

TECHNICAL MANUAL

RiboMAX™ AOF RNA Production System

Instructions for Use of Product
P2000

RiboMAX™ AOF RNA Production System

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1. Description

The successful development and optimization of large-scale manufacturing of high-quality RNA requires optimizing an in vitro transcription (IVT) reaction specific to the target RNA molecule, using reaction components that can be easily scaled through development stages, from research to commercial production, without compromising on performance.

The RiboMAX™ AOF RNA Production System is an in vitro transcription system designed to facilitate the optimization of IVT reactions for cell-free production of large quantities of RNA. The system includes animal-origin-free (AOF), cGMP-manufactured, in vitro transcription reagents to ensure that the same materials used in development are suitable for the large-scale production of RNA.

All product components are AOF. The materials used in the manufacture of the product are from appropriate origins and comply with the relevant revision of the CPMP/CVMP Guideline EMA/410/01 (Note for Guidance on Minimizing the Risk of Transmitting Animal Spongiform Encephalopathy Agents via Human and Veterinary Medicinal Products).

No animal or human materials were used during the manufacture of these products. During the recombinant protein production process (fermentation and protein purification), the products did not contain, use or come into contact with animal-derived materials.

Table 1. Component Formulations. No animal-derived materials were included.

Kit Component	Description
10X Transcription Buffer (–Mg)	800mM HEPES (pH 7.5), 20mM spermidine
T7 RNA Polymerase, AOF (400u/μl)	Enzyme formulated at specified concentration in enzyme storage buffer: 20mM potassium phosphate buffer (pH 7.7 at 25°C), 1mM EDTA, 10mM DTT, 0.1M NaCl, 0.1% Triton® X-100, 50% (v/v) glycerol
Inorganic Pyrophosphatase, AOF (2u/μl)	Enzyme formulated at specific concentration in low-Triton® enzyme storage buffer: 20mM potassium phosphate buffer (pH 7.7 at 25°C), 1mM EDTA, 10mM DTT, 0.1M NaCl, <0.1% Triton® X-100, 50% (v/v) glycerol.
RNasin® Plus Ribonuclease Inhibitor, AOF (40u/μl)	Enzyme formulated at specified concentration in enzyme storage buffer: 20mM HEPES-KOH (pH 7.6), 50mM KCl, 8mM DTT, 50% (v/v) glycerol.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT. #
RiboMAX™ AOF RNA Production System	1 system	P2000

Each system contains sufficient reagents for 5 × 1ml reactions. Includes:

- 275µl T7 RNA Polymerase, AOF (400u/µl)
- 55µl Inorganic Pyrophosphatase, AOF (2u/µl)
- 250µl RNasin® Plus Ribonuclease Inhibitor, AOF (40u/µl)
- 1ml 10X Transcription Buffer (–Mg)
- 1ml 1M Magnesium Chloride (MgCl₂)
- 1ml 1M Magnesium Acetate Mg(OAc)₂
- 1.125ml 1M DTT
- 525µl rATP (100mM)
- 525µl rGTP (100mM)
- 525µl rCTP (100mM)
- 525µl rUTP (100mM)
- 2 × 1.25ml Nuclease-Free Water
- 50µl T7 Linear Control DNA

Storage Conditions: Store all components at –30°C to –10°C.

Note: “–Mg” indicates no magnesium.

3. Guidelines for Preparing In Vitro Transcription Reaction DNA Templates

3.A. Linearized Plasmid DNA

Plasmid DNA is linearized prior to IVT to produce RNA transcripts of defined length. Plasmid DNA can be linearized by digestion with an appropriate restriction endonuclease and then purified prior to use in an IVT reaction.

Avoid the use of restriction enzymes that produce 3' overhangs (Table 2). Extraneous transcripts, in addition to the expected transcript, have been reported when such templates are transcribed (1). These extraneous transcripts can contain sequences complementary to the expected transcript as well as sequences corresponding to the plasmid vector DNA. If these enzymes must be used, the ends of the linearized plasmid DNA template can be made blunt prior to IVT using DNA Polymerase I Large (Klenow) Fragment or T4 DNA polymerase.

Table 2. Commonly Used Restriction Enzymes That Generate 3' Overhangs.

AatII	Apal	BanII
BglI	Bsp1286I	BstXI
CfoI	HaeII	HgiAI
HhaI	KpnI	PstI
PvuI	SacI	SacII
SfiI	SphI	

3.A. Linearized Plasmid DNA (continued)

Table 3. Reliable Enzymes for Linearization.

Blunt-End Cutters (Preferred for Use)	
EcoRV (GAT↓ATC)	Often used due to its simple blunt-end cut, minimizing unwanted extensions.
ScaI (AGT↓ACT)	Sometimes used depending on the plasmid design.
5' Overhang Cutters (Ok to Use; Blunt-End Cutters are Preferred)	
XhoI (C↓TCGAG)	Frequently used for linearization before T7 transcription.
BamHI (G↓GATCC)	Commonly used in vectors designed for easy excision of inserts.
BspQI (GCTCTTCN↓)	Use for preparing scarless in vitro transcription templates from plasmid DNA.
Sall (G↓TCGAC)	Often used in SP6 or T7-based transcription systems.

3.B. PCR-Generated DNA

PCR-generated DNA containing the T7 phage promoter can be used in transcription reactions. The phage promoter sequences can be incorporated into DNA by using primers that flank the phage promoter sequences in the vector or by having the promoter sequence within the 5' oligomer used in the PCR. The resulting PCR-generated DNA should be purified prior to use in an IVT reaction.

4. In Vitro Transcription Reaction Protocol

1. Assemble the reaction on ice. Combine reaction components in the order shown.

Note: In the table below, two sample reaction sizes are provided, 100µl and 1ml.

Reaction Component	Stock Concentration	Sample Reaction		Control Reaction	Final Concentration
Nuclease-Free Water		Yµl	Yµl	28.25–32.25µl	
10X Transcription Buffer (–Mg)	10X	10µl	100µl	10µl	1X
1M Mg(OAc) ₂ or 1M MgCl ₂	1M	3µl	30µl	3µl	30mM
1M DTT (Optional)	1M	0–1µl	0–10µl	0–1µl	0–10mM
Linear Template DNA	Xµl	Xµl	---	50ng/µl	
T7 Linear Control DNA	---	---	5µl	50ng/µl	
100mM rATP	100mM	10µl	100µl	10µl	10mM
100mM rGTP	100mM	10µl	100µl	10µl	10mM
100mM rCTP	100mM	10µl	100µl	10µl	10mM
100mM rUTP	100mM	10µl	100µl	10µl	10mM
RNasin® Plus Ribonuclease Inhibitor	40u/µl	3.75µl	37.5µl	3.75µl	1.5u/µl
Inorganic Pyrophosphatase	2u/µl	1µl	10µl	1µl	0.02u/µl
T7 RNA Polymerase	400u/µl	5µl	50µl	5µl	20u/µl
final reaction volume		100µl	1ml	100µl	

2. Mix gently and incubate at 37°C for 1 hour.

Note: Do not freeze the transcription reaction. After the transcription reaction is complete, proceed directly to DNase step (Section 5.E).

5. Guidelines for Optimizing the In Vitro Transcription Reaction

Each component of an IVT reaction serves an important purpose in RNA synthesis. However, a few key, interdependent reaction components have the greatest impact on IVT reaction performance:

- a) the linearized DNA template
- b) T7 RNA Polymerase
- c) the Mg^{2+} -to- rNTP molar ratio

Therefore, we recommend optimizing the IVT reaction for each DNA template, to maximize yield and minimize the formation of double-stranded RNA (dsRNA) specific to your RNA target.

During reaction optimization, transcript yield and RNA integrity can be monitored using capillary electrophoresis tools like the TapeStation (Agilent) or LabChip® GXII Touch (Caliper Life Sciences). dsRNA formation can be evaluated using the Lumit® dsRNA Detection Assay (Cat.# W2041, W2042) without requiring transcript purification.

5.A. DNA Template Quality

Residual salts (especially NaCl) used to precipitate DNA template may inhibit RNA polymerase activity. Use template DNA that has been purified using column chromatography to minimize salt carryover from DNA template into the IVT reaction.

Nonlinearized template DNA may produce larger than expected RNA transcripts. Analyze template DNA by gel electrophoresis or capillary electrophoresis to evaluate for nonlinearized DNA. If undigested vector is noted, redigest with the appropriate restriction enzyme.

Protruding 3' termini on the DNA template can result in the synthesis of longer than expected transcripts that are initiated at the terminus of the template (1). If it's impossible to avoid linearizing with a restriction enzyme that results in a protruding 3' termini, the DNA template ends should be made blunt using DNA Polymerase I Large (Klenow) Fragment before use in a transcription reaction.

5.B. Enzyme Concentrations

T7 RNA Polymerase

Optimal T7 RNA Polymerase concentrations in an IVT reaction can depend on the size of the DNA template, and reaction incubation time and temperature. The reaction protocol provided starts with a linearized DNA-to-T7 RNA polymerase ratio of 5µg:2,000 units in a 100µl reaction volume with an incubation time of 1 hour at 37°C. Generally, an excess of T7 RNA polymerase is used relative to template DNA to drive the transcription reaction. However, too much or too little enzyme relative to DNA template can result in decreased RNA yield and integrity. Therefore, optimizing the DNA template-to-T7 RNA Polymerase ratio is recommended.

The T7 Linear Control DNA provided with this product will yield a 1,800 base mRNA transcript.

Inorganic Pyrophosphatase and Ribonuclease Inhibitors

Pyrophosphate is a byproduct of an IVT reaction and inhibits polymerase activity by precipitating Mg^{2+} ions (2). Inorganic pyrophosphatase is added to an IVT reaction to catalyze the hydrolysis of pyrophosphates into inactive single orthophosphate ions that do not bind magnesium ions. Including inorganic pyrophosphatase in an IVT reaction has been shown to improve the overall yield of in vitro transcription reactions using T7 RNA polymerase (3). While inorganic pyrophosphatase is an important component of high-yield IVT reactions, adjusting the concentration of the enzyme from the concentration in the standard protocol does not have a significant impact on IVT reaction yield.

RNases are ubiquitous in the environment and pose a significant risk to the in vitro synthesis of RNA transcripts. RNase activity is blocked by including an RNase inhibitor in the IVT reaction. The concentration of RNasin® Plus Ribonuclease Inhibitor present in the standard reaction protocol described above should be sufficient to prevent RNA degradation in an IVT reaction from RNases that could be environmentally introduced into the IVT reaction.

5.C. Magnesium and Nucleotide Concentrations

Magnesium

Either magnesium acetate or magnesium chloride can supply the important Mg^{2+} that is required for T7 RNA polymerase activity. We recommend starting with magnesium acetate in the IVT reaction. For most linear DNA templates, a reaction containing acetate ions results in a higher yield compared to IVT reaction containing chloride ions (4).

Magnesium-to-rNTP Ratio

The magnesium ion is required for both T7 RNA Polymerase activity and nucleoside triphosphates (rNTPs) stabilization during RNA synthesis. Magnesium can also influence the folding structure of nucleic acids. The reaction protocol provided starts with a Mg^{2+} -to-rNTP ratio of 0.75 and an IVT reaction incubation at 37°C for 1 hour. However, this ratio may not be optimal for all DNA templates and T7 RNA polymerase enzyme concentrations. Determining the optimal Mg^{2+} -to-rNTP ratio specific to the DNA template and quantity of T7 RNA polymerase in the reaction is important for an optimally performing IVT reaction that maximizes yield while minimizing dsRNA formation (4).

Incorporating Modified Nucleotides

Modified nucleotides can be incorporated into an IVT reaction to enhance the stability, function and performance of the RNA transcript (5). A modified nucleotide can be included to:

- **Improve RNA stability.** RNA is prone to degradation by nucleases. A modified nucleotide can reduce transcript recognition by RNA-degrading enzymes, therefore prolonging the RNA half-life.
- **Reduce immunogenicity.** Unmodified RNA can be recognized by the immune system as foreign, triggering an inflammatory response. Modified nucleotides can reduce immune response activation.

To replace an rNTP with a modified nucleotide in an IVT reaction, use the modified nucleotide at the same concentration that was determined when optimizing the magnesium-to-rNTP ratio for the unmodified nucleotide.

5.D. Cotranscriptional Capping

Most eukaryotic mRNAs contain a m⁷G(5')ppp(5')G cap at the 5' end, which is important for binding translation initiation factors and contribute to mRNA stability. The following protocol provides initial reaction conditions for cotranscriptional capping in an IVT reaction. This protocol is intended as a starting point. The cap analog-to-total rNTPs ratio will need to be empirically determined to balance the percentage of capped RNA transcript product with the transcription reaction efficiency. Furthermore, incorporating a cap analog can impact the efficiency of the IVT reaction, requiring re-optimization of the DNA template, T7 RNA polymerase and Mg²⁺-to-rNTP ratio in the IVT reaction.

1. Assemble the reaction on ice. Combine reaction components in the order shown.

Note: In the table below, two sample reaction sizes are provided, 100μl and 1ml.

Reaction Component	Sample Reaction		Final Concentration
Nuclease-Free Water	Yμl	Yμl	
10X Transcription Buffer (–Mg)	10μl	100μl	1X
1M Mg(OAc) ₂ or 1M MgCl ₂	3μl	30μl	30mM
1M DTT (Optional)	0–1μl	0–10μl	0–10mM
Linear Template DNA	Xμl	Xμl	50ng/μl
100mM rATP	10μl	100μl	10mM
100mM rGTP	10μl	100μl	10mM
100mM rCTP	10μl	100μl	10mM
100mM rUTP	10μl	100μl	10mM
Cap Analog (100mM)	8μl	80μl	8mM
RNasin® Plus Ribonuclease Inhibitor	3.75μl	37.5μl	1.5u/μl
Inorganic Pyrophosphatase	1μl	10μl	0.02u/μl
T7 RNA Polymerase	5μl	50μl	20u/μl
final reaction volume	100μl	1ml	

2. Mix gently and incubate at 37°C for 1 hour.

Note: Do not freeze transcription reaction. After the transcription reaction is complete, proceed directly to DNase treatment (Section 5.E).

5.E. DNA Template Removal

The DNA template can be removed by digestion with DNase after the transcription reaction. The following is an example of a post-transcription DNase treatment protocol:

1. After performing the IVT reaction, add RNase-Free DNase to a concentration of 1 unit per microgram of template DNA.
2. Incubate for 15 minutes at 37°C.
3. Purify and analyze the RNA by capillary electrophoresis.

6. Guidelines for Evaluating In Vitro Transcription Reaction Performance

6.A. RNA Purification

LiCl precipitation is an inexpensive and simple method for mRNA purification.

1. After DNase treatment, bring the IVT reaction to a final concentration of 2.5M LiCl (from a stock solution of 7.5M lithium chloride, 50mM EDTA).
2. Incubate the solution at -20°C for 30 minutes to overnight, then centrifuge at 16,000rpm for 15 minutes at 4°C.
3. Discard the supernatant and wash the pellet with cold 70% ethanol.
4. Centrifuge at 16,000rpm for 15 minutes at 4°C.
5. Discard the 70% ethanol and resuspend the RNA pellet in nuclease-free water or TE buffer. The RNA solution may need to be heated at 65°C for 3 minutes to resuspend the RNA.

LiCl precipitation will not remove dsRNA contamination. If the RNA contains a polyA tail, then use polyA RNA isolation, such as PolyATtract® mRNA Isolation System (Cat.# Z5210), which uses biotinylated-oligo(dT) probe along with streptavidin paramagnetic particles to separate full-length polyA mRNA from small molecular weight RNA, such as truncated transcripts and dsRNA that does not contain a poly A tail.

6.B. RNA Purity and Concentration

Spectrophotometric measurement is widely used to quantify and assess the quality of RNA samples. A spectrophotometric A_{260}/A_{280} ratio of around ~2.0 generally indicates high RNA purity. An A_{260}/A_{230} ratio between 2.0–2.2 suggests minimal contamination from organic compounds or chaotropic salts often used in RNA purification procedures. If the ratio is significantly lower than these, repurification of the RNA may be required.

6.C. RNA Yield and Integrity

Electrophoresis is widely used to evaluate RNA yield and size. An in vitro transcript can be evaluated by denaturing gel electrophoresis or capillary electrophoresis (e.g., TapeStation [Agilent] or LabChip® GXII Touch [Caliper Life Sciences]). Capillary electrophoresis has an advantage over denaturing gel electrophoresis because it can be used to evaluate RNA yield and size without requiring transcript purification (1:50–1:100 dilution of IVT). Sanger sequencing (Spectrum Compact CE System, Cat.# CE1304) with reverse-transcribed RNA transcript (cDNA) can be performed to verify the RNA transcript sequence integrity.

6.D. Double-Stranded RNA Contamination

IVT reactions can generate double-stranded RNA (dsRNA) byproducts. This contaminant can have negative effects such as high immunogenicity. Sensitive and quantitative dsRNA assessment is an important tool to evaluate dsRNA byproducts in an IVT reaction. The Lumit[®] dsRNA Detection Assay (Cat.# W2041, W2042) can sensitively and accurately quantify dsRNA in IVT samples independent of sequence and size with an accurate linear range of 0.04–2.5ng/ml.

6.E. In Vitro Translation

In vitro translation studies are important for determining whether the RNA transcript successfully directs the expression of the target protein it encodes. Furthermore, analysis of the target protein can provide insight to optimizing untranslated regions (UTRs), codon optimization or selecting genetic modifications that may enhance translation of the IVT RNA transcript to protein. In vitro translation systems, including Rabbit Reticulocyte Lysate System, Nuclease Treated (Cat.# L4960) and Wheat Germ Extract (Cat.# L4380), are optimized for translation and can be driven by mRNA. Both systems have been shown to generate full-length proteins using both uncapped and capped RNA with and without modified nucleotides. The wheat germ system is more stringent and prefers capped mRNA. In vitro translated proteins (such as Transcend[™] Non-Radioactive Translation Detection System (Cat.# L5061) or FluoroTect[™] Green_{Lys} In Vitro Translation Labeling System (Cat.# 5001) can be used to nonradioactively detect and analyze in vitro translated proteins.

6.F. Cell-Based Translation/Expression

The integrity of mRNA, both with and without modified nucleotides, can be evaluated in cells (HEK293 or CHO). Using the ViaScript[™] mRNA Transfection Reagent (Cat.# EV4961), mRNA can be transfected into cells, and subsequent protein analysis by western blotting or functional assays performed.

6.G. Functional Assay

Reporter bioassays and cell health assays can be used to characterize RNA therapies for biological functions, such as Fc effector activity, Immune checkpoint modulation, T cell retargeting, cytokine and growth factor signaling and tumor antigen-specific cell killing. Reporter bioassays help drug developers understand a drug's mechanism of action (MOA) by measuring the potency and stability throughout an RNA therapeutic development workflow.

7. References

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3. Cunningham, P.R. and Ofengand, J. (1990) Use of inorganic pyrophosphatase to improve the yield of in vitro transcription reactions catalyzed by T7 RNA polymerase. *BioTechniques* **9**, 713–4.
4. Boman, J. *et al.* (2024) Quality by design approach to improve quality and decrease cost of in vitro transcription of mRNA using design of experiments. *Biotechnol. Bioeng.* **121**, 3415–27.
5. Liu, A. and Wang, X. (2022) The pivotal role of chemical modification in mRNA therapeutics. *Front. Cell Dev. Biol.* **10**, 901510.

8. Related Products

Animal-Origin-Free, cGMP-Manufactured, In Vitro Transcription Reagents

When transitioning to large-scale RNA production, the components of this kit are individually available and can be formatted specifically to your workflow needs. Please enquire for pricing and availability at:

<https://www.promega.com/global/forms/raw-materials-for-mrna-manufacturing-inquiry-form/>

or email: custom@promega.com

Product	Size	Part#
T7 RNA Polymerase, 80U/μl	Dispensed to order	P618X
T7 RNA Polymerase, 400U/μl	Dispensed to order	P617X
T7 RNA Polymerase, 400U/μl (low Triton® X-100)	Dispensed to order	BX260X
RNasin® Plus Ribonuclease Inhibitor, 40U/μl	Dispensed to order	N373X
RNasin® Plus Ribonuclease Inhibitor, 500U/μl	Dispensed to order	N372X
Pyrophosphatase, Inorganic, 2U/μl	Dispensed to order	M934X
Ribonucleotides (rATP, rUTP, rGTP, rCTP), 100mM	Dispensed to order	BX139X
		BX140X
		BX141X
		BX142X
10X Transcription Buffer (–Mg)	Dispensed to order	P211X
Enzyme Mixes: Mixture of T7 RNA Polymerase, RNasin® Plus Ribonuclease Inhibitor and Inorganic Pyrophosphatase	Made to order	
10X Transcription Buffer (+Mg): Transcription Buffer + Magnesium	Made to order	

8. Related Products (continued)

Products to Evaluate IVT Reaction Performance:

RNA Size and Integrity

Product	Size	Cat. #
Agarose, LE, Analytical Grade*	100g	V3121
Agarose, LMP, Preparative Grade*	25g	V2831
Agarose, Low Melting Point, Analytical Grade*	25g	V2111
Ethidium Bromide Solution, Molecular Grade	10ml	H5041
Diamond™ Nucleic Acid Dye	500µl	H1181
RNA Markers	50µl	G3191
Spectrum Compact CE System		CE1304

*Additional sizes available.

dsRNA Contamination

Product	Size	Cat. #
Lumit® dsRNA Detection Assay*	100 assays	W2041

*Additional kit sizes are available.

In Vitro Translation

Product	Size	Cat. #
Rabbit Reticulocyte Lysate, Nuclease-Treated	30 reactions	L4960
Wheat Germ Extract	5 × 200µl	L4380
Transcend™ Non-Radioactive Translation Detection System	30 reactions	L5070
Transcend™ tRNA	30µl	L5061
FluoroTect™ Green _{Lys} in vitro Translation Labeling System	40 reactions	L5001

Cell-Based Translation/Expression

Product	Size	Cat. #
ViaScript™ mRNA Transfection Reagent	10 plates	EV4961
TransfectNow™ HEK295 Cells*	1 × 0.5ml	NC1001

*Additional kit sizes are available.

DNA Template Preparation

Product	Size	Cat. #
DNA Polymerase I Large (Klenow) Fragment	150u	M2201
T4 DNA Polymerase	100u	M4211

pGEM® Cloning Vectors provide a backbone for generating high-quality DNA templates for in vitro transcription (IVT), featuring dual T7 and SP6 promoters for RNA synthesis. To learn more visit:

www.promega.com/products/pcr/pcr-cloning/

Post-IVT RNA Treatment

Product	Size	Cat. #
RQ1 RNase-Free DNase	1,000u	M6101

RNA Isolation

Product	Size	Cat. #
PolyATtract® mRNA Isolation Systems	3 isolations	Z5210

Functional Bioassays

Please visit the Functional Bioassays products page for a full list of reporter bioassays and cell health assays:

www.promega.com/applications/biologics-drug-discovery/functional-bioassays/

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