Optimized method for RNA extraction from leaves of forest tree species

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Abstract. Extraction of ribonucleic acid (RNA) from woody plants is a difficult task due to the peculiarities of plant material rich in polysaccharides and starch. The available techniques are often ineffective, since they result in the absence/reduced quality/reduced amount of RNA in the final preparation. The method we have optimized is based on the use of cetyltrimethyl ammonium bromide (CTAB), purification by phenol-chloroform extraction, use of lithium chloride and ammonium acetate. The method showed high efficiency for the extraction of RNA from the leaves of birch and poplar samples, in vitro and mature plants, in comparison with previously used methods (extraction using NucleoSpin® RNA Plant (Macherey-Nagel, Germany) columns, Su (2009) method, standard guanidine thiocyanate method). Electropherograms of RNA preparations showed its high integrity and concentration (up to 85 ng/μl), significantly higher purity of the preparation (up to 2.7 times). Purification of the preparation in the process of extraction can significantly reduce the yield of desoxyribonucleic acid (DNA). The optimized method is highly reproducible and can be used for further research, complementary DNA (cDNA) synthesis, qualitative and quantitative PCR analysis. The method allows obtaining high-quality RNA from other objects of agricultural and forest plants.

1. Introduction

Birch and poplar are forest-forming species and are of high ecological and economic importance. A promising direction is testing and selection of woody plant forms that are resistant to the effects of abiotic, such as drought and salinity, and biotic environmental factors. For that aim test systems, also in in vitro culture are often used [1]. The development of in vitro test systems involves the use of molecular methods, including the study of the expression of resistance marker genes, and ribonucleic acid (RNA) isolation is the initial stage of research.

Extraction of RNA is a routine operation in many biological and medical laboratories. The process of nucleic acids extraction depends on a large number of different factors. Necessary conditions for obtaining high-quality material for research are accurate sampling, their fixation and compliance with storage conditions. It is critically important to strictly observe the conditions of sterility in the laboratory, to exclude the ingress of RNases when using solutions and equipment [2].

There are a huge number of different methods that allow extracting RNA or desoxyribonucleic acid (DNA) from samples of various types of objects. At the same time, the success of used method directly depends on the chemical composition of the sample and its structure. Due to the high content of cell walls, polysaccharides, phenolic compounds and endonucleases, the extraction of nucleic acids from samples of fully formed leaves of woody plants is a rather difficult task [3, 4]. The high content of
ribonucleases (RNases), a large amount of water (relatively low concentration of nucleic acids), as well as the presence of hard-to-destroy tissues, such as lignin, make the extraction process practically individual for a given genotype and type of plant tissue [2]. Exposure to stress such as drought, low temperature stress and salinity leads to the active accumulation of high molecular weight polysaccharides and secondary metabolites, which further complicates the process of RNA extraction [5]. Problems associated with extraction are expressed in the absence of extracted RNA/DNA as such, or the extracted product may be completely or partially degraded.

The need to use a technique that allows obtaining free of impurities RNA is driven by its application for further research, including obtaining cDNA, reverse transcription of polymerase chain reaction (PCR), RNA and DNA sequencing, Northern blot analysis and genetic cloning [5-9].

The most of currently existing methods are a variation of the guanidinethiocyanate-phenol-chloroform method [10]. Many of the proposed commercial kits are based on the use of Trizol or guanidine thiocyanate (GTC), which have limitations for complex samples and are often expensive [6, 11]. Methods based on the use of Trizol or guanidine thiocyanate as the main component of the extraction medium have been well established in many model plants such as thale cress, tomatoes, corn, tobacco, and others [2]. However, extraction of RNA from starch-rich seeds is difficult because guanidine thiocyanate induces starch hardening. Loss of RNA occurs as a result of its coprecipitation with polysaccharides/starch due to their structural similarity [5].

The use of standard cetyltrimethylammonium bromide (CTAB) buffer gives good results for a wide range of samples, including woody plants and most plant tissues. Initially, CTAB-buffered techniques were developed for pine tissue samples [12], but have since come to be used to extract RNA from a wide variety of polyphenol and polysaccharide rich objects [3]. The use of CTAB and sodium dodecyl sulfate (SDS) makes it possible to inhibit RNase activity to some extent, depending on the type of sample. CTAB is used as a disintegrator of cell membranes, while β-mercaptoethanol (β-ME) added to the buffer directly during extraction prevents oxidation reactions [13, 14]. The standard purification protocol includes the use of phenol to separate from proteins, and the use of lithium chloride (LiCl) to precipitate RNA, but the procedure for manipulating these components often changes depending on the specific conditions [13]. Insoluble polyvinylpolypyrrolidone (PVPP) is added to the recovery medium to bind and remove phenolic compounds [13], which can be especially useful when working with some woody plant specimens. However, in some cases, when using coarse leaves of species such as oak and birch, modifications of the technique are required, which mainly consist in changing the concentration and composition of the components of the extraction medium.

It was previously reported that the use of a modified CTAB method based on phenol-chloroform extraction using polyvinylpyrrolidone (PVP) and PVPP gives good results in the extraction of RNA from various tissues of Hibiscus tiliaeus [14] and leaves of Melissa officinalis [3] rich in polysaccharides, secondary metabolites and polyphenols. Another modified protocol based on CTAB buffer without the use of phenol and with the addition of an increased amount of 2-β-ME made it possible to extract RNA from 16 different species of herbaceous and woody plants [15]. The CTAB/PVP protocol developed by Barbier et al. made it possible to extract RNA from macadamia, avocado and mango tissues [16]. To work with samples rich in humic and fulvic acids, cations (Ca²⁺, Mg²⁺, Na⁺, K⁺), the CTAB-Trizol protocol has been developed [17]. The modified SDS-LiCl RNA extraction method proposed by Vennapusa et al. allows obtaining high-quality RNA from seeds, leaves and roots of wheat samples subjected to various types of stress, as well as corn and sorghum rich in starch, proteins and fiber [5]. Successful extraction of RNA using 3% SDS buffer was carried out from the leaves of seedlings of Avicennia germinans [18], however, extraction from other tissues of A. germinans and L. racemosa resulted in degraded RNA containing brown sediment and unsuitable for further use [13]. The use of the SDS method also did not show good results in the extraction of RNA from samples of Feijoa sellowian [19], as well as some mangrove species [13].

Since there is still no universal method for extracting RNA from different plant species, the goal of our work was to develop a protocol suitable for working with woody objects of various origins.
contaminated with secondary metabolites, and for extracting RNA suitable for further qualitative and quantitative analyzes.

2. Methods and materials

2.1. Plant material and experimental design

The objects of the study were the leaves of the downy birch hybrid 15-1 obtained by Isakov Yu. N. Samples were selected on the territory of the Semiluksky forest nursery. Birch leaves were sampled in the third week of June 2019, during a period with a reduced average rainfall compared to the previous 10 years. Leaves of birch plants used as a control were sampled during the optimum period in terms of air temperature and precipitation, observed in mid-July.

Another object was poplar leaves of the 'Pyramidal-Osokorevy Kamyshevinskij' variety obtained by Albensky A.V. Poplar cuttings were harvested at a collection-brood plantation, established in 2016. Poplar cuttings 23…25 cm in size were planted in pots with a capacity of 3 l and a height of 20 cm. At the beginning of May, annual poplar plants from the greenhouse complex were transplanted into open ground on an experimental plot of 0.5×0.5 m. To reduce the overheating of the substrate under conditions of high summer temperatures, the growing vessels were buried in the soil. During the growing season, planned care, watering and feeding were carried out. To simulate the drought conditions, the poplars were deprived of watering for 7 days, after which the leaves were selected and fixed.

In vitro birch and poplar plants of the same genotypes were subjected to artificial osmotic stress caused by the introduction of 1% NaCl into the cultivation medium. A pre-autoclaved by 1% NaCl solution, cooled to room temperature, was layered on the culture medium in the ratio of NaCl and the medium – 1 : 5 (v/v). The control group was represented by plants cultivated on a medium without the addition of NaCl. The incubation of the experimental and control groups was carried out under standard conditions, sampling was carried after two days.

All selected material was immediately fixed at -80 °C and used for further manipulations.

2.2. RNA extraction and analysis

2.2.1. Guanidine thiocyanate method. The first method for the extraction of RNA from poplar and birch samples, in vitro clones and leaves of mature plants exposed to stress was the standard phenol-chloroform method using GTC as the extraction buffer (EB). 12M LiCl was used as a precipitant [10].

2.2.2. NucleoSpin® RNA plant column set. Using the NucleoSpin® RNA Plant Column Set (Macherey-Nagel, Germany), RNA was extracted according to the manufacturer's protocol. The RA1 buffer containing 30-60% guanidine thiocyanate and the RAP buffer containing 50…66% guanidine hydrochloride were used for testing on all the poplar and birch samples.

2.2.3. Selection by Su method. RNA was extracted using 2% CTAB and the addition of SDS to the extraction medium according to the authors' protocol [20].

2.2.4. Extraction by modified CTAB method. The method we modified was based on the protocol proposed by Rubio-Piña et al., using 2% CTAB and 3% SDS as EB [13]. The modified protocol included the following stages. Using a mortar and pestle, 200 mg samples of the test material were homogenized with 1500 μl of EB I (2% (w/v) CTAB; 0.1 M Tris-HCl (pH 8.0); 1.4 M NaCl; 20 mM EDTA (pH 8.0); 2 % (w/v) PVP) or EB II (3% SDS (w/v); 0.5 mM EDTA (pH 8.0); 0.1 mM Tris-HCl (pH 8.0); 2% (w/v) PVP). 150 μl of β-ME was added to the samples during homogenization. Then followed the incubation step with the EB at 65°C for 10 min with constant stirring. An equal volume of chloroform was added to the samples and stirred for 30 sec, centrifuged at 10000 rpm for 10 min at 4°C. The supernatant was transferred to a new tube and an equal volume of phenol/chloroform mixture (1: 1) was added. Probes were mixed for 30 s and centrifuged at 10000 rpm for 10 min at 4°C. Then the supernatant
was transferred into a new test tube, an equal volume of chloroform/isoamyl alcohol mixture (24: 1) was added, and centrifugation was carried out under the same parameters. The supernatant was transferred to a new tube, 1/3 of the sample volume of 12M LiCl was added. The probes were incubated at -20 °C overnight. The samples were centrifuged at 10000 rpm for 10 min at 4°C. The supernatant was removed and the precipitate was washed with 96% and 70% ethanol. The samples were dried at room temperature and the precipitate was dissolved in 250 μl of deionized water. RNA precipitation was performed by adding 0.1 volume of 3M sodium acetate and 2 volumes of ethanol. Samples were incubated at -20°C for 2 h, then centrifuged at 13000 rpm for 15 min at 4°C. The precipitated RNA was washed with 70% ethanol and dissolved in 50 μl of deionized water.

2.3. Qualitative assessment of RNA
The quality of the obtained RNA was assessed by electrophoresis in 1% agarose and subsequent visualization using the Infinity VX2 1126MX X-Press gel documenting system (Vilber Lourmat, France) (figures 1-5). The purity of the obtained preparations was assessed spectrophotometrically on an SF-102 device (Avkilon, Russia).

2.4. Reverse transcription and real-time PCR parameters
Reverse transcription to obtain cDNA was performed using 0.5…1 μg of total RNA with a standard reagent kit containing MMLV reverse transcriptase according to the manufacturer's instructions (DiaM, 2020, Russia). To quantify the extracted RNA, the obtained cDNA was amplified with a standard set of reagents containing SYBR Green I dye (Evrogen, 2020, Russia), with primers for the DREB2 gene (dehydration-responsive element binding) on a LightCycler® 480 II device (Roche, Switzerland). The GAPDH gene (glyceraldehyde 3-phosphate dehydrogenase) was used as a reference for birch and 18S rRNA for poplar (table 1). The reaction protocol included the following stages: denaturation at 95°C for 3 min, then 40 cycles from stages 95°C for 10 s, 60°C for 30 s, 72°C for 30 s, then the final elongation of 72°C for 2 min.

| Gene | Sequence |
|------|----------|
| Birch gene primers | |
| GAPDH | F: CAGCCGAAGATGTCAATGCA  
         R: GGCACCTTGTGTGCTACCAC |
| DREB2 | F: AGGCAGAGAACATGGGGAAA  
        R: GAAAGTTGAGGCGAGCGTAA |
| Poplar gene primers | |
| 18S | F: GGCTCTGCCGCTTGCTCT  
     R: CGTCACCAGTCACCACCA |
| DREB2 | F: TGTATGCTCGTATGCTCGT  
       R: TCCTCATAACCGAGACCTC |

2.5. Statistical data processing
The relative level of transcripts was determined using the 2-ΔΔCt method using LightCycler® 480 II software v 1.5.1 (Roche, Switzerland). Statistical analysis was performed using the Statistica software. All experiments were carried out in triplicate.

3. Results and discussion
Poplar and birch RNA samples were extracted using various techniques based on the use of guanidine thiocyanate/guanidine hydrochloride, CTAB and SDS as extraction media.

Initially, poplar and birch RNA samples were extracted using a 47% guanidine thiocyanate buffer using a standard procedure with purification steps using phenol, chloroform, and isoamyl alcohol. Electrophoretic analysis showed that RNA extracted from poplar in vitro samples (figure 1-1a) and
leaves of mature plants (figure 1-Ib) has an integral structure and is present in an amount sufficient for use in further work.

**Figure 1.** Electropherograms of RNA extracted from samples of poplar (I) and birch (II) using the GTC method: a) mature plants; b) in vitro plants.

Analysis of the electropherogram showed that the use of the GTC method did not allow the extraction of RNA from birch samples, both in vitro (figure 1-Ia) and from the leaves of mature plants (figure 1-IIb). Using the NucleoSpin® RNA Plant column set with RA1 extraction buffer, high-quality RNA was obtained from in vitro poplar (figure 2-Ia) and mature plant samples (figure 2-Ib).

Despite the fact that the use of both RA1 and RAP buffers in the column set allowed for the extraction of high-quality RNA from poplar samples, no positive results were obtained for birch with any of the used EBs (figure 2-IIa, 2-IIb). Since GTC-based methods did not harvest any results, we used CTAB-EB based techniques. Protocol proposed by Su et al. allowed us to obtain RNA at a high concentration and quality from both in vitro plants (figure 3-Ia) and mature leaves (figure 3-Ib) of poplar samples. However, RNA extracted from birch samples was at a reduced concentration (figure 3-II-ab).

**Figure 3.** Electropherograms of RNA extracted from samples of poplar (I) and birch (II) using Su protocol: a) mature plants; b) in vitro plants.

The protocols for existing CTAB-based techniques vary considerably. The composition of the EBs can differ in the concentration of CTAB (1-3%). Various authors recommend using soluble PVP [15] or PVPP [13], or both [14]. The method involves heating the homogenate with the EB at 65°C, which removes the polysaccharides, making them more soluble [2].
Our results for woody plants such as birch and poplar indicate the possibility of using PVP for these tree species. Gambino et al. increased the concentration of PVP-40 and LiCl, with a simultaneous decrease in the weight of grape tissues, which made it possible to reduce the incubation time for RNA precipitation [21]. The stage of purification from the remnants of destroyed cell walls and proteins involves the use of a mixture of chloroform and isoamyl alcohol (24:1), which is reflected in the protocols developed by various authors [14, 19]. In addition, acidic phenol used in the purification step [2, 3, 10, 13] significantly increases the quality of purification of woody plant samples during extraction by the CTAB method. Since none of the approved methods made it possible to obtain RNA from birch samples of sufficient quality, we used a modified method based on the Rubio-Piña et al. protocol using CTAB and SDS-based EBs. The main modifications consisted in using the increased ratio (1:7.5) of the sample in the amount of 200 mg to the isolation buffer in the volume of 1500 μl to avoid solidification of the homogenate and better interaction of the plant tissue with the isolation medium. Samples were thoroughly homogenized and incubated for no more than 10 minutes, according to the authors’ protocol. According to our observation, presence or absence of β-ME in EB didn’t influence obtained results. It was fundamental to carry out all stages of centrifugation at 4°C and to mix samples thoroughly at each isolation step. The advantage of the method used is sequential purification using phenol, chloroform and isoamyl alcohol in different ratios, which results in cleaner preparations. Increased concentration of lithium chloride (12M) was used to enhance precipitation with ethanol [22].

Plants, as they grow and develop, accumulate secondary metabolites necessary for exchange with the environment and protection from its adverse effects, which is especially characteristic of woody plants. Microclones represent the juvenile stage of plant development, and the content of secondary metabolites in them is reduced. The accumulation of alkaloids, isoprenoids, phenolates in the organs and tissues of adult plants significantly complicates the extraction of RNA, and its quality and volume decrease.

The modified CTAB-method made it possible to extract RNA of high concentration and significant integrity from both the in vitro clones of poplar (figure 4-Ia) and birch (figure 4-IIb) and mature leaves with the high level of secondary metabolites (figure 4-Ib, 4-IIb). The modified CTAB-method has also been successfully tested on the leaves of other woody objects (oak) and leaves of herbaceous plants, such as corn (data not shown). At the same time, when using 2% SDS in EB, the RNA samples were partially (poplar in vitro samples (figure 5-Ia) and mature leaves (figure 5-Ib) or fully (birch samples (figure 5-IIa, 5-IIb) degraded, the precipitate was heavily contaminated and had a brown color.

Figure 5. Electropherograms of RNA extracted from samples of poplar (I) and birch (II) using the modified SDS-method: a) mature plants; b) in vitro plants.
Determination of the purity of the obtained samples, extracted by various methods, was carried out by measuring their optical density (table 2).

**Table 2.** Comparative evaluation of 260/280 and 260/230 ratios of RNA extracted from poplar and birch *in vitro* plants and mature leaves with the use of different methods.

| Sample      | Guanidine thiocyanate method | NucleoSpin® RNA Plant | Su method | Optimized CTAB-method |
|-------------|------------------------------|-----------------------|-----------|-----------------------|
| A260/280 ratio |                               |                        |           |                       |
| Pop_in v_exp | 0.50±0.40                    | 1.54±0.13             | 2.23±0.20 | 2.00±0.18             |
| Pop_in v_ctrl | 0.92±0.08                   | 1.63±0.02             | 2.75±0.27 | 1.82±0.16             |
| Pop_m_exp    | 1.43±0.07                    | 1.63±0.02             | 2.00±0.20 | 2.17±0.09             |
| Pop_m_ctrl  | 2.00±0.25                    | 1.61±0.04             | 1.90±0.10 | 1.72±0.11             |
| Bet_in v_exp | 0.50±0.10                    | 1.00±0.00             | 1.83±0.15 | 1.95±0.14             |
| Bet_in v_ctrl | 0.46±0.15                   | 1.00±0.00             | 2.00±0.07 | 1.88±0.15             |
| Bet_m_exp    | 0.75±0.20                    | 1.00±0.00             | 1.63±0.04 | 1.46±0.08             |
| Bet_m_ctrl  | 0.81±0.15                    | 0.00±0.00             | 1.65±0.02 | 1.50±0.10             |

| A260/230 ratio |                               |                        |           |                       |
| Pop_in v_exp | 0.85±0.10                    | 1.13±0.05             | 2.00±0.14 | 1.84±0.05             |
| Pop_in v_ctrl | 0.95±0.15                   | 1.18±0.05             | 2.75±0.30 | 1.78±0.07             |
| Pop_m_exp    | 1.25±0.20                    | 1.15±0.05             | 1.42±0.05 | 1.91±0.09             |
| Pop_m_ctrl  | 1.90±0.18                    | 1.00±0.18             | 2.10±0.10 | 1.98±0.10             |
| Bet_in v_exp | 0.76±0.13                    | 1.00±0.00             | 2.75±0.12 | 1.81±0.07             |
| Bet_in v_ctrl | 0.88±0.10                   | 1.00±0.00             | 2.67±0.15 | 1.90±0.06             |
| Bet_m_exp    | 0.95±0.12                    | 0.50±0.00             | 2.70±0.23 | 1.79±0.05             |
| Bet_m_ctrl  | 0.89±0.12                    | 0.00±0.00             | 2.69±0.17 | 1.98±0.08             |

It can be seen from the table that all samples extracted by GTC methods are contaminated with proteins and phenolic compounds (A260/A280 <1.7 and A260/230 < 1.8). At the same time, the use of CTAB methods makes it possible to extract purer RNA, free of contaminations.

The calculated concentration of RNA in the samples indicates the applicability of methods based on the use of GTC for working with poplar samples; however, these methods showed no efficiency for isolation from birch samples (table 3).

**Table 3.** Concentrations of RNA extracted from poplar and birch *in vitro* plants and mature leaves with the use of different methods.

| Sample      | Guanidine thiocyanate method | NucleoSpin® RNA Plant | Su method | Optimized CTAB-method |
|-------------|------------------------------|-----------------------|-----------|-----------------------|
| Concentration, ng/µl |                               |                        |           |                       |
| Pop_in v_exp | 40.0±2.8                     | 55.5±3.1              | 33.0±2.2  | 54.0±3.4              |
| Pop_in v_ctrl | 45.0±2.0                    | 60.0±2.9              | 30.0±2.8  | 58.1±4.2              |
| Pop_m_exp    | 35.0±3.1                     | 49.0±2.7              | 20.0±1.9  | 55.4±2.9              |
| Pop_m_ctrl  | 40.0±3.6                     | 55.0±1.9              | 22.9±1.8  | 59.6±3.6              |
| Bet_in v_exp | 0.0                          | 0.0                   | 16.5±2.5  | 63.9±2.8              |
| Bet_in v_ctrl | 0.0                         | 0.0                   | 20.5±3.4  | 65.8±3.1              |
| Bet_m_exp    | 0.0                          | 0.0                   | 15.6±2.7  | 62.2±4.2              |
| Bet_m_ctrl  | 0.0                          | 0.0                   | 12.5±2.8  | 75.6±3.8              |
The efficiency of the extraction of RNA from using different techniques varies depending on the type of plant. Extraction of RNA from samples rich in polysaccharides and polyphenols is very difficult, since their physicochemical properties are close to RNA, and there is a high probability of their coprecipitation during RNA isolation. A decrease in the efficiency of isolation leads to the loss of RNA, which affects its concentration [23].

Using both GTC and the NucleoSpin® RNA Plant on birch samples results in zero RNA yield (table 3). The concentration of RNA obtained by the method proposed by Su in all birch and poplar samples was significantly reduced relative to other protocols, yielding from 12.5 to 33.0 ng/µl which does not allow the use of RNA preparations in further quantitative PCR analysis. At the same time, modified CTAB method allows to obtain RNA in a high concentration (up to 75.6 ng/µl), sufficient for quantitative analyzes, both from in vitro material and from the leaves of mature plants. The use of CTAB in the extraction buffer promotes efficient destruction of cell walls, and the addition of PVP prevents the oxidation of polyphenols in the cell walls and extracellular matrix.

Real-time PCR analysis showed the presence of characteristic curves of product accumulation during amplification of the studied genes from the cDNA template, which indicates the presence of the used RNA template in sufficient quality and quantity (figure 6a).

![Figure 6](image)

**Figure 6.** Quantitative real-time PCR with RNA samples extracted by the modified CTAB method. a) DREB2 gene amplification curves in birch and poplar samples; b) Expression of the DREB2 gene in birch samples; c) Expression of the DREB2 gene in poplar samples. Bet – birch samples, Pop – poplar samples, in v – in vitro plants, m – mature plants, exp – samples exposed to stress, ctrl – control samples.

The study of the impact of abiotic stress revealed an increase in the expression of the DREB2 gene in birch samples, both in vitro clones and mature plants (figure 6b), the same was observed for poplar samples (figure 6c). DREB2 is a marker gene used to assess the development of resistance to abiotic stress, including drought and salinity [24, 25]. An increase in the expression of this gene indicates the development of adaptation to the stress involving DREB transcription factors in the studied in vitro plants and mature leaves of poplar and birch genotypes. The results obtained indicate the possibility of
using RNA isolated by the modified CTAB method in quantitative analyzes, such as determining the expression of resistance genes.

4. Conclusion

The use of the standard GTC method and NucleoSpin® RNA Plant gives good results for poplar samples of various origins. However, they turned out to be absolutely inapplicable for \textit{in vitro} samples and leaves of adult birch plants due to the high content of polyphenols, proteins and polysaccharides in their cells. The CTAB methods are applicable to a large number of different objects, including woody plants. Though, the reaction protocol is highly dependent on the type of tissue used and the origin of the sample. The modified CTAB-based RNA extraction method is applicable to samples of woody plants such as poplar and birch, including \textit{in vitro} and leaves of mature plants. Other objects to which this protocol can be applied without further modification are oak leaves as well as herbaceous plants (maize). The method allows to obtain RNA of high quality and quantity, applicable for the synthesis of cDNA and use in the quantitative analysis of the determination of gene expression.

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