CELLSCRIPT™
RNA for Translation in Cells

INCOGNITO™ T7 mScript™ Complete Ψ-mRNA Production System

Cat. No. **IMCY250125**

INTRODUCTION

The INCOGNITO™ T7 mScript™ Complete Ψ-mRNA Production System* provides all enzymes and enzyme-related reagents for making pseudouridine (Ψ)-containing, 5'-capped, 3'-polyadenylated mRNA with greatly reduced double-stranded RNA (dsRNA) content, lower than what can be achieved with special T7 RNA polymerase mutants. The kit includes modules for (i) *in vitro* transcription of linear double-stranded DNA templates using the T7 mScript Enzyme Solution, the canonical nucleotides ATP, CTP, GTP and the modified nucleotide pseudouridine-5'-triphosphate (ΨTP), (ii) enzymatic capping of the RNA using the ScriptCap™ Cap 1 Capping System (contains both ScriptCap Capping Enzyme and 2'-O-Methyltransferase) for making mRNA with a Cap 1 cap structure, (iii) A-Plus™ Poly(A) Polymerase for adding a 3'-poly(A) tail, (iv) enzymatic removal of dsRNA content using the Min-Immune™ Gold dsRNA Removal system and (v) 5 M NH_4OAc as a convenient RNA/mRNA purification method.

Post-transfection, capped and tailed mRNA has increased stability and translation efficiency in most eukaryotic cell lines. Additionally, it has been shown that Ψ-mRNAs are translated into protein at higher levels and induce lower innate immune responses in human and other mammalian cells that express various RNA sensors compared to corresponding unmodified mRNAs.[1-6] The INCOGNITO mScript System generates modified mRNA with virtually 100% transcript capping and user-defined poly(A) tail length. Poly(A) tail lengths can be generated much longer than is possible using a template-encoded tail, even greater than 300-A's. Additionally, the removal of dsRNA from mRNA preparations has been shown to be essential for reducing the innate immunogenic response to the mRNA in cells.[7-10] Alternative dsRNA removal methods, such as reverse-phase HPLC[11], hydroxyapatite chromatography[12] and cellulose chromatography[13], are associated with high capital costs as well as reduced final product yields. The Min-Immune Gold dsRNA Removal system provides an easy to use method for removing the dsRNA content of mRNA preps without a reduction of the single-stranded mRNA yield. INCOGNITO Complete Ψ-mRNA is suitable for use in transfection and microinjection experiments as well as *in vitro* translation systems.

* see patent and license information on page 30.

726 Post Road, Madison, WI 53713 • 888-826-5586 (U.S. only) • (608) 442-6484 • Fax (608) 442-6187

MATERIALS

Materials Supplied

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

T7 mScript™ *In vitro* Transcription Module **INCOGNITO™ T7 mScript™ Complete Ψ-mRNA Production System Kit Contents**		(Module 1 of 5) 25 reactions
Kit Module	Kit Component	Reagent Volume
In Vitro Transcription	T7 mScript™ Enzyme Solution	50 µl
	10X T7 mScript™ Transcription Buffer II	50 µl
	100 mM Dithiothreitol (DTT)	50 µl
	ΨTP PreMix 25 mM each GTP, ATP, ΨTP, CTP	180 µl
	RNase-Free DNase I, 1 U/µl	25 µl

ScriptCap™ Cap 1 Capping Module **INCOGNITO™ T7 mScript™ Complete Ψ-mRNA Production System Kit Contents**		(Module 2 of 5) 25 reactions
Kit Module	Kit Component	Reagent Volume
Post-Transcriptional Capping	ScriptCap™ Capping Enzyme, 10 U/µl	100 µl
	ScriptCap™ 2'-O-Methyltransferase,100 U/µl	100 µl
	10X ScriptCap™ Capping Buffer 0.5 M Tris-HCl (pH 8.0), 60 mM KCl and 12.5 mM $MgCl_2$	250 µl
	20 mM *S*-adenosyl-methionine (SAM)	125 µl
	20 mM GTP	125 µl

A-Plus™ Poly(A) Tailing Module **INCOGNITO™ T7 mScript™ Complete Ψ-mRNA Production System Kit Contents**		(Module 3 of 5) 25 reactions
Kit Module	Kit Component	Reagent Volume
Poly(A)-Tailing	A-Plus™ Poly(A) Polymerase, 4 U/µl	130 µl
	10X A-Plus™ Poly(A) Tailing Buffer 0.5 M Tris-HCl (pH 8.0), 2.5 M NaCl and 100 mM $MgCl_2$.	300 µl
	20 mM ATP	150 µl

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

Component list continued on next page.

Min-Immune™ Gold dsRNA Removal Module INCOGNITO™ T7 mScript™ Complete Ψ-mRNA Production System Kit Contents		(Module 4 of 5) 25 reactions
Kit Module	Kit Component	Reagent Volume
dsRNA Removal	Min-Immune™ Gold RNase III (20X)	150 µl
	Min-Immune™ Gold 10X RNase III Treatment Buffer	300 µl

INCOGNITO™ T7 mScript™ Complete Ψ-mRNA Production System Kit Contents		(Module 5 of 5) 25 reactions
Kit Module	Kit Component	Reagent Volume
Common Usage	ScriptGuard™ RNase Inhibitor, 40 U/µl	165 µl
	RNase-Free Water	12 ml
	5 M Ammonium Acetate	12 ml

T7 Control Template DNA: Is a linearized 4.1 kb plasmid that contains a T7 promoter followed by a phage lambda dsDNA insert that encodes a 1,375 base runoff transcript. The Control Template DNA is provided at a concentration of 0.5 µg/µl in $T_{10}E_1$ Buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA).

Materials Required, but not Supplied

- A DNA template for transcription of your RNA of interest.
- Materials or kits for purification of the RNA product if not using the kit provided NH_4OAc. (For suggestions, see RNA Purification, pages 25-26).
- RNase-free TE Buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) **for final mRNA resuspension-only**, if not using the kit provided RNase-Free Water.
- 70% ethanol

Optional Materials

- dsRNA-specific detection system including:
  - dsRNA-specific antibody (e.g., J2 antibody [Absolute Biotech-Exalpha])
  - Dot/slot blotting system for use with the antibody
  - An image analyzer for blot visualization and/or quantification
  - dsRNA standards
- TE saturated phenol/chloroform, 0.5-1 M EDTA

SPECIFICATIONS

Storage Buffers

RNase-Free DNase I is provided in a 50% glycerol solution containing 50 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$ and 0.1% Triton® X-100.

A-Plus Poly(A) Polymerase and Min-Immune Gold RNase III are provided in a 50% glycerol solution containing 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 1 mM DTT, 0.1 mM EDTA and 0.1% Triton X-100.

ScriptGuard RNase Inhibitor is provided in a 50% glycerol solution containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM DTT, 0.1 mM EDTA and 0.1% Triton X-100.

All other enzymes are provided in a 50% glycerol solution containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, 0.1 mM EDTA and 0.1% Triton X-100.

Unit Definitions

One INCOGNITO T7 mScript Complete Ψ-mRNA Production System reaction produces 60 μg of Ψ-containing, 5'-capped, 3'-poly(A)-tailed, dsRNA-depleted mRNA.

One unit of RNase-Free DNase I digests 1 μg of pUC19 DNA to oligodeoxynucleotides in 10 minutes at 37°C.

One unit of ScriptCap Capping Enzyme releases 1 nmole of inorganic phosphate from GTP in 10 minutes at 37°C under standard assay conditions.

One unit of ScriptCap 2'-O-Methyltransferase methylates one picomole of a control Cap 0 RNA in 1 hour at 37°C under standard assay conditions.

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.

One unit of ScriptGuard RNase Inhibitor results in 50% inhibition of 5 ng of RNase A.
Activity is measured by the inhibition of hydrolysis of cyclic 2',3'-CMP by RNase A.

Functional Testing

The INCOGNITO T7 mScript Complete Ψ-mRNA Production System is functionally tested under standard reaction conditions using the T7 Control Template DNA. The *in vitro* transcription module must produce at least 50 μg of RNA from 1 μg of the T7 Control Template DNA in 20 minutes at 37°C. A-Plus Poly(A) Polymerase is functionally tested in 1X A-Plus Poly(A) Tailing Buffer with 1 mM ATP and a 1.4 kb transcript. The capping and dsRNA removal module enzymes are tested independently using non-T7 control transcript RNA to assay for completeness of reaction.

Contaminating Activity Assays

All components of the INCOGNITO T7 mScript Complete Ψ-mRNA Production System are free of detectable RNase and DNase activity, except for the inherent activity of the RNase-Free DNase I and Min-Immune Gold RNase III components.

BEFORE YOU START: IMPORTANT TIPS FOR OPTIMAL RESULTS

♦ **Cap 0- vs. Cap 1-mRNA**:

The difference between Cap 0- and Cap 1-mRNA is the addition of a methyl group at the 2'-O position of the penultimate (second from the 5' end) nucleotide of the transcript (see Figures 1A and 1B). This methylation is part of the natural capping process in higher eukaryotic cells and in some but not all cases improves *in vivo* translation versus the corresponding Cap 0-mRNA.

The ScriptCap Capping Enzyme and ScriptCap 2'-O-Methyltransferase work together to produce the Cap 1 structure. To obtain a Cap 0 structure, simply omit the ScriptCap 2'-O-Methyltransferase from the reaction. When using a new cell line or translation system, we recommend performing a comparison between Cap 0- and Cap 1-mRNA translation efficiencies to determine the optimal cap structure for that system.

Figure 1A Cap 0-mRNA

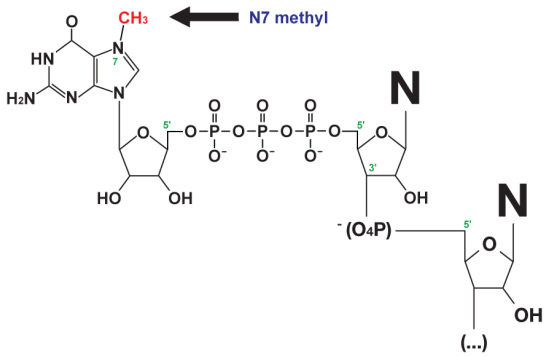
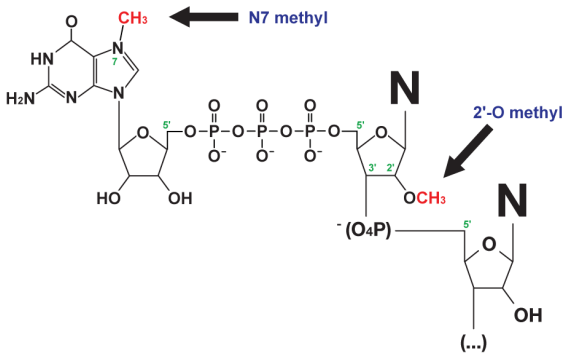


Figure 1B Cap 1-mRNA



♦ **Maintaining an RNase-Free Environment**:

Highly stable RNases are ubiquitous, including on human skin.

Creating an RNase-free work environment and maintaining RNase-free solutions is critical for successful work with RNA.

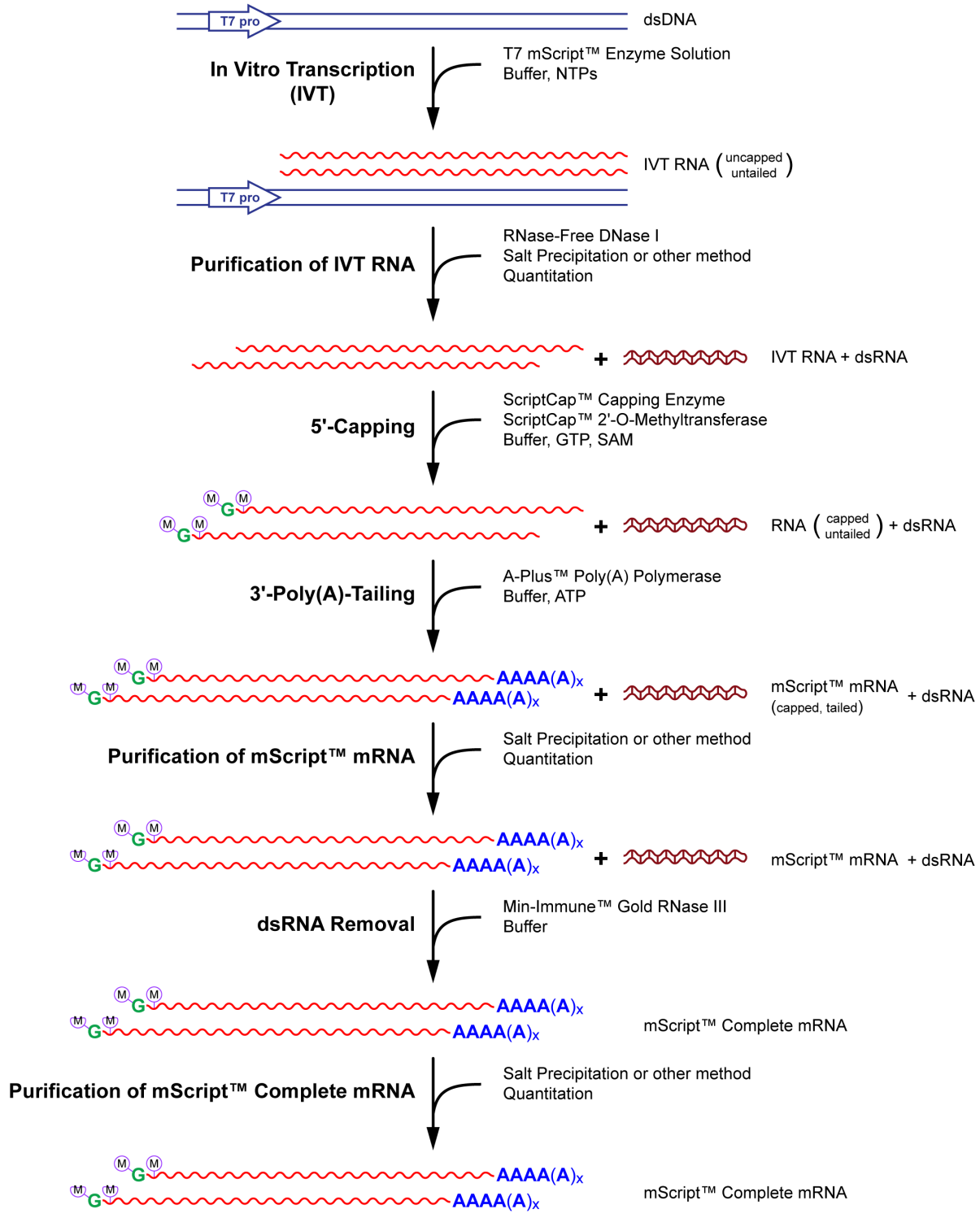
We strongly recommend to:

– Use RNase-free tubes and pipette tips.

– Always wear gloves when handling kit components or samples containing RNA and change gloves frequently, especially after touching potential sources of RNase contamination such as doorknobs, pens, pencils and human skin. Do not touch any kit component or tube containing RNA with an ungloved hand.

– Keep all kit components tightly sealed when not in use. Keep all tubes containing RNA tightly sealed during the incubation steps.

Figure 2. INCOGNITO T7 mScript Complete Ψ-mRNA Production System Procedure



INCOGNITO T7 mSCRIPT Complete Ψ-mRNA PRODUCTION PROCEDURE OUTLINE

Synthesis of IVT Ψ-RNA
DNase I Treatment of IVT Reaction
Purification of the Transcription Product
Synthesis of Capped Ψ-RNA
Synthesis of Poly(A)-Tailed Ψ-RNA
Purification of the Capped & Tailed mScript Ψ-mRNA
Min-Immune Gold dsRNA Removal Treatment
Purification of the Capped & Tailed mScript Complete Ψ-mRNA

An abbreviated procedure for experienced users of this kit can be found in the Technical Appendix.

BEFORE YOU START: IMPORTANT TIPS FOR OPTIMAL *IN VITRO* TRANSCRIPTION

- **Template Requirements**:

  The optimal templates for *in vitro* transcription are linear double-stranded DNA (dsDNA) molecules with 5'-protruding ends. DNA templates with blunt ends are less preferable and **templates with 3'-protruding ends should not be used**.

  Transcription templates can be prepared from clones of the DNA to be transcribed in plasmids or other circular dsDNA vectors by linearizing the vectors downstream of the cloned DNA using a suitable restriction endonuclease or other means.

  Alternatively, transcription templates can be generated by PCR amplification of RNA or DNA of interest using a strategy that results in joining of a T7 promoter to the appropriate end of the PCR product (e.g., wherein, the T7 promoter is either joined to the DNA or RNA that is amplified or is incorporated into one of the PCR primers).

- **Template Efficiency and Incubation Time**:

  60 μg of IVT RNA are recommended for treatment in the subsequent capping and tailing reactions. The T7 Control Template DNA produces >60 μg of a ~1.4 kb RNA per 1 μg of DNA template in a 30 minute reaction.

  However, yields vary for different templates based on the template sequence, structure, length, purity and the sequence and length of the particular RNA polymerase promoter. Examples of contaminants that can affect transcription yield include RNase, phenol, trace metals and SDS. See the Technical Appendix for suggestions related to template purification.

PROCEDURE

A. Synthesis of IVT Ψ-RNA

The *In Vitro* Transcription and Common Usage Modules are required for this portion of the procedure.

1. Set up the transcription reaction **at room temperature** by adding the reagents **in the order indicated below**:

INCOGNITO T7 mScript IVT Reaction	
Component	Amount
RNase-Free Water	x µl
Linearized template DNA with T7 RNAP promoter	1 µg
10X T7 mScript Transcription Buffer II	2 µl
ΨTP PreMix	7.2 µl
100 mM DTT	2 µl
ScriptGuard RNase Inhibitor	0.5 µl
T7 mScript Enzyme Solution	2 µl
Total Reaction Volume	20 µl

2. Incubate at 37°C for 15-30 minutes.

Important Assemble transcription reactions at room temperature in the order indicated at left. Assembly of transcription reactions at <22°C or in an alternate order, can result in the formation of an insoluble precipitate.

Transcription Buffer stored at –70°C may result in the formation of a white precipitate. To dissolve it, heat the tube at 37°C for 5 minutes and mix thoroughly.

One microgram of DNA template is recommended for most reactions. If the DNA template is <0.16 µg/µl, concentrate it, then resuspend in the appropriate amount of RNase-Free Water.

Important Use a 30 minute incubation when transcribing a template for the first time. Shorter incubation times can be used for subsequent transcription reactions once the yield characteristics of the template have been defined.

B. DNase I Treatment of IVT Reaction

1. DNase I treatment is used to remove the DNA template from the IVT reaction.

DNase I Treatment of IVT Reaction	
Component	Amount
IVT Reaction (from Step A)	20 µl
RNase-Free DNase I	1 µl
Total Reaction Volume	21 µl

2. Incubate for 15 minutes at 37°C.

3. Proceed to Purification of the Transcription Product.

C. Purification of the Transcription Product

The Common Usage Module is required for this portion of the procedure.

This step involves purification by ammonium acetate precipitation. It selectively precipitates RNA, while leaving most of the protein, DNA and unincorporated NTPs in the supernatant. Note: for this method, the RNA to be purified must be >100 bases in size. For alternative RNA purification methods, see Technical Appendix, RNA Purification, pages 25-26.

Ammonium Acetate Precipitation

1. Adjust reaction volume to 50 µl total using RNase-Free Water (add 29 µl to the reaction).
2. Add one volume (50 µl) of 5 M ammonium acetate. Mix well.
3. Incubate for 10-15 minutes on ice or at 4°C or at –20°C.
4. Pellet the RNA by centrifugation at >10,000 x g for 10 minutes at room temperature or at 4°C.
5. Remove the supernatant with a pipette and gently rinse the pellet with 70% ethanol to remove residual ammonium acetate.
6. Remove the 70% ethanol with a pipette without disturbing the RNA pellet.
7. Allow the RNA pellet to air dry, then resuspend in 50-75 µl of RNase-Free Water for quantitation. **Do not resuspend the RNA in an EDTA-containing solution.**

Over dried pellets will be difficult to resuspend.

8. Quantitate the RNA by spectrophotometry or fluorimetry.

If desired, the RNA can now be frozen and stored overnight at –20°C.

BEFORE YOU START: IMPORTANT TIPS FOR OPTIMAL CAPPING

- **Cap 0- vs. Cap 1-mRNA**:

  The difference between Cap 0- and Cap 1-mRNA is the addition of a methyl group at the 2'-O position of the penultimate (second from the 5' end) nucleotide of the transcript (see Figures 1A and 1B). This methylation is part of the natural capping process in higher eukaryotic cells and in some but not all cases improves *in vivo* translation versus the corresponding Cap 0-mRNA.

  The ScriptCap Capping Enzyme and ScriptCap 2'-O-Methyltransferase work together to produce the Cap 1 structure. To obtain a Cap 0 structure, simply omit the ScriptCap 2'-O-Methyltransferase from the reaction. When using a new cell line or translation system, we recommend performing, a comparison between Cap 0- and Cap 1-mRNA translation efficiencies to determine the optimal cap structure for that system.

- **SAM**:

  SAM slowly degrades over time at room temperature and above.
  Keep thawed SAM solutions on ice at all times.

- **RNA Source**:

  RNA produced in an *in vitro* transcription reaction should be purified and resuspended in RNase-Free Water prior to use in the ScriptCap Capping Enzyme System. **Do not resuspend the RNA in an EDTA-containing solution**.

- **RNA Secondary Structure**:

  Some RNA transcripts can form stable secondary structures (homodimers and hairpins) involving the 5'-most nucleotides of the transcript severely limiting access of the 5'-most nucleotide to the ScriptCap Capping Enzyme. In order to increase the capping efficiency of such RNAs, use longer or hotter heat denaturation conditions than those noted in the protocol. Times and temperatures required will vary. In extreme cases, or when a heat denaturation step is not a viable option, reengineering of the 5' end sequence may be necessary to alleviate the secondary structure. This is often accomplished with a single point mutation within the first 5 bases of the transcript (non-coding region). Contact CELLSCRIPT Technical Services for suggestions and recommendations.

D. Synthesis of Capped Ψ-RNA

The Post-Transcriptional Capping and Common Usage Modules are required for this portion of the procedure.

1. The protocol below was designed to build a Cap 1 structure on the 5' end of 50-60 μg of uncapped Ψ-RNA. **If a Cap 0 structure is desired**, replace the ScriptCap 2'-O-Methyltransferase in Step 3 with an equivalent volume of RNase-Free Water.

   Combine the following reagents:

INCOGNITO mScript Capping Reaction (step 1)	
Component	Amount
RNase-Free Water	x μl
In vitro transcribed Ψ-RNA, 50-60 μg	≤69.5 μl
Total Volume	69.5 μl

Heat-denaturation of the RNA is an optional step, but it is strongly recommended for RNAs which have not previously been characterized for their ease of enzymatic capping.

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.

INCOGNITO mScript Capping Reaction (step 3)	
Component	Amount
10X ScriptCap Capping Buffer	10 μl
20 mM GTP	5 μl
20 mM SAM	5 μl
ScriptGuard RNase Inhibitor	2.5 μl
ScriptCap 2'-O-Methyltransferase, (100 U/μl optional)	4 μl
Total Volume	26.5 μl

Important Do not include the ScriptCap Capping Enzyme in this mix.

Important Keep the thawed stock SAM solution on ice.

A white precipitate may form in the 10X ScriptCap Capping Buffer upon storage. To dissolve it, heat the tube at 37°C for 5 minutes and mix thoroughly.

If a Cap 0 structure is desired, replace the ScriptCap 2'-O-Methyltransferase with RNase-Free Water.

4. ***Just prior to starting the reaction,*** add the ScriptCap Capping Enzyme to the Cocktail from Step 3 and then combine this with the uncapped RNA solution from Step 1.

INCOGNITO mScript Capping Reaction (step 4)	
Component	Amount
Cocktailed reaction components (from step 3)	26.5 μl
ScriptCap Capping Enzyme (10 U/μl)	4 μl
Heat-denatured RNA (from step 1)	69.5 μl
Total Reaction Volume	100 μl

The efficiency of 2'-O-methylation can be lower if the RNA 5' end is structured. If your RNA is not completely 2'-O-methylated, we recommend increasing the incubation time to 2 hours. Also, since the concentration of methylation sites for a given mass is higher for small RNA than for large RNA, increase the reaction time for small RNA. For example, we suggest to increase the reaction time to 2 hours if your RNA is <730 nucleotides in length.

5. Incubate at 37°C for 30 minutes.
   Longer incubation times, if desired, are acceptable.

6. Proceed directly to 3'-Poly(A)-Tailing. Purification of the RNA prior to the tailing step is **NOT** necessary. Alternatively, the unpurified capped RNA can be frozen and stored overnight at –20°C.

E. Synthesis of Poly(A)-Tailed Ψ-RNA

The Poly(A)-Tailing and Common Usage Modules are required for this portion of the procedure.

1. The protocol below was designed to produce ~150 b long poly(A) tails on 60 μg of capped Ψ-RNA. Combine the following reagents:

INCOGNITO mScript Poly(A)-Tailing Reaction	
Component	Amount
5'-Capped *In vitro* transcribed Ψ-RNA (from Step D6)	100 μl
ScriptGuard RNase Inhibitor	0.5 μl
10X A-Plus Poly(A) Tailing Buffer	12 μl
20 mM ATP	6 μl
A-Plus Poly(A) Polymerase (4 U/μl)	5 μl
Total Volume	123.5 μl

Important **Do not** heat-denature the 5'-Capped *In vitro* transcribed RNA.

2. Incubate at 37°C for 30 minutes.

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

3. Stop the reaction using any one of the following methods:
   a) Proceed directly to Step F, Purification of the Capped and Tailed Ψ-mRNA (page 13).
   b) Immediate storage at –20°C or –70°C.
   c) Addition of EDTA to a final concentration of >11 mM.

Important **Do not** stop the reaction by heat denaturation because it may degrade the RNA.

If desired, the RNA can now be frozen and stored overnight at –20°C.

F. Purification of the Capped and Tailed Ψ-mRNA

The Common Usage Module is required for this portion of the procedure.

This step involves purification by ammonium acetate precipitation. It selectively precipitates RNA, while leaving most of the protein, DNA and unincorporated NTPs in the supernatant. Note: for this method, the RNA to be purified must be >100 bases in size. For alternative RNA purification methods, see Technical Appendix, RNA Purification, pages 25-26.

1. Add one volume (124 µl) of 5 M ammonium acetate to the reaction tube. Mix well.
2. Incubate for 10-15 minutes on ice or at 4°C or at –20°C.
3. Pellet the RNA by centrifugation at >10,000 x g for 10 minutes at room temperature or at 4°C.
4. Remove the supernatant with a pipette and gently rinse the pellet with 70% ethanol to remove residual ammonium acetate.
5. Remove the 70% ethanol with a pipette without disturbing the RNA pellet.
6. Allow the RNA pellet to air dry, then resuspend in 60 µl RNase-Free Water for quantitation. **Do not resuspend the RNA in an EDTA-containing solution if proceeding to the dsRNA removal step.**

   Over dried pellets will be difficult to resuspend.
7. Quantitate the RNA by spectrophotometry or fluorimetry.

If desired, the RNA can now be frozen and stored overnight at –20°C.

BEFORE YOU START: IMPORTANT TIPS FOR dsRNA REMOVAL

- **RNA Source**:

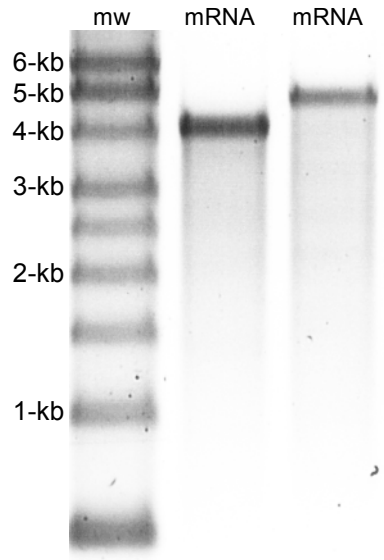
  It is important that the RNA is resuspended in RNase-free Water and NOT in an EDTA-containing solution such as $T_{10}E_1$ buffer.

- **Treatment of single-stranded RNAs (ssRNA) containing inherent double-stranded regions**:
  The Min-Immune Gold dsRNA Removal system was designed to remove the contaminating dsRNA-content, produced as a transcription reaction byproduct, from the desired ssRNA transcription reaction product. However, some RNAs naturally contain dsRNA regions or structures within them. Some of these are vital functional components of the RNA (e.g., cellular factor binding sites). The lengths of these dsRNA regions dictate their sensitivity to both Min-Immune Gold RNase III cleavage (see Example Data on the following pages) as well as to recognition by intracellular dsRNA-specific pattern recognition receptors. **Because of this, we recommend that all new RNA sequences to be treated with the Min-Immune Gold dsRNA Removal system be characterized for Min-Immune Gold RNase III cleavage sensitivity by performing a 1X (or smaller) reaction treatment followed by analysis via denaturing agarose gel electrophoresis of treated versus non-treated samples prior to treatment of larger amounts of the same RNA.** See Example Data on the following pages.

Example Data:

Figure 3

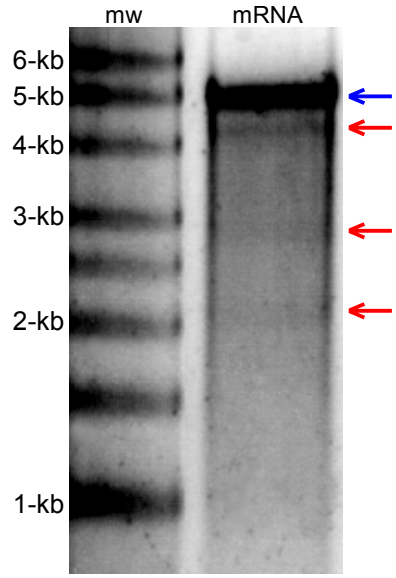
Formaldehyde-denaturing 1% agarose gel.
Two different treated Ψ-mRNA samples.
Standard image exposure (Syngene® G:Box).



Min-Immune Gold dsRNA Removal system treated RNAs, in this example: pseudouridine-containing mRNAs, should appear as single full-length bands of ssRNA lacking any background banding pattern that was not present in the untreated RNA sample.

Figure 4

Formaldehyde-denaturing 1% agarose gel.
Treated Ψ-mRNA sample.
Image over exposure (Syngene G:Box).

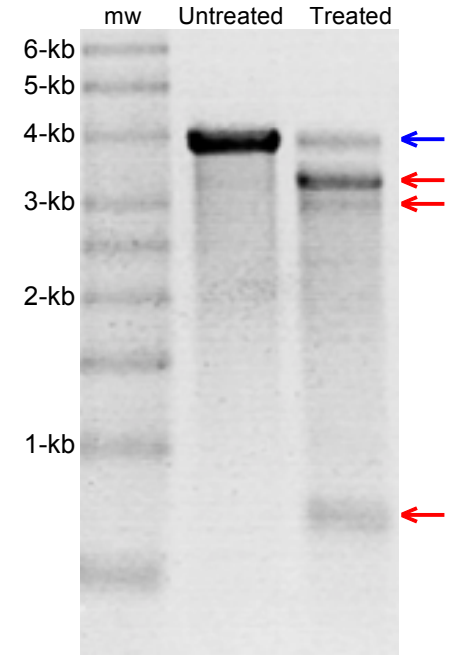


Some RNA samples can form transient dsRNA regions (molecular breathing) during the reaction which are substrates for Min-Immune Gold RNase III cleavage. These will appear as faint less than full length background bands.

- **Blue** arrow = full length mRNA.
- **Red** arrows = cleaved RNA fragments from transient Min-Immune Gold RNase III sensitive sites.

This does not affect the bulk functionality of the treated RNA.

Figure 5
Formaldehyde-denaturing 1% agarose gel.
Treated & Untreated Ψ-mRNA sample.
Standard image exposure (Syngene G:Box).

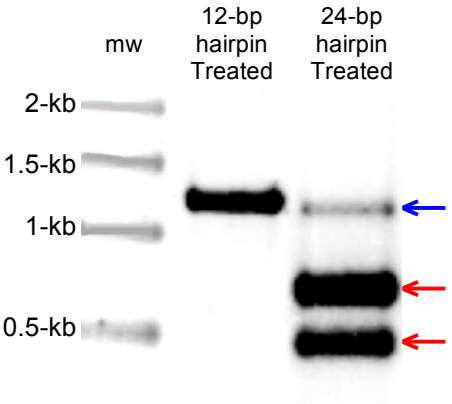


Some RNA samples contain inherent stable dsRNA regions due to the sequence of the RNA which are substrates for Min-Immune Gold RNase III cleavage. These will appear as strong less than full length background bands along with a diminished full length RNA band.

- **Blue** arrow = full length mRNA.
- **Red** arrows = cleaved RNA fragments from inherent Min-Immune Gold RNase III sensitive sites.

This will greatly affect the bulk functionality of the treated RNA. See the Troubleshooting section for options on how to handle such RNAs.

Figure 6
Formaldehyde-denaturing 1% agarose gel.
Treated U-IVT RNA samples.
Standard image exposure (Syngene G:Box).



A 1.25-kb RNA construct was designed to contain an inherent dsRNA region which included either a 12-bp or 24-bp hairpin in the secondary structure, and treated with Min-Immune Gold RNase III.

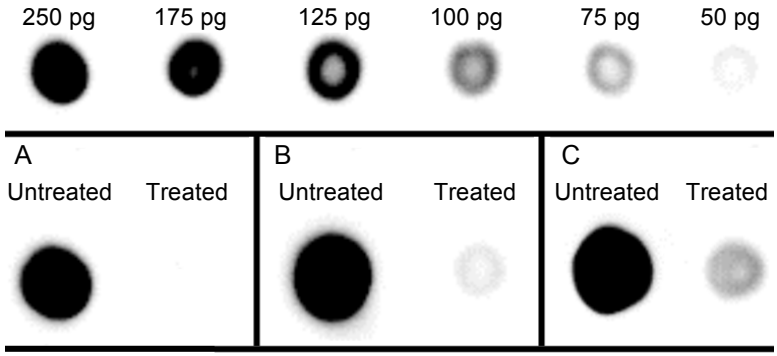
- **Blue** arrow = full length RNA.
- **Red** arrows = cleaved RNA fragments from Min-Immune Gold RNase III sensitive sites.

Duplexes of 12-bp dsRNA are not recognized by Min-Immune Gold RNase III while duplexes of 24-bp dsRNA are recognized.

Figure 7

dsRNA immuno-dot blot assayed with dsRNA-specific J2 antibody detection using horseradish peroxidase and chemiluminescent detection.

1 μg of Untreated or Treated mRNA was blotted for each experimental sample.



Pseudouridine-containing dsRNA quantification standards

Untreated and treated pseudouridine-containing mRNA samples.

A) Example result of an mRNA prep where the dsRNA content is totally removed by treatment. This mRNA can be used for downstream applications.

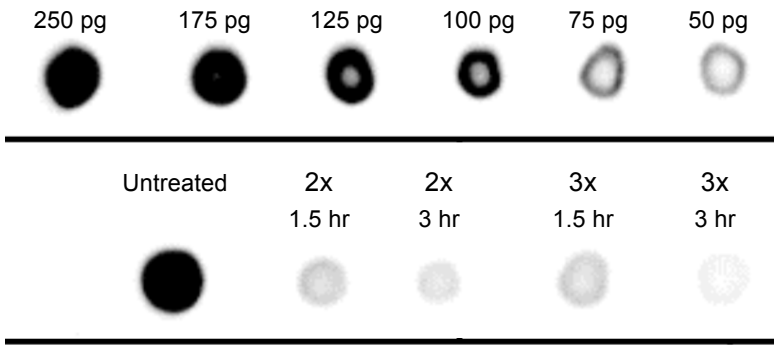
B) Example result of an mRNA prep where the mRNA contains an inherent dsRNA region which is not recognized by Min-Immune Gold RNase III but is recognized by the J2 antibody. This mRNA can be used for downstream applications. See Figure 8 below.

C) Example result of an mRNA prep where the dsRNA content is not totally removed by treatment. This mRNA would require retreatment prior to many downstream applications.

Figure 8

dsRNA immuno-dot blot assayed with dsRNA-specific J2 antibody detection using horseradish peroxidase and chemiluminescent detection.

1 μg of Untreated or treated mRNA was blotted for each experimental sample.



Pseudouridine-containing dsRNA quantification standards

Untreated and treated pseudouridine-containing mRNA samples.

Example result of an mRNA prep where the mRNA contains an inherent dsRNA region which is not recognized by Min-Immune Gold RNase III but is recognized by the J2 antibody. The dsRNA region could not be removed with two (2x) or three (3x) Min-Immune Gold RNase III treatments even with extended incubation times (1.5 & 3 hrs). This mRNA can be used for downstream applications.

G. Min-Immune Gold dsRNA Removal Treatment

1. Recommended: save an amount of untreated RNA to run on a denaturing agarose gel versus the treated RNA for post treatment RNA analysis.

   For the RNA to be treated, combine the following reagents at room temperature in the order indicated below:

Min-Immune Gold dsRNA Removal Treatment Reaction	
Component	Amount
RNase-Free Water	x µl
Min-Immune Gold 10X RNase III Treatment Buffer	12 µl
ScriptGuard RNase Inhibitor	3 µl
60 µg mScript Ψ-mRNA (from Step F7, page 13)	y µl
Min-Immune Gold RNase III (20X)	6 µl
Total Volume	120 µl

2. Incubate at 37°C for 60 minutes.

3. Proceed to Purification of the Capped & Tailed mScript Complete Ψ-mRNA.

H. Purification of the Capped & Tailed mScript Complete Ψ-mRNA

Before use in *in vivo* and *in vitro* translation systems, the capped and tailed Ψ-mRNA needs to be purified. We recommend the following protocol. This step involves purification by ammonium acetate precipitation. It selectively precipitates RNA, while leaving most of the protein, DNA and unincorporated NTPs in the supernatant. Note: for this method, the RNA to be purified must be >100 bases in size. For alternative RNA purification methods, see Technical Appendix, RNA Purification, pages 25-26.

1. Add one volume (120 µl) of 5 M ammonium acetate to the reaction tube. Mix well.

2. Incubate for 10-15 minutes on ice or at 4°C or at –20°C.

3. Pellet the RNA by centrifugation at >10,000 x g for 10 minutes at room temperature or at 4°C.

4. Remove the supernatant with a pipette and gently rinse the pellet with 70% ethanol to remove residual ammonium acetate.

5. Remove the 70% ethanol with a pipette without disturbing the RNA pellet.

6. Allow the RNA pellet to air dry, then resuspend in 30-60 µl RNase-Free Water, $T_{10}E_1$ or other suitable buffer for quantitation.

   Over dried pellets will be difficult to resuspend.

7. Quantitate the RNA by spectrophotometry or fluorimetry.

8. While usually unnecessary, steps 1-7 may be repeated a second time for even cleaner RNA.

TROUBLESHOOTING

Synthesis of IVT RNA

Symptom	Solution
Low yields or less than full-length transcripts	Cleanup the templates to remove any RNase or other contaminants (see Technical Appendix for procedure).
	Verify that ScriptGuard RNase Inhibitor was added to the reaction.
	Extend the incubation time. Do not extend the reaction time beyond 3 hours.
	Increase the template concentration.
	Increase the reaction temperature to 42°C.
Assembled reaction formed an insoluble precipitate	Repeat assembly of the reaction at >22°C.
White precipitate in reaction buffer	Incubate the reaction buffer at 37°C for 5 minutes then mix thoroughly to dissolve the precipitate.
	Do not store the kit at –70°C.

Synthesis of Capped RNA

Symptom	Solution
Low capping efficiency	Cleanup the templates to remove any RNase or other contaminants.
	Verify that ScriptGuard RNase Inhibitor was added to the reaction.
	SAM slowly degrades at room temperature and above. Keep SAM solutions on ice at all times.
	Increase the capping reaction incubation time. For example, up to 3 hours at 37°C.
	Some RNAs form stable structures (e.g., homodimers, hairpins) at the 5' end, limiting access by Capping Enzyme or 2'-O-Methyltransferase. Analyze the sequence and increase the RNA denaturation temperature to above the T_m (e.g., to 65°C for 20 min, 75°C for 10 min, 85°C for 5 min). If the 5' end is highly structured, it might be necessary to modify the 5' end sequence using molecular biology techniques. Often this can be accomplished by making a single point mutation within the first 5 bases of the DNA template for the RNA transcript (non-coding region). Contact CELLSCRIPT Technical Services for suggestions and recommendations.
White precipitate in reaction buffer	Incubate the reaction buffer at 37°C for 5 minutes then mix thoroughly to dissolve the precipitate.
	Do not store the kit at –70°C.

TROUBLESHOOTING

Synthesis of Poly(A)-Tailed RNA

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.
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.
No Poly(A) tails are observed	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.

dsRNA Removal

Symptom	Solution
Faint, minor, less than full length background bands are seen on the gel post-treatment	RNA samples were not fully denatured. • Be sure to be running a denaturing agarose gel. • Rerun the samples with an increased heat denaturing step prior to gel loading.
	RNA sample formed transient dsRNA regions (molecular breathing) during the reaction which were substrates for Min-Immune Gold RNase III cleavage. • See example Fig 4, page 15. • If very minor, no further treatment nor treatment changes are needed. • If minor, treat subsequent samples of this RNA more gently. Reduce incubation to 30 minutes or reduce the amount of Min-Immune Gold RNase III used by 50%.
Strong, major, less than full length background bands are seen on the gel post-treatment	RNA sample contained an inherent stable dsRNA region of sufficient length to be a substrate for Min-Immune Gold RNase III cleavage. • See example Figs 5 & 6, page 16. • The Min-Immune Gold dsRNA Removal system is not appropriate for treating this particular RNA sequence. If the dsRNA structure is crucial to RNA functionality, use an alternative method for dsRNA removal. If the dsRNA structure is not crucial to RNA functionality, mute the dsRNA structure to remove the duplex formation by altering the RNA sequence and retreat the new RNA.

TROUBLESHOOTING

dsRNA Removal (continued)

Symptom	Solution
dsRNA assessment indicates remaining dsRNA content	RNA contains a region of dsRNA. • See example Figs 7 & 8, page 17. • dsRNA region may be inherent to the RNA sequence and recognizable by the dsRNA-specific antibody but may not be recognizable by the Min-Immune Gold RNase III or intracellular dsRNA-specific pattern recognition receptors. • If very minor, no further treatment nor treatment changes are needed. • If minor, the RNA sample may be retreated with Min-Immune Gold RNase III. If the dsRNA assessment still shows dsRNA content after retreatment, then the dsRNA region is most likely inherent to the RNA sequence. Try the RNA in the downstream application. If the dsRNA assessment now shows virtually no dsRNA, the next time that specific RNA is to be made, more strongly treat the RNA by extending the incubation time to 2 or 3 hours or increasing the amount of Min-Immune Gold RNase III used in the reaction.
	Abnormally high amounts of dsRNA were present in the RNA sample. • Retreat the RNA sample. • The next time that specific RNA is to be made, more strongly treat the RNA by extending the incubation time to 2 or 3 hours or increasing the amount of Min-Immune Gold RNase III used in the reaction. • Consider using an altered transcription protocol that would produce less dsRNA during the IVT reaction.
	Min-Immune Gold dsRNA Removal reaction was inefficient or failed. • Retreat the RNA sample. If the problem persists contact CELLSCRIPT Technical service.

RELATED PRODUCTS

– EZ-QC™ mRNA Cap 1 Efficiency Assay Kit
– EZ-QC™ mRNA Capping Efficiency Assay Kit
– EZ-QC™ mRNA Poly(A) Tail Length Assay Kit
– EZ-QC™ XBG mRNA Capping Efficiency Assay Kit
– INCOGNITO™ T7 mScript™ Complete N1meΨ-mRNA Production System
– T7 mScript™ Complete Standard mRNA Production System

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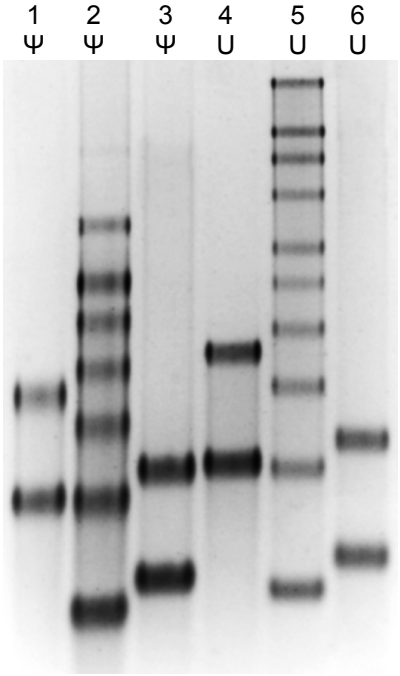
TECHNICAL APPENDIX

A. Electrophoretic Mobility of Ψ-RNA in Denaturing Agarose

Ψ-RNA displays altered mobility during electrophoresis as compared to the comparable U-RNA of identical sequence. This is relevant when comparing Ψ-RNA transcript size to a U-RNA molecular weight ladder.



2% denaturing agarose gel.

Lane 1) Ψ-RNAs, 1,875 & 1,077 bases.
Lane 2) Ψ-RNA Molecular Weight Markers
(0.5, 1.0, 1.5, 2.0, 2.5, 3.0 & 4.0 kilobases).
Lane 3) Ψ-RNAs, 1,203 & 673 bases.

Lane 4) U-RNAs, 1,875 & 1,077 bases.
Lane 5) U-RNA Molecular Weight Markers
(0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0 & 9.0 kilobases).
Lane 6) U-RNAs, 1,203 & 673 bases.

B. ABBREVIATED INCOGNITO T7 mScript COMPLETE Ψ-mRNA PRODUCTION PROCEDURE

for experienced kit users.

Synthesis of IVT Ψ-RNA and DNase I Treatment of IVT Reaction

1. Combine the following at room temperature in the order given.

x	µl	RNase-Free Water
1	µg	linearized template DNA with T7 promoter
2	µl	10X T7 mScript Transcription Buffer II
7.2	µl	ΨTP PreMix
2	µl	100 mM DTT
0.5	µl	ScriptGuard RNase Inhibitor
2	µl	T7 mScript Enzyme Solution
20	µl	Total reaction volume.....Incubate at 37°C for 15-30 minutes.

2. Add 1 µl RNase-Free DNase I, Incubate at 37°C for 15 minutes.

IVT RNA Purification

3. Add 29 µl of RNase-Free Water and mix; add 50 µl of 5 M ammonium acetate and mix; incubate on ice, 4°C, or –20°C for 10-15 minutes; Collect by centrifugation; Wash pellet with 70% ethanol; Resuspend RNA in 50-75 µl RNase-Free Water; Quantitate RNA.

Synthesis of Capped Ψ-RNA

4. Heat Denature the RNA, In one tube, combine the following reaction components:

x	µl	RNase-Free Water
≤69.5	µl	*In vitro* transcribed Ψ-RNA (50-60 µg RNA)
69.5	µl	Total volume

5. Incubate at 65°C for 5-10 minutes. Transfer the tube immediately to ice.

6. While the heat-denatured RNA is cooling on ice, make a Cocktail by combining the following reaction components together in a separate tube.

10	µl	10X ScriptCap Capping Buffer
5	µl	20 mM GTP
5	µl	20 mM SAM
2.5	µl	ScriptGuard RNase Inhibitor
4	µl	ScriptCap 2'-O-Methyltransferase (optional)
26.5	µl	Total volume

7. ***Just prior to*** combining the mixtures from Steps 4 and 6, add the ScriptCap Capping Enzyme to the mixture from Step 6, then combine this mixture with the mixture from Step 4.

26.5	µl	Cocktailed reaction components (from step 6)
4	µl	ScriptCap Capping Enzyme
69.5	µl	Heat-denatured RNA (from step 4)
100	µl	Total reaction volume — Incubate at 37°C for ≥30 minutes.

Synthesis of Poly(A)-Tailed Ψ-RNA

8. Combine the following at room temperature in the order given.

100	µl	5'-Capped *In vitro* transcribed RNA (from Step 7)
0.5	µl	ScriptGuard RNase Inhibitor
12	µl	10X A-Plus Poly(A) Tailing Buffer
6	µl	20 mM ATP
5	µl	A-Plus Poly(A) Polymerase
123.5	µl	Total reaction volume.....Incubate at 37°C for 30 minutes.

Purification of the Capped and Tailed Ψ-mRNA

9. Add 124 µl of 5 M ammonium acetate and mix; incubate on ice, 4°C, or –20°C for 10-15 minutes; Collect by centrifugation; Wash pellet with 70% ethanol; Resuspend RNA in 60 µl RNase-Free Water; Quantitate RNA.

Min-Immune Gold dsRNA Removal Treatment

10. Save an amount of untreated RNA to run on a denaturing agarose gel versus the treated RNA for post treatment RNA analysis.

    Combine the following at room temperature in the order given.

x	µl	RNase-Free Water
12	µl	Min-Immune Gold 10X RNase III Treatment Buffer
3	µl	ScriptGuard RNase Inhibitor
y	µl	60 µg mScript Ψ-mRNA
6	µl	Min-Immune Gold RNase III (20X)
120	µl	Total reaction volume.....Incubate at 37°C for 60 minutes.

Purification of the Capped & Tailed mScript Complete Ψ-mRNA

11. Add 120 µl of 5 M ammonium acetate and mix; incubate on ice, 4°C, or –20°C for 10-15 minutes; Collect by centrifugation; Wash pellet with 70% ethanol; Resuspend RNA in 30-60 µl RNase-Free Water; Quantitate RNA.

C. Clean-up of Problematic IVT Templates

Templates that give low yields or less than full-length transcripts may contain RNase or other contaminants. Such templates usually give better results after the following treatment:[14] Alternatively, see RNA Purification Section III (next page), skip step 4.

1) Add Proteinase K to 100-200 µg/ml and SDS to 0.5%.
2) Incubate for 30-60 minutes at 37°C.
3) Extract with an equal volume of a 1:1 mixture of TE-saturated phenol/chloroform.
4) Ethanol precipitate.
5) Gently remove the supernatant and rinse the pellet with 70% ethanol.
6) Resuspend in RNase-Free TE Buffer.

D. RNA Purification

Purify the RNA by your preferred method. The method chosen should remove residual proteins and unincorporated NTPs 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 NTPs in the supernatant. Note: for this method, the RNA to be purified must be >100 bases in size.

1) Add one volume 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 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 NTPs 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, 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 NTPs from the RNA.

1) Add one volume 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 NTPs by spin column chromatography.[14] For commercially-available columns, follow the manufacturer's instructions for this step. Recover the RNA in ~100 µl.
5) Add one-tenth volume of 3 M sodium acetate and 2.5 volumes 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 manufacturer's recommended protocol. The final resuspension of RNA should be in RNase-Free Water, TE or other suitable buffer.

E. Synthesis of IVT Ψ-RNA with Minimal Amounts of T7 mScript Enzyme Solution

The *In Vitro* Transcription and Common Usage Modules are required for this portion of the procedure.

1. Set up the IVT reaction at room temperature.
   Add the reagents in the order listed below.

Alternate INCOGNITO mScript IVT Reaction	
Component	Amount
RNase-Free Water	x µl
Linearized template DNA with T7 RNAP promoter	1 µg
10X T7 mScript Transcription Buffer II	2 µl
ΨTP PreMix	7.2 µl
100 mM DTT	2 µl
ScriptGuard RNase Inhibitor	0.5 µl
T7 mScript Enzyme Solution	0.6 µl
Total Reaction Volume	20 µl

2. Incubate at 37°C for 1-2 hours.

Important Assemble transcription reactions at room temperature in the order indicated at left. Assembly of transcription reactions at <22°C or in an alternate order, can result in the formation of an insoluble precipitate.

Transcription Buffer stored at –70°C may result in the formation of a white precipitate. To dissolve it, heat the tube at 37°C for 5 minutes and mix thoroughly.

One microgram of DNA template is recommended for most reactions. If the DNA template is <0.16 µg/µl, concentrate it, then resuspend in the appropriate amount of RNase-Free Water.

Important Use a 2 hour incubation when transcribing a template for the first time. Shorter incubation times can be used for subsequent transcription reactions once the yield characteristics of the template have been defined.

F. Synthesis of Capped Ψ-RNA with Minimal Amounts of ScriptCap Capping Enzymes

The Post-Transcriptional Capping and Common Usage Modules are required for this portion of the procedure.

1. The protocol below was designed to build a Cap 1 structure on 5' end of 50-60 μg of uncapped RNA. **If a Cap 0 structure is desired**, replace the ScriptCap 2'-O-Methyltransferase in Step 3 with an equivalent volume of RNase-Free Water.

   Combine the following reagents:

Alternate INCOGNITO mScript Capping Reaction (step 1)	
Component	Amount
RNase-Free Water	x μl
In vitro transcribed Ψ-RNA, 50-60 μg	≤74.5 μl
Total Volume	74.5 μl

Heat-denaturation of the RNA is an optional step, but it is strongly recommended for RNAs which have not previously been characterized for their ease of enzymatic capping.

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.

Alternate INCOGNITO mScript Capping Reaction (step 3)	
Component	Amount
10X ScriptCap Capping Buffer	10 μl
20 mM GTP	5 μl
20 mM SAM	5 μl
ScriptGuard RNase Inhibitor	2.5 μl
ScriptCap 2'-O-Methyltransferase (100 U/μl optional)	2 μl
Total Volume	24.5 μl

Important Do not include the ScriptCap Capping Enzyme in this mix.

Important Keep the thawed stock SAM solution on ice.

A white precipitate may form in the 10X ScriptCap Capping Buffer upon storage. To dissolve it, heat the tube at 37°C for 5 minutes and mix thoroughly.

If a Cap 0 structure is desired, replace the ScriptCap 2'-O-Methyltransferase with RNase-Free Water.

4. ***Just prior to starting the reaction,*** add the ScriptCap Capping Enzyme to the Cocktail from Step 3 and then combine this with the uncapped RNA solution from Step 1.

Alternate INCOGNITO mScript Capping Reaction (step 4)	
Component	Amount
Cocktailed reaction components (from step 3)	24.5 μl
ScriptCap Capping Enzyme (10 U/μl)	1 μl
Heat-denatured RNA (from step 1)	74.5 μl
Total Reaction Volume	100 μl

The efficiency of 2'-O-methylation can be lower if the RNA 5' end is structured. If your RNA is not completely 2'-O-methylated, we recommend using the standard protocol. Also, since the concentration of methylation sites for a given mass is higher for small RNA than for large RNA, we recommend using the standard protocol if your RNA is <730 nucleotides in length.

5. Incubate at 37°C for 2 hours.

6. Proceed directly to 3'-Poly(A)-Tailing. Purification of the RNA prior to the tailing step is NOT necessary. Alternatively, the unpurified capped RNA can be frozen and stored overnight at –20°C.

G. Synthesis of Poly(A)-Tailed RNA with Minimal Amounts of A-Plus Poly(A) Polymerase

The Poly(A)-Tailing and Common Usage Modules are required for this portion of the procedure.

1. The protocol below was designed to produce ~150 b long poly(A) tails on 60 μg of capped RNA. Combine the following reagents:

Alternate mScript Poly(A)-Tailing Reaction	
Component	Amount
5'-Capped *In vitro* transcribed RNA (from Step D6, page 12 or Step F6, page 29)	100 μl
ScriptGuard RNase Inhibitor	0.5 μl
10X A-Plus Poly(A) Tailing Buffer	12 μl
20 mM ATP	6 μl
A-Plus Poly(A) Polymerase (4 U/μl)	1.8 μl
Total Volume	123.5 μl

Important **Do not** heat-denature the 5'-Capped *In vitro* transcribed RNA.

To extend the poly(A) tail to >200 b, we recommend using the standard protocol.

2. Incubate at 37°C for 2 hours.

3. Stop the reaction using any one of the following methods:
   a) Proceed directly to Step F, Purification of the Capped and Tailed Ψ-mRNA (page 13).
   b) Immediate storage at –20°C or –70°C.
   c) Add of EDTA to a final concentration of >11 mM.

Important **Do not** stop the reaction by heat denaturation because it may degrade the RNA.

H. Min-Immune Gold dsRNA Removal Treatment

We do not recommend a minimal enzyme treatment for the removal of dsRNA.
Follow the standard protocol for this step. See page 18.

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