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CELLSCRIPT™
RNA for Translation in Cells

INCOGNITO™ T7-FlashScribe™ N1meΨ-RNA Transcription Kit

Cat. No. **IFMY240625**

INTRODUCTION

The INCOGNITO™ T7-FlashScribe™ N1meΨ-RNA Transcription Kit* is specially formulated to enable users to obtain the maximum possible yields of N1-methyl-pseudouridine-containing RNA (N1meΨ-RNA) from an *in vitro* transcription (IVT) reaction. Although yield varies with the DNA template and other factors, a standard 60 minute, 20 µl reaction will yield up to 160 µg of RNA from 1 µg of the control template. These yields are made possible by the high-performance properties of the T7-FlashScribe enzyme. The standard reaction can be scaled up to produce milligram amounts of RNA containing the canonical nucleotides ATP, CTP, GTP and the modified nucleotide N1-methyl-pseudouridine-5'-triphosphate (N1meΨTP).

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]

INCOGNITO T7-FlashScribe IVT N1meΨ-RNA can be further processed into mRNA (5'-capped and 3'-poly[A] tailed). Options include: post-transcriptional capping using CELLSCRIPT's ScriptCap™ Cap 1 Capping System (contains both ScriptCap Capping Enzyme and 2'-O-Methyltransferase); post-transcriptional tailing using CELLSCRIPT's A-Plus™ Poly(A) Polymerase Tailing Kit; and/or co-transcriptional tailing through the use of a template-encoded tail.

MATERIALS

Materials Supplied

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

INCOGNITO™ T7-FlashScribe™ N1meΨ-RNA Transcription Kit Contents (25 reactions)	
Kit Component	Reagent Volume
T7-FlashScribe™ Enzyme Solution	50 µl
10X T7-FlashScribe™ Transcription Buffer II	50 µl
100 mM GTP	45 µl
100 mM ATP	45 µl
100 mM N1meΨTP	45 µl
100 mM CTP	45 µl
100 mM Dithiothreitol (DTT)	50 µl
RNase-Free DNase I, 1 U/µl	25 µl
ScriptGuard™ RNase Inhibitor, 40 U/µl	15 µl
T7 Control Template DNA, 0.5 µg/µl	10 µl
RNase-Free Water	1.4 ml

* see patent and license information on page 10.

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

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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 Section C "Purification of the Transcription Product")
- RNase-free TE Buffer (10 mM Tris-HCl, pH 7.5, 1 mM 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. ScriptGuard RNase Inhibitor is supplied 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 Definition

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

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-FlashScribe N1meΨ-RNA Transcription Kit is functionally tested under standard reaction conditions using the T7 Control Template DNA. The kit must produce at least 120 μg of RNA from 1 μg of the T7 Control Template DNA in 60 minutes at 37°C.

Contaminating Activity Assays

All components of the INCOGNITO T7-FlashScribe N1meΨ-RNA Transcription Kit 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 *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**:

In vitro transcription of 1 μg of the T7 Control Template DNA using the INCOGNITO T7-FlashScribe N1meΨ-RNA Transcription Kit yields ~160 μg of ~1.4-kb RNA in 60 minutes at 37°C in a standard 20 μl reaction. The table below summarizes our experiences with 1 μg of the control template DNA in a standard reaction.

Incubation Time (minutes)				
20	30	60	90	120
78-80 μg	100-109 μg	120-175 μg	120-172 μg	121-199 μg

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.

♦ **Optimizing the Reaction**:

The recommended reaction conditions should give excellent yields of RNA with most templates.

However, the yield may be improved for some templates by extending the reaction time (e.g., to 1-2 hours), increasing the amount of template in the reaction, or increasing the reaction temperature from 37°C to 42°C.

♦ **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.

PROCEDURE

A. Synthesis of IVT N1meΨ-RNA

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

INCOGNITO T7-FlashScribe N1meΨ-RNA Transcription Reaction (Step A)	
Component	Amount
RNase-Free Water	x µl
Linearized template DNA with T7 RNAP promoter	1 µg
10X T7-FlashScribe Transcription Buffer II	2 µl
100 mM ATP	1.8 µl
100 mM CTP	1.8 µl
100 mM N1meΨTP	1.8 µl
100 mM GTP	1.8 µl
100 mM DTT	2 µl
ScriptGuard RNase Inhibitor	0.5 µl
T7-FlashScribe Enzyme Solution	2 µl
Total Reaction Volume	20 µl

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.

2. Incubate at 37°C for 30 minutes.

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 (Step B)	
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

Purify the RNA using 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. For long-term storage, RNA can be stored as an ethanol pellet.

I) **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 (21 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.
8) 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** if the RNA will later be enzymatically capped (e.g., with CELLSCRIPT's ScriptCap Cap 1 Capping System).
9) Quantitate the RNA by spectrophotometry or fluorimetry. If desired, adjust the concentration of the RNA with RNase-Free Water. The RNA can now be frozen and stored at –20°C or –70°C.

II) **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) 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** if the RNA will later be enzymatically capped (e.g., with CELLSCRIPT's ScriptCap Cap 1 Capping System).
10) Quantitate the RNA by spectrophotometry or fluorimetry. If desired, adjust the concentration of the RNA with RNase-Free Water. The RNA can now be frozen and stored at –20°C or –70°C.

Continued on next page.

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

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) 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.
6) 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.
7) Incubate for 15 minutes on ice.
8) Pellet the RNA by centrifugation at >10,000 x g for 15 minutes at 4°C.
9) Remove the supernatant with a pipette and gently rinse the pellet with 70% ethanol.
10) Remove the 70% ethanol with a pipette without disturbing the RNA pellet.
11) 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** if the RNA will later be enzymatically capped (e.g., with CELLSCRIPT's ScriptCap Cap 1 Capping System).
12) Quantitate the RNA by spectrophotometry or fluorimetry. If desired, adjust the concentration of the RNA with RNase-Free Water. The RNA can now be frozen and stored at –20°C or –70°C.

IV) **RNA-Binding Purification Column**: Several options are available commercially from multiple vendors. Follow the manufacturer's recommended protocol.

1) Follow the manufacturer's recommended protocol.
2) The final resuspension of RNA should be in RNase-Free Water for quantitation. **Do not resuspend the RNA in an EDTA-containing solution** if the RNA will later be enzymatically capped (e.g., with CELLSCRIPT's ScriptCap Cap 1 Capping System).
3) Quantitate the RNA by spectrophotometry or fluorimetry. If desired, adjust the concentration of the RNA with RNase-Free Water. The RNA can now be frozen and stored at –20°C or –70°C.

TROUBLESHOOTING

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 4 hours.
	Increase the template concentration.
	Increase the reaction temperature to 42°C.
	Check for stable secondary structure (T_m >37°C) in the DNA template which can cause T7 RNAP pausing or dissociation from the template.
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.

RELATED PRODUCTS

– A-Plus™ Poly(A) Polymerase Tailing Kit
– INCOGNITO™ T7-FlashScribe™ Ψ-RNA Transcription Kit
– Min-Immune™ Gold dsRNA Removal Kit
– ScriptCap™ 2'-O-Methyltransferase Kit
– ScriptCap™ Cap 1 Capping System
– ScriptCap™ m^{7}G Capping System
– T7-FlashScribe™ Transcription Kit V2

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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. Clean-up of Problematic 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 Purification of the Transcription Product Section III, skip step 5.

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.

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