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Study on a Rapid Extraction and Detection Method for 16S rRNA of Intestinal Flora

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Abstract: This study established a rapid extraction method for the 16S rRNA gene of intestinal flora. Combined with next-generation sequencing (NGS) technology, this method can be applied to intestinal microbial ecology analysis. The fecal sample was mixed with lysis buffer, incubated at 90°C for 15 minutes, vortexed, and then centrifuged. The supernatant was collected, and specific primers were added for PCR amplification. The PCR products were purified, and index tags were established for the targets to obtain libraries. After library purification and quality inspection, the libraries could be loaded for sequencing. Under optimized conditions: when the number of PCR cycles was 25 and the initial amount of DNA for library construction was 12.5 ng, fewer chimeras were generated. The library size was 500–700 bp, and there were no primer dimers of approximately 120 bp. This method is rapid, accurate, and sensitive, and can be used for the analysis and detection of the 16S rRNA gene of intestinal flora.

Keywords: Extraction; Intestinal flora; Sequencing; 16S rRNA

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1. Introduction

A complex microbial ecosystem exists in the human intestine, mainly including bacteria, fungi, archaea, and viruses, which are collectively referred to as intestinal flora [1]. These intestinal floras perform important physiological functions such as digestion, nutrition, immunity, and symbiosis. A large number of studies have shown that intestinal microorganisms have an important impact on intestinal function [2]. From a genetic perspective, the human genome carries approximately 25,000 genes, while the total number of genes encoded by human intestinal microorganisms is about 150 times that of human genes. All genetic information of human intestinal microorganisms is called the human intestinal metagenome [3]. Therefore, studying the composition of intestinal flora and its correlation with the occurrence of diseases through intestinal microbial metagenomics has become a research focus nowadays.

At present, the main methods for extracting microbial DNA include thermal lysis, chemical method, and

enzymatic lysis. Thermal lysis directly heats the sample for lysis to extract genomic DNA. This method is simple with high recovery rate, but the purity of microbial DNA is low. The chemical method generally adopts the phenol/chloroform extraction method, which has complex operation steps, requires a large amount of sample, and has low DNA yield. The enzymatic lysis uses lysozyme for microbial wall breaking, with mild conditions and high recovery rate of microbial DNA, but more impurities.

Currently, commercial kits generally use the enzymatic lysis method, followed by centrifugal filtration with a silica column membrane. This method can obtain microbial DNA with high purity, but has complex operation, long time consumption, and low yield. In this study, a method for extracting the 16S rRNA gene of intestinal flora was established by combining the chemical method and thermal lysis method. Combined with next-generation sequencing technology, the difference between this method and the conventional spin column method was analyzed, aiming to establish a detection method suitable for large-scale sample extraction and analysis of the 16S rRNA gene.

2. Materials and methods

2.1. Materials and instruments

The materials are as follows:

(1) PCR Amplification Mix Kit: Nanjing Vazyme Biotechnology Co., Ltd;

(2) Taq Enzyme, dNTP, Agarose, D2000 Ladder: Products of Beijing Solarbio Science & Technology Co., Ltd;

(3) Qubit 3.0 Fluorometer (with Qubit dsDNA HS Assay Kit): Product of Thermo Fisher Scientific, USA;

(4) 2200 High Sensitivity D1000 Assay Kit: Product of Agilent Technologies, USA;

(5) Nextera Index Kit – PCR Primers: Product of Illumina, USA;

(6) AMPure XP Beads: Product of Beckman Coulter, USA;

(7) Fecal DNA Extraction Kit: Product of Fujian Xilong Biotechnology Co., Ltd;

(8) Primers: Synthesized by Fuzhou Shangya Biotechnology Co., Ltd.;

(9) Other reagents: All of analytical grade.

The instruments are as follows:

(1) T100-PCR Amplifier;

(2) Mini-sub Cell GT System Electrophoresis Apparatus;

(3) Gel Doc XR+ Gel Imaging Analyzer: All products of Bio-Rad Laboratories, USA;

(4) Miseq Sequencer: Product of Illumina, USA.

2.2. Detection methods

2.2.1. Extraction method of fecal sample DNA

The lysis buffer was prepared according to Song *et al.* [4]. A total of 1.2 mL of lysis buffer was added to the fecal sample and oscillated intermittently for 1–2 minutes until the sample was mixed uniformly, It was incubated at 90°C for 15 minutes, vortexed for 30 seconds, and centrifuged at 12,000 rpm for 10 minutes. After centrifugation, 700 μL of the supernatant was transferred to a new centrifuge tube. The spin column method of the kit was operated with reference to the instruction manual.

2.2.2. PCR amplification and electrophoresis

The sequence of the primers are as follows:

(1) Upstream primer F: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACTCCTACGGGA GG -CAGCAG

(2) Downstream primer R: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACH -VGGGTWTCTAAT

For the PCR reaction system, 2.5 μL of DNA extracted from the above sample, 1 μL of primer mixture, 12.5 μL of PCR Amplification Mix (2X), and 9.0 μL of ddH_2O was mixed and gently pipetted 10 times to mix uniformly. It was then placed in the PCR amplifier, and the reaction conditions are as follows: initial denaturation at 95°C for 3 minutes; followed by 25–30 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 45 seconds; final extension at 72°C for 5 minutes; and holding at 4°C.

After the reaction, 2 μL of the PCR product was taken for agarose gel electrophoresis. The electrophoresis conditions were: 1% agarose, D2000 Ladder, 120V, and 20 minutes.

2.2.3. Purification of PCR products

The AMPure XP beads were took out from the 4°C refrigerator in advance and equilibrated at room temperature for 30 minutes, and oscillated to mix well before use. A new 1.5 mL centrifuge tube was taken, 18.4 μL of beads were added and 23 μL of the PCR product from the previous step was added. The mixture was gently pipetted to mix well, and stood at room temperature for 5 minutes. After centrifugation, the centrifuge tube was placed on a magnetic rack for 10 minutes until the liquid became clear. The supernatant was carefully removed, and the beads were not aspirated as much as possible. The centrifuge tube was kept on the magnetic rack, and 200 μL of 80% ethanol was slowly added along the tube wall, pipetted and stood for 30 seconds. The supernatant was removed, the washing step was repeated once, and any residual ethanol was removed as much as possible when removing the supernatant. The centrifuge tube was kept on the magnetic rack, and stood for 4 minutes at room temperature to dry the beads. After that, 32 μL of resuspension buffer was added and pipetted to mix the beads well, and stood for 2 minutes at room temperature. Following instantaneous centrifugation, the centrifuge tube was placed on the magnetic rack until the liquid became clear, while 30 μL of the supernatant was transferred to a new 1.5 mL centrifuge tube.

2.2.4. Establishment of index tags

PCR amplification mix (2X) was mixed with 10 μmol/μL Indexed PCR primer i5 and Indexed PCR primer i7, and they were thawed on ice. Only one set of Index sequences was listed below; the remaining sequences can be found in the Nextera Index Kit-PCR Primers.

i5: AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAGCGT;
i7: CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGG.

The reaction system was prepared in a 0.2 mL centrifuge tube as follows: 5 μL of purified PCR product, 5 μL of Indexed PCR primer i5 (10 μmol/μL), 5 μL of Indexed PCR primer i7 (10 μmol/μL), 25 μL of PCR Amplification Mix (2X), and 10 μL of ddH_2O. The mixture was gently pipetted 10 times to mix well, and placed in a PCR amplifier. The conditions were the same as described above, with the number of cycles set to 8.

2.2.5. Library purification and quality inspection

A new 1.5 mL centrifuge tube was taken, and 50 μL of beads equilibrated at room temperature was added. The PCR product from the previous step was added, pipetted 10 times to mix well, and stood for 5 minutes at room temperature. After instantaneous centrifugation, the centrifuge tube was placed on a magnetic rack until the liquid became clear. The supernatant was carefully removed, and the PCR tube was kept on the magnetic rack. A total of 200 μL of 80% ethanol was added, pipetted, and stood for 30 seconds. The supernatant was removed, washing step was introduced once, and any residual ethanol was removed as much as possible when removing the supernatant. The centrifuge tube was kept on the magnetic rack, and stood at room temperature for 4 minutes to dry the beads. A volume of 27 μL of resuspension buffer was added and pipetted to mix the beads well, followed by standing at room temperature for 2 minutes. After instantaneous centrifugation, the centrifuge tube was placed on the magnetic rack until the liquid became clear. The supernatant (25 μL) was transferred to a new 1.5 mL centrifuge tube. Only 1 μL of the supernatant was taken to detect the concentration using a Qubit 3.0 Fluorometer (Qubit dsDNA HS Assay Kit) and the concentration was not recorded. The Agilent 2200 High Sensitivity D1000 kit was used to detect the band distribution of the library.

2.2.6. Library sequencing

According to the requirements of sequencing data volume, this experiment was set to 30,000 tags per sample, and the libraries were mixed. After mixing, perform quality inspection using Qubit 3.0 and Agilent 2200 Bioanalyzer, and dilute the library to the on-machine concentration with Hyb Buffer.

The library was denatured with 0.2N NaOH for 5 minutes, neutralized with 0.2 M Tris-HCl (pH 7.0), and diluted with Hyb buffer. The final on-machine dilution of the library was performed with Hyb buffer, where the on-machine diluted library was put into the reagent tank, placed into the Miseq sequencer together with the chip. The sample sheet was imported, and sequencing was performed.

3. Results and analysis

3.1. Comparison of sample extraction methods

The 16S rRNA genes of fecal samples were extracted using the lysis and the spin column method respectively, and the results are shown in **Table 1**.

Table 1. Comparison of extraction effects between lysis and column-passing method

Sample	DNA concentration (ng/μL)		Total DNA amount (ng)	
	Lysis	Column-passing	Lysis	Column-passing
1	2.43	2.75	127.2	133.5
2	2.89	3.12	158.7	165.3
3	2.64	2.95	169.1	177.6

The DNA concentration extracted by the lysis method is lower than that by the column-passing method. This is mainly because the column-passing method adsorbs the extracted DNA on the column and then elutes it, which plays a concentration role and significantly increases the DNA concentration. In terms of the total amount of DNA extracted from the samples, there is no significant difference between the two methods. However, the lysis method

can greatly shorten the extraction time and improve the experimental efficiency. In this experiment, the 16S rRNA of the samples extracted above was used for subsequent amplification experiments.

3.2. Experimental results of PCR cycle number optimization

A higher number of PCR cycles leads to the generation of more chimeras, which reduces the amount of valid data. Therefore, in this experiment, 3 different cycle numbers were designed to explore the experimental system and investigate the effect of PCR cycle number on the products. The experimental results are shown in **Table 2**. The results indicate that when the PCR cycle number is 25, fewer chimeras are generated; thus, 25 PCR cycles were selected.

Table 2. Experimental results of different PCR cycle numbers

Cycle number	Total library amount (ng)	Assembly rate (%)	Chimera rate (%)
25	29.5	67.48	1.41
28	175	64.41	1.57
30	400	68.39	3.01

3.3. Electrophoretogram of the first-round PCR products

The DNA of the aforementioned PCR products was analyzed by electrophoresis, and the results are shown in the figure below.

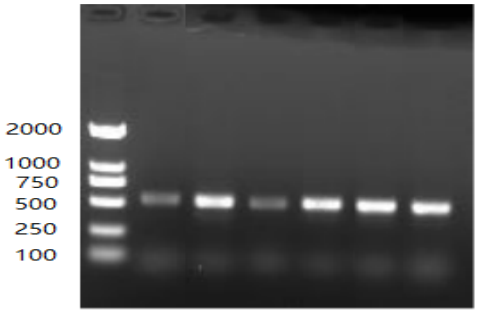

Figure 1. Electrophoretogram of PCR amplification products.

The first lane is Marker DL2000, and the band sizes from top to bottom are 2000, 1000, 750, 500, 250, and 100 bp in sequence. The electrophoresis bands of the post-PCR products are all clear, and the DNA molecular weight is approximately 500 bp.

3.4. Optimization of Initial DNA Amount for Library Construction

The results of exploring the experimental systems with initial DNA amounts of 12.5 ng and 25 ng for library construction are shown in **Table 3**. When the initial DNA amount was 12.5 ng, fewer chimeras were generated; therefore, 12.5 ng was finally selected as the initial DNA amount for library construction.

Table 3. Experimental results of different initial DNA amounts for library construction

Initial amount (ng)	Total library amount	Assembly rate (%)	Chimera rate (%)
12.5	25	66.97	1.12
25	31.25	67.62	1.86

3.5. Electrophoresis of the second-round PCR

The DNA of the aforementioned PCR products was analyzed by electrophoresis, and the results are shown in the figure below.

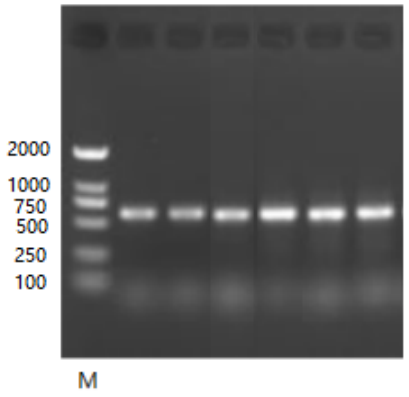

Figure 2. Electrophoretogram of the second-round PCR amplification products.

In the above figure, the first lane is Marker DL2000, and the band sizes from top to bottom are 2000, 1000, 750, 500, 250, and 100 bp in sequence. The electrophoresis bands of the post-PCR products are all clear, and the DNA molecular weight is approximately 600 bp.

3.6. Library purification and quality inspection results

After the library was purified by the bead method, primer dimers could be removed. The constructed library was subjected to quality inspection using an Agilent 2200 Bioanalyzer, and the results are shown in **Figure 3**. The library size was 500–700 bp, and there were basically no primer dimers of approximately 120 bp. The quality of the library constructed from DNA obtained by the lysis method was basically consistent with that of the library obtained from DNA extracted by the bead method or spin column method. Therefore, lysis method can be used to obtain DNA for library construction.

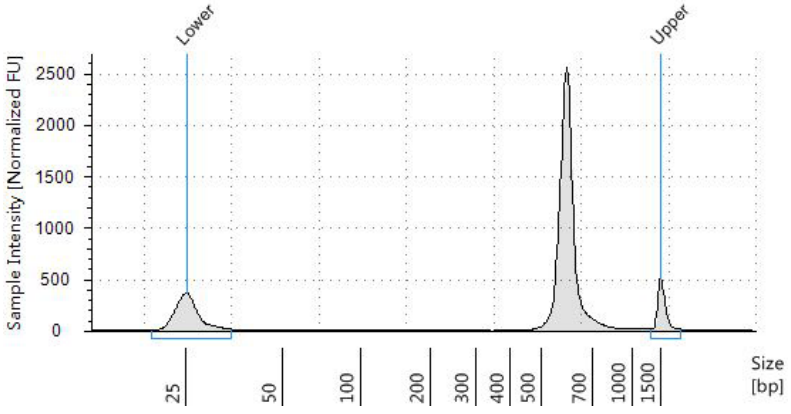

Figure 3. Library quality inspection results

3.7. Comparison of sequencing results between lysis and column-passing method

The Miseq PE250 sequencing results of the lysis method and column-passing method were compared, and

the findings are presented in **Table 4**. As indicated in the table, there was no significant difference in library concentration between the two methods. For the lysis method, the number of valid Tags obtained via Miseq PE250 ranged from 32,000 to 35,000, with Q30 values between 78% and 81% and an average of 79.13%. For the column-passing method, the valid Tags obtained by Miseq PE250 were in the range of 33,000 to 35,000, and the Q30 values varied from 77% to 80%, with an average of 78.53%. Throughout the entire sequencing run, all Q30 values exceeded 75%, demonstrating good base quality. It is evident that the sequencing results of the lysis method and column-passing method showed no significant difference. However, the lysis method reduced experimental steps, shortened sample pretreatment time, and significantly improved experimental efficiency.

Table 4. Comparison of sequencing results between lysis and column-passing method

Sample	Library concentration (ng/μL)		Number of tags		≥ Q30 (%)	
	Lysis	Column-passing	Lysis	Column-passing	Lysis	Column-passing
1	12.3	11.37	32364	33679	78.42	79.24
2	9.34	10.81	33701	33177	78.54	77.80
3	15.8	15.61	34943	34506	80.42	78.54

4. Discussion

With the rapid development of biological sciences, research methods for intestinal flora have advanced from traditional microbial isolation and culture to molecular biology-based detection. The normal intestinal flora consists of 500 to 1,500 different bacterial species, with the vast majority being anaerobic bacteria. The genome encoded by the human intestinal flora can be regarded as the "second human genome"; it participates in human nutrition, metabolism, and immune processes, and is one of the most important factors affecting human health [5]. Due to the large quantity and high diversity of intestinal flora, traditional microbiological detection is time-consuming, easily affected by operating methods, and cannot fully reflect the true interactions between intestinal microorganisms and between microorganisms and the host. The field of microbiology increasingly relies on molecular biology techniques to study the diversity and complexity of intestinal microorganisms [6].

The latest research by Alexandre *et al.* showed that 1,952 uncultured candidate bacterial species were identified by reconstructing 92,143 metagenomically assembled genomes from 11,850 human gut microbiomes [7]. Samuel *et al.* isolated 737 bacterial strains for culture, growth, and DNA sequencing studies, which were classified into 273 different bacterial groups. This included 173 bacterial types that had not been sequenced before, and among these 173 types, 105 had not been successfully isolated by researchers previously [8]. The complexity and diversity of intestinal microorganisms are closely related to human physiological and metabolic functions. By studying the composition of intestinal microbial flora, we can improve the analysis of the relationship between intestinal microorganisms and various diseases. Currently, intestinal microorganisms have been found to be associated with obesity, diabetes, intestinal diseases, allergies, autism, and mental illnesses [9].

The 16S rRNA gene is suitable for large-scale sample screening but provides limited taxonomic and functional resolution [10]. High-throughput 16S rRNA sequencing technology has many advantages: it can not only conduct in-depth analysis of intestinal flora structure and diversity but also combine with other "omics" technologies to further understand the genetic functions and metabolic pathways of intestinal microorganisms [5,11].

It is currently one of the most important detection methods. The 16S rRNA gene is suitable for large-scale sample screening, and the extraction method of the 16S rRNA gene from intestinal flora is one of the key technologies at present.

Tian *et al.* used the thermal lysis column-passing method and direct thermal lysis method to extract and detect the bacterial DNA of *Staphylococcus aureus* from pure cultured and artificially contaminated fish samples, with the kit method used as a control for DNA extraction. The results showed that the thermal lysis column-passing method was only one gradient lower than the kit method, demonstrating its good stability, efficiency, and practicality [12]. Peng *et al.* compared the differences between the thermal lysis method and commercial kit method in extracting fecal microbial DNA for 16S rRNA sequencing. OTU (Operational Taxonomic Unit) determination results showed that the thermal lysis method was highly consistent with the commercial kit method. The thermal lysis method can be used for fecal microbial determination, significantly reducing sample preparation costs and improving sample processing efficiency [13].

In this study, a combination of lysis buffer and thermal lysis method was used. This method features simple operation, high DNA purity, and high extraction rate. There was no significant difference in sequencing results between the lysis method and the column-passing method. Additionally, the lysis method shortened sample pretreatment time and greatly improved experimental efficiency.

5. Conclusion

In this study, the method of direct fecal lysis was used to obtain DNA from intestinal flora. This method is more simple and rapid, and the quality of the constructed library is notable, which is basically consistent with the quality of libraries constructed from DNA obtained by traditional DNA extraction methods. It shortens the sample pretreatment time and greatly improves the experimental efficiency.

Disclosure statement

The authors declare no conflict of interest.

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