MJAVL
Manas Journal of Agriculture Veterinary and Life Sciences
ISSN/e-ISSN 1694-7932
Volume 15 (Issue 2) (2025) Pages 194-199

Research Article

Optimization of the DNA Extraction Protocol from the Leaves of *Fritillaria eduardii* Regel

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ABSTRACT

Fritillaria eduardii Regel is a rare endemic species of high conservation and scientific value, yet molecular studies on this plant are often hampered by difficulties in DNA extraction due to the high content of polysaccharides, phenolic compounds, and secondary metabolites in its tissues. In this study, we tested and optimized a modified CTAB protocol for obtaining high-quality DNA from leaf tissues of *F. eduardii*. An initial protocol including polyvinylpyrrolidone (PVP) was evaluated, but the DNA yields and purity were inconsistent. Several key modifications were introduced, including exclusion of PVP from the composition, adjustment of buffer composition, replacement of chloroform–octanol with chloroform–isoamyl alcohol, the addition of a CTAB/NaCl purification step, and the use of a high-salt TE buffer for resuspension.Spectrophotometric analysis confirmed that the optimized protocol significantly improved DNA quality and stability. Among the initial nine test samples, only one fully met the optimal purity ratio (A260/A280 = 1.8–2.0), while others showed acceptable but suboptimal values, and three were unsuitable without additional purification. After protocol optimization, over 50 DNA samples were successfully obtained, with concentrations ranging from 548.7 to 4022.9 ng/µl and purity values between 1.80 and 2.09, confirming reproducibility and efficiency.The modified CTAB protocol provides reliable and reproducible DNA suitable for PCR amplification, sequencing, and phylogenetic studies. This approach can be recommended for *F. eduardii* and potentially adapted for other *Fritillaria* species rich in polysaccharides and phenolic compounds.

ARTICLE INFO

Received: 03.10.2025
Accepted: 20.10.2025

Keywords: *F. eduardii,* DNA extraction,CTAB, spectrophotometric analysis

Fritillaria eduardii Regel жалбыректарынан ДНК бөлүп алуу протоколун оптималдаштыруу Regel

АБСТРАКТ

Fritillaria eduardii Regel — сейрек кездешүүчү субэндемикалык түр, биологиялык ар түрдүүлүктү сактоо жана илимий изилдөөлөр үчүн чоң мааниге ээ. Бирок, бул өсүмдүктүн ткандарында полисахариддердин, фенолдук кошулмалардын жана экинчилик метаболиттердин көлөмү жогору болгондуктан, ДНК изоляциясы кыйынчылыктарды жаратат. Бул изилдөөдө *F. eduardii* жалбырак ткандарынан сапаттуу ДНК алуу үчүн модификацияланган CTAB протоколу сыналып оптималдаштырылды. Поливинилпирролидон (PVP) камтыган баштапкы протоколду колдонууда ДНКнын чыгышы жана тазалыгы туруктуу болгон жок. Ошондуктан бир нече өзгөртүүлөр киргизилди: буфердин курамын тууралоо, хлороформ–октанолду хлороформ–изоамил спирти менен алмаштыруу, CTAB/NaCl тазалоо этабын киргизүү жана ДНКны кайра эритүү үчүн туздуу TE буферин колдонуу.Спектрофотометриялык анализдин жыйынтыктары оптималдаштырылган протокол ДНКнын сапатын жана туруктуулугун бир кыйла жогорулатканын тастыктады. Баштапкы тогуз үлгүнүн ичинен бирөө гана оптималдуу тазалык катышына (A260/A280 = 1,8–2,0) дал келди, калгандары жакын, бирок субоптималдуу маанилерди көрсөттү, үч үлгү болсо кошумча тазалоосуз колдонууга жараксыз болду. Протокол оптималдашкандан кийин 50дөн ашык үлгү ийгиликтүү алынып, концентрациялары 548,7–4022,9 нг/мкл диапазонунда, тазалык көрсөткүчтөрү болсо 1,80–2,09 аралыгында болду. Бул протоколдун ишенимдүүлүгүн жана натыйжалуулугун далилдейт.Модификацияланган CTAB протоколу ПЧР амплификациясы, секвенирлөө жана филогенетикалык изилдөөлөр үчүн ылайыктуу, сапаттуу жана туруктуу ДНКны камсыз кылат. Бул ыкма *F. eduardii* үчүн сунушталат жана полисахариддерге жана фенолдук кошулмаларга бай башка *Fritillaria* түрлөрүнө да ыңгайланышы мүмкүн.

ARTICLE INFO

Received: 03.10.2025
Accepted: 20.10.2025

Ачкыч сөздөр: *F. eduardii,* ДНК экстракциясы,CTAB,спектрофотометриялык анализ

Cite this article as: Kydyralıeva, B., & Kurmanbekova, G. (2025). Optimization of the DNA Extraction Protocol from the Leaves of *Fritillaria eduardii* Regel. *Manas Journal of Agriculture Veterinary and Life Sciences*, 15(2), 194-199. https://doi.org/10.53518/mjavl.1796304

INTRODUCTION

The genus *Fritillaria* L. (Liliaceae) includes more than 160 species of perennial bulbous plants that inhabit the temperate regions of the Northern Hemisphere - from Europe and North Africa to East Asia and North America (Wietsma et al., 2011; Day et al., 2014). These plants have high morphological and genetic diversity, which is due to both the wide ecological amplitude and phylogenetic diversity within the genus.

Within Central Asia, representatives of *Fritillaria* are presented by both endemic and widespread species. In particular, according to botanical studies, at least 7 species grow on the territory of Kyrgyzstan, among which special attention is paid to *F. eduardii* Regel - a local subendemic with a limited range on the northern slope of Mount Aigul-Tash (Sennikov & Lazkov, 2013, Kimsanaliev et.al, 2022).

Modern molecular genetic studies of the genus *Fritillaria* are actively developing in the context of both systematics and phylogenetics, as well as biotechnological applications (Ambrožová et al., 2011; Day et al., 2014; Karimov et al., 2024). However, the process of isolating high-quality DNA from the tissues of representatives of this genus often presents methodological difficulties. The main problem in isolating DNA from *Fritillaria* tissues is the presence of high concentrations of polysaccharides, polyphenols, secondary metabolites and mucus-forming compounds, which interfere with the pure extraction of nucleic acids (Aydın et al., 2018; Lin et al., 2001; Rashid & Yaqoob, 2021; Wang et al., 2025).

Extraction of DNA from bulbs is a particularly challenging task, since bulb tissues are characterized by a high content of phenolic compounds and reserve carbohydrates that actively interact with nucleic acids. When using standard commercial kits (e.g., CTAB protocols), DNA degradation, low yield, or the presence of inhibitors that reduce the efficiency of subsequent PCR analysis are often observed (Li et al., 2018). Therefore, optimization of sample preparation conditions and modification of existing extraction protocols to obtain high-quality DNA from various *Fritillaria* organs remain a relevant area.

Various modified protocols have been described previously, including antioxidant pretreatment, use of polyvinylpyrrolidone (PVP), β-mercaptoethanol, increasing EDTA concentrations, as well as additional purification steps with isoamyl alcohol and RNase trituration (Rønsted et al., 2005; Li et al., 2018; Koçak et al., 2020). The selection of the optimal protocol often depends not only on the plant species but also on the tissue used, developmental stage, and storage conditions of the samples (Day et al., 2014).

Thus, given the complex chemical composition of the tissues of *Fritillaria* species, the choice of an optimal DNA extraction method requires the individual adjustment of conditions for each species and organ, ensuring the isolation of high-quality genetic material suitable for molecular-genetic and phylogenetic studies. The aim of this study was the adaptation and optimization of the CTAB protocol for obtaining high-quality DNA from the leaves of *F. eduardii*.

MATERIALS AND METHODS

Plant material

Samples of *Fritillaria eduardii* Regel were collected from a natural population on the northern slope of Aigul-Tash Mountain (Gissaro-Alai mountain system, Kyrgyz Republic; coordinates: 39.952533 N, 70.956234 E). Frozen leaf samples of the plant were used for DNA isolation. Prior to extraction, the material was stored at –20°C.

DNA extraction

Initially, DNA isolation was carried out using a modified CTAB+PVP protocol, adapted for tissues rich in phenolic compounds and polysaccharides. Cetyltrimethylammonium bromide (CTAB) is a surface-active agent commonly employed for DNA extraction from tissues containing high amounts of polysaccharides. The CTAB buffer facilitates cell lysis and prevents the interference of secondary metabolites in the DNA extraction process. In this protocol, CTAB binds polysaccharides and removes them from the solution.

Main steps:

- CTAB + PVP (polyvinylpyrrolidone), liquid nitrogen – homogenization
- Water bath at +65°C for 1 hour – denaturation of proteins and cell membranes
- Chloroform–octanol (24:1) – phase separation
- Centrifugation at 13,000 rpm for 15 min – collection of the supernatant
- Isopropanol, NaCl – DNA precipitation
- Incubation at –20°C for 1 hour – to inhibit enzymatic activity and increase DNA yield
- Centrifugation at 13,000 rpm for 10 min – pellet collection
- Ethanol – DNA washing/precipitation
- Centrifugation at 13,000 rpm for 3 min – pellet collection
- The last two steps were repeated twice.

The concentration and quality of the extracted DNA were assessed spectrophotometrically using NanoDrop (Thermo NanoDrop 2000). DNA samples were stored in a freezer at –20°C.

RESULTS AND DISCUSSION

The initial method of DNA extraction was performed strictly according to the modified CTAB protocol, i.e., with the addition of PVP (polyvinylpyrrolidone). Homogenization of the samples (0.5 g) was carried out using 2 ml CTAB and 0.05 g PVP (1 M Tris-HCl; 0.5 M EDTA, pH 8.0; 5 M NaCl; 2.2% (w/v) CTAB) in the presence of liquid nitrogen . The obtained mixture was incubated in a water bath at 65 °C for 1 hour, which ensured protein denaturation and the disruption of cell membranes. Subsequently, a chloroform–octanol mixture (24:1) was added for phase separation, followed by centrifugation at 13,000 rpm for 15 minutes, after which the supernatant was collected. DNA precipitation was carried out using isopropanol in the presence of NaCl, with subsequent incubation at –20°C for 1 hour to inhibit enzymatic activity of DNAse and increase DNA yield. The mixture was then centrifuged at 13,000 rpm for 10 minutes to obtain a pellet. Additional DNA precipitation was performed using ethanol, followed by centrifugation at 13,000 rpm for 3 minutes. The last two steps of washing and centrifugation were repeated twice.

The quality and concentration of the extracted DNA were assessed spectrophotometrically using a NanoDrop spectrophotometer (Thermo NanoDrop 2000). The extracted DNA samples were stored in a freezer at –20°C.

As a result of the extraction, 9 DNA samples were obtained (Table 1).

Table 1. *Results of spectrophotometric analysis of DNA*

Sample No.	DNA concentration (ng/µl)	A260/A280
1	1756.0	1.75
2	621.0	1.55
3	532.5	0.64
4	831.5	1.63
5	477.05	1.13
6	548.0	1.96
7	2000.0	1.65
8	610.5	1.58
9	1674.0	1.60

The optimal values of DNA purity (A260/A280) are considered to be in the range of 1.8–2.0, which indicates pure DNA without protein contamination. Among the nine samples analyzed, only sample No. 6 (1.96) fell within this optimal range. Several samples were close to the expected values, including No. 1 (1.75), No. 7 (1.65), No. 4 (1.63), No. 8 (1.58), and No. 9 (1.60). Although these values are acceptable, they indicate the possible presence of proteins or phenolic compounds. In contrast, samples No. 2 (1.55), No. 3 (0.64), and No. 5 (1.13) showed significantly lower ratios, suggesting contamination with proteins or phenolics, and were considered unsuitable for PCR-based downstream applications without additional purification.

As some authors suggest, PVP buffers could not meet the need to remove abundant and chemically diverse intracellular metabolites in some species (Zhang et al., 2013), which can prevent extraction of high concentrations of DNA. We suggest that a similar mechanism may occur in *Fritillaria* species.

The relatively low yield of DNA with optimal purity for subsequent PCR-based methods necessitated modifications to several stages of the extraction procedure. After a number of modifications to different steps of the original DNA extraction protocol, the optimized DNA extraction protocol is as follows:

Approximately 50 mg of tissue, pre-cooled in liquid nitrogen, was ground and homogenized, then mixed with 350 µl of extraction buffer (100 mM Tris-HCl; 25 mM EDTA, pH 8.0; 1,4 M NaCl; 2% (w/v) CTAB) preheated to 65°C. Samples were incubated in a water bath at 60°C for at least 1 hour. After incubation, an equal volume (~500 µl) of chloroform:isoamyl alcohol (24:1) was added, followed by shaking for 5 minutes and centrifugation at 12,000 rpm for 20 minutes at room temperature.

The upper aqueous phase (~500 µl) was transferred to a new 1.5 ml tube, mixed with an equal volume of CTAB/NaCl solution, and incubated in a water bath at 60°C for 10 minutes. The tubes were then cooled on ice for 5 minutes, followed by another extraction with chloroform:isoamyl alcohol (24:1), shaking for 5 minutes, and centrifugation at 12,000 rpm for 20 minutes at room temperature.

The aqueous phase (~500 µl) was transferred to a fresh tube, mixed with an equal volume of chilled isopropanol, and incubated at –20°C for 30 minutes to precipitate DNA. Samples were then centrifuged at 13,500 rpm for 10 minutes at room temperature. The supernatant was discarded, and the pellet was washed with 70% chilled ethanol, followed by centrifugation at 13,500 rpm for 5 minutes. A second ethanol wash was performed, after which the pellet was air-dried at room temperature for 5–10 minutes.Finally, the pellet was resuspended in 50 µl of high-salt TE buffer (Tris 10 mM; NaCl 1,0 M; EDTA 0.1 mM; pH 8.00) and incubated at 37°C for 30 minutes to allow complete dissolution.

As a result of the extraction, more than 50 DNA samples with relatively similar parameters were obtained. We present 15 samples that show the full range of DNA obtained (Table 2).

Table 2. *Results of the second spectrophotometric analysis of DNA*

Sample No.	DNA concentration (ng/µl)	A260/A280
1	555.5	1.98
2	669.9	1.63
3	686.7	1.83
4	816.2	1.09
5	874.7	1.78
6	990.3	1.99
7	1057.9	1.96
8	1494.5	1.97
9	1506.0	1.96
10	1769.8	2.04
11	2055.7	2.07
12	3426.5	2.05
13	4022.9	2.00
14	5588.0	2.01
15	7063.9	2.04

The result shown under sample No. 1 (555.5 ng/µl) in Table 2 represented the lowest DNA yield. For most samples, the concentration was between 669.9 and 4022.9 ng/µl. Two samples showed extremely high values (5558.0 ng/µl and 7063.9 ng/µl). With respect to DNA purity (A260/A280), the values were generally within the optimal range of 1.83–2.07. The purity value for two samples was acceptable (1.63 and 1.78) although indicated the possible presence of proteins or phenolic compounds. And just one sample showed the unacceptable value - 1.09.

CONCLUSION

When the original DNA extraction protocol (with PVP) was performed, only a few samples met the optimal purity parameters (A260/A280 = 1.8–2.0), in particular sample No. 6 (1.96). Several samples had values close to the acceptable range but indicated the possible presence of proteins or phenolic compounds, while samples No. 2, No. 3, and No. 5 were unsuitable for further molecular analyses without additional purification. The relatively low DNA yield at optimal purity levels necessitated modifications to the extraction protocol. After optimization of the protocol, more than 50 DNA samples with stable quality and concentration parameters were obtained. The main range of DNA concentrations was 669.9 - 4022.9 ng/µl; the extreme high values were equal to 5558.0 and 7063.9 ng/µl and the lowest result was 555.5 ng/µl. Regarding DNA purity (A260/A280), most samples fell within the optimal range (1.83–2.07), confirming the efficiency of the modified protocol. Only three samples with ratios of 1.09, 1.63 and 1.78 demonstrated non-optimal parameters, and even in this case two of them could still considered acceptable.

We conclude that the modified DNA extraction protocol ensures stable and reproducible results, yielding samples with optimal purity and sufficient concentration for subsequent molecular analyses. Thus, the method can be recommended for processing *Fritillaria eduardii* tissues and potentially adapted for other species of the genus *Fritillaria*, which are rich in polysaccharides and phenolic compounds. This protocol provides DNA of sufficient quality for PCR assays, sequencing, and phylogenetic studies.

AUTHOR CONTRIBUTION

All authors contributed equally.

CONFLICT OF INTEREST

The authors certify that they have no conflict of interest.

FUNDING

The research was conducted as part of the KTMU-BAP-2023.FB.01 project funded by the Kyrgyz-Turkish Manas University.

COMPLIANCE WITH ETHICAL STANDARDS

This work does not contain any human or animal subjects.

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