CELLSCRIPT™
RNA for Translation in Cells

A-Plus™ Poly(A) Polymerase Tailing Kit

Cat. No. **C-PAP5104H**

INTRODUCTION

The A-Plus™ Poly(A) Polymerase Tailing Kit uses ATP as a substrate for template-independent addition of adenosine monophosphates to the 3'-hydroxyl termini of RNA. The standard protocol produces a poly(A)-tail length of ~150 b on 40-60 μg of RNA. Polyadenylation increases the stability of RNA in eukaryotic cells and enhances its ability to be translated after transfection or microinjection.[1-3] A Poly(A) tail is useful to provide a priming site for first-strand cDNA synthesis in certain applications, and can be used to end-label[4] or quantify[5] mRNA.

MATERIALS

Materials Supplied

Important Store at –20°C in a freezer without a defrost cycle. Do not store at –70°C.

A-Plus™ Poly(A) Polymerase Tailing Kit Contents (50 reactions)	
Kit Component	Volume
A-Plus™ Poly(A) Polymerase, 4 U/μl in 50% glycerol, 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 1 mM dithiothreitol (DTT), 0.1 mM EDTA and 0.1% Triton® X-100.	100 μl
10X A-Plus™ Poly(A) Tailing Buffer 0.5 M Tris-HCl, pH 8.0, 2.5 M NaCl and 100 mM $MgCl_2$.	500 μl
10 mM ATP	500 μl
RNase-Free Water	2 x 1.4 ml

Materials Required, but not Supplied

- Materials or kits for purification of the RNA product. (For suggestions, see RNA Purification, page 4)
- RNase-free TE Buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA)
- Optional: TE saturated phenol/chloroform, 0.5-1 M EDTA, ScriptGuard™ RNase Inhibitor

SPECIFICATIONS

Unit Definition

One unit of A-Plus Poly(A) Polymerase converts 1 nmole of ATP into acid-insoluble material in 10 minutes at 37°C under standard assay reaction conditions.

Functional Testing

The A-Plus Poly(A) Polymerase Tailing Kit is functionally tested in 1X A-Plus Poly(A) Tailing Buffer with 1 mM ATP and a 1.4 kb transcript.

Contaminating Activity Assays

All components of the A-Plus Poly(A) Polymerase Tailing Kit are free of detectable RNase and DNase activity.

For more information, consult the appropriate safety data sheet (SDS) at www.cellscript.com/products.html.

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BEFORE YOU START: IMPORTANT TIPS FOR OPTIMAL POLY(A)-TAILING

♦ **Poly(A)-Tail Length**:

The standard protocol generates poly(A)-tails approximately 150 b long.

Assuming all other reaction parameters remain constant, poly(A)-tail length increases with the following parameter changes:

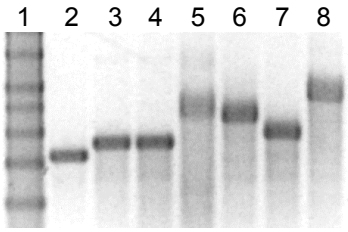
– **Units of A-Plus Poly(A) Polymerase**: Increase from 2 U, to as much as 16 U (standard is 4 U).

– **Incubation time**: Increase from 10 minutes, to as long as 60 minutes (standard is 30 minutes).

– **Quantity of substrate RNA**: Decrease from 60 μg, to as little as 1 μg (standard is 60 μg).

– **Reaction volume**: Decrease from 100 μl, to as little as 10 μl (standard is 100 μl).

To find the best reaction conditions for the desired poly(A)-tail length, set-up several test reactions covering a range of the changing parameter (Figure 1).



Lane 1: RNA MW markers (sizes top to bottom: 4.0, 3.0, 2.5, 2.0, 1.5 and 1.0 kb).
Lane 2: non-poly(A) tailed RNA.
Lane 3: 60 μg RNA, 16 U enzyme, 30 min., 100 μl rxn volume.
Lane 4: 60 μg RNA, 6 U enzyme, 60 min., 100 μl rxn volume.
Lane 5: 1 μg RNA, 2 U enzyme, 60 min., 50 μl rxn volume.
Lane 6: 1 μg RNA, 2 U enzyme, 30 min., 10 μl rxn volume.
Lane 7: 10 μg RNA, 4 U enzyme, 60 min., 50 μl rxn volume.
Lane 8: 1 μg RNA, 2 U enzyme, 60 min., 10 μl rxn volume.

♦ **Stopping the Reaction**:

Depending upon the subsequent reactions the poly(A)-tailed RNA will be subjected to, the tailing reaction may be stopped by any one of the following means:

– Immediate freezing of the completed reaction at –20°C or –70°C.

– Removal of the enzyme by phenol/chloroform extraction.

– Chelation of the Mg^{2+} with EDTA.

– Do **not** heat denature the enzyme to stop the reaction, because it may degrade the RNA. **Important**

♦ **Addition to an *In Vitro* Translation Reaction**:

Prior to use in *in vitro* translation systems or *in vivo* experiments, poly(A)-tailed RNA should be purified by one of the following methods: (see RNA Purification, page 4)

– Ammonium acetate precipitation.

– Phenol/Chloroform extraction followed by ethanol precipitation.

– Spin columns.

PROCEDURE

A. Synthesis of Poly(A)-Tailed RNA

1. The standard reaction will produce ~150 b long poly(A)-tails on 40-60 μg of capped or uncapped RNA. RNA from *in vitro* transcription reactions and from co-transcriptional capping reactions need to be purified before addition to the poly(A)-tailing reaction. Completed capping reactions from CELLSCRIPT's ScriptCap™ Cap 1 Capping System may be added directly to the poly(A)-tailing reaction without purification (see Technical Appendix, page 7).

   Combine the following reagents:

Standard A-Plus Poly(A)-Tailing Reaction (step 1)	
Component	Amount
RNase-Free Water	x μl
RNA, 40-60 μg	y μl
Total Volume	75.5 μl

Heat-denaturation of the RNA is an optional step.

Important Only heat-denature the RNA and water components. **Do not** include any other reagent in this step.

2. Incubate at 65°C for 5-10 minutes, then transfer to ice.

3. While the heat-denatured RNA is cooling on ice, prepare a "Cocktail" of the following reaction components together in a separate tube.

Standard A-Plus Poly(A)-Tailing Reaction (step 3)	
Component	Amount
10X A-Plus Poly(A) Tailing Buffer	10 μl
10 mM ATP	10 μl
ScriptGuard RNase Inhibitor (sold separately)	2.5 μl
A-Plus Poly(A) Polymerase, 8 Units	2 μl
Total Volume	24.5 μl

Addition of ScriptGuard RNase Inhibitor is optional but highly recommended.

4. After cooling the RNA on ice, combine the components from Steps 1 & 3 (above) together.

Standard A-Plus Poly(A)-Tailing Reaction (step 4)	
Component	Amount
Heat-denatured RNA (from step 1)	75.5 μl
Cocktailed reaction components (from step 3)	24.5 μl
Total Reaction Volume	100 μl

5. Incubate at 37°C for 60 minutes.

To extend the poly(A)-tail to >200 b, increase the incubation time to 90 minutes.

6. Stop the reaction using any one of the following methods:

   a) immediate storage at –20°C or –70°C.
   b) Add of EDTA to a final concentration of >11 mM.
   c) Phenol/chloroform extract then precipitate using salt/alcohol. See RNA Purification (page 4).

B. RNA Purification

Purify the RNA by your preferred method. The method chosen should remove residual proteins and unincorporated ATP from the RNA. Several options are listed below. RNA can be stored at –20°C or –70°C. If the RNA is to be stored indefinitely, store the RNA as an ethanol pellet.

I) **Organic Extraction / Ammonium Acetate Precipitation**: Removes all proteins and selectively precipitates RNA, leaving most of the unincorporated ATP in the supernatant. Note: for this method, the RNA to be purified must be >100 bases in size.

1) Add one volume (100 µl) of TE-saturated phenol/chloroform. Vortex vigorously for 10 seconds.
2) Spin in a microcentrifuge at >10,000 x g for 5 minutes to separate the phases.
3) Remove the aqueous (upper) phase with a pipette and transfer to a clean tube.
4) Add one volume (100 µl) of 5 M ammonium acetate, mix well then incubate for 15 minutes on ice.
5) Pellet the RNA by centrifugation at >10,000 x g for 15 minutes at 4°C.
6) Remove the supernatant with a pipette and gently rinse the pellet with 70% ethanol.
7) Remove the 70% ethanol with a pipette without disturbing the RNA pellet.
8) Allow pellet to dry, then resuspend in RNase-Free Water, TE or other suitable buffer.

II) **Ammonium Acetate Precipitation**: Selectively precipitates RNA, while leaving most of the protein and unincorporated ATP in the supernatant. Note: for this method, the RNA to be purified must be >100 bases in size.

1) Add one volume of 5 M ammonium acetate (100 µl for the standard reaction), mix well.
2) Incubate for 15 minutes on ice.
3) Pellet the RNA by centrifugation at >10,000 x g for 15 minutes at 4°C.
4) Remove the supernatant with a pipette and gently rinse the pellet with 70% ethanol.
5) Remove the 70% ethanol with a pipette without disturbing the RNA pellet.
6) Allow pellet to dry, then resuspend in RNase-Free Water, TE or other suitable buffer.
7) While usually unnecessary, steps 1-6 may be repeated a second time for even cleaner RNA.

III) **Organic Extraction / Chromatography / Ethanol Precipitation**: Removes all proteins and unincorporated ATP from the RNA.

1) Add one volume (100 µl) of TE-saturated phenol/chloroform. Vortex vigorously for 10 seconds.
2) Spin in a microcentrifuge at >10,000 x g for 5 minutes to separate the phases.
3) Remove the aqueous (upper) phase with a pipette and transfer to a clean tube.
4) Remove unincorporated ATP by spin column chromatography.[6] For commercially-available columns, follow the manufacturer's instructions for this step. Recover the RNA in ~100 µl.
5) Add one-tenth volume (10 µl) of 3 M sodium acetate and 2.5 volumes (250 µl) of 95% ethanol to the tube, mix well.
6) Incubate for 15 minutes on ice.
7) Pellet the RNA by centrifugation at >10,000 x g for 15 minutes at 4°C.
8) Remove the supernatant with a pipette and gently rinse the pellet with 70% ethanol.
9) Remove the 70% ethanol with a pipette without disturbing the RNA pellet.
10) Allow pellet to dry, then resuspend in RNase-Free Water, TE or other suitable buffer.

IV) **RNA-Binding Purification Column**: Several options are available commercially from multiple vendors. Follow the manufacture's recommended protocol. The final resuspension of RNA should be in RNase-Free Water, TE or other suitable buffer.

TROUBLESHOOTING

Symptom	Solution
Poly(A)-tails are longer than expected	Decrease the time of incubation of the reaction.
	Decrease the amount of A-Plus Poly(A) Polymerase used in the reaction.
	Increase the concentration of substrate RNA in the reaction by using more RNA, or decreasing the total reaction volume.
Poly(A)-tails are shorter than expected	Increase the time of incubation of the reaction.
	Increase the amount of A-Plus Poly(A) Polymerase used in the reaction.
	Decrease the concentration of substrate RNA in the reaction by using less RNA, or increasing the total reaction volume.
No Poly(A)-tails are observed	The RNA 3' end may be buried in secondary structure. Perform the RNA heat-denaturation step.
	The RNA substrate is contaminated. Repurify the RNA substrate (see Technical Appendix).
	Enzyme is inactive. Store only at –20°C. Keep on ice when not in the freezer.
	ATP is hydrolyzed. Do not expose to elevated temperatures.

RELATED PRODUCTS

– Cap-Clip™ Acid Pyrophosphatase
– INCOGNITO™ T7-FlashScribe™ Ψ-RNA Transcription Kit
– INCOGNITO™ T7-FlashScribe™ N1meΨ-RNA Transcription Kit
– INCOGNITO™ T7 mScript™ Ψ-mRNA Production System
– INCOGNITO™ T7 mScript™ N1meΨ-mRNA Production System
– INCOGNITO™ SP6 Ψ-RNA Transcription Kit
– INCOGNITO™ T7 Ψ-RNA Transcription Kit
– INCOGNITO™ T7 5mC- & Ψ-RNA Transcription Kit
– INCOGNITO™ T7 ARCA 5mC- & Ψ-RNA Transcription Kit
– MessageMAX™ T7 ARCA-Capped Message Transcription Kit V2
– ScriptCap™ 2'-O-Methyltransferase Kit
– ScriptCap™ Cap 1 Capping System
– ScriptCap™ m^7G Capping System
– ScriptGuard™ RNase Inhibitor
– SP6-Scribe™ Standard RNA IVT Kit
– T7-FlashScribe™ Transcription Kit
– T7 mScript™ Standard mRNA Production System
– T7-Scribe™ Standard RNA IVT Kit

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TECHNICAL APPENDIX

A. Repurification of Problematic RNA Substrates

RNA substrates which fail to be poly(A)-tailed, may contain contaminants. The RNA can be repurified by following the "Organic Extraction/Ammonium Acetate Precipitation" protocol (Protocol I), presented on page 4.

B. Poly(A)-Tailing with Minimal Amounts of A-Plus Poly(A) Polymerase

1. This protocol produces results (~140 b long poly(A)-tails on 40-60 μg of capped or uncapped RNA) comparable to those of the standard poly(A)-tailing reaction (page 3), while minimizing the amount of enzyme required in the reaction.

   Combine the following reagents:

Minimum Enzyme A-Plus Poly(A)-Tailing Reaction (step 1)	
Component	Amount
RNase-Free Water	x μl
RNA, 40-60 μg	y μl
Total Volume	77 μl

Heat-denaturation of the RNA is an optional step.

Important Only heat-denature the RNA and water components. **Do not** include any other reagent in this step.

2. Incubate at 65°C for 5-10 minutes, then transfer to ice.
3. While the heat-denatured RNA is cooling on ice, prepare a "Cocktail" of the following reaction components together in a separate tube.

Minimum Enzyme A-Plus Poly(A)-Tailing Reaction (step 3)	
Component	Amount
10X A-Plus Poly(A) Tailing Buffer	10 μl
10 mM ATP	10 μl
ScriptGuard RNase Inhibitor (sold separately)	2.5 μl
A-Plus Poly(A) Polymerase, 2 Units	0.5 μl
Total Volume	23 μl

Addition of ScriptGuard RNase Inhibitor is optional but highly recommended.

4. After cooling the RNA on ice, combine the components from Steps 1 & 3 (above) together.

Minimum Enzyme A-Plus Poly(A)-Tailing Reaction (step 4)	
Component	Amount
Heat-denatured RNA (from step 1)	77 μl
Cocktailed reaction components (from step 3)	23 μl
Total Reaction Volume	100 μl

5. Incubate at 37°C for 2 hours.
6. Stop the reaction as indicated in Step 6, page 3.

D. Poly(A)-Tailing of ScriptCap Cap 1 Capping System Reaction RNA

1. The reaction will produce ~150 b long poly(A)-tails on 40-60 μg of ScriptCap Cap 1 Capping System Reaction RNA. Completed reactions are added directly to the poly(A)-tailing reaction without purification.

   Combine the following reagents in the order given:

ScriptCap m^7G RNA Poly(A)-Tailing Reaction	
Component	Amount
ScriptCap Cap 1 Capping System Reaction	100 μl
ScriptGuard RNase Inhibitor *	0.6 μl
10X A-Plus Poly(A) Tailing Buffer	13.2 μl
10 mM ATP	13.2 μl
A-Plus Poly(A) Polymerase, 20 Units	5 μl
Total Volume	132 μl

Important **Do not** heat-denature the ScriptCap m^7G capping reaction RNA.

* ScriptGuard RNase Inhibitor is a component of the ScriptCap m^7G Capping System.

To extend the poly(A)-tail to >200 b, increase the incubation time to 60 minutes.

2. Incubate at 37°C for 30 minutes.

3. Stop the reaction as indicated in Step 6, page 3.

The performance of this product is guaranteed for one year from the date of purchase.

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