

Rapid preparation of RNA samples for NMR spectroscopy and X-ray crystallography

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ABSTRACT

Knowledge of the three-dimensional structures of RNA and its complexes is important for understanding the molecular mechanism of RNA recognition by proteins or ligands. Enzymatic synthesis using T7 bacteriophage RNA polymerase is used to prepare samples for NMR spectroscopy and X-ray crystallography. However, this run-off transcription method results in heterogeneity at the RNA 3'-terminus. For structural studies, RNA purification requires a single nucleotide resolution. Usually PAGE purification is used, but it is tedious, time-consuming and cost ineffective. To overcome these problems in high-throughput RNA synthesis, we devised a method of RNA preparation that uses *trans*-acting DNAzyme and sequence-specific affinity column chromatography. A tag sequence is added at the 3' end of RNA, and the tagged RNA is picked out using an affinity column that contains the complementary DNA sequence. The 3' end tag is then removed by sequence-specific cleavage using *trans*-acting DNAzyme, the arm lengths of which are optimized for turnover number. This purification method is simpler and faster than the conventional method.

INTRODUCTION

The increasing interest in the structure and function of RNA has created the demand for a high-throughput RNA purification method. For example, X-ray crystallography and NMR spectroscopic studies require milligram quantities of pure RNA in order to obtain useful structural information. The bacteriophage protein T7 RNA polymerase is usually used for *in vitro* transcription reactions using a synthetic DNA template (1). However, T7 RNA polymerase requires a sequence that starts with G, and some sequences in the first six residues do not give an acceptable yield of RNA transcripts (1,2).

RNA purification requires single-nucleotide resolution to separate the transcript of the correct length (N) from aborted

($N-1$) or 'add-on' ($N+1$, $N+2$) transcripts, which are usually present in comparable amounts. In most cases, PAGE is used to resolve the correct RNA transcript followed by size-exclusion liquid chromatography if required. However, the loading capacity of the conventional slab PAGE gel is ~ 60 OD, so the protocols used to obtain milligram quantities of pure RNA for NMR studies require at least four runs on $40 \times 60 \times 0.3$ cm³ polyacrylamide gels. In addition, PAGE purification of RNA is tedious, time-consuming and cost ineffective.

Immobilized oligonucleotides are widely used in molecular biology, clinical analysis and other biotechnological fields that require the identification of specific DNA sequences (3). Most of these applications are based on hybridization between the immobilized oligonucleotide and complementary sequences in a sample. By constructing columns with a single-stranded polynucleotide covalently attached to the support, the column retains RNA or DNA that can base pair with the attached sequence. This sequence-specific polynucleotide separation technique is called DNA affinity chromatography. The ideal approach for DNA immobilization is covalent binding on a solid surface, via a single-point attachment (4).

The type of DNAzyme described here was developed by Santoro and Joyce (5) through *in vitro* selection. These molecules are DNA oligonucleotides with a small catalytic core that anneals to RNA targets by hybridizing two flanking arms of 7–13 nt, which each depend on the sequence. On the addition of Mg²⁺ or other divalent ions, the DNAzyme cleaves the RNA specifically at the designated purine–pyrimidine junction. A highly reactive catalytic core has been optimized and kinetically characterized in elegant studies (5–7). DNAzyme can be targeted to cleave many different RNA sequences efficiently under multiple-turnover conditions (6).

In this study, we describe a high-throughput method of preparing RNA samples using DNA affinity chromatography and DNAzyme. To make the purification steps simple and fast, we used sequence-specific affinity column chromatography and a *trans*-acting DNAzyme. The affinity column chromatography was used to separate abortive transcripts from the correct transcript. The *trans*-acting DNAzyme eliminated the 3'-heterogeneity caused by add-on transcripts. This new method is highly efficient and reliable; 5 mg quantities of an RNA sample can be prepared in three days.

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MATERIALS AND METHODS

Oligonucleotide preparation

DNAzyme, complementary DNA oligonucleotides and DNA templates for the T7 RNA polymerase transcription reaction were obtained from Alpha DNA (Canada) and used without further purification. An RNA oligonucleotide, 5'-GAGAG-UGCUGAUACUGGCCUCUGUAAGAAGCCCUUCAG-3', was synthesized by *in vitro* transcription with T7 RNA polymerase using unlabeled NTPs and ^{13}C , ^{15}N -labeled UTP (1). T7 RNA polymerase and the labeled NTPs were prepared using previously published methods (8,9). The affinity resin was synthesized chemically on 1 g of Oligo Affinity Support (Glen Research). After synthesis, the oligo resin was deprotected in ammonium hydroxide. The concentration of each substrate RNA and DNAzyme was determined by the absorbance at 260 nm.

Affinity column

The affinity resin was packed into columns for affinity chromatography. The column was equilibrated with five column volumes of binding buffer (250 mM NaCl, 20 mM sodium phosphate buffer, pH 7.0). The synthesized RNA sample was loaded onto the affinity column and incubated at room temperature for 1 h. The column was washed with five column volumes of binding buffer. The bound RNA was eluted with the elution buffer (20 mM sodium phosphate buffer, pH 7.0). The column was regenerated for the next use by rinsing it several times with elution buffer followed by equilibration with binding buffer.

DNAzyme reaction

The substrate RNA was combined with DNAzyme in 0.1× reaction buffer (15 mM NaCl, 4 mM Tris-HCl, pH 7.5). The solution was heated at 95°C for 3–4 min to denature the RNA and DNA, and then placed on ice for 5 min. The sample was then incubated at 25°C for 10 min. The cleavage reaction was carried out in 150 mM NaCl, 40 mM Tris-HCl (pH 7.5) at 37°C. The reaction was initiated by adding MgCl_2 to give a concentration of 60 mM. After the reaction, the DNAzyme DNA was eliminated by digestion with RNase-free DNase I (Promega) followed by size-exclusion chromatography using Superdex 75 (Amersham Biosciences). The products were analyzed by electrophoresis on a 15% polyacrylamide-7 M urea denaturing gel.

NMR spectroscopy

The purified RNA sample was dialyzed extensively against 10 mM sodium phosphate/0.01 mM EDTA (pH 6.8). It was then freeze-dried and dissolved in 0.2 ml of 99.96% D_2O . NMR spectra were recorded on a Bruker DMX600 MHz spectrometer, and processed using XWINNMR 3.1 (Bruker). A two-dimensional (2D) ^1H - ^{13}C constant time heteronuclear single quantum correlation (ct-HSQC) (10) spectrum was recorded for the ^{13}C , ^{15}N -UTP-specific labeled RNA sample at 303 K.

RESULTS

High-throughput RNA sample preparation

The procedure used for high-throughput RNA sample preparation is illustrated in Figure 1. The *in vitro* transcription procedure using T7 RNA polymerase and synthetic DNA template has been reported previously (1). After *in vitro* transcription, the reaction mixture was desalted on a NAP-25 column (Amersham Biosciences). Affinity column chromatography was used to separate abortive transcripts from correct length and add-on transcripts. The transcribed RNA, which contains an affinity tag located at its 3' end, binds to the complementary oligonucleotide on the affinity column. Abortive transcripts were washed away because they do not have the affinity tag and cannot bind to the affinity column. The RNA with the affinity tag was collected at the final elution step. The RNA was sequence-specifically cleaved using the *trans*-acting DNAzyme to remove the affinity tag. The substrate RNA and DNAzyme DNA were incubated at 37°C for 3 h. The cleaved affinity tag was removed using the affinity column once more. The target RNA was released into the flow-through with the DNAzyme DNA, whereas the affinity tag bound to the column. The DNA was digested with DNase I for 1 h. The final products were purified using size-exclusion chromatography.

SLI RNA purification

We used our method to synthesize the SLI domain of the 3'-non-translated region of the hepatitis C virus RNA

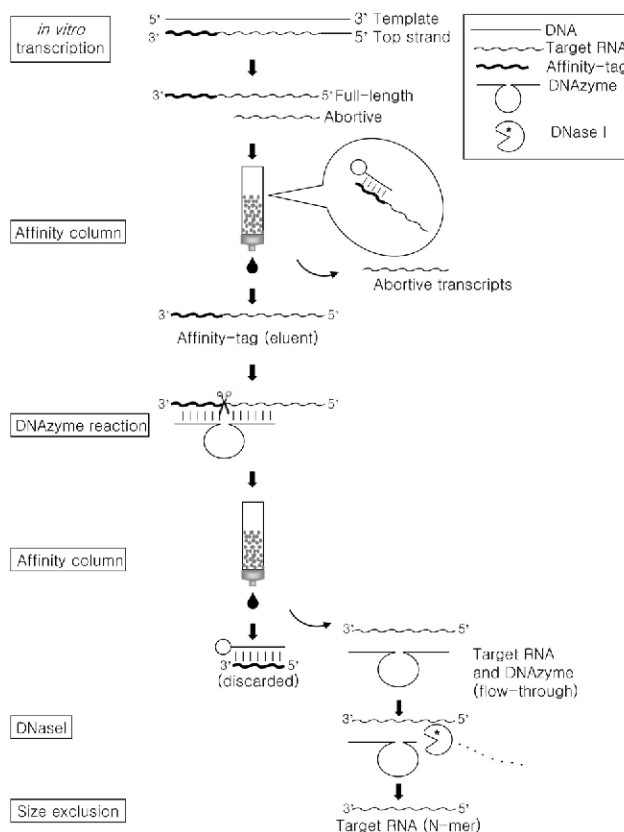


Figure 1. Schematic protocol for preparing high-throughput RNA samples.

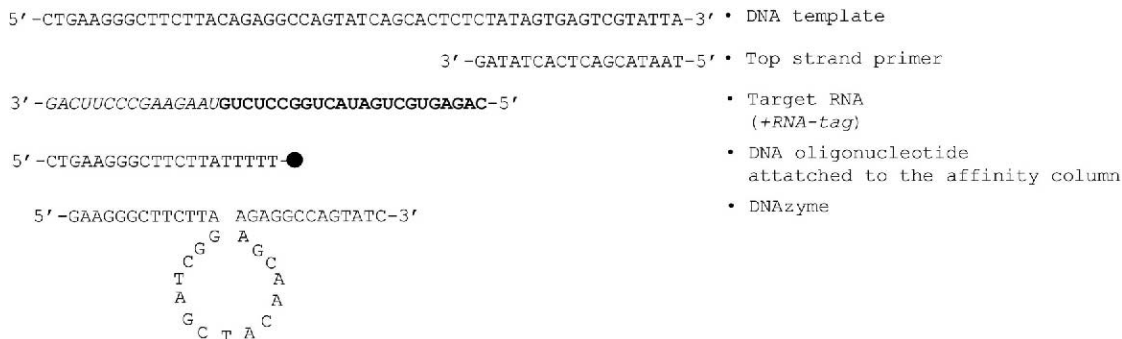


Figure 2. The sequence of the oligonucleotides used to prepare an RNA sample.

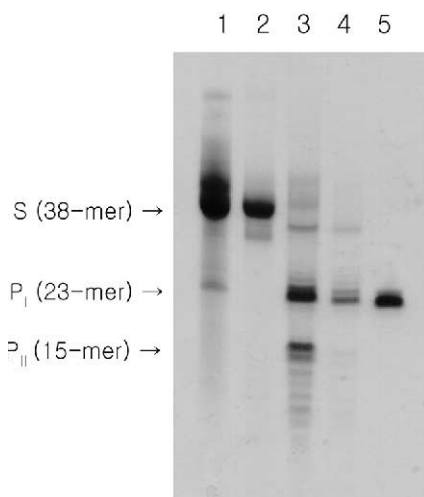


Figure 3. Purification of RNA with an affinity column and DNAzyme. Lane 1, crude RNA transcription mixture; lane 2, RNA purified using the affinity column; lane 3, the DNAzyme reaction products; lane 4, DNAzyme and product I purified using the affinity column; and lane 5, RNA purified using size-exclusion chromatography. The products were separated by electrophoresis on a 15% polyacrylamide-7 M urea denaturing gel and were detected using toluidine blue.

genome (11). The SLI domain is believed to function in the initiation and regulation of viral RNA replication by interacting with components of the viral replicase complex (12,13). Figure 2 shows the sequences of the target RNA, DNA template, top strand, the oligonucleotide attached to the affinity column and DNAzyme.

The results of high-throughput RNA sample preparation are shown in Figure 3. Lane 1 shows the crude transcription mixture containing the hepatitis C virus target RNA. This mixture includes the DNA template, primer, mono- or triphosphate ribonucleotides, T7 RNA polymerase, reaction buffer and RNA transcription products, which contain the full-length RNA, a number of abortive transcripts and possibly add-on transcripts ($N + 1$ or $N + 2$).

As shown in Figure 3, lane 2, the tagged RNA was picked out by the affinity column, which bound the complementary DNA sequence. Abortive transcripts were washed away.

The affinity tag was removed by sequence-specific cleavage using the *trans*-acting DNAzyme. The arm lengths of the DNAzyme were optimized to 13 nt each for the maximum turnover number under the reaction conditions. The ratio of

DNAzyme to substrate RNA is 1:10. Figure 3, lane 3, shows the composition of the cleavage mixture obtained after a 3 h incubation of the RNA substrate and DNAzyme. The 38 nt RNA substrate was cleaved into two products: the 23 nt target RNA and 15 nt affinity tag. The DNAzyme cleaved the substrate at the predicted site.

The cleavage mixture was separated using the affinity column once more (Figure 3, lane 4). The flow-through of the affinity column included the target RNA and DNAzyme DNA, while the affinity tag was retained in the column. The DNAzyme was removed by DNase I, followed by size exclusion. After the final size-exclusion chromatography, the product was very pure, >99%, as shown in Figure 3, lane 5. The efficiency and reproducibility of this method should greatly facilitate rapid purification of RNA in milligram quantities.

NMR spectroscopy

Preliminary NMR spectroscopy demonstrated that the ^{13}C , ^{15}N -UTP-specific labeled nucleotides were incorporated into the sample and that the sample was adequately concentrated. The 23 nt RNA of the SLI domain contains six uracils. In the 2D ^1H - ^{13}C HSQC spectrum, there should be 12 peaks corresponding to the six uracil C5s and C1's. Figure 4 clearly shows six uracil C5s with ^{13}C chemical shifts at 85–88 ppm and six C1's at 72–76 ppm. There is one broad uracil C1' peak ~ 75 ppm, which suggests that the residue is in a dynamic environment.

DISCUSSION

T7 RNA polymerase is very amenable to large-scale RNA synthesis, and can be obtained readily in large quantities by using overexpression and purification techniques (1). RNA is synthesized by *in vitro* transcription using T7 RNA polymerase. However, RNA purification suffers from heterogeneity at the 3' end, which can be a serious matter for NMR and X-ray crystallography. One method uses the hepatitis delta virus (HDV) ribozyme to produce *in vitro* transcripts with a homogeneous 3' end (14). In this study, we added an affinity tag at the 3' end. The affinity tag was cleaved using DNAzyme, which is more convenient and cost-effective than ribozyme. The cleavage reaction is very efficient. There is only one requirement for a specific sequence upstream of the reaction site: there must be a purine at the 3' end.

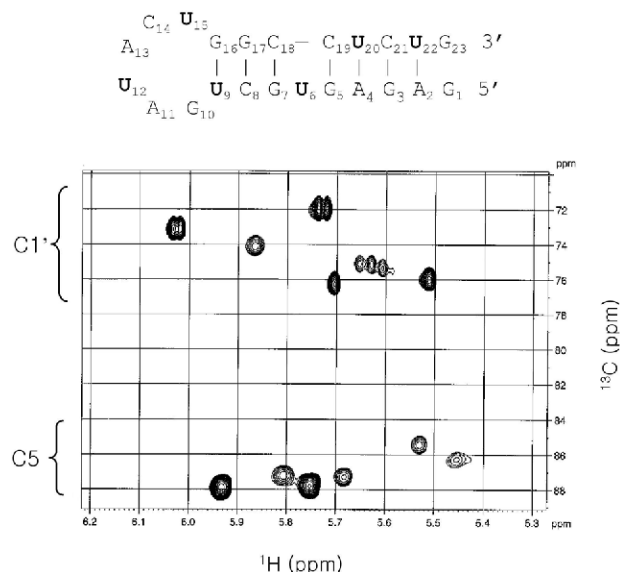


Figure 4. A section of the constant-time ^1H - ^{13}C HSQC spectrum of the ^{13}C , ^{15}N -UTP-specific labeled RNA recorded at 30°C . The concentration of RNA is 0.8 mM. The sample is in 99.96% D_2O solvent and 10 mM phosphate buffer (pH 6.8). The ^1H dimension is on the horizontal axis, and the ^{13}C dimension is on the vertical axis. The C1's of the uracils resonate at 72–76 ppm, and the C5s at 85–88 ppm. The ^{13}C , ^{15}N -UTP-specific labeled U residues in the 23 nt RNA are indicated in boldface type.

The most common approach to purifying RNA from a transcription mixture is based on PAGE. This technique comes at a high price in terms of time, labor, cost and toxicity. The separation itself may take hours and the final resolution may be inadequate. Preparative-scale electrophoresis is plagued by a loss of resolution due to the frequent practice of overloading. To overcome this, we devised the affinity purification of RNA transcripts followed by DNAzyme cleavage of the affinity tag. The affinity column synthesized using 1 g of Oligo Affinity Support contained ~ 40 μmol equivalents of oligo, which is sufficient for purifying 1 μmol of RNA transcripts. This method can also overcome polyacrylamide contamination of the RNA sample. In addition, the affinity column is reusable. Therefore, the affinity column system is less costly than PAGE per run. The affinity column method is convenient and efficient.

The first nucleotides at the 5' end of the RNA product are important in determining how efficiently the reaction will proceed. Typically, the sequence at the 5' end of the RNA begins with a guanosine and is purine rich (1). Therefore, RNA prepared with T7 RNA polymerase for use in structural studies often contains modifications of the 5' end sequence to maximize transcription yield, a compromise that sometimes must be avoided because of sequence effects on physical properties and structure. The 5' end sequence limitation can be overcome by using DNAzyme (15) or hammerhead ribozyme (16,17). We also added a 5' tag to increase the transcription yield and used DNAzyme to cleave the 5' tag (data not shown). Affinity purification could be used to separate the cleaved RNA products from the 5' tag.

We described a rapid purification method for RNA transcripts using an affinity column and DNAzyme. We showed that the method is very efficient and that it can be scaled up to

large quantities for NMR spectroscopy or X-ray crystallography. Our method is superior to PAGE in terms of time, safety and, most importantly, purity. Within three days, 5 mg quantities can be purified.

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