Ion-pair reverse-phase liquid chromatography analysis for separating and quantifying RNA generated via in vitro transcription reactions

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Run-off RNA transcripts of in vitro transcription reactions have traditionally been analyzed using denaturing polyacrylamide gel electrophoresis (PAGE) and detection of labeled-UTP incorporated into the transcripts. Although multiple products from in vitro transcription reactions can be detected, quantitative analysis of RNA transcripts by denaturing PAGE is difficult. Ion-pair reverse-phase high performance liquid chromatography (IP RP HPLC) is a high-resolution technique that can separate nucleic acids based on size. Using denaturing conditions, RNA run-off transcript samples of mixed sizes can be separated and quantified. IP RP HPLC improves the ability in detecting run-off transcription products, and establishes a better quantitative method for comparing transcripts of different length.

Introduction

The transcription of DNA to RNA via RNA polymerases is a fundamental process in cellular systems. In eukaryotes, we observe transcription in the nucleus (genomic DNA) as well as in the mitochondrial matrix (mitochondrial DNA). There are many tools available to investigate nuclear transcription; however, few tools exist to study mitochondrial transcription. Recently an in vitro transcription system using recombinantly expressed and purified mitochondrial transcription proteins has been developed (*1*). Ion-pair reverse-phase high performance liquid chromatography (IP RP HPLC) has long been used as a high-resolution technique in separating DNA based on size (*2,3*). *Our aim is to develop a method using IP RP HPLC to separate RNA based on size and quantify RNA transcripts generated from in vitro transcription systems*. Ultimately we would like to apply this method to the mitochondrial in vitro transcription system to quantitatively distinguish between promoter utilization.

Methods and Materials

All samples were run using a TMC Basic S-3 (4.6 x 150mm) column. The column was run on an LC-2010HT Liquid Chromatography System by Shimadzu.

To develop an RNA method for IP RP HPLC using our system, we used an RNA ladder (Sigma-Aldrich, RNA ladder 0.28-6.6kb) as shown in Figure 1. Samples consist of a 100 fold dilution in 0.25x TAE. All samples were heated at 65°C, then directly set in ice for one minute. The column was equilibrated at 90% solvent A and 10% solvent B at 60°C. Solvent A: 0.1M triethylammonium acetate (TEAA), in diethyl pyrocarbonate (DEPC) treated water, and solvent B: 0.07M TEAA with 25% acetonitrile (ACN). Run-off RNA transcripts were generated in vitro using T7 RNA Polymerase (Promega) and a linear DNA template generated from the pGEM Express Positive Control plasmid (Promega, see panel below). RNA transcript samples were analyzed using the same method developed for the RNA ladder (Figure 2). Varying amounts of DNA template were used with the T7 RNA Polymerase in vitro system to show quantification.

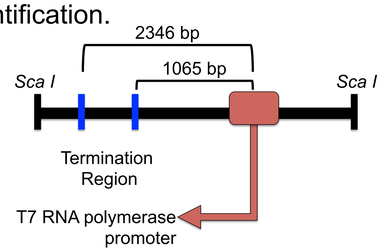
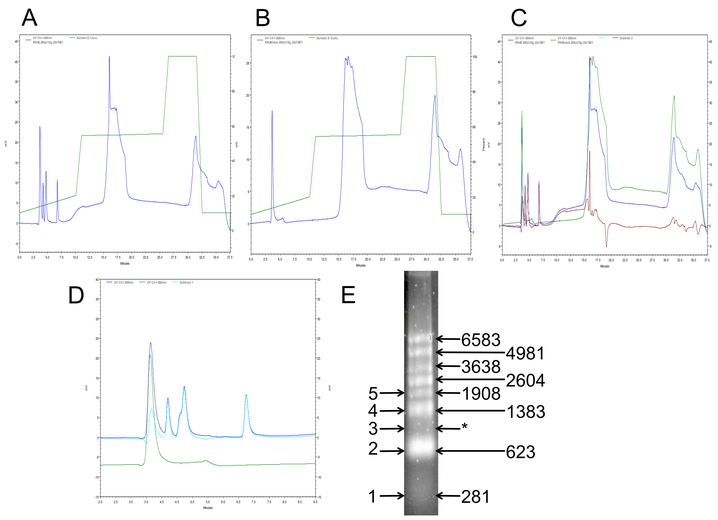


Figure 1: RNA Ladder Sample



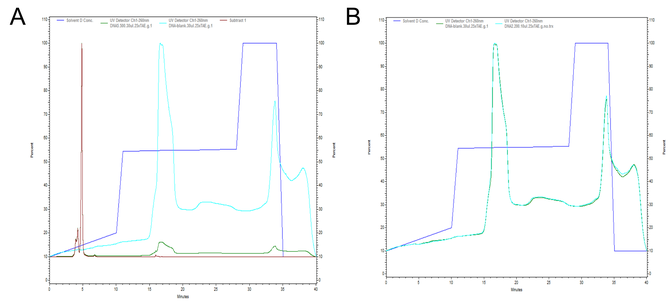
Panels A & B show absorption traces (A_{260}; blue line) and solvent B concentration (0.07M TEAA with 25% ACN; green line) vs. retention time (minutes). **A)** A 100 fold dilution of mixed size RNA in 0.25x TAE. **B)** Blank sample, 0.25x TAE. **C)** An overlay of the blank sample (green line) and the mixed RNA sample (blue line). The red line represents the difference between the two samples. Panel **D)** is an enlargement of the key peaks from retention time of 2 - 9 minutes as seen in panel C. The light blue line represents the difference between the RNA sample and the blank sample**.** ***Importantly, this indicates the mixed RNA sample can be separated based on size using IP RP HPLC***. Panel **E)** is an agarose gel separation of the mixed RNA sample. The right indicate the number of base pairs in each RNA, and the left represents the peaks seen in D corresponding to the light blue trace.

Quantification of RNA Run-off Transcript

Amount DNA Template	Area Under Curve	Normalized Area
4 μL	886298	0.82
8 μL	1076294	1.00
3 μL	741350	0.71
6 μL	860227	0.82
12 μL	1047468	1.00

Using peak area, RNA run-off transcripts can be quantified based on different amounts of DNA template with all other parameters held constant.

Figure 2: in vitro Transcription RNA Run-off Transcript

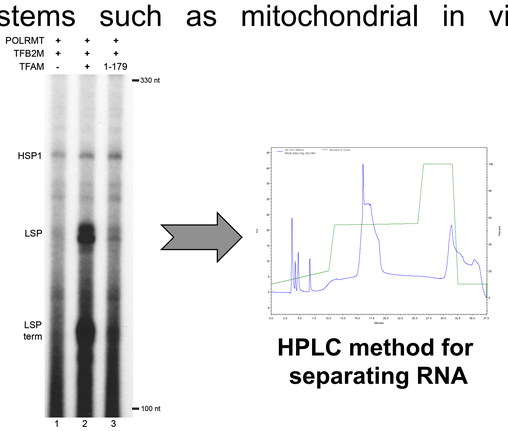


A) In vitro transcription RNA run-off transcript analysis. A_{260} of the in vitro generated RNA sample (green), the blank sample (no enzyme added, blue), and the red line represents the difference. ***These data indicate that RNA run-off transcripts can be separated in the size range of 281-1908 bp using this IP RP HPLC method***. **B)** In vitro transcription blank sample and DNA template overlay.

Future Directions

- Apply the developed IP RP HPLC method to additional in vitro transcription systems: other simple systems with alternate RNA polymerases; and more complex systems such as mitochondrial in vitro transcription.

Mitochondrial in vitro Transcription: Denaturing Acrylamide gel with ^{32}P-uTP for separating RNA transcripts



HPLC method for separating RNA

Acknowledgements

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References

1. Bestwick M.L. & Shadel G.S. (2013) *Trends. Biochem. Sci.* **38**, 283-91
2. Azarani A. & Hecker K.H. (2001) *Nuc. Acids Res.* **29**, e7.
3. Dickman M.J. & Hornby D.P. (2006) *RNA* **12**, 691-696
4. Wysoczynski C.L., et al. (2013) *Nuc. Acids Res.* **41**, e194.