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Purification of In Vitro Transcribed RNA using the SV Total RNA Isolation System

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Abstract

Purification of RNA following in vitro transcription is required to remove unincorporated nucleotides that can affect concentration determination, to remove the enzymes used in the reaction and to exchange buffers. Traditional methods of RNA purification from in vitro transcription reactions have involved the use of phenol/chloroform extraction or lithium/chloride precipitation. These methods are often inefficient, and the yield is highly variable. Here we investigate using the SV Total RNA Isolation System for purification of RNA following in vitro transcription.

The SV Total RNA Isolation System is attractive for purification of in vitro transcribed RNA, because it results in essentially pure RNA in water without the need for organic extractions or precipitations.

Introduction

The SV Total RNA Isolation System[a,b] was designed for isolation of high-quality RNA from tissue and cultured cells. This system uses a lysis buffer containing guanidine thiocyanate and β-mercaptoethanol to disrupt cells and precipitate cellular proteins away from RNA released into solution. Following centrifugation to remove cellular debris, RNA is ethanol precipitated and bound to the silica membranes in the spin column format. The membrane is treated with RNase-free DNase I to remove contaminating cellular DNA, and impurities are separated from the RNA during three washes. In a final step, the RNA is eluted from the column by applying RNase-free water (1).

For in vitro transcriptions, linearized template DNA that possesses either an SP6 or T7 DNA-dependent RNA polymerase promoter is combined in a reaction with ribonucleotides, transcription buffer, an enzyme mix containing RNA polymerase (SP6 or T7), ribonuclease inhibitor and pyrophosphatase. Incubation of this reaction at 37°C for more then 1 hour results in milligram quantities of RNA in a solution containing unincorporated nucleotides as well as residual proteins and DNA (2).

Many applications for in vitro transcribed RNA require that the RNA be purified away from reaction components. The SV Total RNA Isolation System is attractive for purification of in vitro transcribed RNA, because it results in essentially pure RNA eluted in water without the need for organic extractions or precipitations. In the experiments described here, we have tested a modified protocol of the SV Total RNA Isolation System for purification of in vitro transcribed RNA. In addition, we have investigated which buffers are required for this procedure.

RNA Purification

RNA was in vitro transcribed from linearized plasmid template using the RiboMAX™ T7 Large Scale RNA Production System[c,d,e,f] (Cat.# P1300) as described in the protocol (2). Aliquots of 50, 100, 200 or 400µl of the in vitro transcription reaction were dispensed to separate tubes. SV RNA Lysis Buffer (with β-mercaptoethanol), blue dilution buffer and 95% ethanol was added to each tube as listed in Table 1. No centrifugation was required after the addition of lysis buffer and blue dilution buffer because of the low level of proteins in the reaction.

Table 1. Volume of Buffers and Ethanol Added to In Vitro Transcription Reactions.

Transcription Reaction	Lysis Buffer	Blue Dilution Buffer	95% Ethanol
50µl	87.5µl	175µl	100µl
100µl	175µl	350µl	200µl
200µl	350µl	700µl	400µl
400µl	700µl	1,400µl	800µl

Immediately after addition of ethanol, the components were mixed by pipetting, and no more than 825µl was transferred to a spin column assembly. Spin columns were centrifuged using a Microfuge® 18 centrifuge (Beckman Coulter) at top speed (14,000 rpm) for 1 minute. The flowthrough was discarded, and this process was repeated until the entire volume was applied to the spin basket. Spin columns were washed twice by adding 600µl wash buffer containing ethanol and centrifuging at top speed for 1 minute. One final wash was performed using 250µl wash buffer and centrifugation at top speed for 2 minutes. The spin baskets were transferred to elution tubes, and 100µl RNase-free water was applied directly to the membrane. The assembly was incubated at room temperature for approximately 1 minute, and RNA was eluted by centrifugation at top speed for 1 minute. The elution step was repeated once using a fresh tube.

One microliter from each 100µl elution, along with 1µl of unpurified RNA, was analyzed by electrophoresis on a 1% formaldehyde gel (Figure 1). The gel is overloaded in the first elution from each volume of transcription reactions, but the RNA appears intact.

RNA concentrations and purity were analyzed using a DU® 520 spectrophotometer (Beckman Coulter) and a 1:100 dilution of the eluted RNAs (Table 2). The expected A_{260}/A_{280} ratio for pure DNA and RNA is 1.8 and 2.0 (3), respectively. The A_{260}/A_{280} ratio for these samples is less than the expected value and probably reflects the fact that the transcription reaction was not treated to remove DNA template.

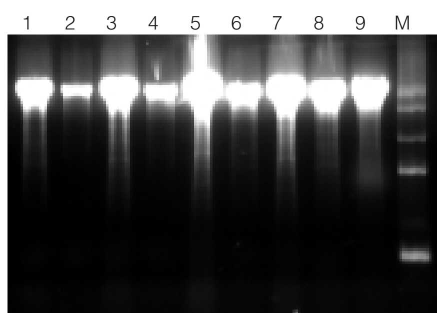

Figure 1. Formaldehyde gel analysis of RNA purified using the SV Total RNA Isolation System. One microliter of RNA was combined with 4µl RNA sample loading buffer (Sigma Chemical Co.) and incubated at 65°C for 10 minutes. After quenching on ice, the samples were loaded on a 1.4% agarose gel containing 1% formaldehyde and 1X formaldehyde gel running buffer (Geno Technology, Inc.). Lane 1, 50µl elution 1; lane 2, 50µl elution 2; lane 3, 100µl elution 1; lane 4, 100µl elution 2; lane 5, 200µl elution 1; lane 6, 200µl elution 2; lane 7, 400µl elution 1; lane 8, 400µl elution 2; lane 9, unpurified RNA; lane M, 0.24–9.5kb RNA ladder (Invitrogen).

Table 2. RNA Yield from In Vitro Transcription Reactions Following Purification with the SV Total RNA Isolation System.

Reaction Volume	Elution No.	A_{260}	A_{260}/A_{280}	Conc. (µg/µl)	Total RNA (µg)
50µl	1	0.647	1.9	2.6	260
	2	0.047	1.9	0.2	20
100µl	1	0.763	1.9	3	300
	2	0.429	1.9	1.7	170
200µl	1	0.887	1.9	3.5	350
	2	0.619	1.9	2.5	250
400µl	1	1.069	1.9	4.3	430
	2	0.733	1.9	2.9	290

elution volumes = 100µl

Nearly all RNA was recovered from 50µl of in vitro transcription reaction after only one elution with water. As the volume of in vitro transcription reaction applied to the column increased, the yield from the first elution increased only slightly, while a much greater yield was observed from the second elution. These results are clearly demonstrated on the formaldehyde gel. The yield from 100µl of transcription reaction was nearly double that of the 50µl load volume when two elutions were performed. The total RNA yield did not increase linearly with only two elutions when load volumes of 200µl and 400µl were tested. In another set of experiments, the yield from a 200µl in vitro transcription reaction applied to a single RNA purification column was increased after three and four elutions at 100µl each; however, the best yields were achieved when only 100µl of in vitro transcription reaction was used per column (data not shown).

To determine essential components for RNA purification, various combinations of SV RNA lysis buffer, blue dilution buffer and ethanol were combined with 100µl of in vitro transcribed RNA (Table 3). RNA purification was performed using the procedure described above using a single spin column for each mixture. Two elutions (100µl each) were performed into a single elution tube, and dilutions were analyzed using a spectrophotometer. A_{260}/A_{280} ratios ranged from 1.8 to 1.9. Overall yields were not significantly different for samples 1 through 6. For sample 2, addition of ethanol alone to the transcription reaction resulted in a white precipitate, and after purification, the total RNA yield appeared to increase significantly. However, analysis of the RNA on a formaldehyde gel indicated that RNA purified using ethanol alone was far less concentrated (data not shown). It is likely that unincorporated nucleotides were included in the white precipitate observed after addition of ethanol. This would affect the absorbance readings from the sample but would not show up on a gel. Applying RNA to the column alone (sample 8) resulted in a substantial loss of the in vitro transcription product, indicating additional components are needed.

Table 3. Buffer Requirements for RNA Purification from In Vitro Transcription Reactions Using the SV Total RNA Isolation System.

Sample No.	1	2	3	4	5	6	7	8
Transcription Volume (100µl)	+	+	+	+	+	+	+	+
Lysis Buffer (175µl)	+	+	+	+	–	–	–	–
Blue Dilution Buffer (350µl)	+	+	–	–	+	+	–	–
95% Ethanol (200µl)	+	–	+	–	+	–	+	–
A_{260}/A_{280} Ratio	1.8	1.8	1.8	1.8	1.8	1.9	1.9	1.7
Conc. (µg/µl)	1.1	1	1.4	0.9	1.1	1.2	4.7	0.1
Total yield (µg)	220	200	280	180	220	240	940	20

(+) = Reagent added, (–) = reagent absent

Conclusion

The SV Total RNA Isolation System provides an efficient means of purifying RNA from in vitro transcription reactions. We obtained the best and most consistent results when 100µl of transcription reaction is combined with all three solutions (SV RNA Lysis Buffer, blue dilution buffer and 95% ethanol) and purified over a single column. When greater quantities of RNA are required, the reaction can be split over multiple columns.

The SV Total RNA Isolation System also can be used to concentrate in vitro transcribed RNA. Not all templates perform well in an in vitro transcription reaction, and results between in vitro transcription reactions can vary. By analyzing the RNA on a 1% formaldehyde gel prior to purification, the volume of transcription reaction applied to a column, as well as the number of elutions and volume of elutions, can be adjusted. Although not tested here, contaminating template DNA may be removed using this system by treating RNA on the column with DNase I prior to elution. Most importantly, RNA purified using the SV Total RNA Isolation System has behaved similarly to RNA purified using other means in a functional assay (data not shown).

Acknowledgments

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References

1. *SV Total RNA Isolation System Technical Manual* #TM048 (2001) Promega Corporation.
2. *RiboMAX™ Large Scale RNA Production Systems — SP6 and T7 Technical Bulletin* #TB166 (2001) Promega Corporation.
3. Sambrook and Russell (2001) Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Press.

Protocol

◆ *SV Total RNA Isolation System Technical Manual* #TM048, Promega Corporation. (www.promega.com/tbs/tm048/tm048.html)

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Ordering Information

Product	Size	Cat.#
SV Total RNA Isolation	50 preps	Z3100
System[a,b]*	10 preps	Z3101
RiboMAX™ Large Scale RNA Production System—T7[c,d,e,f]*	1 system	P1300
SV RNA Lysis Buffer*	50ml	Z3051
Vac-Man® Laboratory Vacuum Manifold, 20-sample capacity	1 each	A7231
Vacuum Adapters	20 each	A1331

* For Laboratory Use.

[a] U.S. Pat. No. 6,218,531, Australian Pat. No. 745185 and other patents pending.

[b] Australian Pat. No. 730718 and other patents and patents pending.

[c] U.S. Pat. No. 5,552,302, Australian Pat. No. 646803 and other patents.

[d] The RiboMAX™ Large Scale RNA Production System–T7 (Cat.# P1300) is covered by U.S. Pat. No. 5,256,555, 6,586,218, 6,586,219 and other pending patents and is sold under a license from Ambion, Inc. It is intended for research use only. Parties wishing to use this product for other applications should contact Ambion, Inc.

[e] The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673. A license (from Promega for research reagent products and from The Regents of the University of California for all other fields) is needed for any commercial sale of nucleic acid contained within or derived from this product.

[f] U.S. Pat. Nos. 4,966,964, 5,019,556 and 5,266,687, Australian Pat. Nos. 616881 and 641261 and other pending and issued patents, which claim vectors encoding a portion of human placental ribonuclease inhibitor, are exclusively licensed to Promega Corporation.

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