

## Original Article

# Analysis of different methods of extracting DNA and RNA from paraffin-embedded tissues

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**Abstract:** The aim of this research study was to identify the optimal method of extracting DNA and RNA from formalin-fixed, paraffin-embedded (FFPE) tissues, and to improve amplification of gene fragments with the method. Twenty FFPE specimens from 2018 were randomly selected. Total DNA was extracted using a kit method and phenol/chloroform method, and total RNA was extracted using a kit and Trizol method. DNA and RNA concentrations and OD<sub>260</sub>/OD<sub>280</sub> ratio were determined using a NanoDrop-2000 spectrophotometer. The human  $\beta$ -globin gene was amplified using a LightCycler 480 Real-Time PCR System. Gel electrophoresis was performed using 1% agarose for PCR amplification products. One-way ANOVA was used to assess the DNA and RNA quality extracted by different techniques. The experimental results showed that the concentration and purity of DNA and RNA and the success rate of fluorescence quantitative PCR amplification were all higher when extraction was performed using the kit. This shows that the quality of DNA and RNA extracted from the FFPE tissue samples using the kit method is reliable and the method can be used for clinical amplification of some gene fragments.

**Keywords:** FFPE tissues, DNA and RNA extraction, internal reference gene amplification, comparison of extraction methods

## Introduction

Formalin-fixed, paraffin-embedded (FFPE) tissues are the most widely used and easily obtained specimens in clinicopathological diagnosis [1]. Extraction of DNA and RNA from the samples not only solved the problem of the small number of cases, but it also effectively combined the results of gene detection with retrospective analysis of tumors [2]. However, formaldehyde immobilization and paraffin embedding can easily cause DNA and RNA to degrade and encourage cross-linking of histones. Thus, it is difficult to amplify PCR products, which limits the research work. Studies have shown [3] that the purity, concentration, and PCR amplification rate of DNA and RNA obtained by different extraction methods are quite different. In this paper, two DNA extraction methods (kit method and phenol/chloroform method) and two RNA extraction methods (kit method and Trizol method) were compared

in order to select the most suitable methods of DNA and RNA extraction.

## Materials and methods

### Reagents

Faure Marin, ethanol, xylene, phenol, chloroform and isoamyl alcohol were purchased from the National Pharmaceutical Corporation (Shanghai, China). DNA/RNA extract kits were gotten from Qiagen (Germany). The primers were synthesized by Sangon Biotech Co., Ltd (Shanghai, China). 5× All-In-One MasterMix are purchased from abm (Zhenjiang, China). Premix Ex Taq™ II were bought from TaKaRa (Japan).

### Tissue specimens

Twenty FFPE specimens diagnosed uterine leiomyoma and collected during 2018 were selected. They were collected during surgery and kept in the archives in Department of Pathology,

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Jiading District Central Hospital Affiliated Shanghai University of Medicine & Health Sciences. Paraffin blocks were selected to carry out complete serial sections, according to the archived HE staining sections. All of the specimens were fixed using 10% neutral formalin solution and embedded in conventional paraffin. The selected specimens were preserved completely, and these were observed under a microscope with no tissue autolysis, necrosis, or massive hemorrhage. All of the articles were sterilized and 4-6 sections of FFPE 20- $\mu$ m thick were placed in a 1.5-mL EP pipe for backup.

### DNA extraction

*Phenol/chloroform extraction:* ① Dewaxing: 1 mL of dimethylbenzene was added to FFPE tissues in the 1.5-mL EP pipe and thoroughly shaken using 3000 rpm centrifugation for 2 minutes. Then, the supernatant was discarded and 1 mL of absolute ethanol was put in to mix, with 3000 rpm centrifugation for 2 minutes. Subsequently, the supernatant was discarded and the specimen was repeatedly dewaxed. Finally, 100%, 95%, and 75% gradient ethanol was affiliated in turn to wash the residual toluene. ② Digestion: The dewaxed tissues were incubated in a 37°C incubator for 15-30 minutes, and then 200  $\mu$ L STE solution (100 mmol/L Tris-HCl, pH 8.0, 20 mmol/L EDTA, 0.8% (W/V) SDS), was added. The mixture was heated in water bath at 90°C for 10 minutes, then 20 g/L protease K was affiliated and incubated in water bath at 55°C for 3 hours until the floc disappeared. After that, the mixture underwent 3000 rpm centrifuge for 1 minute and then the supernatant was obtained. ③ Purification: DNA was extracted using saturated phenol, and phenol/chloroform/isoamyl alcohol (25:24:1), successively. Then, 2 times volume of anhydrous ethanol and 1 times volume of 3% sodium acetate was added differentially. Finally, the purified product was stored in -20°C refrigerator for the night, and DNA was precipitated. ④ Dissolution: 10,000 rpm centrifugation for 8 minutes, then DNA was dissolved with TE buffer (100  $\mu$ L pH 8.0), and reserved at -20°C.

*Kit method:* DNA was extracted and purified according to the instructions of QIAGEN All Prep® DNA/RNA FFPE Kit. The main steps were as follows: ① Xylene dewaxing. ② Cell lysis + protease digestion. ③ Adsorption column for DNA. ④ TE buffer for DNA dissolution.

### RNA extraction

*Trizol method:* ① 10  $\mu$ L Proteinase K was added to the EP tube containing tissue slices for digestion. ② 1 ml Trizol was used according per 50-100 mg of tissue samples and transferred into the centrifugal tube. ③ Centrifugation for 5 minutes at 12,000 rpm to discard the precipitation, then 200  $\mu$ L chloroform was added accordingly to 1 mL Trizol, shaken and mixed for 15 seconds, and stored at room temperature for 10 minutes. ④ Centrifugation for 15 minutes at 12,000 rpm, then the upper water phase was absorbed. Then, 0.6 mL isoamyl alcohol was added accordingly to 1 mL Trizol, mixed, and stored for 5-10 minutes at room temperature. ⑤ Centrifugation at 4°C for 10 minutes at 12,000 rpm and supernatant was discarded, then 1 mL 75% ethanol was mixed accordingly to 1 mL Trizol, oscillated gently, and suspended. ⑥ RNA was dried at room temperature, dissolved with 50  $\mu$ L RNA-free ddH<sub>2</sub>O, and reserved at -20°C.

*Kit method:* RNA was extracted and purified according to the instructions of QIAGEN All Prep® DNA/RNA FFPE Kit. The main steps were as follows: ① Xylene dewaxing. ② Cell lysis + protease digestion. ③ Adsorption column for RNA; ④ RNA-free ddH<sub>2</sub>O for DNA dissolution.

### DNA and RNA quality control

Concentration and purity of DNA and RNA: The yield and quality ( $OD_{260}/OD_{280}$ ) of the DNA and RNA products were measured by a spectrophotometer (NanoDrop-2000, Thermo Scientific). When  $OD_{260}/OD_{280}$  is  $1.8\pm 0.1$ , the total DNA extracted is qualified, while  $OD_{260}/OD_{280}$  is  $2.0\pm 0.1$ , the total RNA extracted is qualified.

DNA and RNA electrophoresis: electrophoresis on 1% agarose gels stained with ethidium bromide. Degradation of DNA and RNA, and amplification of internal reference genes were observed using BTS-20.M automatic digital imaging system and LUV-260D ultraviolet projector (Tanon 1600, Tanon).

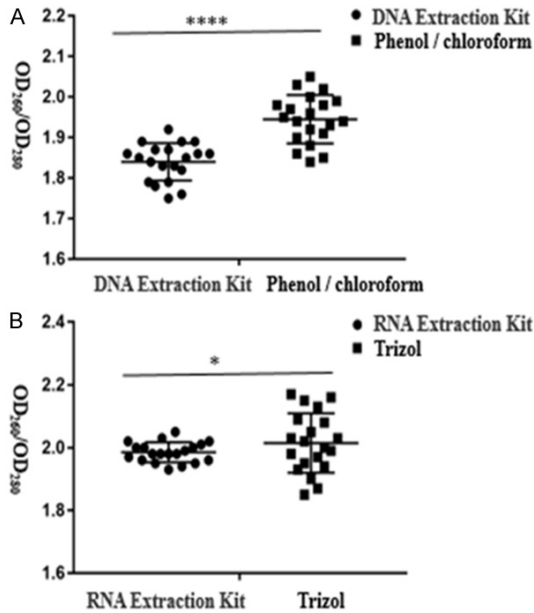
### Amplification of internal reference gene

RNA was transcribed into cDNA using abm® 5 $\times$  All-In-One MasterMix (with AccuRT Genomic DNA Removal Kit) and then amplified by real-time PCR (LightCycler 480, Roche, Germany).

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**Table 1.** Primer sequences and fragments of amplified products of  $\beta$ -globin gene

Primers	Sequences	Products
Forward Primer	5'-GAAGAGCCAAGGACAGGTAC-3'	268 bp
Reverse Primer	5'-CAACTTCATCCACGTTACC-3'	



**Figure 1.** Purity of DNA and RNA extracted by different methods. A. The purity of DNA extracted using the kit and phenol/chloroform was compared to that of DNA extracted using other methods. B. The purity of RNA extracted using the kit and Trizol method was compared to that of RNA extracted using other methods. \* $P < 0.05$  and \*\*\*\* $P < 0.0001$ .

Fluorescence quantitative PCR amplification was performed using TaKaRa SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> II (Tli RNaseH Plus). The cycling protocol was as follows: denaturation at 95°C for 30 seconds, 30 cycles of amplification (5 seconds at 95°C, and 20 seconds at 60°C). After PCR, the amplification of internal reference genes was analyzed by curve and Cp value of PCR amplification. Meanwhile, the amplified products of PCR were electrophoresed on 1% agarose gel containing ethidium bromide. A 100 bp DNA ladder marker was used as the standard reference (Marker). Pictures were taken after electrophoresis to observe whether the size of the electrophoretic band met the experimental requirements. Primer sequences and fragments of amplified products of  $\beta$ -globin gene were seen in **Table 1**.

### Data analysis

Data are represented as the mean  $\pm$  standard error of the mean. Statistical significance was analyzed by unpaired Student's *t* test or oneway analysis of variance, using GraphPad v5.0 software (GraphPad Software, Inc., La Jolla, CA, US).  $P < 0.05$  was considered to indicate a statistically significant difference.

### Results

#### *OD<sub>260</sub>/OD<sub>280</sub> ratio of specimens in each group*

The  $OD_{260}/OD_{280}$  ratio of DNA extracted by the two methods is shown in **Figure 1A**:  $OD_{260}/OD_{280}$  of DNA extracted by kit was  $1.84 \pm 0.01$ ; while  $OD_{260}/OD_{280}$  of DNA extracted by phenol/chloroform was  $1.945 \pm 0.01$ . The  $OD_{260}/OD_{280}$  ratio of RNA extracted by the two methods is shown in **Figure 1B**:  $OD_{260}/OD_{280}$  of RNA extracted by kit was between 1.9 and 2.1; while  $OD_{260}/OD_{280}$  of RNA extracted using the trizol method differed significantly between 1.85 and 2.20. These results show that the purity of DNA and RNA extracted using the kit method was greater than by the other method, and the difference was statistically significant.

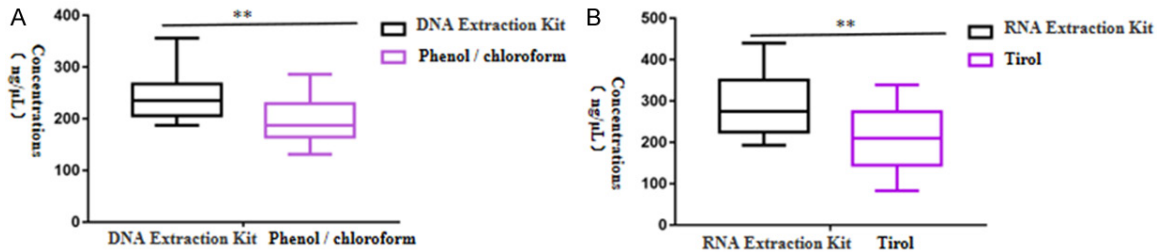
#### *Comparison of DNA and RNA concentration in each group*

The concentration of DNA extracted by the two methods is shown in **Figure 2A**. The concentration of DNA extracted using the kit was  $244.7 \pm 10.89$  ng/ $\mu$ L, while the concentration of DNA extracted using phenol/chloroform was  $195.3 \pm 9.11$  ng/ $\mu$ L. The concentration of RNA extracted by the two methods is shown in **Figure 2B**. The concentration of RNA extracted by kit was  $291.7 \pm 16.81$  ng/ $\mu$ L, and the concentration of RNA extracted using the Trizol method was  $212 \pm 16.21$  ng/ $\mu$ L. The results show that the concentration of DNA and RNA extracted by the kit method was higher, and the difference was statistically significant.

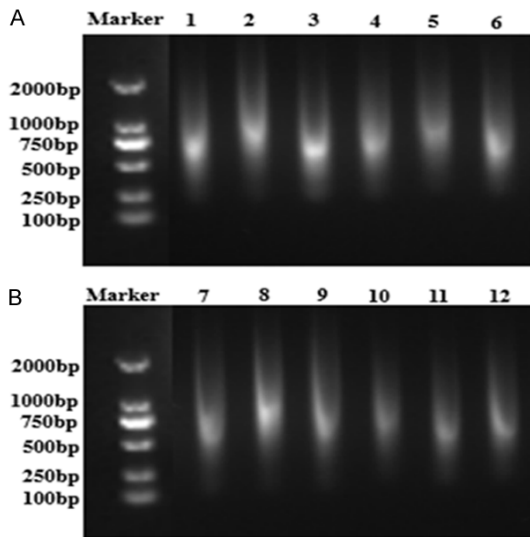
#### *Electrophoresis of DNA and RNA with different extraction methods*

The molecular weight of DNA and RNA obtained using the kit extraction method and the traditional extraction method were not found to be significantly different upon electrophoresis. Both of them were 2000-100 bp diffusion bands.

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**Figure 2.** The concentration of DNA and RNA extracted by different methods. A. The concentration of DNA extracted by kit and phenol/chloroform was compared. B. The concentration of RNA extracted by kit and Trizol method was compared. \*\* $P < 0.01$ .



**Figure 3.** Degradation of DNA and RNA extracted by different methods. A. Agarose gel electrophoresis for DNA extraction, 1, 2, 3: electrophoretic bands for DNA extraction by kit method; 4, 5, 6: electrophoretic bands for DNA extraction by phenol/chloroform. B. Agarose gel electrophoresis for RNA extraction, 7, 8, 9: electrophoretic bands for RNA extraction using the kit method; 10, 11, 12: electrophoretic bands for RNA extraction by the Trizol method.

As shown, the extracted DNA and RNA were all degraded seriously (**Figure 3A, 3B**).

### Amplification results of human $\beta$ -globin gene

Human  $\beta$ -globin gene in DNA and RNA extracted using different methods was amplified by fluorescence quantitative PCR, and the kinetic curves (**Figure 4A-D**) and CP values (**Figure 4E, 4F**) were analyzed. The CP value of amplification products from DNA extracted using the kit and phenol/chloroform was partly  $20.85 \pm 0.16$  and  $21.71 \pm 0.15$  and the CP value of amplification products from RNA extracted by kit and Trizol method was  $20.96 \pm 0.09$  and  $21.55 \pm 0.12$ ,

respectively. The results show that the Cp value of DNA and RNA extracted with kit was smaller and the success rate of amplification was higher.

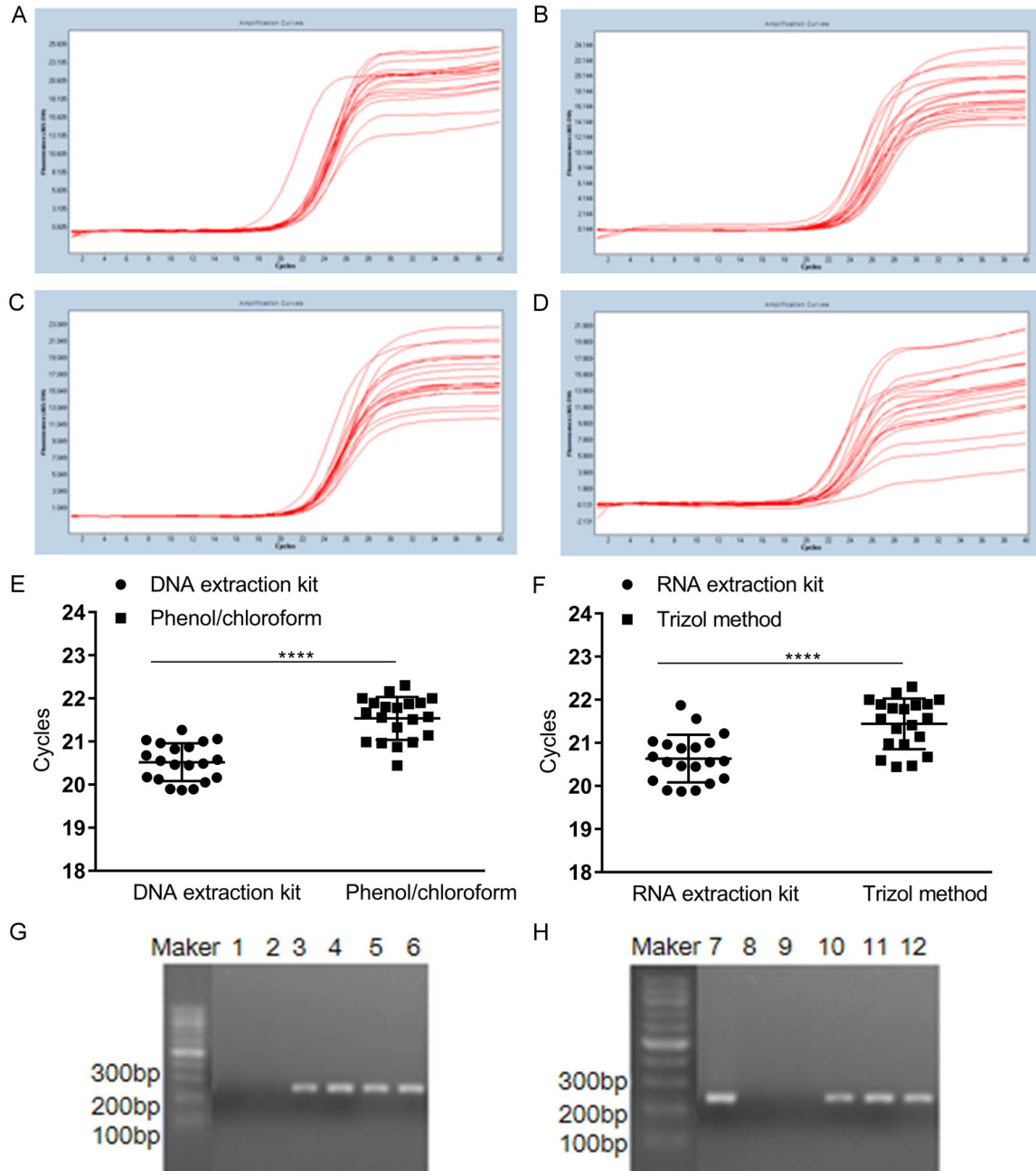
PCR products of human  $\beta$ -globin gene were detected using 1% agarose gel. Clear internal reference bands of DNA and RNA extracted by the kit were found at 268 bp (**Figure 4G, 4H**). The internal reference bands of DNA and RNA extracted using traditional phenol/chloroform extraction and Trizol extraction were fuzzy or invisible (**Figure 4G, 4H**). The results show that the success rate of amplification of DNA and RNA extracted by kit was higher.

### Discussion

The large number of FFPE tissues collected by hospital Pathology Departments make up an important source of research materials. The most difficult problem of DNA and RNA extraction from FFPE tissues is the degradation of DNA and RNA and protein cross-linking [4-6]. Most scholars believe that the cause of DNA and RNA degradation is that formalin is easily oxidized into formic acid in the air [7, 8], and formic acid has a strong degradative effect on DNA and RNA. Moreover, tissue immobilization causes extensive cross-linking of nucleic acids and histones, forming a strong complex, which can easily cause DNA and RNA breakage.

Through experiments and literature review, the following conclusions have been made. ① Selection: It is advisable to avoid performing examination of parts of the sample containing hemorrhage, necrosis, or autolysis, and select densely packed areas. ② The thickness of slices: The tissues fixed by formalin and embedded in paraffin become tough and difficult to homogenize. In the past, FFPE tissues were cut into

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**Figure 4.** Amplification results of human  $\beta$ -globin gene in DNA and RNA extracted by different methods. Amplification curve of human  $\beta$ -globin gene in DNA extracted using the kit (A) and phenol/chloroform (B); amplification curve of human  $\beta$ -globin gene in RNA extracted using the kit (C) and that Trizol method (D). Comparison of Cp value of DNA (E) and RNA (F) extracted in different ways. (G) Agarose gel electrophoresis of amplification products in DNA extracted by different methods. 1, 2, 3: electrophoretic bands for DNA extracted by phenol/chloroform; 4, 5, 6: electrophoretic bands for DNA extracted using the kit method; (H) Agarose gel electrophoresis of amplification products in RNA extracted by different methods. 7, 8, 9: electrophoretic bands for RNA extracted using the Trizol method; 10, 11, 12: electrophoretic bands for RNA extracted by kit method. \*\*\*\* $P < 0.0001$ .

3-5- $\mu$ m slices. However, some studies have suggested that [9] DNA and RNA are easily switched off if the slices of FFPE tissues are so thin. It also affects the acquisition of high-

molecular-weight DNA and RNA. Therefore, it is best to be with a thickness of 15-20  $\mu$ m. In this experiment, the thickness of the slices was 20  $\mu$ m, and the quantity and quality of DNA and



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RNA were all better. ③ Other factors: Repeatability of PCR amplification and complex operation conditions are difficult to control using traditional organic solvents for extraction of DNA and RNA. Furthermore, the extracted DNA and RNA have poor practicability. In contrast, DNA and RNA extracted by the kit method are more conducive to PCR amplification [10]. In this experiment, DNA and RNA were extracted by a Qiagen All Prep® DNA/RNA FFPE Kit. This kit can extract DNA and RNA simultaneously. The purity of DNA and RNA basically met the experimental requirements. Over 99% of the samples were able to amplify the internal reference bands.

In summary, the broken fragments of nucleic acids extracted from FFPE are arbitrary, which depends on many factors, such as the time intervals from sampling to fixing in the agent, the time of preservation of wax blocks, the types of fixer, the fixed time and the fixed temperature. These factors are influential in molecular biology research of FFPE specimens.

DNA was extracted using the kit method and phenol/chloroform method and RNA was extracted using the kit method and Trizol method, both from 20 FFPE tissues in this research. The results show that the purity and concentration of DNA and RNA extracted using the kit method are higher than those of another traditional extraction method. The 1% agarose gel electrophoresis showed that the DNA and RNA extracted using different methods all had different degrees of degradation. Fluorescence quantitative PCR was used to amplify human  $\beta$ -globin gene in the extracted DNA and RNA. It was found that the Cp values of DNA and RNA extracted by kit method were smaller and the amplified bands were clearer. In summary, the extraction of DNA and RNA by kit is more successful than traditional extraction methods.

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### Disclosure of conflict of interest

None.

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