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# DNA EXTRACTION FROM LEAVES OF WOODY PLANTS WITHOUT LIQUID NITROGEN

**Abstract.** This article describes a method for the rapid and convenient isolation of genomic DNA from leaves of woody plants. In this article, there is tested a standard method of isolating genomic DNA from 45 plant species. The selected plants are rare and endemic representatives of the West Kazakhstan region flora and collectible exotic specimens of the Mangyshlak experimental botanical garden. The research involves a comparison with the modified for these species protocol for DNA extraction from plant tissues. Three methods of DNA analysis are used: visual (presence or absence of white precipitate, contamination), electrophoresis and spectrophotometry. Based on the results of electrophoregrams and spectrophotometry, a decision was made to modify the isolation protocol. Modified method of DNA extraction is based on the absence of liquid nitrogen, mercaptoethanol and RNAse A or T1 are used instead it. Due to the fact that the studied species contain a sufficiently large amount of tannins, an increase of incubation and centrifugation time had a positive effect on the result of DNA isolation. The entire process takes no more than 3 hours, and does not require the use of liquid nitrogen. This method allows to obtain high quality preparations with a DNA average concentration of 140.32 μg. The resulting DNA product can be used in plants molecular genetic identification and certification studies.

**Key words:** DNA, leaves, extraction, spectrophotometry, electrophoresis.

**Introduction.** Nowadays along with traditional methods of studying plants in breeding, genetic and taxonomic studies, methods based on elucidating the variability of DNA and its structure are becoming increasingly important. These methods make it possible to determine the nature of the variability of genetic material with high accuracy, which makes it possible to identify individuals with the most pronounced useful qualities at the early stages of plant development [1]. However, for conducting molecular genetic studies related to the study of the structure of genes, in the first stage it is necessary to obtain a pure DNA preparation without signs of degradation and impurities.

It has been experimentally established that the best results are obtained when DNA is extracted from the buds and young leaves of the plant [2], while in all dead cells (for example, in the xylem of angiosperms), DNA degradation is observed, which makes further analysis impossible. Particular attention should be given to the species mentioned down, since plants of different species are characterized by different physiological and biochemical features, such as the presence of various substances (polysaccharides, tannins, polyphenols and their quinone oxidized products), which create significant impurities in nucleic preparations. Physicochemical properties of such substances to a certain extent coincide with the properties of nucleic acids, which makes it difficult to completely separate them from DNA and causes a poor quality of samples, which makes unfit their further use.

The necessity to maintain intraspecific variability, both at inter-population and intrapopulation levels, is not sufficiently taken into account when studying rare plant species. The population approach remains the least developed in the field of plant biodiversity conservation, since there are still no generally accepted methods for identifying not only population but even specific features of gene pools.

It is also known that the Birch family (*Betulaceae*) contains diarylheptanoids, polyphenols, flavonoids, terpenoids, steroids and other compounds, and the Rose family (*Rosaceae*) contains flavonoids, tannins and ellagic acid, which must be taken into account when carrying out molecular genetic studies of plants of this kind that require the isolation of DNA [3, 4].

The purpose of this article was to investigate the possibility of using a standard technique for isolating DNA from plants on representatives of the above mentioned families and optimizing this technique for a particular object.

**Methods.** As objects of research, there are taken plants representing rare and endemic species of the natural flora of Western Kazakhstan Region and collectible exotic plants of the Mangyshlak experimental botanical garden.

The objects of our research were plants of the following families: 10 species of the Birch family (*Betulaceae*), 8 species of the Rose family (*Rosaceae*), 2 species of the Pine family (*Pinaceae*), 1 species of the Yew family (*Taxaceae*), 1 species of the Cypress family (*Cupressaceae*), 1 species of the Gymnosperms family (*Ginkgodaceae*), 2 species of the Soapberry family (*Sapindaceae*), 4 species of the Barberry family (*Berberidaceae*), 1 species of the Celtis family (*Cannabaceae*), 1 species of the Walnut family (*Juglandaceae*), 2 species from *Platanaceae* and *Salicaceae* families, 1 species of the Moschatel family (*Adoxaceae*), 1 species of *Peganaceae* family, 1 species of the Madder family (*Rubiaceae*), 1 species from *Nitrariaceae*, *Paulowniaceae, Acanthaceae, Fabaceae, Fagaceae, Rhamnaceae* and *Oleaceae* families.

The DNA was isolated according to the standard procedure proposed by S. Porebski [5].

The purpose of our study was the development of a protocol for the isolation of DNA from the leaves without the use of liquid nitrogen. We used the modified CTAB method. For DNA extraction there were used sample 100 mg of fresh leaves hinge was triturated in a mortar in the presence of 300 μl for extraction (120 mM Tris-HCl pH 8.0, 20 mM EDTA, pH 8.0, 1.4 M NaCl, 2.5% CTAB). Trituration options were used without liquid nitrogen. The number of replicates was 6-8 times. The resulting homogenate was incubated at 58-60°C for 60 minutes in a thermostat, vortexing alternately, centrifuged for 10 minutes at 10,000 g. An equal volume of chloroform and isoamyl alcohol was added to the aqueous phase, the mixture was centrifuged for 10 minutes at 10,000 g. The DNA precipitate was washed with 70% ethanol. The incubation was continued for 30 minutes at -20°C. The DNA was reprecipitated with propanol and dissolved in 10 μl TE buffer. The assays were performed in 8-fold replication. The qualitative and quantitative characteristics of DNA are important for molecular genetic analysis. Because possible impurities can inhibit the process of PCR.

Quantitative and qualitative evaluation of the isolated DNA was performed using a DNA-photometer (BioPhotomer Plus, Eppendorf, Germany) and electrophoretic analysis. For photometric analysis, the adsorption of aqueous DNA solutions was measured at three wavelengths: 260/280 nm and 260/230 nm [6]. The size of DNA molecules varied from 7 to 1381 ng/μl. Reusable Eppendorf cuvettes allow the measurement of samples with a volume of only 50 μl, and with the Eppendorf μCuvette G1.0 cuvette the sample volume can be reduced to 1.5 μl. To assess the purity and quality of nucleic acids in spectrophotometric measurement, the purity of the sample is determined based on the ratio of optical densities at wavelengths of 230, 260 and 280 nm. The extinction ratio of 260 nm / 280 nm allows us to judge the purity of the nucleic acid. Pure DNA preparations have a ratio of at least 1.67 [6].

The degradation of the molecules of the obtained preparations was carried out by electrophoresis in 2% agarose gel. Visualization of DNA, RNA was carried out using the Quantum-ST5-1100 Gel-Documentation System, Vilber Lournat, France.

**Results and discussion.** In the first series of experiments, a comparative evaluation of the DNA extraction procedure was carried out in 26 plant species (table 1). DNA of 19 plant species was isolated only by a modified protocol due to the insufficiency of plant material, since these species are endangered.

It was shown that with the use of a standard protocol for the DNA isolation from *Berberis thunbergii, Berberis iliensis, Berberis spp., Celtis caucasica, Malococarpus crithmifolius* and *Nitraria shoberi* the quantity and quality of the isolated DNA were unsatisfactory. To improve the quality of DNA, the isolation procedure was further optimized, involving the exclusion of mercaptoethanol.

Using a standard technique for DNA isolation by the CTAB method from plants (the entire isolation process takes an average of 7-8 hours) allowed us to obtain total DNA preparations. A spectrophotometric

Table 1 – DNA isolation protocols from the studied species

| Sample number | Objects | Pro-tocol | Sample # | Objects | Pro-tocol | Sample number | Objects | Pro-tocol |
|---|---|---|---|---|---|---|---|---|
| 1 | *Pinus eldarica* | S&M | 16 | *Platanus spp.* | S&M | 31 | *Erythrina crista-galli* | M |
| 2 | *Pinus strobus* | S&M | 17 | *Sambucus spp.* | S&M | 32 | *Betula ulmifolia* | M |
| 3 | *Taxus baccata* | S&M | 18 | *Malococarpus crithmifolius* | S&M | 33 | *Betula tianschanica* | M |
| 4 | *Juniperus polycarpos var. turcomanica* | S&M | 19 | *Crataegus ambigua* | S&M | 34 | *Betula maximowicziana* | M |
| 5 | *Ginkgo biloba* | S&M | 20 | *Populus diversifolia* | S&M | 35 | *Betula turkestanica* | M |
| 6 | *Acer campestre* | S&M | 21 | *Rubia cretacea* | S&M | 36 | *Betula microphylla* | M |
| 7 | *Acer henryi* | S&M | 22 | *Nitraria schoberi* | S&M | 37 | *Betula pendula var. carelica* | M |
| 8 | *Berberis iliensis* | S&M | 23 | *Betula pendula* | S&M | 38 | *Betula platyphylla* | M |
| 9 | *Berberis thunbergii* | S&M | 24 | *Malus sieversii* | S&M | 39 | *Betula ajanensis* | M |
| 10 | *Berberis spp.* | S&M | 25 | *Malus niedzwetzkyana* | S&M | 40 | *Quercus spp.* | M |
| 11 | *Berberis Karkaralensis* | S&M | 26 | *Prunus armeniaca* | S&M | 41 | *Paliurus spina-christi* | M |
| 12 | *Corylus avellana* | S&M | 27 | *Prunus mandshurica* | M | 42 | *Cotoneaster melanocarpus* | M |
| 13 | *Celtis caucasica* | S&M | 28 | *Populus tremula L.* | M | 43 | *Cotoneaster multiflorus* | M |
| 14 | *Pterocarya pterocarpa* | S&M | 29 | *Paulownia tomentosa* | M | 44 | *Cotoneaster nitens* | M |
| 15 | *Platanus orientalis* | S&M | 30 | *Acanthus mollis* | M | 45 | *Fraxinus angustifolia subsp. syriaca* | M |

*Note:* S – standard protocol; M – modified protocol.

study of the purity of the obtained samples showed that the absorption ratio at 260 nm / 280 nm was equal to an average of 1.25 (table 2). This indicates that the obtained DNA samples contain impurities in large quantities. Therefore there were a repurification with the addition 50 µl of MEK (mercaptoethanol) and 5 µl of RNAse T1 to the CTAB.

Table 2 – The amount of DNA isolated using standard protocol

| Sample number | Concentration of DNA, ng/microL | $A_{260}/A_{280}$ | Sample number | Concentration of DNA, ng/microL | $A_{260}/A_{280}$ |
|---|---|---|---|---|---|
| 1 | 86 | 2.29 | 14 | 431 | 1.15 |
| 2 | 1431 | 1.06 | 15 | 296 | 1.04 |
| 3 | 607 | 1.29 | 16 | 215 | 0.93 |
| 4 | 898 | 1.17 | 17 | 565 | 1.08 |
| 5 | 712 | 1.13 | 18 | 94 | 1.14 |
| 6 | 372 | 1.32 | 19 | 616 | 1.01 |
| 7 | 1455 | 0.98 | 20 | 13 | 1.36 |
| 8 | 28 | 0.84 | 21 | 283 | 1.57 |
| 9 | 914 | 1.09 | 22 | 881 | 1.08 |
| 10 | 766 | 1.07 | 23 | 52 | 1.32 |
| 11 | 520 | 1.25 | 24 | 42 | 1.86 |
| 12 | 131 | 1.44 | 25 | 73 | 1.30 |
| 13 | 429 | 1.72 | 26 | 526 | 1.05 |

Summarizing the data of the table, we can say that the amount of DNA in the samples is high enough, which can not be said about the ratio $A_{260}/A_{280}$. Only *Celtis caucasica* and *Malus Sieversii* have a pure DNA sample. The *Pinus eldarica* has big amount free nucleic acids in its DNA sample. Despite the high concentration (it varies from 13 ng/microL to 1455 ng/microL), the purity, and hence the quality of DNA, is very low, which prompted us to modify the isolation protocol for the above species.

The presence of degradation in the samples can lead to an incorrect spectrophotometric evaluation of the concentration of DNA, to its overestimation due to the phenomenon of hyperchromism. In addition, these drugs are of little use in the future to work on the study and manipulation of large DNA fragments.

Summarizing the obtained data, we can conclude that this method of DNA extraction is not effective for studied plants.

Further optimization of the standard DNA isolation protocol was performed by compilation the optimal temperature, time and buffer volume that made it possible to obtain a high concentration DNA preparation with no degradation and impurities, which is confirmed by spectrophotometric and electrophoregram in the agarose gel. The obtained samples had an absorption ratio at 260 nm / 280 nm equal, on average, to 1.88, which indicates the purity of the obtained DNA preparations. The DNA samples were measured in three replicates. The $A_{260}/A_{280}$ ratio for pure nucleic acids should be within the range of 1.67 - 2.2 and optimally is about 1.8 and ~ 2.0 for DNA and RNA, respectively. A value of less than 1.67 may indicate contamination of the sample with polypeptides, more than 2 for possible degradation and the presence of free nucleotides. According to the data obtained with BioPhotometer Plus, it can be seen that the DNA samples of *Rubia cretacea* and *Betula microphylla* were destroyed and there are many free nucleotides in the samples (table 3).

Table 3 – The amount of DNA isolated using modified protocol

| Sample number | Concentration of DNA, ng/microL | $A_{260}/A_{280}$ | Sample number | Concentration of DNA, ng/microL | $A_{260}/A_{280}$ |
|---|---|---|---|---|---|
| 1 | 13 | 2.11 | 25 | 25 | 1.86 |
| 2 | 7 | 1.79 | 26 | 31 | 1.75 |
| 3 | 275 | 1.88 | 27 | 240 | 2.1 |
| 4 | 73 | 1.77 | $28D_2$ | 24 | 1.67 |
| 5 | 10 | 1.93 | $28D_3$ | 15 | 1.70 |
| 6 | 25 | 1.69 | $28D_3$ | 11 | 1.45 |
| 7 | 28 | 1.21 | 28T | 61 | 1.66 |
| 8 | 16 | 1.71 | 29 | 17 | 1.94 |
| 8 | 35 | 1.74 | 30 | 19 | 1.84 |
| 9 | 27 | 1.67 | 31 | 198 | 1.79 |
| 10 | 62 | 1.02 | 32 | 34 | 1.83 |
| 11 | 204 | 1.99 | 33 | 138 | 1.91 |
| 12 | 836 | 1.96 | 34 | 39 | 0.96 |
| 13 | 13 | 1.42 | 35 | 553 | 2.19 |
| 14 | 19 | 1.96 | 36 | 5 | 3.49 |
| 15 | 100 | 2.00 | 37 | 195 | 1.98 |
| 16 | 29 | 1.83 | 38 | 137 | 1.77 |
| 17 | 23 | 1.83 | 39 | 670 | 1.83 |
| 18 | 7 | 0.98 | 40 | 1381 | 2.23 |
| 19 | 14 | 1.03 | 41 | 485 | 2.16 |
| 20 | 8 | 1.77 | 42 | 587 | 2.07 |
| 21 | 7 | 6.17 | 43 | 40 | 1.68 |
| 22 | 45 | 1.87 | 43 | 37 | 1.66 |
| 23 | 15 | 1.60 | 44 | 49 | 1.75 |
| 24 | 24 | 1.74 | 45 | 110 | 1.94 |

The table shows the spectrophotometry data of all the species studied, *Berberis iliensis,* diploid *Populus tremula L.* and *Betula maximowicziana* were isolated in two replicates. This was due to a poor visual evaluation of the DNA sample. In comparison with DNA samples isolated by a standard method, the results of DNA samples isolated by a modified method are directly opposite. The DNA concentration in the samples is comparatively very small, and ranges from 5 ng/microL to 1381 ng/microL, where samples of only 15 species *(Taxus baccata, Berberis karkaralensis, Corylus avellana, Platanus orientalis, Prunus mandshurica, Erythrina crista-galli, Betula tianschanica, Betula turkestanica, Betula pendula var. carelica, Betula platyphylla, Betula ajanensis, Quercus spp., Paliurus spina-christi, Cotoneaster melanocarpus and Fraxinus angustifolia subsp. syriaca)* have a concentration exceeding the 100 ng/microL. Accordingly, the range of the remaining species ranges from 5 ng/microL to 73 ng/microL.

Ratio $A_{260}/A_{280}$ is another indicator shown in table 3, the purity of DNA is determined by the value of this ratio. The 35 species studied have $A_{260}/A_{280}$ ratios within the DNA purity range of S. Porebski [5]. *Acer henryi, Berberis spp., Celtis caucasica, Malococarpus crithmifolius, Crataegus ambigua, Rubia cretacea, Betula pendula,* diploid *Populus tremula L., Betula maximowicziana* and *Betula microphylla* have samples with contaminated DNA, *Rubia cretacea* and *Betula microphylla* in samples have a large number of nucleic acids, that means destruction of DNA.

To determine the degree of degradation of molecules in the resulting preparations, we performed electrophoresis in a 2% agarose gel with the addition of ethidium bromide. Figure shows the electrophoregrams of DNA isolated according to a standard and modified protocol.

Above in figure 1, the electrophoregrams of the samples isolated by the standard method and modified are shown. Comparison can be made even in a visual way. Figure 1a shows that *Platanus spp.* and *Sambucus spp.,* isolated by the standard method, are brightly expressed on the electrophoregram, whereas, *Malococarpus crithmifolius, Crataegus ambigua* and *Populus diversifolia,* isolated by the standard protocol, are weakly expressed. DNA samples of *Taxus baccata, Nitraria shoberi* and *Prunus mandshurica*, isolated by a modified method, have a very good electrophoresis result. Also these three types have good spectrophotometry: *Taxus baccata* (275 ng/microL, $A_{260}/A_{280}$=1.88), *Nitraria shoberi* (45 ng/microL, $A_{260}/A_{280}$ =1.87) and *Prunus mandshurica* (240 ng/microL, $A_{260}/A_{280}$=2.1).

DNA samples of *Berberis thunbergii, Berberis spp.* and *Prunus armeniaca* have a small amount. However, DNA samples isolated by a modified method show fairly good results. Especially *Berberis thunbergii, Berberis spp., Berberis Karkaralensis* and *Pterocarya pterocarpa. Berberis Karkaralensis* and *Pterocarya pterocarpa* results of spectrophotometry are 204 ng/microL, $A_{260}/A_{280}$=1.99 and 19 ng/microL, $A_{260}/A_{280}$=1.96.

DNA samples of *Betula ulmifolia, Betula microphylla* and *Betula platyphylla* isolated by standard method are insignificant, oppositely DNA samples isolated by modified method show sufficiently good results. There is especially distinguished a *Erythrina crista-galli.* Its spectrophotometry result is 198 ng/microL and $A_{260}/A_{280}$ =1.79.

The results of DNA samples isolated by the standard method are negative. As for DNA samples isolated by the modified method, the species have a pronounced result. We want to note the following samples: *Quercus spp.* (7 ng/microL, $A_{260}/A_{280}$=1.79)*, Piunus strobus* (73 ng/microL, $A_{260}/A_{280}$=1.77)*, Juniperus polycarpos var. Turcomanica* (10 ng/microL, $A_{260}/A_{280}$ =1.93)*, Ginkgo biloba* (25 ng/microL, $A_{260}/A_{280}$ =1.69)*, Acer campestre* (28 ng/microL, $A_{260}/A_{280}$ =1.21) and *Acer henryi* (1381 ng/microL, $A_{260}/A_{280}$ =2.23).

Analysis of electrophoregrams of DNA studied species shows efficiency of application of the modified DNA extraction protocol.

In general, we improved the protocol of DNA extraction as follows:

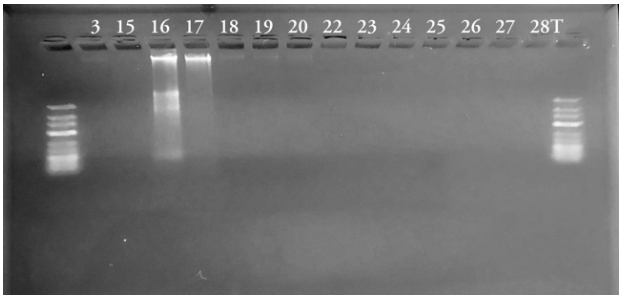
1. The tissue sample is thoroughly triturated with a pestle for 5 minutes in 1000 μl pre-heated to 65°C CTAB buffer;

2. Incubate in a thermostat at 65°C for 30 minutes, periodically mixing the solution by rotating the tube (it is not recommended to shake the tube, because the buffer foam, DNA is destroyed);

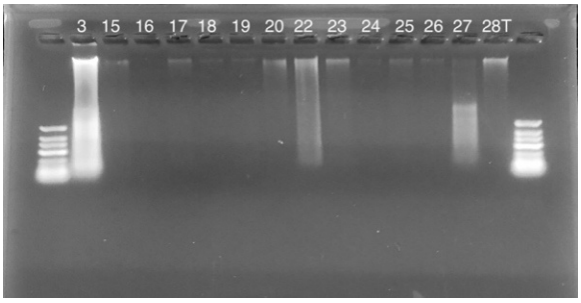
3. After cooling the tube to room temperature, add an equal volume of chloroform-isoamyl alcohol mixture and stir slowly with a shaking for 20 minutes;

4. Separate the phases by centrifugation for 15 minutes (12000 *g);

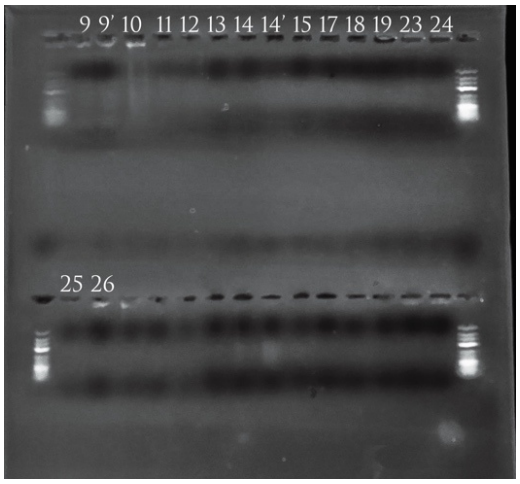
5. The supernatant is transferred to a clean 2 ml tube;




Standard protocol

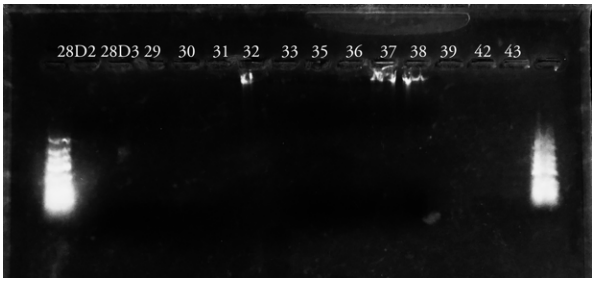



Modified protocol




Standard protocol


Modified protocol

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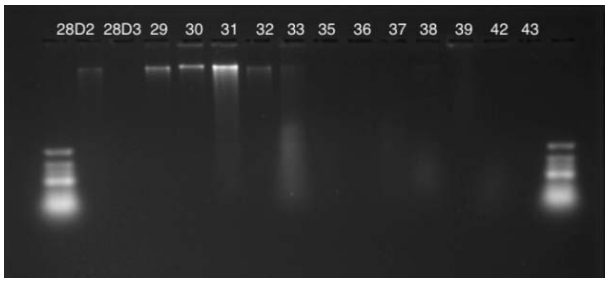


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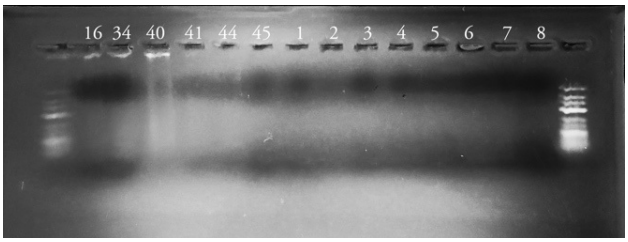
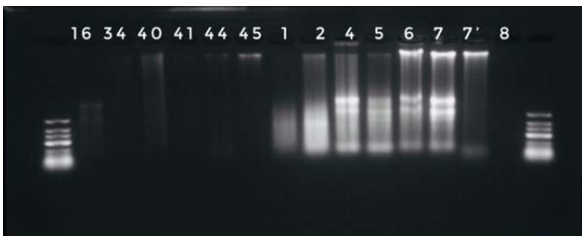



Standard protocol




Modified protocol

Electrophoregrams of DNA isolated according to a standard and modified protocol

6. Add 2/3 of the volume of isopropanol;
7. Leave for 1 hour at -20°C to precipitate the DNA;
8. Centrifuge for 15 minutes (13 000*g), isopropanol is taken off;
9. Add two volumes of 80% ethanol, incubate for 15 minutes, centrifuge for 15 minutes (13 000*g);
10. The DNA precipitate is dissolved in water or TAE buffer (0.01 M Tris-HCl, pH 7.4, 0.1 mM EDTA) until the precipitate disappears completely.

**Conclusions.** The use of a standard procedure for isolating DNA from plants (Table 1) showed the presence of a significant amount of impurities in the resulting preparations, as well as DNA degradation, which was confirmed by electrophoresis and spectrophotometrically. Incomplete removal of inhibitors, polysaccharides and polyphenols leads to inhibition of subsequent enzymatic reactions in the PCR process and causes DNA degradation after prolonged storage. For further analysis, these preparations were unsuitable.

In order to obtain a qualitative preparation of total DNA, the standard isolation procedure was modified and optimal temporal, temperature and concentration conditions were selected, some stages were changed, which allowed to fully achieve the goal.

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## ДНК-НЫҢ СИПАТТАМАСЫ НИТРОГЕНДІҢ ЖАБДЫҚ ӨСІМДЕРІНДЕГІ ДЕҢГЕЙІНДІ ТАҢДАУ

**Аннотация.** Мақалада тез әрі ыңғайлы әдісімен ағаш өсімдіктердің жапырақтарынан геномдық ДНҚ-ны алу туралы сипатталған. Ғылыми мақалада өсімдіктердің 45 түрінен алынған геномдық ДНҚ-ның стандартты әдісімен алуын тексеріу жүргізілді. Іріктеп алынған өсімдіктер Батыс Қазақстан аумағының сирек және эндемиктері мен Маңғышлақ эксперименттік ботаниқалық бағының жинақталған экзотикалық түрлері болып табылады. Бұл жұмыста өсімдік ұлпасынан ДНҚ бөлу үшін салыстырмалы модификациялық хаттама түрін усынады. Зерттеу барысында ДНҚ талдау әдісінің 3 түрі қолданылды: визуалды (ақ тунбаның бар немесе жоқ болуы, ластаныу), электрофорез және спектрофотометрия нәтижесінде. ДНҚ-ны алудың модификациялық әдісі сұйық азотты қолданбауға негізделген. Баарлық процесс 3 (үш) сағаттан кем уақыт алады. Алынған түрлерде таниннның жоғары мөлшеуде болғаны, инкубацияның уақытын көбейгені және центрифугалау ДНҚ-ның алуын жағымды әсер қалдырады. Бұл әдіс жоғары сапалы ДНҚ-дан 140,32 мкг концентрациясымен препараттарды алуға болады. Алынған ДНҚ өнімін молекулярды генетикалық сәйкестендіру және өсімдіктің сертификаттау үшін қолданұға болады.

**Түін сөздер:** ДНҚ, жапырақтар, алу, спектрофотометрия, электрофорез.

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## ВЫДЕЛЕНИЕ ДНК ИЗ ЛИСТЬЕВ ДРЕВЕСНЫХ РАСТЕНИЙ БЕЗ ЖИДКОГО АЗОТА

**Аннотация.** В статье описывается метод быстрого и удобного выделения геномной ДНК из листьев древесных растений. В научной статье проводится проверка стандартного метода выделения геномной ДНК из 45 видов растений. Отобранные растения являются редкими и эндемичными представителями флоры Западно-Казахстанского региона и коллекционными экзотическими особями Мангышлакского экспериментального ботанического сада. Работа предполагает сравнение с модифицированным для данных видов протоколом выделения ДНК из тканей растений. В работе используются три метода анализа ДНК: визуальный (наличие или отсутствие белого осадка, загрязнение), электрофорез и спектрофотометрия. Основываясь на результатах электрофореграмм и спектрофотометрии, было принято решение для модификации протокола выделения. Модифицированный метод выделения ДНК основан на отсутствии применения жидкого азота. Весь процесс занимает не более 3 часов. В связи с высоким содержанием танинов в изучаемых видах, увеличение времени инкубации и центрифугирования благоприятно влияют на результат выделения ДНК. Данный метод позволяет получить препараты высокого качества с концентрацией ДНК 140.32 мкг. Полученный продукт ДНК можно использовать в исследованиях молекулярной генетической идентификации и сертификации растений.

**Ключевые слова:** ДНК, листья, выделение, спектрофотометрия, электрофорез.

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