Research Article

Short Term Protocol for the Isolation and Purification of DNA for Molecular Analysis

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ABSTRACT

The tremendous potential of genomics and other recent advances in molecular biology, the role of science to improve reliable DNA extraction and quantization methods is more relevant than even before. The ideal process for genomic DNA extraction demands high quantities of pure, integral and intact genomic DNA from the biological sample is the vital primary step to succeed in various molecular biological techniques such as Polymerase Chain Reaction (PCR), restriction enzyme analysis, mutation detection, genotyping as well as linkage analysis. Moreover DNA extraction from blood samples is the most important requirement for the determination of genetic abnormalities, epigenetic studies and various diagnostic and preventive tests. Conventional genomic DNA extraction protocols need expensive and hazardous reagents for decontamination of phenolic compounds from the extracts and are only suited for certain types of tissues. We developed a simple, rapid, less hazardous, cost-effective and high throughput protocol for extracting high quality DNA from blood of various fishes, using GenElute DNA extraction kit for genomic DNA. Unlike most DNA preparations methods that require multiple steps and special handling, which increases the risk of error or contamination, the protocol employs a single purification step to remove contaminating compounds, using a column preparation solution, wash solution and elution solution. Quality and quantity of the extracted DNA were checked by UV Spectrophotometer. By measuring the 260/230 and 260/280 nm absorbance ratios (A_{260}/A_{230} and A_{260}/A_{280}), the DNA concentration, purity and protein contamination of samples were determined (> 1.8). To evaluate the probability of DNA degradation, agarose gel electrophoresis was carried out by loading 5µl of extracted DNA on 1.2% agarose gel. The UV Spectrophotometric and gel electrophoresis analysis resulted in high intactness of DNA. In conclusion, this study represents a reliable approach for intact pure DNA extraction method from fish blood.

Keywords: Fish, blood, DNA-extraction, quantization, GenElute Genomic DNA Extraction Kit.

INTRODUCTION

DNA is reported to be the most appropriate molecule for the detection and identification of fish species in processed food products, offering numerous advantages over the analysis of proteins [1]. DNA is present in all tissue types, has a greater stability at high temperatures, and the diversity afforded by the genetic code allows differentiation of closely related species [2, 3].

Traditionally, DNA extraction protocols based on the addition of organic solvents, such as phenol and chloroform, have been frequently used to isolate genomic DNA from animal species[4]. Although such methods produce acceptable results for samples of diverse origins, they are time consuming and require the use of reagents that can not only chemically contaminate the extracted DNA, but which are also a health hazard [5]. More recently, a number of commercial kits have been introduced for DNA extraction from different foods, employing either a variety of solvents and/or specialised columns containing DNA-binding substances. The limited reports comparing protocols for DNA extraction from fish have mainly focused on fish fins and larvae[6], or museum fish specimens preserved in formalin or ethanol [7]. The aim of this study was to identify the most feasible method for the extraction of high quantity and quality DNA from fish blood and scales. With this objective, the efficiency of commercially available kit was compared for their ability to extract high yields of pure DNA suitable for PCR amplification from three fish species namely *Cyprinus carpio specularis, Cyprinus carpio communis* and *Oncorhynchus mykiss* available in Kashmir.

A number of simplified protocols for DNA extraction have been reported, such as a salting out procedure[8], microwave-based extraction[9], silica–guanidinium thiocyanate method[10], a CTAB procedure [11], a boiling method [12], and Chelex-based extraction [13]. However, the majority of these methods were developed for samples collected either from plants or from human or other mammalian sources. Genetic, haematology, and biochemistry studies employed in human clinic, demand blood samples in order to recognize and prevent human serious disorders [14, 15]. DNA extraction from blood samples is the most requirements for the determination of genetic abnormalities, epigenetic studies and various diagnostic and preventive tests [16, 17].

DNA extraction demands a simple, rapid and economical protocol with minimum co-extraction of inhibitors for downstream processes. Furthermore, it should recover high amounts of pure and integral gDNA. To reach this aim, several DNA extraction protocols from blood samples have been developed and commercialized [18]. However, not even a single protocol meets all these criteria [19]. Most of conventionally used protocols for genomic DNA isolation require an overnight incubation with proteinase K enzyme for cell lysing and protein digest that can lead to some nuclease degradation in 37^0 C. Besides, RNA contamination can routinely be removed

by using of RNase A enzyme, which makes these protocols time and cost-consuming [20].

Due to increasing demands for simple, rapid and cost-effective DNA extraction protocol in different fields of biology, medical biotechnology and diagnosis of human disorders, the present study has aimed to introduce a novel, simple, rapid and cost-effective protocol for DNA extraction for further molecular analysis. It provides high yields of remarkably pure DNA from blood samples of fishes which is suitable for most downstream processes.

MATERIALS AND METHODS

Blood Samples from the Fishes

Blood samples were freshly collected from the three fish species namely *Cyprinus carpio specularis, Cyprinus carpio communis* and *Oncorhynchus mykiss* by puncturing the caudal vein with a micro syringe or by decapitating the caudal portion and exposing the blood vessel. This fresh blood was collected in anticoagulant EDTA tubes.

Reagents and solutions

Blood DNA extraction kit (**GenElute Blood Genomic DNA Kit, Sigma Aldrich; Cat. No. NA2000**) was used for isolation of genomic DNA from blood samples with some modifications.

- Resuspension solution
- Lysis solution C
- Column preparation solution
- Prewash solution
- Wash solution
- Elution solution (10mM Tris-HCl, 0.5mM EDTA, pH 9.0)
- Proteinase K
- RNase A Solution
- Ethanol (95-100%)

DNA extraction procedure

Concisely, the extraction procedures for fresh samples were done in the following manner:

1. The blood of the three fishes namely *Cyprinus carpio specularis, Cyprinus carpio communis* and *Oncorhynchus mykiss* was collected by puncturing a micro syringe into the caudal vein and transferred to EDTA anticoagulant tubes.
2. The blood was then equilibrated to room temperature before beginning preparation.
3. 200µl of blood was transferred to 1.5ml microcentrifuge tube and 20µl of Proteinase K solution was added to the tube.
4. For RNA-free genomic DNA, 20µl of RNase A solution was added.
5. 200µl of Lysis Solution C was added to the sample and vortex thoroughly for 15 seconds.
6. The sample was then incubated for at 55^0 C for 10 minutes in a water bath.
7. 500 µl of Column preparation solution was added to each pre-assembled GenElute Miniprep Binding Column and centrifuge at 12,000g for 1 minute. The flow was discarded through liquid.
8. 200µl of ethanol was added to lysate (step 6). The solution was mixed thoroughly by vortexing for 5-10 seconds.
9. Transfer entire content (of step 8) into the treated column (Step 5).
10. The Column was centrifuge at 6500g for 1 minute and discard the tube containing flow-through liquid and place the column in a new 2 ml collection tube.
11. First Wash of solution was done by adding 500 µl of Prewash Solution to the column and centrifuge at 6500g for 1 minute.
12. For second Wash, 500µl of Wash Solution was added to the column.
13. The Column was centrifuge for 3 minutes at 12000-16000g.
14. DNA was eluted by adding 200µl of the Elution Solution directly into the centre of the Column.
15. The Column was then centrifuged for 1 minute at 6500g.

To increase elution efficiency, the Column was incubated for 5 minutes at room temperature after adding the Elution Solution, then centrifuge.

Elute contains pure genomic DNA. The DNA can be stored for short term at 2-8° C and for long term storage, -20° C is recommended.

Quantification of extracted DNA by spectrophotometer

Known volumes of DNA extracts were diluted to 2 ml in double distilled water and aliquots of the diluted DNA were transferred to separate quartz cuvettes. The diluted DNA solutions were quantified and assessed for impurities by measuring the absorbance at 260 nm (A260) and 280 nm (A280) in a spectrophotometer. DNA concentrations were calculated by multiplying the A260 measurement by the dilution factor and then by 50, based on the relationship that an A260 of 1.0 equals 50 mg/ml pure DNA[21]. DNA yields were calculated by multiplying the DNA concentration value by the final volume of DNA extracted with each method. DNA purities were determined by calculating the A260/A280 ratios. Samples calculated to have A260/ A280 ratios of approximately 1.8-2.0 were assumed to be pure samples, free from protein and/or RNA contamination[22-25].

Electrophoresis of extracted genomic DNA

Amplified DNA fragments were separated by electrophoresis at 100 V on 1.2% agarose gel with Tris-borate-EDTA buffer[21]. The DNA is visualized in the gel by addition of ethidium bromide. This binds strongly to DNA by intercalating between the bases and is fluorescent meaning that it absorbs invisible UV light and transmits the energy as visible orange light. Pour 1.2 g of agarose into a 250 mL conical flask and 100 mL of 0.5xTBE were added, swirl to mix. It is good to use a large container, as long as it fits in the microwave, because the agarose boils over easily. Microwave for about 1 minute to dissolve the agarose. The agarose solution boils over very easily. It is good to stop it after 45 seconds and give it a swirl. Leave it to cool on the bench for 5 minutes down to about 60°. Pour the gel slowly into the tank. Push any bubbles away to the side using a disposable tip. The comb was inserted and checked that it is correctly positioned. Pour 0.5x TBE buffer into the gel tank to submerge the gel to 2–5 mm depth. An appropriate amount of loading buffer was added into each tube and leave the tip in the tube. The loading buffer gives colour and density to the sample to make it easy to load into the wells. The first and the last well were loaded with 5 µl of the marker namely step up 50 kb DNA ladder (Merck). This ladder consists of 20 bands of double stranded DNA fragments ranging from 50-1000 bp with size increment of 50 bp (Fig. 1). The size of the ladder ranges from 0.15kb to 1kb. The other wells were loaded with 5 µl of DNA. The gel tank was closed, switched on the power-source and ran the gel at 100 V.

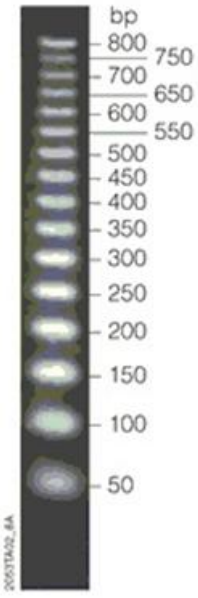

Figure 1: Step up 50 kb DNA ladder (Merck)

RESULTS AND DISCUSSION

The conventional genomic DNA extraction protocols need expensive and hazardous reagents for decontamination of phenolic compounds from the extracts and are only suited for certain types of tissues. This is a simple, rapid, less hazardous, cost-effective and high throughput protocol for extracting high quality DNA from blood of fishes by using GenElute DNA extraction kit for genomic DNA. Unlike most DNA preparations methods that require multiple steps and special handling, which increases the risk of error or contamination, this protocol employs a single purification step to remove contaminating compounds, using a column preparation solution, wash solution and elution solution.

In molecular biology, quantitation of nucleic acids is commonly performed to determine the average concentrations of DNA or RNA present in a mixture, as well as their purity. Reactions that use nucleic acids often require particular amounts and purity for optimum performance. There are several methods to establish the concentration of a solution of nucleic acids, including Spectrophotometric quantification and UV fluorescence in presence of a DNA dye.

Spectrophotometric and Electrophoretic analysis of extracted genomic DNA

Nucleic acids absorb ultraviolet light in a specific pattern. In a spectrophotometer, a sample is exposed to ultraviolet light at 260 nm, and a photo-detector measures the light that passes through the sample. The more light absorbed by the sample, the higher the nucleic acid concentration in the sample. It is common for nucleic acid samples to be contaminated with other molecules (i.e. proteins, organic compounds, other). The ratio of the absorbance at 260 and 280nm ($A_{260/280}$) is used to assess the purity of nucleic acids. For pure DNA, $A_{260/280}$ is ~1.8. The Spectrophotometric comparison of absorbance at A_{260} / A_{280} nm provided a purity factor of 1.85-1.9, indicating its good quality (table. 1)

Table 1: Absorbance ratio and Purity of the Isolated DNA

Fish Species	OD at 260nm	OD at 280nm	$A_{260/280}$
Cyprinus carpio communis	0.028	0.015	1.86
Cyprinus carpio specularis	0.026	0.014	1.85
Oncorhynchus mykiss	0.023	0.012	1.9

The isolated DNA had no signs of degradation which is validated by sharp and unified bands on 1.2% agarose gel electrophoresis (Fig. 2). The DNA size has been compared with step up 50 kb DNA ladder. The size of the DNA has been compared with the ladder having size of 1000 bp, 950 bp, 900 bp, 850 bp, 800 bp, 750 bp, 700 bp, 650 bp, 600 bp, 550 bp, 500 bp, 450 bp, 400 bp, 350 bp, 300 bp, 250 bp, 200 bp, 150 bp, 100 bp and 50 bp. The size of the extracted DNA as compared with the ladder is 1000bp.

Accurate understanding of material function in molecular biology has enhanced the capability of the scientist to develop the alternative methods for DNA extraction from a variety of samples. It is obvious that the choice of the methodology will depend on several factors, including cost, time, simplicity, and robustness.

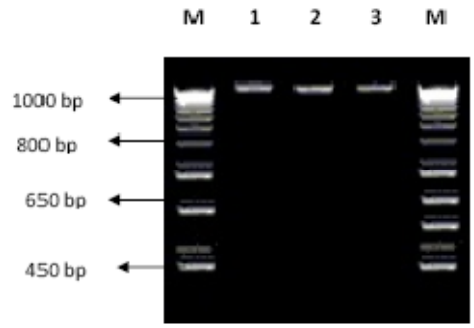

Figure 2: A typical DNA banding pattern resolved in 1.2% agarose gel and stained with Ethidium bromide. Template DNA was from Step up 50 kb DNA ladder (Lane M), *Cyprinus carpio specularis* (Lane 1), *Cyprinus carpio communis* (Lane 2) and *Oncorhynchus mykiss* (Lane 3).

Numerous genomic DNA isolation protocols have been optimized for blood samples[16, 20, 18,] most of which were verified to be reproducible and yielded sufficiently high-quality DNA for genetic analysis. However, in most of these methods, enzymes (such as proteinase K and RNAse A) or toxic organic solvents (such as phenol or guanidine isothiocyanate) have been exploded[18]. There are only a few methods that are non-enzymatic and do not employ hazardous organic solvents, therefore, the standard protocols for DNA extraction remain time-consuming. Since these enzymes are expensive and most of the materials that are used routinely are toxic, it is reasonable to apply an efficient DNA extraction procedure that does not undergo these steps. In an attempt to attain these aims and simplify the procedure, in this study we described a very simple, inexpensive, rapid and less-hazardous protocol for extracting high quality DNA from fish blood by using GenElute Genomic DNA Extraction Kit.

DNA samples isolated by the present method from the blood of the fishes were successfully used in the amplification of sequences using random primers. Reproducible fragments of different lengths were amplified cleanly in almost all the species of fishes. Moreover, PCR amplification indicated that the quality of the extracted DNA is good enough to allow PCR amplification of desired length fragment without further purification.

CONCLUSION

In conclusion, this study represents a reliable approach for DNA extraction from blood of the fishes. This method does not use any hazardous chemical, so it is more reliable and safe than the other methods. By practising this protocol, we reached to pure and high throughput yield from a small amount of sample which makes current method applicable in medical laboratories as well as research centres particularly where the downstream processes are to be followed.

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