CELLSCRIPT™
RNA for Translation in Cells

INCOGNITO™ T7 ARCA 5mC- & Ψ-RNA Transcription Kit

Cat. No. **C-ICTAMY110510**

INTRODUCTION

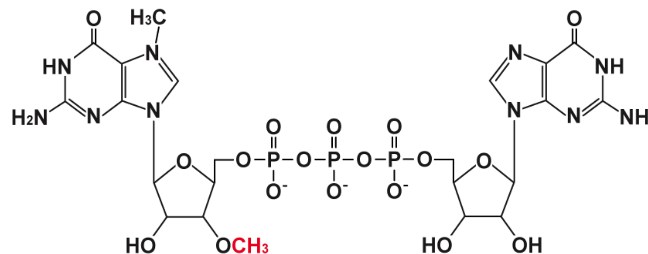
The INCOGNITO™ T7 ARCA 5mC- & Ψ-RNA Transcription Kit* is optimized for high-yield synthesis of 5-methyl-cytidine- pseudouridine-containing anti-reverse cap analog (ARCA: $m_2^{7,3'-O}$G[5']ppp[5'])-capped RNA (ARCA-5mCΨ-RNA) from an *in vitro* transcription (IVT) reaction. A 3 hour, 20 μl reaction yields up to 40 μg of ARCA-5mCΨ-RNA from 1 μg of the 1.4 kb standard T7 control template DNA. Because ARCA contains a 3'-O-methyl group on the m^7G nucleotide (Figure 1), ARCA can only be incorporated in the correct orientation at the 5' end of the RNA during an *in vitro* transcription/capping reaction.[1-3] This is not true for the standard cap analog (m^7G[5']ppp[5']G). Thus, ARCA incorporation results in the synthesis of capped RNA that is more efficiently translated *in vivo* than standard cap analog.

The ARCA/GAΨ5mC PreMix contains all four ribonucleotides (GAΨ5mC) and the ARCA. The PreMix ensures the optimal concentration of each NTP and ratio of ARCA to GTP (4:1), maximizing transcript capping (~80%) and yield. Because the concentration of GTP in the reaction is limiting, the ARCA is preferentially incorporated as the first or 5'-terminal G of the transcript. Such RNA has been used in the landmark publication by Warren *et al*. (2010), to successfully reprogram multiple human cell types into induced pluripotent stem cells.[4]

It has been shown that Ψ-mRNAs and Ψ5mC-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.[5-8]

INCOGNITO ARCA-5mCΨ-RNA can be further processed into mRNA for expression in cells. Options include: post-transcriptional capping using CELLSCRIPT's ScriptCap™ 2'-O-Methyltransferase (for conversion to a Cap 1 structure); 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.

Figure 1. ARCA Structure.



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.

INCOGNITO™ T7 ARCA 5mC- & Ψ-RNA Transcription Kit Contents (10 reactions)	
Kit Component	Volume
T7-Scribe™ Enzyme Solution	20 µl
10X T7-Scribe™ Transcription Buffer	20 µl
ARCA/GAΨ5mC PreMix (18.75 mM ATP, ΨTP & 5mCTP; 3.75 mM GTP & 15 mM ARCA)	80 µl
20 mM GTP	20 µl
100 mM Dithiothreitol (DTT)	20 µl
RNase-Free DNase I, 1 U/µl	10 µl
ScriptGuard™ RNase Inhibitor	10 µl
T7 Control Template DNA, 0.5 µg/µl	10 µl
RNase-Free Water	1.4 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 Section C "Purification of the ARCA-5mCΨ-RNA Transcription Product")
- RNase-free TE Buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA)
- Optional: TE saturated phenol/chloroform, 0.5-1 M EDTA

* see patent and license information on page 11.

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

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 Definitions

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 ARCA 5mC- & Ψ-RNA Transcription Kit is functionally tested under standard reaction conditions using the T7 Control Template DNA. The kit must produce at least 35 μg of RNA from 1 μg of the T7 Control Template DNA in 3 hours at 37°C.

Contaminating Activity Assays

All components of the INCOGNITO T7 ARCA 5mC- & Ψ-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 TRANSCRIPTION AND CAPPING

- **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**:

  In vitro transcription of 1 μg of the T7 Control Template DNA using the INCOGNITO T7 ARCA 5mC- & Ψ-RNA Transcription Kit yields approximately 40 μg of ~1.4-kb RNA in 3 hours at 37°C in a standard 20 μl 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.

- **Reaction Yields**:

  The standard reaction conditions give excellent results with most templates. The protocol may need to be modified to increase yields of some templates. To increase yields of some templates the protocol can be modified as follows:

  1) Extend the incubation to four hours. **Do not** extend the reaction time beyond 4 hours.
  2) Raise the template concentration.
  3) Increase the reaction temperature to 42°C.

- **Optimizing Yields for Long Templates**:

  Synthesis of transcripts ≥5 kb may require the addition of 1-2 μl of 20 mM GTP to the standard protocol. While this may decrease the percentage of capped transcript to 50-60%, it will increase the yield of full length transcript.

- **Optimizing Yields for Short Templates**:

  Synthesis of transcripts <500 b requires reaction times of 4 hours to maximize yields.

- **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 Capped RNA

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

Standard INCOGNITO T7 ARCA 5mC- & Ψ-RNA Transcription Reaction	
Component	Amount
RNase-Free Water	x µl
Linearized template DNA with T7 RNAP promoter	1 µg
10X T7-Scribe Transcription Buffer	2 µl
ARCA/GAΨ5mC PreMix	8 µl
100 mM DTT	2 µl
ScriptGuard RNase Inhibitor	0.5 µl
T7-Scribe Enzyme Solution	2 µl
Total Reaction Volume	20 µl

2. Incubate at 37°C for 3 hours for transcripts >500 bases. Incubate at 37°C for 4 hours for transcripts <500 bases.

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..

10X T7-Scribe 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 <0.19 µg/µl, concentrate it, then resuspend in the appropriate amount of RNase-Free Water.

B. DNase I Treatment of IVT Reaction

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

Standard 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 ARCA-5mCΨ-RNA 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, DNA 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 30-50 μl of RNase-Free Water for quantitation. **Do not resuspend the RNA in an EDTA-containing solution** if the RNA will later be enzymatically converted to Cap 1 RNA (e.g., with CELLSCRIPT's ScriptCap 2'-O-Methyltransferase Kit).
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, while leaving most of the DNA and 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 50 μl total using RNase-Free Water (add 29 μl to the reaction).
2) Add one volume (50 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 (50 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 30-50 μl of RNase-Free Water for quantitation. **Do not resuspend the RNA in an EDTA-containing solution** if the RNA will later be enzymatically converted to Cap 1 RNA (e.g., with CELLSCRIPT's ScriptCap™ 2'-O-Methyltransferase Kit).
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, digested DNA, and unincorporated NTPs from the RNA.

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 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 digested DNA and unincorporated NTPs by spin column chromatography.[8] For commercially-available columns, follow the manufacturer's instructions for this step. Recover the RNA in 50-100 μl.
6) Add one-tenth volume (5-10 μl) of 3 M sodium acetate and 2.5 volumes (125-250 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 30-50 μl of RNase-Free Water for quantitation. **Do not resuspend the RNA in an EDTA-containing solution** if the RNA will later be enzymatically converted to Cap 1 RNA (e.g., with CELLSCRIPT's ScriptCap 2'-O-Methyltransferase Kit).
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 30-50 μl of RNase-Free Water for quantitation. **Do not resuspend the RNA in an EDTA-containing solution** if the RNA will later be enzymatically converted to Cap1 RNA (e.g., with CELLSCRIPT's ScriptCap™ 2'-O-Methyltransferase Kit).
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.

REFERENCES

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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.

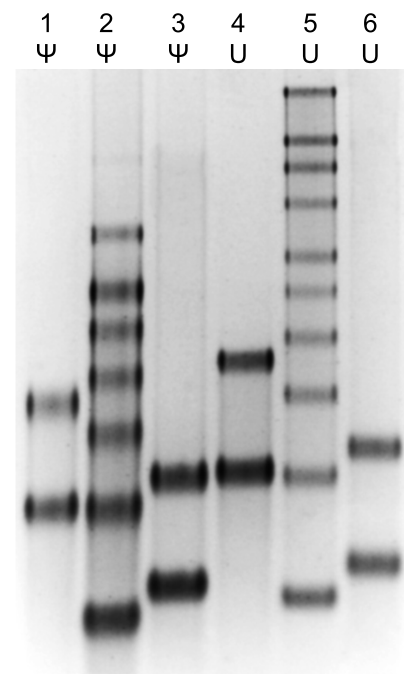
RELATED PRODUCTS

– A-Plus™ Poly(A) Polymerase Tailing Kit
– INCOGNITO™ T7-FlashScribe™ N1meΨ-RNA Transcription Kit
– INCOGNITO™ T7-FlashScribe™ Ψ-RNA Transcription Kit
– INCOGNITO™ T7 mScript™ N1meΨ-mRNA Production System
– 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
– Min-Immune™ Gold dsRNA Removal Kit

TECHNICAL APPENDIX

A. Electrophoretic Mobility of 5mCΨ-RNA in Denaturing Agarose

5mCΨ-RNA displays altered mobility during electrophoresis as compared to the comparable CUF-RNA of identical sequence. This is relevant when comparing 5mCΨ-RNA transcript size to a CU-RNA molecular weight ladder. The data shown below is for Ψ-RNA vs U-RNA but the same concept holds true for 5mCΨ-RNA as well.



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. 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:[9] 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.

Research Use Label License

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