

Evaluation of the effectiveness of DNA extraction methods from sheep ear tissue samples for WGS

Shynggys D. Orkara¹ , Zhangylsyn M. Khamzina¹ , Botagoz O. Kulzhanova² , Darkhan B. Smagulov³ 
Aigerim K. Khamzina¹ 

¹Kazakh National Agrarian Research University, Almaty, Republic of Kazakhstan

²LLP Scientific and Educational Center “Qazyna”, Almaty, Republic of Kazakhstan

³West Kazakhstan Innovation and Technological University, Uralsk, Republic of Kazakhstan

Corresponding author: Aigerim K. Khamzina: aigerim.khamzina55@gmail.com

Coauthors: (1: ShO) orkarashynggys@gmail.com; (2: ZKh) zhangylsyn.khamzina@gmail.com
(3: BK) botagoz-89@mail.ru; (4: DS) dark.smagul@gmail.com

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Abstract

Background and Aim. Whole-genome sequencing is a powerful tool for studying the genetic structure of livestock populations. Effective implementation of WGS is largely determined by the quality of the source genomic DNA. Sheep ear notches obtained in the field are a convenient source of biomaterial; however, their high connective tissue density makes DNA extraction challenging. The aim of this study was to compare the CTAB method and the commercial PureLink Genomic DNA Kit for DNA extraction from ear notches of Kazakh fat-tailed sheep.

Materials and Methods. The study was conducted in two stages. CTAB lysis buffer was used in the first stage, and the commercial PureLink Genomic DNA Kit (Thermo Fisher Scientific) was used in the second stage. Quality control was performed using a NanoDrop2000 spectrophotometer and gel electrophoresis.

Results. The average DNA concentration isolated by the CTAB method was 293 ng/μl, and the A260/280 ratio ranged from 1.70 to 1.86 (mean 1.78 ± 0.04), indicating heterogeneous purity. The PureLink kit demonstrated consistently high quality: concentration 350.2 ± 96.9 ng/μl, A260/280 1.86 ± 0.01, and A260/230 2.13 ± 0.08.

Conclusion. The commercial PureLink Genomic DNA Kit is recommended for DNA extraction from sheep ear notches, as it provides consistent quantitative and qualitative data that meet WGS requirements.

Keywords: DNA extraction; Kazakh fat-tailed sheep breed; PureLink Genomic DNA Kit; sheep ear notches; CTAB; whole genome sequencing.

Introduction

Whole-genome sequencing (WGS) occupies a key position in modern agricultural genetics, as it offers unique opportunities for the comprehensive analysis of genomic variability and the identification of genes associated with economically significant traits [1]. With the advancement of Illumina and other platform technologies, WGS of farm animals has become accessible even to relatively small laboratories [2].

Efficient implementation of WGS is largely determined by the quality of the original genomic DNA [3]. High-throughput sequencing technologies require DNA of sufficient concentration (typically at least 10 ng/μL), high purity (A260/280 ratio in the range of 1.8–2.0 and A260/230 in the range of 2.0–2.2), and preserved molecular integrity [4]. The presence of impurities, inhibitors, or DNA fragmentation can lead to incomplete library preparation and reduced sequencing data quality [4].

One common source of biomaterial for molecular genetic studies of sheep is ear pinna tissue, obtained by collecting ear notches. This type of biomaterial is convenient for field collection and does not require invasive procedures. However, the ear pinna is characterized by a high density of connective tissue and such components as collagen, which can reduce the efficiency of nucleic acid extraction and inhibit PCR, requiring optimization of extraction protocols. Therefore, choosing the optimal method for DNA extraction from ear notches remains a pressing issue.

Currently, various approaches are used to isolate genomic DNA: classical laboratory protocols, in particular the CTAB-based (cetyltrimethylammonium bromide, Sigma-Aldrich, St. Louis, MO, USA), method [5, 6], and commercial DNA extraction kits [7]. The CTAB method, based on the cationic detergent cetyltrimethylammonium bromide, effectively removes polysaccharide and protein contaminants, which is especially important when working with tissues rich in secondary metabolites. However, the CTAB method is significantly more time-consuming compared to commercial kits, and each step of the protocol is subject to modification, creating reproducibility issues in the absence of a detailed description of the extraction conditions [8].

Commercial kits, such as the PureLink Genomic DNA Kit (Thermo Fisher Scientific), offer standardized, reproducible procedures that can ensure more consistent DNA quality and reduce the impact of human error [9]. However, a direct comparison of the effectiveness of the CTAB method and a commercial kit for a specific type of biomaterial (sheep ear notches) remains insufficiently studied.

This task is particularly relevant in the Republic of Kazakhstan, where indigenous sheep breeds, particularly the Kazakh fat-tailed coarse-wool, remain insufficiently studied at the genomic level, and WGS data for them is virtually nonexistent [10]. Building a reliable genetic base for such populations requires dependable and reproducible methods for obtaining DNA suitable for WGS.

The aim of this study was to evaluate the effectiveness of the CTAB method and the commercial PureLink Genomic DNA Kit for extracting genomic DNA from ear notches of Kazakh fat-tailed sheep based on the quantitative and qualitative characteristics of the obtained DNA and to verify its suitability for WGS.

Materials and Methods

Ethical approval

Institutional Review Board Statement: The study was approved by the Bioethics Commission of PHPEI "West Kazakhstan Innovation and Technology University", Kazakhstan (approval date: 02 September 2024, protocol number 15/09).

Sampling

Ear tissue samples were collected from 40 sheep, including 20 Kazakh coarse-wool fat-tailed (KCWFT) and 20 Kazakh semi-coarse-wool fat-tailed (KSCWFT) sheep, at the Bepen farm in the Beskaragay District, East Kazakhstan Region, Republic of Kazakhstan. One ear tissue sample was collected from each animal using sterile instruments and placed individually in a sterile, labeled tube. Thus, a total of 40 biological samples were obtained. For the paired comparison of the two DNA extraction methods, two portions were taken from the same ear tissue sample of each animal; no additional tissue samples were collected. For each extraction method, approximately 0.025 g of tissue was used. Consequently, DNA obtained by the two methods represented paired measurements from the same individual animal. Both DNA extraction procedures were performed on the same day, thereby eliminating differences in storage duration between the two methods. The samples were stored at -20°C until DNA extraction.

Stage 1: DNA Extraction Using CTAB

In the first stage of the study, genomic DNA was extracted from ear tissue samples using a CTAB-based method. Approximately 25 mg (0.025 g) of each tissue sample was placed in a sterile 1.5-mL microcentrifuge tube, followed by the addition of 200 μL of PBS. The tissue was mechanically minced into small pieces using sterile scissors to facilitate subsequent lysis. After homogenization, 500 μL of CTAB buffer was added to the samples. (CTAB: 20.00 g/L (2% w/v), EDTA- $\text{Na}_2 \times 2\text{H}_2\text{O}$: 7.44 g/L (20 mM), Tris: 12.11 g/L (100 mM), Sodium chloride: 81.82 g/L (1.4 M)) и 4 μL of proteinase K (20 mg/ml). Incubation was carried out in a thermo-shaker at 56°C for 4 hours at 1000 rpm.

After lysis, 500 μL of a 24:1 mixture of chloroform and isoamyl alcohol were added to the samples, thoroughly mixed with a vortex, and centrifuged at $11,400 \times g$ for 5 minutes. The upper aqueous phase

was carefully transferred to a clean tube, avoiding the interfacial protein film. To precipitate the DNA, sodium acetate (1/10 the supernatant volume) and 350 µl of isopropanol were added. The mixture was then incubated for 10 minutes at 23 °C temperature and centrifuged at 12,100 x g for 5 minutes.

The resulting DNA precipitate was washed with 70% ethanol, centrifuged again, and any residual liquid was removed. The precipitate was dried at 23 °C temperature for 10–15 minutes and then dissolved in 50 µl of deionized water. The resulting DNA samples were stored at +4 °C or –20 °C until further use. The concentration and purity of the isolated DNA were assessed using a NanoDrop 2000 spectrophotometer (ThermoScientific).

Step 2: DNA Extraction with a Commercial PureLink Kit

Genomic DNA extraction from ear notch samples was also performed using the PureLink Genomic DNA Mini Kit according to the manufacturer's recommendations with minor modifications.

For DNA extraction, 0.025 g of ear notch tissue was minced into small fragments using sterile scissors and placed in 1.5 ml tubes containing 180 µl of Digestion Buffer and 20 µl of Proteinase K. The samples were incubated in a thermo-shaker at 56 °C for 4 hours at 1000 rpm until complete tissue lysis.

After incubation, the samples were centrifuged at 11,400 x g for 3 minutes to pellet insoluble particles. The supernatant was transferred to a new tube, and 20 µl of RNase was added. The mixture was briefly vortexed and incubated at 23 °C temperature for 2 minutes.

Then, 200 µl of lysis buffer was added to the samples, mixed thoroughly, and incubated at 70 °C for 10 minutes without shaking. Then, 200 µl of 96–100% ethanol was added and vortexed. The resulting mixture was transferred to filtration columns and centrifuged at 6,200 x g for 2 minutes. If necessary, additional centrifugation was performed to ensure complete passage of the liquid through the membrane. During the wash step, 500 µl of Wash Buffer 1 was added to the column and centrifuged at 6,200 x g for 2 minutes. Then, 500 µl of Wash Buffer 2 was added and centrifuged at 11,400 x g for 1 minute. After washing, the filtration column was transferred to a clean 1.5 ml tube.

To elute the DNA, 70 µl of Elution Buffer, preheated to 70 °C, was applied to the column membrane, incubated for 2 minutes, and centrifuged at 11,400 x g for 1 minute. The resulting DNA samples were stored at +4 °C or –20 °C until further use. Quantitative and qualitative assessment was performed using three methods: NanoDrop 2000 spectrophotometry (concentration, A260/280 and A260/230); Qubit 4 fluorimetry with the Qubit dsDNA BR Assay complex; agarose gel electrophoresis (1% agarose gel, 100 V voltage, TAE buffer) to assess DNA integrity.

Preparation for Whole Genome Sequencing

The isolated DNA was sent to Novogene (London, UK) for library construction and whole genome sequencing on the Illumina platform. The laboratory tested the DNA for library preparation, including assessing its concentration, integrity, and the absence of inhibitors. Based on the analysis results, all samples were deemed suitable for sequencing, and FASTQ files were generated.

Statistical analysis

Statistical analysis was performed using Microsoft Excel. Descriptive statistics were calculated for DNA concentration and purity parameters (A260/280 and A260/230 ratios) and are presented as mean ± standard deviation (SD), together with the minimum and maximum values. Differences between the KCWFT and KSCWFT breeds were assessed using an independent-samples Student's t-test. A p-value < 0.05 was considered statistically significant.

Results

Isolation of CTAB with buffer

In the first stage of the study, DNA was isolated from sheep ear notches (n=40) using the CTAB method. Analysis on a NanoDrop2000 spectrophotometer revealed that the average KCWFT breed DNA concentration was 293.5 ± 47.8 ng/µL (range 221.8–421.8 ng/µL). The A260/280 ratio ranged from 1.70 to 1.86 (mean 1.78 ± 0.04), indicating the presence of protein impurities in some samples. The A260/230 ratio was significantly lower— 1.52 ± 0.13 (range 1.28–1.84), indicating a significant content of organic compounds and residual amounts of CTAB buffer. DNA concentrations for the KCWFT are shown in Table 1.

Table 1. Qualitative parameters of the KCWFT breed DNA isolated by the CTAB method

Sample	Concentration, ng/μL	A260/280	A260/230
1	285.6	1.79	1.52
2	308.1	1.82	1.58
3	262.3	1.75	1.41
4	341.5	1.83	1.65
5	294.2	1.80	1.54
6	415.3	1.84	1.72
7	253.8	1.74	1.38
8	301.9	1.81	1.56
9	328.6	1.82	1.61
10	244.1	1.73	1.35
11	278.5	1.78	1.48
12	365.4	1.84	1.68
13	309.7	1.81	1.59
14	232.5	1.72	1.32
15	268.8	1.77	1.45
16	339.2	1.83	1.64
17	221.8	1.70	1.28
18	288.4	1.79	1.51
19	303.5	1.80	1.57
20	255.9	1.76	1.43
Mean (n=20)	294.4 ± 48.0	1.77 ± 0.05	1.53 ± 0.14

When analyzing the breeds separately, no statistically significant differences were found ($p > 0.05$). For the KCWFT breed (n=20), the indicators were: concentration 294.4 ± 48.0 ng/μL, A260/280 1.77 ± 0.05 , A260/230 1.53 ± 0.14 .

For the KSCWFT breed (n=20): concentration 292.6 ± 48.1 ng/μL, A260/280 1.79 ± 0.04 , A260/230 1.52 ± 0.12 . (Table 2).

Table 2. Qualitative parameters of DNA of the KSCWFT breed, isolated by the CTAB method

Sample	Concentration, ng/μL	A260/280	A260/230
21	281.3	1.78	1.50
22	312.4	1.81	1.60
23	258.7	1.74	1.39
24	345.2	1.82	1.66
25	290.5	1.79	1.53
26	408.6	1.85	1.73
27	249.3	1.73	1.36
28	298.4	1.80	1.55
29	332.1	1.81	1.62
30	240.6	1.72	1.34
31	275.2	1.77	1.46
32	358.9	1.83	1.67
33	305.3	1.80	1.58

34	228.9	1.71	1.31
35	264.2	1.76	1.44
36	335.7	1.82	1.63
37	225.4	1.70	1.29
38	284.9	1.78	1.52
39	300.1	1.79	1.56
40	251.3	1.75	1.42
Mean $\pm$ SD	292.6 $\pm$ 48.1	1.79 $\pm$ 0.04	1.52 $\pm$ 0.12

Agarose gel electrophoresis (1% agarose, TAE buffer, 100 V, 30 min) of DNA isolated from ear notches of Kazakh fat-tailed sheep using the CTAB method demonstrated significant variability in quality between individual samples. All wells of the gel contained visible DNA bands, but their morphology varied. Samples #4 and #11 and #12 were characterized by relatively compact bands of high-molecular-weight DNA, while samples #2, #10, and #11 demonstrated pronounced smearing in the range of low-molecular-weight fragments, indicating fragmentation of genomic DNA during the extraction process (Figure 1). Sample #9 was additionally distinguished by a broad, smeared band, indicating heterogeneity of the fractional composition.

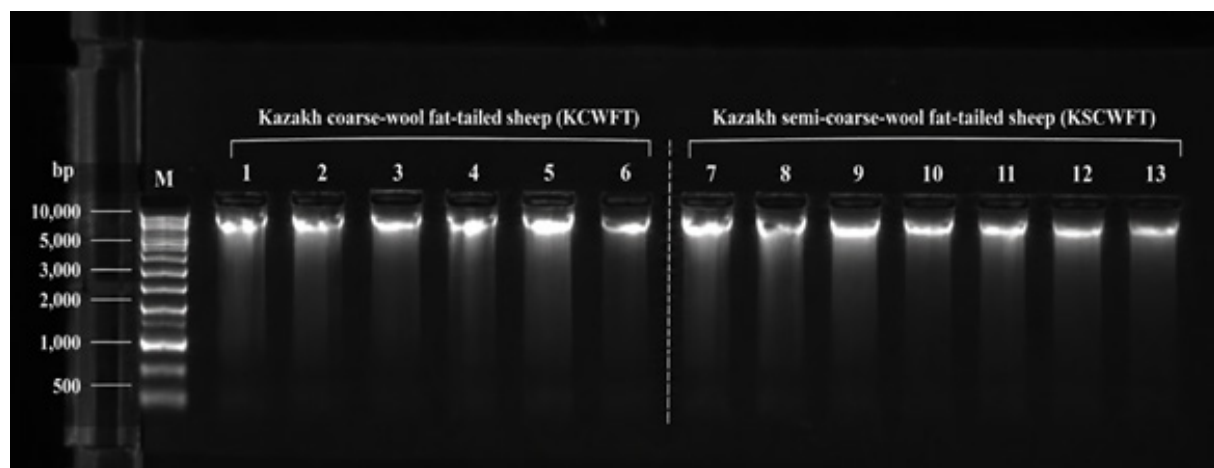


Figure 1. Agarose gel electrophoresis of extracted genomic DNA samples of two sheep breeds. High-molecular-weight DNA bands are visible near the wells, with varying degrees of DNA smearing among the samples

Based on these results, it was decided that a commercial kit was needed to obtain DNA with higher and more stable purity

DNA Extraction with the PureLink Commercial Kit

In the second stage, the commercial PureLink Genomic DNA Mini Kit was used to extract DNA from the ear notches of all 40 sheep. The isolated DNA concentration ranged from 131.5 to 585.4 ng/ μ L and averaged 350.2 ± 96.9 ng/ μ L. The A260/280 ratio was 1.86 ± 0.01 , indicating high DNA purity without significant protein or phenol impurities. The A260/230 ratio was 2.13 ± 0.08 , indicating minimal organic impurities. Data for the KCWFT breed are presented in Table 3.

Table 3. Qualitative parameters of KCWFT breed DNA isolated using the PureLink Genomic DNA Kit

Sample	Concentration, ng/ μ L	A260/280	A260/230
1	386.4	1.88	2.16
2	425.4	1.88	2.21
3	444.0	1.88	2.23

Continuation of Table 3

4	444.6	1.87	2.21
5	365.8	1.88	2.20
6	585.4	1.85	2.21
7	342.3	1.87	2.17
8	281.4	1.87	2.16
9	389.8	1.87	2.11
10	273.2	1.86	2.08
11	266.9	1.85	2.18
12	371.1	1.86	2.15
13	335.4	1.86	2.09
14	242.2	1.86	2.05
15	263.7	1.86	2.01
16	357.8	1.86	1.98
17	245.8	1.86	2.03
18	131.5	1.88	2.20
19	338.8	1.87	2.02
20	325.0	1.86	2.18
Mean $\pm$ SD	340.8 $\pm$ 96.5	1.87 $\pm$ 0.01	2.13 $\pm$ 0.08

No statistically significant differences were revealed in the separate analysis of breeds. For the KCWFT breed (n=20), the indicators were: concentration 340.8 $\pm$ 96.5 ng/ μ L, A260/280 1.87 $\pm$ 0.01, A260/230 2.13 $\pm$ 0.08 (Table 3). For the KSCWFT breed (n = 20): concentration 359.6 $\pm$ 97.6 ng/ μ L, A260/280 1.86 $\pm$ 0.01, A260/230 2.13 $\pm$ 0.09 (Table 4).

Table 4. Qualitative parameters of DNA of the KSCWFT breed isolated by the PureLink Genomic DNA Kit

Sample	Concentration, ng/ μ L	A260/280	A260/230
1	498.0	1.86	2.23
2	351.3	1.87	2.17
3	476.1	1.87	2.21
4	404.8	1.86	2.20
5	309.5	1.86	2.10
6	159.0	1.87	2.15
7	292.6	1.85	1.90
8	446.1	1.85	2.08
9	361.5	1.87	2.22
10	466.6	1.85	2.13
11	396.9	1.85	2.14
12	348.4	1.85	2.14
13	300.1	1.86	2.22
14	414.9	1.85	2.19
15	428.7	1.86	1.98
16	390.5	1.85	2.16
17	329.2	1.86	2.13

Continuation of Table 4

18	189.7	1.87	2.21
19	503.8	1.85	2.04
20	325.0	1.86	2.18
Mean $\pm$ SD	359.6 $\pm$ 97.6	1.86 $\pm$ 0.01	2.13 $\pm$ 0.09

Agarose gel electrophoresis revealed the presence of a high-molecular-weight DNA fraction in all samples obtained using the PureLink kit, indicating that the molecular integrity of the DNA was preserved during the extraction process (Figure 2).

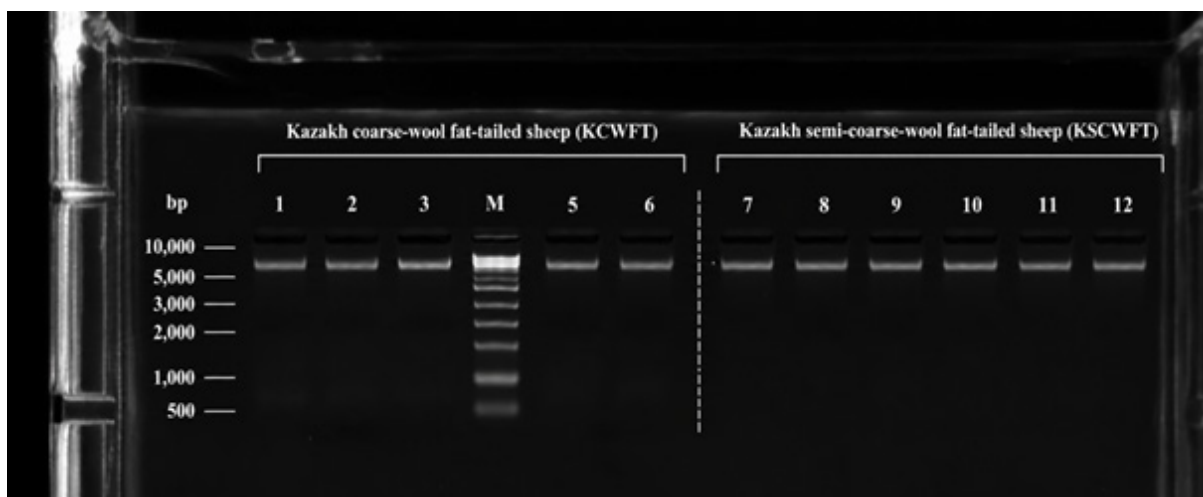


Figure 2. Agarose gel electrophoresis of extracted genomic DNA samples of two sheep breed. High-molecular-weight DNA bands are visible near the wells, with varying degrees of DNA smearing among the samples

All DNA samples isolated using the PureLink kit were sent to a commercial laboratory for library construction. Based on quality control performed at the lab, all 40 samples were deemed suitable for library preparation and sequencing. The resulting FASTQ files demonstrate the functional suitability of the isolated DNA for WGS.

Discussion

Comparative DNA Yield Analysis

The results of this study demonstrate that both the CTAB method and the commercial PureLink Genomic DNA Kit provide sufficient yields of genomic DNA from sheep ear notches for subsequent WGS. The average DNA concentration, determined spectrophotometrically, was 293.5 ng/ μ L for samples isolated by the CTAB method and 350.2 ng/ μ L for samples obtained with the PureLink kit. Both values significantly exceed Illumina's minimum library construction requirements, which are 100–500 ng of total DNA, or $\sim$ 10 ng/ μ L concentration at a typical eluate volume [<https://knowledge.illumina.com/library-preparation/dna-library-prep/library-preparation-dna-library-prep-faq-list/000001262>].

Literature data show that DNA yields are generally higher using the CTAB method than commercial kits. For example, a study on DNA extraction from grape leaves showed that the CTAB method yields higher DNA concentrations compared to commercial kits from Qiagen, VWR, and MP [11]. Similar results were obtained when working with highly processed animal by-products, where the CTAB method demonstrated the greatest efficiency in DNA recovery [8]. In our study, the difference in concentration between the methods was only $\sim$ 19% in favor of PureLink, which is smaller than typical differences described in the literature. This may be explained by the specific nature of ear tissue and the high density of connective tissue, which equally impedes lysis in both protocols.

DNA Purity Assessment: A260/280 Ratio

The A260/280 absorbance ratio is a key indicator of the presence of protein contaminants in DNA samples. For high-quality genomic DNA, this ratio should be in the range of 1.8–2.0. In our study, the

CTAB method yielded average A260/280 values of 1.77 ± 0.05 (KCWFT) and 1.79 ± 0.04 (KSCWFT), which is at the lower end of the acceptable range and, in some samples, was outside it (the minimum value was 1.63). This indicates the presence of residual protein contaminants in DNA preparations isolated using the CTAB method. In contrast, the PureLink Genomic DNA Kit provided significantly higher and more consistent A260/280 values: 1.87 ± 0.01 (KCWFT) and 1.86 ± 0.01 (KSCWFT), fully meeting Illumina requirements.

Coefficient of variation (CV) analysis showed that the spread of A260/280 values using the CTAB method was 2-fold higher (CV ~3.4%) than using PureLink (CV ~1.6%). This confirms the data of *Schenk et al.*, 2023 [8], who noted that “numerous modifications to the CTAB protocol create a critical problem, as studies become neither transparent nor reproducible.” The high variability of the CTAB protocol is due to the fact that each step from mechanical tissue disruption to sediment conditioning is subject to individual variations. At the same time, commercial kits such as PureLink offer standardized, reproducible procedures, which reduces the influence of human factors and ensures interlaboratory comparability of results.

A260/230 Ratio Analysis

The most significant differences between the two methods were revealed in the A260/230 ratio, which reflects the presence of organic impurities. For DNA suitable for high-throughput sequencing, this ratio should exceed 2.0, while values below 1.5 indicate high concentrations of contaminants that can negatively impact enzymatic reactions. In our study, the CTAB method yielded average A260/230 values of 1.53 ± 0.14 (KCWFT) and 1.52 ± 0.12 (KSCWFT), which are significantly below the recommended threshold. However, in a number of samples (Nos. 3, 7, 10, 11, 14, and 15), this ratio fell below 1.5, indicating significant organic impurities. These data are consistent with publications reporting that classical CTAB protocols are prone to leaving polysaccharide contaminants in DNA preparations.

In contrast, the PureLink Genomic DNA Kit provided high and CTAB A260/230 values: 2.13 ± 0.08 (KCWFT) and 2.13 ± 0.09 (KSCWFT), fully meeting the requirements of Illumina (2.0–2.2) and Novogene (OD260/230 without specific limitations, but with the requirement of no degradation) (Novogene Sample Requirements). A key advantage of the PureLink kit is its use of a silica gel column purification, which effectively removes polysaccharides, oligosaccharides, and other polar compounds remaining after CTAB extraction.

DNA Integrity Analysis by Agarose Gel Electrophoresis

Agarose gel electrophoresis is the method of choice for assessing genomic DNA integrity prior to high-throughput sequencing.

DNA isolated by CTAB was characterized by significant fragmentation. Minor blotches were observed in the gel, along with a major band of high-molecular-weight DNA. This may indicate degradation of the genomic DNA during the extraction process.

Unlike CTAB, the PureLink Genomic DNA Kit recovered high-molecular-weight DNA in all 40 samples (100%). Clear, compact bands were visualized on the gels, with no signs of blotches or low-molecular-weight fragments. This demonstrates that PureLink effectively inactivates endogenous nucleases early in the extraction process, while column purification minimizes mechanical stress on DNA molecules. In a study comparing DNA extraction methods from grape leaves, the CTAB method demonstrated a predominance of high-molecular-weight DNA with minimal smear, while commercial kits using mechanical homogenization (bead beating) showed significant fragmentation. However, in our case, the CTAB method yielded the opposite result of fragmentation, which is likely due to the specific nature of the animal tissue (dense connective tissue).

All 40 samples (20 KCWFT and 20 KSCWFT) isolated with the Purelink kit were sent to the Novogene commercial laboratory for library creation and sequencing. Based on Novogene's internal QC, all samples isolated with the PureLink kit were deemed suitable for sequencing, and FASTQ files were generated.

The data obtained in this study have important practical implications for the development of genomic studies of indigenous sheep breeds in the Republic of Kazakhstan. Validation of the method for obtaining high-quality genomic DNA from ear notches opens the possibility of large-scale WGS studies of Kazakh fat-tailed breeds, which will enable: (1) the construction of reference genomes for the main indigenous breeds; (2) the identification of genetic variants associated with economically valuable

traits (meat productivity, wool, adaptation to climatic conditions); (3) conduction of a comparative analysis of the genomic structure of Kazakh breeds with global sheep populations; (4) the development of molecular markers for breeding programs. Thus, this study provides a methodological foundation for the formation of a reliable genetic database of indigenous sheep breeds in Kazakhstan, which is of strategic importance for the conservation of genetic resources and the development of sheep farming in the region.

Conclusion

The study demonstrated that the commercial PureLink Genomic DNA Kit provides more CTABLE and reproducible DNA quality parameters from sheep ear notches compared to the classical CTAB method. The concentration, purity, and integrity of DNA obtained using the PureLink kit met the requirements for WGS.

Authors' contributions

ShO, and AKh: conceptualized and designed the study, conducted a comprehensive literature search, analyzed the collected data, and prepared the manuscript. ZKh, BK, DS: conducted final editing and proofreading of the manuscript. All authors read, reviewed, and approved the final version of the manuscript.

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Conflict of Interest

The authors declare that they have no known financial interests or personal relationships that could have influenced the results presented in this work.

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