DATA SHEET

Jena Bioscience

HighYield T7 mRNA Uridine Modification Testkit (ARCA)

Synthesis of differentially uridine-modified (m)RNA with Cap 0 structure

Cat. No.	Amount
RNT-137	1 kit

For general laboratory use.

Shipping: shipped on blue ice

Storage Conditions: store at -20 °C

Additional Storage Conditions: avoid freeze/thaw cycles

Shelf Life: 12 months after date of delivery

Description:
HighYield T7 mRNA Uridine Modification Testkit is designed to produce large amounts of differentially uridine-modified (m)RNA via *in vitro* transcription with T7 RNA polymerase. Included Cap analog ARCA allows additional co-transcriptional introduction of a Cap 0 moiety. The resulting 5'-capped (m)RNA can subsequently be used for microinjection, transfection or *in vitro* translation experiments. Epigenetic internal mRNA modifications e.g. (pseudo)uridine methylation or thiolation as well as 5'-capping have been shown to increase translation efficiency and to reduce immunogenicity [1]-[7] however, the optimal combination needs to be individually termined for each mRNA target.

Internal modifications are introduced by correspondingly labeled nucleotides. Anti-reverse Cap analog (ARCA, $m^{7,3'\text{-}O}GP_3G$) co-transcriptionally introduces a 7-methylguanosine moiety (m^7G, Cap 0 structure) required for efficient translation and increased stability of eukaryotic mRNA. 3'-O-methylation of the m^7G moiety allows incorporation in the correct ("anti-reverse") orientation only resulting in a 100 % translatable capped (m)RNA population.

Nukleotide	Reference
Pseudo-UTP	[1]-[4]
N^1-Methylpseudo-UTP	[5],[6]
5-Methoxy-UTP	[7]
2-Thio-UTP	[1]-[3]
ARCA	[3][4]

The kit contains sufficient reagents for 25 ARCA-capping reactions à 20 µl (6 mM ARCA, 1.5 mM GTP, 7.5 mM CTP, 7.5 mM UTP, 7.5 mM ATP). Amounts of each modified UTP are sufficient for 6 reactions (7.5 mM) à 20 µl each. An individual optimization of Cap Analog concentration or incorporation of modified nucleotides (e.g. Pseudo-UTP) up to 100% substitution can easily be achieved with the single nucleotide format.

A 20 µl of ARCA-capping reaction without modified uridine yields about 30-50 µg RNA after 30 min incubation (1 µg T7 control template (G-initiating), 1.4 kb RNA transcript). Yields may however vary depending on the template (promotor design, sequence length, secondary structure formation)) as well as on the combination and substitution rate of modified uridines.

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Content:

HighYield T7 RNA Polymerase Mix
incl. RNase inhibitor and 50 % glycerol (v/v)
2x 40 µl

HighYield T7 Reaction Buffer
1x 200 µl (10x), HEPES-based

ATP - Solution
1x 100 µl (100 mM)

GTP - Solution
1x 100 µl (100 mM)

CTP - Solution
1x 100 µl (100 mM)

UTP - Solution
1x 100 µl (100 mM)

ARCA - Solution
4x 10 µl (100 mM)

Pseudo-UTP
1x 10 µl (100 mM)

N^1-Methylpseudo-UTP
1x 10 µl (100 mM)

5-Methoxy-UTP
1x 10 µl (100 mM)

2-Thio-UTP
1x 10 µl (100 mM)

T7 G-initiating control template (1.4 kbp)
1x 10 µl (200 ng/µl), 1.4 kbp PCR fragment plus T7 class III phi6.5 promotor (G-initiating) resulting in 1400 nt RNA transcript

PCR-grade water
1x 1.2 ml

DTT
1x 100 µl (100 mM)

To be provided by user
T7 Promotor-containing DNA template
RNA purification tools
RNAse-free DNAse I

Important Notes (Read before starting)

Prevention of RNAse contamination
Although a potent RNase Inhibitor is included, creating a RNAse-free work environment and maintaining RNAse-free solutions is critical for performing successful *in vitro* transcription reactions. We therefore recommend

- to perform all reactions in sterile, RNAse-free tubes using sterile pipette tips.
- to wear gloves when handling samples containing RNA.
- to keep all components tightly sealed both during storage and reaction procedure.

Template requirements

- **Template type**:

  ARCA-capping: Linearized plasmid DNA or PCR products containing a double-stranded G-initiating T7 class III phi6.5 promotor region upstream of the target sequence.
  Minimum T7 promotor sequences:
  T7 class III phi6.5 promotor (G-initiating)
  5'-TAATACGACTCACTATA**G***NN*...-3'
  Bold: First base incorporated into RNA, *NN*: ideally CG

- **Template quality**: DNA template quality directly influences yield and quality of transcription reaction. Linearized plasmid DNA needs to be fully digested and to be free of contaminating RNase, protein and salts. We recommend selecting restriction enzymes that generate blunt ends or 5´-overhangs and purification by phenol/chloro¬form extraction. A PCR mixture can be used directly however, better yields will usually be obtained with purified PCR products (e.g. via silica-membrane based purification columns).

- **mRNA production**: For the production of functional mRNA, the DNA template needs to encode the following structural features e.g. 3'-UTR, 5'-UTR, correctly orientated target sequence and poly(A)-tail. Alternatively, poly (A)-tailing can post-transcriptionally be performed with Poly(A) polymerase.

***In vitro* Transcription protocol**

The general protocols are set up for 1 µg DNA template (refer to "Important Notes" regarding template requirements), a final NTP concentration of 7.5 mM, a final ARCA concentration of 6 mM and 100% substitution of UTP by a modified UTP (ARCA-capping / No-capping reaction).

ARCA-capping reaction:
Depending on the RNA sequence and final application, individual

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reaction optimization may improve product yield and biological function (e.g. modified UTP/UTP ratio, variation of ARCA:GTP ratio, variation of template amount, variation of incubation time). An optimal balance between reaction and capping efficiency is usually achieved by an ARCA:GTP ratio of 4:1 (approx. 80% capped RNA transcripts). The synthesis of RNA transcripts >/= 5000 nt may require higher GTP concentrations. Lowering the ARCA:GTP ratio (e.g. 2:1) lowers the capping efficiency but may significantly improve the yield of full-length transcripts.

Component	Volume	Final conc.
PCR-grade water	X µl	
HighYield T7 Reaction Buffer (10x)	2 µl	1x
DTT (100 mM)	2 µl	10 mM
ARCA (100 mM)	1.2 µl	6 mM
GTP (100 mM)	0.3 µl	1.5 mM
modified UTP (100 mM)	1.5 µl	7.5 mM
CTP (100 mM)	1.5 µl	7.5 mM
ATP (100 mM)	1.5 µl	7.5 mM
Template DNA	X µl	1 µg
HighYield T7 RNA Polymerase Mix	2 µl	
Total volume	**20 µl**	

- Place HighYield T7 RNA Polymerase Mix on ice.
- Thaw all remaining components at room temperature (RT), mix by voretexing and spin down briefly.
- Assemble all components at RT to a nuclease-free microtube (sterile pipette tips) in the following order:
- Mix PCR-grade water, HighYield T7 Reaction Buffer and DTT by voretexing and spin down briefly.
- Add nucleotide solutions and template DNA, vortex and spin down briefly.
- Add HighYield T7 RNA Polymerase Mix vortex and spin down briefly.
- Incubate for 2h at 37°C in the dark (e.g. PCR cycler). Individual optimization may increase product yield (0.5h–4h at 37°C).

No-capping reaction:
Depending on the RNA sequence and final application, individual reaction optimization may improve product yield and biological function (e.g. modified UTP/UTP ratio, variation of template amount, variation of incubation time). 5'-capping can be achieved posttranscriptionally with capping enzymes (alternativ approach to co-transcriptional capping with cap analoges).

Component	Volume	Final conc.
PCR-grade water	X µl	
HighYield T7 Reaction Buffer (10x)	2 µl	1x
DTT (100 mM)	2 µl	10 mM
GTP (100 mM)	1.5 µl	7.5 mM
modified UTP (100 mM)	1.5 µl	7.5 mM
CTP (100 mM)	1.5 µl	7.5 mM
ATP (100 mM)	1.5 µl	7.5 mM
Template DNA	X µl	1 µg
HighYield T7 RNA Polymerase Mix	2 µl	
Total volume	**20 µl**	

- Place HighYield T7 RNA Polymerase Mix on ice.
- Thaw all remaining components at room temperature (RT), mix by voretexing and spin down briefly.
- Assemble all components at RT to a nuclease-free microtube (sterile pipette tips) in the following order:
- Mix PCR-grade water, HighYield T7 Reaction Buffer and DTT by voretexing and spin down briefly.
- Add nucleotide solutions and template DNA, vortex and spin down briefly.
- Add HighYield T7 RNA Polymerase Mix vortex and spin down briefly.
- Incubate for 2h at 37°C in the dark (e.g. PCR cycler). Individual optimization may increase product yield (0.5h–4h at 37°C).

Please note: Reagents for the following steps are not provided within this kit.

DNA template removal
Depending on the down-stream application, removal of template DNA might be required. We recommend a salt-resistant, high efficiency DNAase such as Turbo™DNAse (ThermoFisher). Follow the manufacturer instructions.

Removal of 5'-triphosphate groups
5'-ends of *in vitro* phosphorylated RNAs carry a triphosphate group that is known to trigger RIG-1 mediated innate immune response in mammalian cells[8,9]. Removal with phosphatases (e.g. CIP) before final purification is therefore recommended for RNA probes intended for transfection experiments. Please refer to the following references for more detailed information: [8],[9].

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(m)RNA purification

Purification of (m)RNA is required prior to transfection or (m)RNA quantitation by absorbance measurement. Spin column purification will remove proteins, salts and unincorporated nucleotides. Please follow the manufacturer instructions and ensure that the columns match with product size and possess a sufficient binding capacity (e.g. RNA Clean & Concentrator™ columns (Zymo Research) or Monarch® RNA Cleanup kit (NEB)). Other RNA purification methods such as LiCl precipitation may work but have not been tested.

(m)RNA quantitation

RNA concentration can be determined by absorbance measurement at 260 nm (A_{260}) according to the Law-of-Lambert-Beer (A_{260} = 1 corresponds to 40 µg/ml ssRNA).

Related Products:

$m_2^{7,3'-O}GP_3G$ (ARCA Cap Analog) - Solution, #NU-855
Pseudo-UTP, #NU-1139
N^1-Methylpseudo-UTP, #NU-890
5-Methoxy-UTP, #NU-972
2-Thio-UTP, #NU-1151

Selected References:

[1] Karikó *et al.*(2005) Suppression of RNA Recognition by Toll-like Receptors: The Impact of Nucleoside Modification and the Evolutionary Origin of RNA. *Immunity***23**:165.
[2] Karikó *et al.*(2008) Incorporation of Pseudouridine into mRNA Yields Superior Nonimmunogenic Vector With Increased Translational Capacity and Biological Stability. *Mol. Ther.***16(11)**:1833.
[3] Kormann *et al.*(2011) Expression of therapeutic proteins after delivery of chemically modified mRNA in mice. *Nature Biotechnology***29(2)**:154.
[4] Warren *et al.*(2011) Highly Efficient Reprogramming to Pluripotency and Directed Differentiation of Human Cells with Synthetic Modified mRNA. *Cell Stem Cell***7**:618.
[5] Svitkin *et al.*(2017) N1-methyl-pseudouridine in mRNA enhances translation through eIF2alpha-dependent and independent mechanisms by increasing ribosome density. *Nucleic Acid Res***45(10)**:6023.
[6] Andies *et al.*(2015) N1-methylpseudouridine-incorporated mRNA outperforms pseudouridine-incorporated mRNA by providing enhanced protein expression and reduced immunogenicity in mammalian cell lines and mice. *J. Control. Release***217**:337.
[7] Li *et al.*(2016) Effects of Chemically Modified Messenger RNA on Protein Expression. *Bioconjugate Chem.***27**:849.
[8] Wienert *et al.* (2018) In vitro transcribed guide RNAs trigger an innate immune response via RIG-I pathway. *PLoS Biol.* **16 (7)** :e2005840.
[9] Kim *et al.* (2018) CRISPR RNAs trigger innate immune responses in human cells. *Genome Res.* **28 (3)**:367.