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ORIGINAL PAPER

A rapid and cost effective method in purifying small RNA

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Abstract Purification of RNA fragments from a complex mixture is a very common technique, and requires consideration of the time, cost, purity and yield of the purified RNA fragments. This study describes the fastest method of purifying small RNA with the lowest cost possible, without compromizing the yield and purity. The technique describes the purification of small RNA from polyacrylamide gel, resulting in a good yield of small RNA with minimum experimental steps in avoiding degradation of the RNA, obviating the use of ethidium bromide and phenol–chloroform extraction, as well as siliconized glass wools to remove the polyacrylamide gel particles. The purified small RNA is suitable for a wide variety of applications such as ligation, end labelling with radio isotope, RT–PCR (Reverse Transcriptase-PCR), Northern blotting, experimental RNomics study and also Systematic Evolution of Ligands by Exponential Enrichment (SELEX).

Keywords Small RNA · Degradation · Purification · Gel elution

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Introduction

RNA plays diverse roles such as recognizing various target molecules and carrying out a myriad of catalytic functions, owing to its capability to form unique and compactly folded structures. These omnipotent roles displayed by RNA are attributable to the presence of the hydroxyl group at the 2' position, which adds to the flexibility of these molecules (Wiegand et al. 1996). Hence, remarkable features manifested by RNA have made these molecules the subject of intense study and analysis in a wide variety of applications, including RNA–protein mapping with nuclease protection assay, toe-printing analyses, RNAse protection assay, boundary analysis, SELEX (Gopinath 2009), RNomics (Tang et al. 2009) and others.

On the other hand, the identification of a wide variety of small non protein coding RNAs (npcRNA) (having the size of 20–30 nucleotides) by experimental and computational approaches that play a major role in gene expression both transcriptionally or post-transcriptionally in eukaryotes, has also become a matter of extreme interest. These small RNA molecules are usually obtained from polyacrylamide gel excision of total RNA, and must be of high purity to ensure successful cloning, sequence characterization and construction of small RNA cDNA libraries for deep sequencing (Tagami et al. 2010; Lu et al. 2007). Moreover, in application such as generation of an RNA aptamer, which is less than 100 bp by SELEX, it is imperative to have a suitable purification method to guarantee the production of the RNA aptamer having high specifity and affinity to the corresponding target molecules.

There are several methods described to obtain highly purified small RNA such as the crush and soak method, electroelution using a dialysis bag, electroelution onto cellulose membrane (Sambrook et al. 1989), electrophoresis of

RNA molecules onto diethylaminoethyl paper (Vorndam and Kerschner 1986; Zassenhaus et al. 1982) centrifugal filtration through filter membranes (Zhu et al. 1985) or siliconized sterile glass wool (Heery et al. 1990). Methods such as crush and soak, which involves the elution of RNA fragments into elution buffer (300 mM Sodium Acetate, 1 mM EDTA (pH 8.0)) requires incubation at 37°C overnight, which is very time-consuming and can cause degradation of the RNA molecules. On the other hand, electroelution methods, which are based on the ability of RNA molecules to be retained within a dialysis membrane also consume time and are tedious, whereas the use of cellulose membrane or diethylaminoethyl paper in purifying RNA is expensive. Futhermore, phenol–chloroform, which effectively removes protein to give a protein-free RNA mixture, is sometimes not completely removed, and can interfere with downstream applications and is not effective in purifying small RNA molecules. In order to obtain a good yield of RNA with the minimal degradation possible, the steps involved should be very short, as RNA is unstable due to the reactivity of the 2′-OH group, leading to the generation of 2′, 3′-cyclic monophosphate, responsible for the degradation of the RNA (Wiegand et al. 1996).

Thus, the critical factor towards attaining a highly purified small RNA is very significant to ensure the success of molecular biology techniques such as quantitative real-time PCR (qRT-PCR), which is very much dependent on RNA with good integrity and purity. The RNA must be of good quality to avoid enzyme inhibition and inhibition imparted by the residual contaminants (Bilgin et al. 2009). Consequently, the choice of RNA elution and purification is crucial to ensure quality of the RNA obtained is good. In this study, a very simplified method is reported in purifying small RNA (20–350 bp) where there is no usage of special equipment, reducing the time of purification up to 90 min while eradicating the need for hazardous reagents such as phenol–chloroform and ethidium bromide, enabling the purified RNA to be used in subsequent downstream applications immediately. Illumination of nucleic acid RNA under the wavelength of 254 nm enables the visualization of nucleic acid (up to 300 ng) due to the absorbance by the ring system in nitrogen bases, thus obviating the need to use ethidium bromide. The RNA visualized appears as a purplish band on a white background on the silica-coated glass plate. Not staining with eithidium bromide has the advantage of maintaining RNA in the native state, which is imperative in subsequent downstream application. The aim of this investigation was to develop a rapid and cost-effective method for purifying small RNA. Different elution temperatures for purifying RNA from polyacrylamide gel were taken into consideration. The integrity and quality of the purified RNA were tested using Reverse Transcription-PCR (RT-PCR).

Materials and methods

Reagents

The template used in the PCR reactions was the ethanol-precipitated PCR product obtained from the third cycle of SELEX performed on the target protein, Protein A (Sigma, USA). Primers were purchased from Biobasic Inc. (Toronto, Canada). Avian myeloblastosis virus (AMV) reverse transcriptase was obtained from Promega while Ampliscribe T7 transcription kit was purchased from Epicentre Technologies, Madison, WI.

Polymerase chain reaction (PCR)

The starting single-stranded (ss) DNA pool template used for the SELEX experiment is 5′-AGC TCA GCC TTC ACT GC(N40) GGC ACC ACG GTC GGA TCC AC with the forward primer, 5′-TCT AAT ACG ACT CAC TAT AGG AGC TCA GCC TTC ACT GC-3′ and the reverse primer, 5′-GTG GAT CCG ACG GTG GTG CC-3′. PCR was carried out in 300 µl of reaction using 30 ng of starting template in 1 × PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl) containing 1.5 mM $MgCl_2$, 200 µM each of four dNTPs (dATP, dGTP,dCTP and dTTP) and 7.5 U Taq DNA polymerase (MBI Fermentas, Lithuania). PCR conditions were an initial denaturation at 95°C for 1 min followed by 25 amplification cycles (45 s denaturation at 94°C; 1 min annealing at 55°C; and 1 min elongation at 72°C) and final elongation at 72°C for 5 min. These PCR products were ethanol precipitated to a final volume of 8 µl and subjected to in vitro transcription.

In vitro transcription and electrophoresis

The PCR product, ethanol-precipitated to a final volume of 8 µl was used directly for an in vitro transcription reaction which would yield RNA having the length of 77 nt. In vitro transcription was carried out using Ampliscribe T7 transcription kit (Epicentre Technologies, Madison, WI). The reaction mixture assembled in 60 µl contains 1 × Ampliscribe T7 reaction buffer, 7.5 mM each of ATP, CTP, UTP, GTP, 10 mM of dithiothreitol (DTT), 60 units of AmpliScribe™ T7 RNA polymerase enzyme (10 units/µl) and 1.5 units of RiboGuard RNase Inhibitor (1 unit/µl). The reaction mixture was incubated at 37°C for 3 h and to it was added an equal volume of 2 × RNA loading dye. This mixture was heated at 95°C for 2 min, cooled on ice and loaded onto 10%, 7M urea-denaturing polyacrylamide gel (PAGE). Electrophoresis was carried out at 140 V for 30 min.

Using different amounts of starting ethanol-precipitated PCR product, ranging from 300, 350, 400, 450 and 500 ng, in vitro transcription reactions were set up for 3 h and the transcripts were consequently purified from 10%, 7M urea-denaturing PAGE. Following loading onto 10%, 7M urea-denaturing PAGE, electrophoresis was carried out at 140 V for 30 min. Upon completion of electrophoresis, one of the glass plates was removed carefully and the gel was covered with a clean plastic wrap. The gel was placed upside down on a flat surface and removed slowly from the glass plate. The gel covered with plastic wrap was placed on top of the silica coated glass plate and visualized by using hand-held u.v. light source. Band of interest visualized as a single prominent band under low-intensity u.v. (254 nm) shadowing on the surface of the silica gel-coated glass plate was excised using a clean razor blade into an 1.5 ml Eppendorf tube and the gel slices were then crushed into tiny particles by using 1 ml pipette tips carefully so as not to spill the gel particles out of the Eppendorf tube.

RNA elution and ethanol precipitation

The crushed gel particles was added with 400 µl of sterile RNAse and DNAse-free water, heated at 50°C for 30 min, followed by the addition of 1 ml of 100% ethanol into the mixture which is then shaked vigorously. The mixture was then centrifuged at maximum speed for 1 min, followed by the collection of supernatant into a fresh microcentrifuge tube and added with 40 µl of 3 M NaOAc, pH 5.2. This mixture was then incubated at −80°C for 15 min, centrifuged at maximum speed at 4°C for 10 min and the supernatant was decanted. After rinsing with 1 ml of 70% ethanol, the pellet was air-dried for 3 min and reconstituted in 25 µl of water followed by concentration measurement. A few microliters of the purified RNA was run again on the 10%, 7M urea-denaturing PAGE gel to check for RNA integrity (Figs. 1, 2).

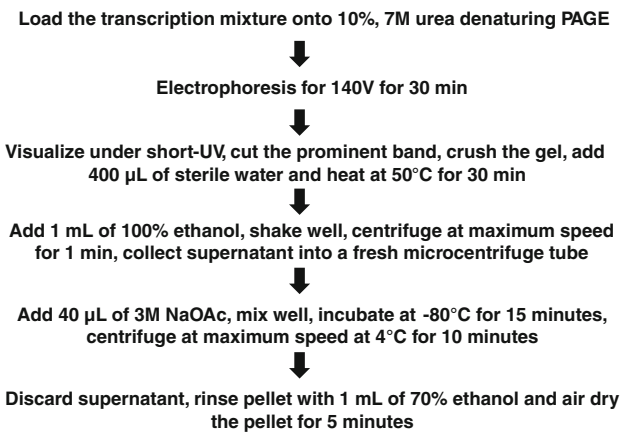

Fig. 1 Flow chart of the RNA purification from polyacrylamide gel

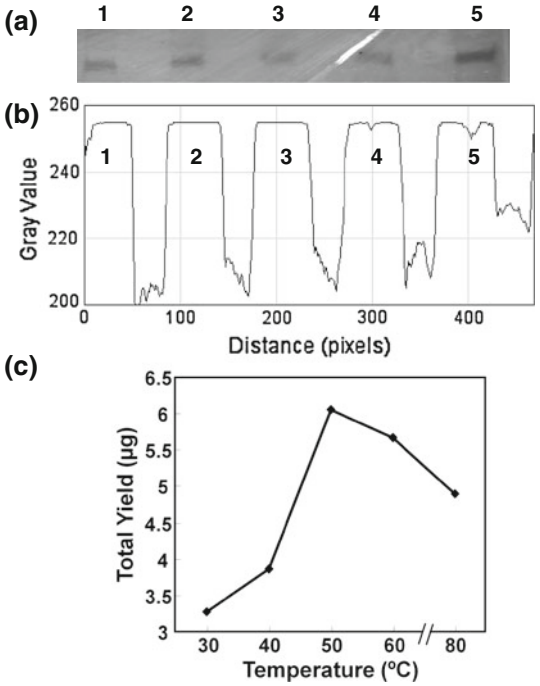

Fig. 2 **a** Loading of the in vitro transcription mixture into 5 different lanes to be eluted at different temperatures. *Lane 1*: 30°C, *Lane 2*: 40°C, *Lane 3*: 50°C, *Lane 4*: 60°C and *Lane 5*: 80°C. **b** Plot profile Image J Analysis of the transcript bands of these 5 different temperatures. **c** The *graph* shows the total yield of the purified RNA at each elution temperature

Elution of small RNA at different temperatures

Elution of RNA was also carried out at different temperatures of 30, 40, 50, 60 and 80°C for 30 min. Following elution, the RNA molecules were recovered as explained and the concentration was measured. Additionally, two sets of elution were carried out, which was at 37°C for 16 h and 55°C for 30 min, followed by purification and concentration measurement.

Reverse transcription-PCR

400–500 ng of purified RNA from each of the corresponding elution temperatures were subjected to RT–PCR. These RNAs were denatured in 16.3 µl of the reverse transcription mixture (1 × reverse transcriptase buffer, 0.4 mM reverse primer) at 95°C for 2 min, followed by cooling to room temperature for 5 min. This was followed by the addition of 3.2 µl of 10 mM dNTP and 0.5 µl of 20 unit/µl of Avian Myeloblastosis Virus (AMV) Reverse Transcriptase enzyme. These mixtures were then incubated at 42°C for 30 min, followed by PCR amplification for 20 cycles, using the PCR parameters mentioned above.

Results and discussion

Small RNAs are associated with the members of Argonaute (Ago) family, comprising two subfamilies: Piwi and Ago, known to perform various functions including mediation of mRNA stability and translation as well as the targeted epigenetic modification of the specific region of the genome (Finnegan and Matzke 2003). Small interfering RNAs target complementary RNA for translational inhibition or transcript cleavage (Tang 2005). Owing to the astounding roles that these small RNAs play in gene regulation, they have become the subject of extensive research. Since they are small in size and easily discernible from the larger fragments of RNA, acquiring these small RNAs is made possible by purification of size-fractionated total RNA from the denaturing polyacrylamide gel. In this study, it has been demonstrated that sufficient yield of the small RNAs can be obtained employing the method described.

RNA degradation is one of the critical problems in all experimental settings including purification of RNA from complex mixture (Opitz et al. 2009), as RNA is very susceptible to chemical and enzymatic degradation (Tenhunen 1989). Susceptibility of the RNA to degradation can be explained by the reactivity of the 2′-OH group that acts as intramolecular nucleophile, attacking the neighbouring phosphorus atom to form a pentacoordinated intermediate. This is followed by the removal of 5′-OH group from this intermediate to form 2′, 3′-cyclic monophosphate. Following hydrolysis of this cyclic monophosphate intermediate, RNA hydrolysis is completed. However, this phenomenon is very different from the hydrolysis of DNA that depends on the intermolecular nucleophilic attack on the phosphorus atom, making RNA hydrolysis faster and efficient compared to DNA hydrolysis. Therefore, additional care and precautions have to be taken to minimize the degradation of RNA.

Temperature and pH, which influence the reactivity of the intramolecular nucleophilic 2′-OH group are the most important factors that facilitate the degradation of RNA. In this study, sterile RNAse and DNAse-free water was chosen as the medium of RNA elution, as the pH is around 7–7.5, maintaining the reactivity of the 2′-OH at very low level, and to ensure the absence of other metal ions such as Mg^{2+} that can mediate the cleavage of RNA, promoting RNA degradation (Breslow and Huang 1991). In this experiment, since the RNA product has the size of 77nt, this RNA product was used as the experimental subject to mimic the elution of small RNAs from denaturing polyacrylamide gel which are within the range of 21–26 nucleotides long. To examine the influence of temperature on elution of small RNA from denaturing polyacrylamide gel, an attempt has been made to carry out elution at different temperatures such as 30, 40, 50, 60 and 80°C. The PCR product which has been in vitro transcribed was then loaded into 5 different lanes of 10%, 7M urea-denaturing PAGE. Image J analysis of the intensity of the transcript bands in these lanes were carried out and showed an average arbitrary value between 200 and 230 from the point of scanning for all the corresponding elution temperatures. In addition, the average peak areas cover the values of 50–60 (Fig. 2a, b).

Since the initial amount of RNA was not known and constituted by the value of Image J analysis, the Image J value (arbitrary value) of the elution temperature 80°C, which is the highest was considered as X and all the other Image J values for the rest of the elution temperatures were equalized using the value X as the reference value. Equalization of these values is very important, as the amount of initial transcripts are not equal, which were implicated by the Image J values itself, so as to determine the total yield of RNA eluted out of the initial transcribed products for all the corresponding elution temperatures. After calculations were made, the amount of RNA obtained for each elution temperature of 30, 40, 50, 60 and 80°C were 3.28, 3.86, 6.05, 5.67 and 4.90 μg respectively (Table 1). This analysis showed that the elution temperature of 50°C was the best choice. This was further substantiated by the graph of the total yield of purified RNA for each elution temperature versus the elution temperature, in which the total yield of the recovered RNA increased up to the elution temperature of 50°C, but decreased, with another further increase of 10°C up to 80°C (Fig. 2c). Even though there was not much difference between the elution temperatures of 30 and 40°C, a drastic increase of RNA yield was observed after the temperature exceeds 40°C, indicating that the ideal range of RNA yield is between the range of 40 and 50°C. This is also further attested by the yield of RNA obtained after the temperature of 40°C. Hence a temperature range between 40 and 50°C is amenable in eluting small RNAs applicable for most of the subsequent downstream applications that entail sufficient RNA.

To corroborate the integrity and quality of the purified small RNA, around 300–500 ng of the purified RNA from each of the elution temperatures was subjected to RT–PCR reaction followed by 20 cycles of PCR. From Fig. 3, it was shown that there were single discrete bands at the expected size of 98 bp, suggesting that all of these purified RNAs were of good quality, suitable for downstream applications. However, though no significant degradation was visible at the elution temperature of 80°C, elution at this temperature is not advisable. The polyacrylamide gel particles can be a source of the trace amounts of metal ions such as metal ion hydrate, drawing a proton from 2′-OH group, inducing anionic attack of the $2'\text{-}O^-$ on the phosphorus atom, promoting degradation of RNA. Temperature as high as 80°C

Table 1 Total yield of RNA for each different elution temperature, Image J values, Equalized values of Image J and the corrected yield of RNA

Elution temperature (°C)	Yield (μg)	Image J value	Equalized image J value	Corrected yield of RNA
30	4.00	205	0.8193X	3.28
40	4.22	210	0.9130X	3.86
50	6.38	218	0.9478X	6.05
60	5.98	218	0.9478X	5.67
80	4.90	230	X	4.90

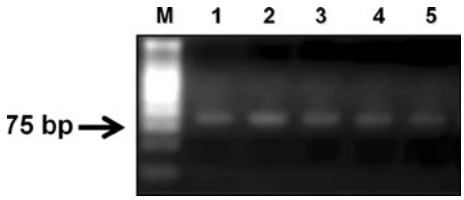

Fig. 3 5 μl of the RT–PCR product from each different elution temperatures was electrophoresed on 4% agarose gel. *Lane M*: 20 bp DNA ladder, *Lane 1*: 30°C, *Lane 2*: 40°C, *Lane 3*: 50°C, *Lane 4*: 60°C and *Lane 5*: 80°C

will definitely increase the hydrolysis rate of the hydrated metal ion on the 2′-OH group, leading to faster degradation at very high temperature. Moreover, 80°C is the melting temperature of most RNA molecules, which can induce single strand cleavage of the RNA in the presence of Mg^{2+}. The increase of the rate of hydrolysis of RNA fragment in accordance to the increase of the temperature of up to 80°C also accounts for the decrease in the yield of the total purified RNA observed after the elution temperature of 50°C.

Investigation of the time-dependent elution of small RNA authenticated that a considerable amount of small RNA can be obtained by carrying out elution at 50°C for 30 min (1.12 μg). However, an increase in the elution time up to 12 h at a much lower temperature of 37°C resulted in a final yield of 2.12 μg (Table 2). It can be concluded that out of the starting material of 5.94 μg, a considerable amount of small RNA can still be obtained by carrying out the elution of small RNA at 50°C for 30 min. This reaction can also be scaled up by increasing the starting amount of RNA up to 20–30 μg to compensate for the loss of RNA as a result of elution from polyacrylamide gel. This was attested by the rise in the amount of purified RNA which corresponds to an increase of the purified PCR product from 350 ng to 500 ng (Table 3). Though overnight elution yielded a greater amount of RNA compared to the elution carried out at 50°C for 30 min, taking into consideration the time factor, elution at 50°C is also very suitable in recovering small RNAs from polyacrylamide gel. This will also greatly minimise the predisposition of the RNA eluted from polyacrylamide gel to possible degradation at longer incubation temperature. Checking of a few aliquots of these purified RNAs on the 10%, 7M urea denaturing PAGE also revealed that both of these RNAs were in good and comparable quality, further validated by the 3D-plot profile of Image J analysis on the bands observed on the polyacrylamide gel, showing an equal level of intensity with sharp bands (Fig. 4a, b).

This purification method greatly reduces the long hours of time spent on the incubation period with the elution buffer and immensely cut the cost involved with the usage of dialysis tubing in purifying RNA. The mere heating of the finely crushed gel particles with sterile water increases the surface area of contact between the gel particles and water, facilitating the accelerated diffusion of the majority of small RNA molecules from the gel to the water, which can be obtained by simple ethanol precipitation method. This also averts the prerequisite for any special buffer which contains metal ions that can promote the degradation of RNA. Moreover, this technique eliminated the need to stain the polyacrylamide gel with ethidium bromide, which is a potent mutagen and proved to interfere with the functions of DNA polymerase and RNA polymerase via intercalation with the template (Eliott 1963; Waring 1964). Owing to its sensitivity, u.v. shadowing enables

Table 2 Total yield of RNA eluted at 37°C for 16 h and 50°C for 30 min

Elution temperature	Amount of RNA input for purification (μg)	Conc. (ng/μl)	Yield (μg)
37°C, 16 h (Overnight)	5.94	212 ng/μl (10 μl)	2.12
50°C for 30 min	5.94	112 ng/μl (10 μl)	1.12

Table 3 Amount of RNA obtained following purification of the in vitro transcribed RNA using different amounts of starting DNA template

Amount of DNA input for in vitro transcription (ng)	RNA purified from polyacrylamide gel (μg)
300	10
350	11.5
400	20
450	22.5
500	30

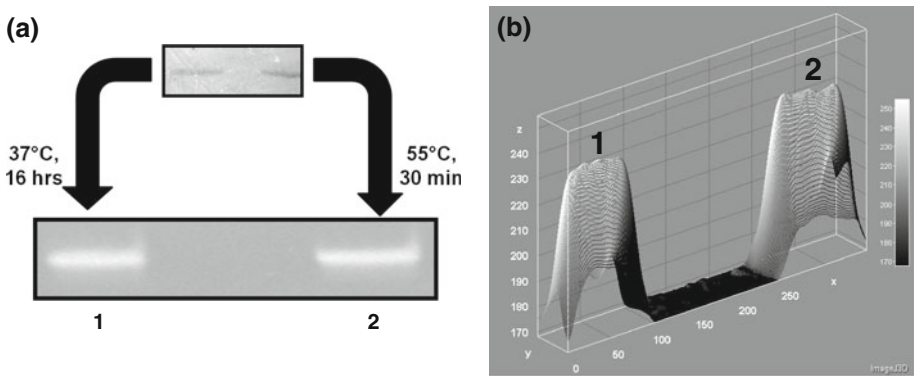

Fig. 4 **a** 5 µl of the purified RNA at two different elution conditions loaded onto 10%, 7M urea denaturing polyacrylamide gel. *Lane 1*: elution at 37°C for 16 h, *Lane 2*: elution at 50°C for 30 m **b** Plot profile of Image J analysis of these purified RNA at two different elution conditions

visualization of low amount of nucleic acid under the u.v. wavelength of 254 nm, which can also exhibit contaminating truncated transcription products, enables precise excision of the desired band appearing as a dark purplish colour, leading to selective purification of the RNA (Fig. 2a) (Farrell 2010). This selective excision of the band of interest is enhanced by the use of the silica gel-coated glass plate, which provides a platform that fluoresces more strongly than the glass plate, giving greater sensitivity for detecting RNA shadow upon illumination with the u.v. light. Moreover, the use of phenol–chloroform which is highly hazardous in nature, can also be avoided (Swati et al. 2008). Urea as an effective chaotropic and protein-solubilizing agent denatures proteins such as T7 RNA polymerase enzyme, which is present in the in vitro transcription mixture, obviating the need to use phenol–chloroform in removing protein (Raspi et al. 1998).

This method is also very amenable for purifying small RNA as low as 2–3 µgs from polyacrylamide gel, which is far in excess of that required for most molecular biology applications and proved to greatly reduce the time of experiment such as SELEX, where each round of the SELEX cycle can be carried out in a day. This method proved to be very useful in purifying small RNA molecules, single stranded oligonucleotides and also chemically synthesized deoxyribonucleotides. In addition, this protocol can be applied to purify small RNA labelled at the ends. The RNA obtained after purification using this method is stable after prolonged storage at −20 and −80°C.

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