CELLSCRIPT™
RNA for Translation in Cells

INCOGNITO™ T7 mScript™ N1meΨ-mRNA Production System

Cat. No. **IMMY240625**

INTRODUCTION

The INCOGNITO™ T7 mScript™ N1meΨ-mRNA Production System* provides all enzymes and enzyme-related reagents for making N1-methyl-pseudouridine (N1meΨ)-containing, 5'-capped, 3'-polyadenylated mRNA. 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 N1-methyl-pseudouridine-5'-triphosphate (N1meΨTP), (ii) enzymatic capping of the RNA using the ScriptCap™ Cap 1 Capping System (contains both ScriptCap Capping Enzyme and 2'-O-Methyl-transferase) for making mRNA with a Cap 1 cap structure, (iii) A-Plus™ Poly(A) Polymerase for adding a 3'-poly(A) tail. and (iv) 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. The mScript System improves upon existing capping methods by ensuring virtually **100% transcript capping**, all caps in the **proper orientation** and the ability to produce **large amounts** of capped RNA at a **reasonable cost**. This mRNA is suitable for use in transfection and microinjection experiments as well as *in vitro* translation systems. It has been shown that N1meΨ-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]

MATERIALS

Materials Supplied

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

INCOGNITO™ T7 mScript™ N1meΨ-mRNA Production System Kit Contents
25 reactions (Module 1 of 4)

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
	N1meΨTP PreMix 25 mM each GTP, ATP, N1meΨTP, CTP	180 µl
	RNase-Free DNase I, 1 U/µl	25 µl

* see patent and license information on page 22.

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

Component list continued on next page.

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INCOGNITO™ T7 mScript™ N1meΨ-mRNA Production System Kit Contents 25 reactions (Module 2 of 4)		
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

INCOGNITO™ T7 mScript™ N1meΨ-mRNA Production System Kit Contents 25 reactions (Module 3 of 4)		
Kit Module	Kit Component	Reagent Volume
Poly(A)-Tailing	A-Plus™ Poly(A) Polymerase, 4 U/μl	130 μl
	10X A-Plus™ 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

INCOGNITO™ T7 mScript™ N1meΨ-mRNA Production System Kit Contents 25 reactions (Module 4 of 4)		
Kit Module	Kit Component	Reagent Volume
Common Usage	ScriptGuard™ RNase Inhibitor, 40 U/μl	90 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. (For suggestions, see RNA Purification, page 17)
- RNase-free TE Buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA)
- Optional: 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 is 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 N1meΨ-mRNA Production System reaction produces 60 μg of 5'-capped, 3'-poly(A)-tailed 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 N1meΨ-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 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 N1meΨ-mRNA Production System are free of detectable RNase and DNase activity, except for the inherent activity of the RNase-Free DNase I component.

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

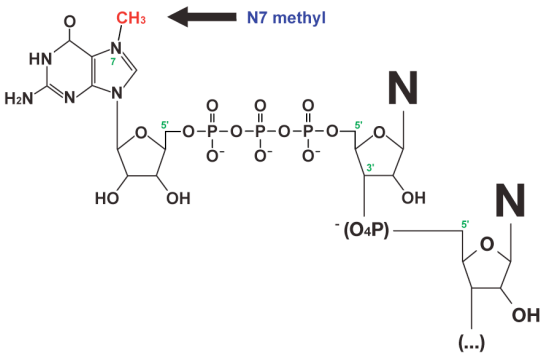
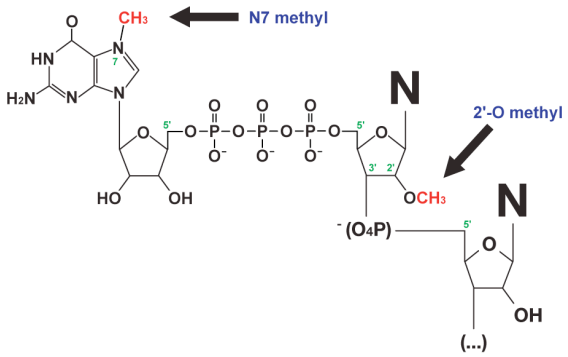


Figure 1B Cap 1-mRNA



♦ **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.

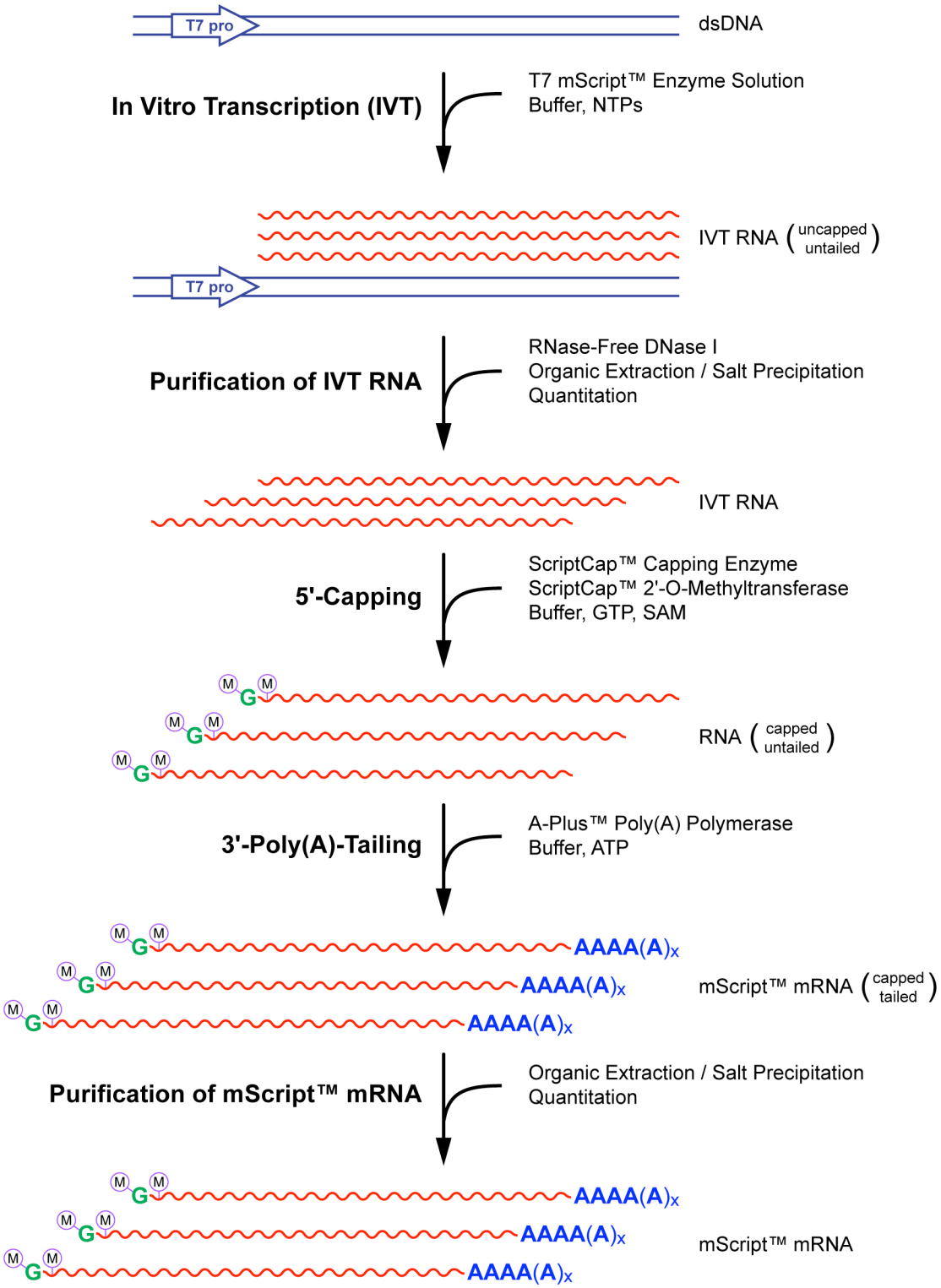
We strongly recommend to:

– 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 N1meΨ-mRNA Production System Procedure



INCOGNITO T7 mSCRIPT N1meΨ-mRNA PRODUCTION PROCEDURE OUTLINE

Synthesis of IVT N1meΨ-RNA
DNase I Treatment of IVT Reaction
Purification of the Transcription Product
Synthesis of Capped N1meΨ-RNA
Synthesis of Poly(A)-Tailed N1meΨ-RNA
Purification of the Capped and Tailed N1meΨ-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 ~100 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 N1meΨ-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
N1meΨTP Solution	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(s) 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 RNA Purification.

C. Purification of the Transcription Product

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

This step involves organic extraction followed by ammonium acetate precipitation. It removes all proteins and selectively precipitates RNA, leaving most of the DNA and unincorporated NTPs in the supernatant.

> Alternative method: **Ammonium Acetate Precipitation**
> See Technical Appendix, RNA Purification, section II (page 17).

1. Adjust reaction volume to 200 µl total using RNase-Free Water (add 179 µl to the reaction).
2. Add one volume (200 µl) of TE-saturated phenol/chloroform. Vortex vigorously for 10 seconds.
3. Spin in a microcentrifuge at >10,000 x g for 5 minutes to separate the phases.
4. Remove the aqueous (upper) phase with a pipette and transfer to a clean tube.
5. Add one volume (200 µl) of 5 M ammonium acetate, mix well then incubate for 15 minutes on ice.
6. Pellet the RNA by centrifugation at >10,000 x g for 15 minutes at 4°C.
7. Remove the supernatant with a pipette and gently rinse the pellet with 70% ethanol.
8. Remove the 70% ethanol with a pipette without disturbing the RNA pellet.
9. Allow the pellet to dry, then resuspend in 50-75 µl of RNase-Free Water for quantitation. **Do not** resuspend the RNA in an EDTA-containing solution. 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 N1meΨ-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 5' end of 50-60 μg of uncapped N1meΨ-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 N1meΨ-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.

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 N1meΨ-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 N1meΨ-RNA.

   Combine the following reagents:

INCOGNITO mScript Poly(A)-Tailing Reaction	
Component	Amount
5'-Capped *In vitro* transcribed N1meΨ-RNA (from Step D6, page 11)	100 µl
ScriptGuard RNase Inhibitor	0.5 µl
10X A-Plus 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 N1meΨ-mRNA (page 12).

   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.

F. Purification of the Capped and Tailed N1meΨ-mRNA

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

Before use in *in vivo* and *in vitro* translation systems, the capped and tailed N1meΨ-mRNA needs to be purified. We recommend the following protocol. This step involves organic extraction followed by ammonium acetate precipitation. It removes all proteins and selectively precipitates RNA, leaving most of the DNA and unincorporated NTPs in the supernatant.

For alternative protocols please refer the Technical Appendix, RNA Purification (page 17).

1. Add RNase-Free Water or $T_{10}E_1$ Buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) to the reaction tube (77 µl for the standard reaction) to achieve a total volume of 200 µl.
2. Add one volume (200 µl) of TE-saturated phenol/chloroform. Vortex vigorously for 10 seconds.
3. Spin in a microcentrifuge at >10,000 x g for 5 minutes to separate the phases.
4. Remove the aqueous (upper) phase with a pipette and transfer to a clean tube.
5. Add one volume (200 µl) of 5 M ammonium acetate, mix well then incubate for 15 minutes on ice.
6. Pellet the RNA by centrifugation at >10,000 x g for 15 minutes at 4°C.
7. Remove the supernatant with a pipette and gently rinse the pellet with 70% ethanol.
8. Remove the 70% ethanol with a pipette without disturbing the RNA pellet.
9. Allow pellet to dry, then resuspend the RNA (~60 µg) in RNase-Free Water, TE or other suitable buffer.


Over dried pellets will be difficult to resuspend.

10. Quantitate the RNA by spectrophotometry or fluorimetry and adjust to the desired concentration.


Purified capped and tailed RNA can now be frozen and stored overnight at –20°C or –70°C.

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.

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.

RELATED PRODUCTS

– A-Plus™ Poly(A) Polymerase Tailing Kit
– Cap-Clip™ Acid Pyrophosphatase
– INCOGNITO™ T7-FlashScribe™ N1meΨ-RNA Transcription Kit
– INCOGNITO™ T7-FlashScribe™ Ψ-RNA Transcription Kit
– INCOGNITO™ T7 mScript™ Ψ-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 V2
– T7 mScript™ Standard mRNA Production System V2
– T7-Scribe™ Standard RNA IVT Kit

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TECHNICAL APPENDIX

A. Electrophoretic Mobility of N1meΨ-RNA in Denaturing Agarose

While Ψ-RNA displays altered mobility during electrophoresis as compared to the comparable U-RNA of identical sequence, the mobility of N1meΨ-RNA is much more comparable to that of U-RNA. Thus N1meΨ-RNA can be sized against a U-RNA molecular weight ladder more accurately.

B. ABBREVIATED INCOGNITO T7 mSCRIPT N1meΨ-mRNA PRODUCTION PROCEDURE

for experienced kit users.

Synthesis of IVT N1meΨ-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	N1meΨTP 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 179 µl of RNase-Free Water and mix; Perform organic extraction; Add 200 µl of 5 M ammonium acetate; mix; incubate on ice for 15 minutes; Collect by centrifugation; Wash pellet with 70% ethanol; Resuspend RNA in 50-75 µl RNase-Free Water; Quantitate RNA.

Synthesis of Capped N1meΨ-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 N1meΨ-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 combing 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 N1meΨ-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 Tailing Buffer
6	µl	20 mM ATP
5	µl	mScript Poly(A) Polymerase
123.5	µl	Total reaction volume.....Incubate at 37°C for 30 minutes.

Purification of the Capped and Tailed N1meΨ-mRNA

9. Add 77 µl of RNase-Free Water or $T_{10}E_1$ Buffer and mix, Perform organic extraction,
Add 200 µl of ice-cold 5 M ammonium acetate, mix, incubate on ice for 15 minutes,
Collect by centrifugation, Wash pellet with 70% ethanol, Resuspend RNA in RNase-Free Water
Quantitate RNA, adjust to a final desired concentration, store at –20°C or –70°C.

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:[7]
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.[7] 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 manufacture's recommended protocol. The final resuspension of RNA should be in RNase-Free Water, TE or other suitable buffer.

E. Synthesis of IVT N1meΨ-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
N1meΨTP 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(s) 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 N1meΨ-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 N1meΨ-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 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 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 11 or Step F6, page 21)	100 μl
ScriptGuard RNase Inhibitor	0.5 μl
10X A-Plus 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, increase the incubation time to 60 minutes.

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 N1meΨ-mRNA (page 12).
   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.

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