungal/Bacterial Miniprep Kit, based on ZR BashingBead<sup>™</sup> Lysis and Zymo-Spin<sup>™</sup> technology containing five reagents with various functions during extraction; cell digestion, membrane washing, and DNA elution.

The CTAB-based manual method has been used to extract DNA from marine invertebrates [13] with a modification to reduce contaminants and boost the DNA purity of *Balanus sp.* [19]. Even though all presently published techniques have proved their efficacy in isolation of DNA convenient for PCR amplification or restricting

digestion, they need long incubations, numerous precipitation procedures, and washing with ethanol to produce efficient genomic DNA with high purity [17]. These further modifications decrease the overall product and might fail to extract large amounts of high-quality DNA [20]. Our modification in the lysis step with liquid nitrogen and purification using Zymo-Spin<sup>™</sup> technology improves the DNA concentration and purity by decreasing time, unlike other previous changes.

### 3.1 DNA quality and quantity measurement

Both the agarose gel electrophoresis and the spectrophotometer Nanodrop were used for assessing the DNA characters. The visualized DNA aliquots on agarose gel are shown in Figure 2. The Nanodrop is helpful for the detection of impurities such as carbohydrates, proteins, and salts that can suppress and intercept DNA usage in molecular genetic analyses. Typically, high purity DNA has A260/A280 ratios of 1.8. The overall DNA yield in the C-TAB-based method was in a 400–1100 ng sample for conducting many PCR reactions. In comparison, the DNA obtained from the Fungal/Bacterial Miniprep Kit was 15-250 ng, per 250 mg of animal homogenized material (Table 2). The DNA absorption was determined at A 260/280 to be in



the range of 1.4 to 2.01.

**Figure 2.** Gel electrophoresis of total genomic DNA of *C. quadricolor* tissues, and 1 Kb DNA ladder, Goldbio. GA.K= Gut-A-Kit, GB.K= Gut-B-Kit, SA.K= Skin-A-Kit, SB.K= Skin-B-Kit, GA.C= Gut-A-CTAB, GB.C= Gut-B-CTAB, SA.C= Skin-A-CTAB, SB.K= Skin-B-CTAB, and ng.con= negative control.

**Table 2:** Summary of DNA concentration and purity for *C. quadricolor* tissues using CTAB method and Zymo Kit.

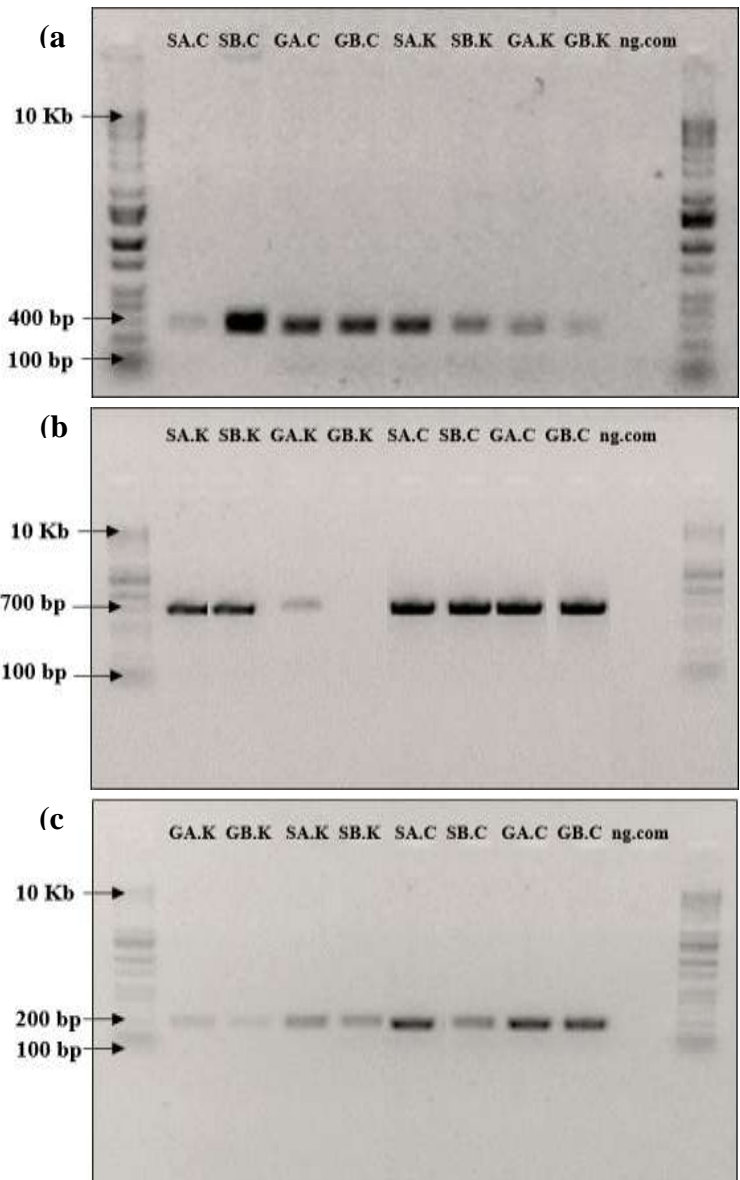
<i>C. quadricolor</i>	CTAB method		Fungal/Bacterial Miniprep Kit	
	DNA Con.[ng/μL]	DNA purity	DNA Con. [ng/μL]	DNA purity
Skin A	884.1	1.86	58.7	1.78
Skin B	505	1.83	234.3	1.83
Gut A	1094	2.01	77.9	1.75
Gut B	436	1.78	15	1.41

### 3.2 Verification of DNA using agarose gel electrophoresis

Here DNA and PCR products were visualized by 1 and 1.5% agarose, respectively. Electrophoresis of DNA aliquots extracted with CTAB showed a single band with a huge molecular weight; it also showed no sheared DNA and was not contaminated with RNA. Electrophoresis of DNA aliquots obtained by Zymo kite also showed high purity but low concentration (Fig. 2). It is well known that many factors affect the efficiency of PCR, DNA concentration, and quality, such as the impurities within the reaction mixture, a thermocycler efficiency, optimal temperature for each cycle, quality of PCR chemical constituent, and primers practicality [21]. All DNA extracts obtained with the CTAB method were suitable for amplifying the PCR, and their amplicons were distinguished with a high concentration and purity. However, Zymo Kit's yield was only sufficient for 50% of the samples with significant differences in concentrations. There were no differences in purity. The 16S rRNA and COI gene amplicons of *C. quadricolor* DNA were obtained with two techniques shown in (Fig. 3). All CTAB DNA extracts had positive COI and 16S rRNA amplification, while the DNA extracted with the Zymo kit amplicons were appeared faint or not amplified.

### 3.3 The efficiency of DNA extracted with two methods in next-generation sequencing

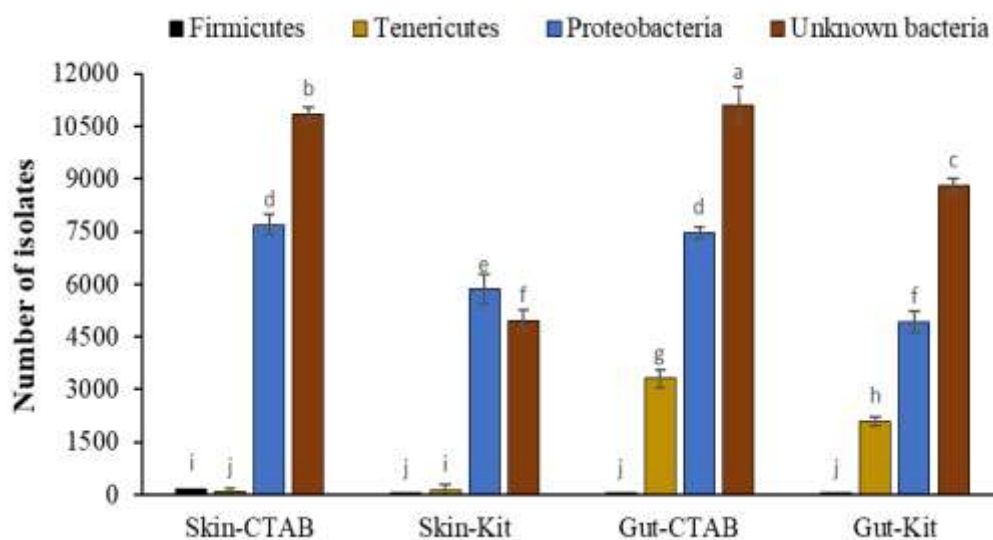
We examined the efficiency of the DNA extracted with two methods by community analysis using next-generation sequencing of 16S rRNA gene fragments, determined by their efficiency in capturing different microorganism populations. The total microbiome isolated using the CTAB method was 43737 compared to 32269 captured using Zymo Kit (Table 3). The number of structural variations (SVs) differed within *C. quadricolor* tissues using the extracted DNA by the two methods (Fig.4). Besides, the microbiome isolated by the CTAB extraction method more variable than produced by the Zymo Kit. Also, the number of microbiome sequences present in the DNA of the two methods was more in the DNA extraction with CTAB. Moreover, many classes were only captured by the CTAB method, for instance, Archaea, Actinobacteria, Cytophagales, Rhodospirillales, Sphingomonadales, Betaproteobacteria, and Salinisphaerales.



**Figure 3.** (a) Amplification of the universal 16S rRNA 331f and 797r primers, (b) Amplification of (COI) 700 bp on Agarose gel, and (c) Amplification of the universal 16S rRNA 331f and 797r primers. Amplification of 16S rRNA for community analysis 300 bp. GA.K= Gut-A-Kit, GB.K=Gut-B-Kit, SA.K= Skin-A-Kit, SB.K=Skin-B-Kit, GA.C= Gut-A-CTAB, GB.C= Gut-B-CTAB, SA.C=Skin-A-CTAB, SB.K= Skin-B-CTAB, and ng.con=negative control. 10 kB HI-LO DNA ladder.

**Table 3:** Number of sequences per sample.

Nudibranch	Animal part	Primers		Instrument code	Number of sequences
		Forward	Reverse		
<i>C. quadricolor</i>	▪ Skin -A (k)	SA506	SA704	24D	8384
	▪ Skin -B (k)	SA507	SA704	14D	6680
	▪ Gut-A (k)	SA507	SA702	210III	1414
	▪ Gut-B (k)	SA507	SA702	12A	16791
	▪ Skin -A ©	SA507	SA706	16F	4060
	▪ Skin -B ©	SA507	SA703	13C	22034
	▪ Gut-A ©	SA506	SA710	27G	4755
	▪ Gut-B ©	SA506	SA706	26F	12921



**Figure 4.** Genomic variations of *C. quadricolor* tissues using DNA extracted by the two methods. Means ( $\pm$ standard error) followed by different letters on the same bars show significant differences according to Fisher's test at  $p < 0.05$ .

The number of sequences and sample variability shows that the CTAB method efficiently isolates the microbiome associated with an invertebrate while demonstrating that the Zymo Kit needs some manipulations to produce more DNA.

Ideally, the protocols of DNA extraction must be quick, efficient, and comfortable to accomplish and obtain sufficient DNA with high quality, appropriate for molecular analysis applications [22]. Traditional techniques such as CTAB commonly take a long time and require poisonous substances [10]. Compared to this, DNA isolation kits have the advantages of limited usage of chemicals, handy methodology, short isolation procedure, and fast results [23]. However, there are some disadvantages to commercial kits, like the high costs of commercial tools. In some studies, the DNA yields and purity are lower than those obtained by conventional methods, as the present study agrees with [23-25].

#### 4. Conclusions

Our study used *C. quadricolor* for DNA extraction, the two used methods gave adequate quality and quantity of DNA for use in the COI and 16S rRNA analyses. The CTAB based method gave the purest product with the lowest level of DNA degradation and contamination. On the other hand, to perform PCR only, relatively small amounts of DNA are required. DNA was also sufficient for other applications, such as high-throughput amplicon sequencing of 16S rRNA gene fragments. Based on our findings, CTAB-based methods are also suitable for preparing materials for use in next-generation sequencing-based applications. Moreover, the time required to use toxic chemicals in a CTAB-based process should be ignored, especially when handling challenging samples.

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