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(71) **Applicants: KUDO BIOTECHNOLOGY, INC.** [US/US]; 117 Kendrick Street, Needham, MA 02494 (US). **KUNO BIOTECHNOLOGY (SHANGHAI) CO. LTD.** [CN/CN]; 1 & 2F, Block E, Han's Enterprise Bay, No. 555 Wanfang Road, Shanghai, 201112 (CN). **RVAC MEDICINES (US), INC.** [US/US]; 880 Winter Street, Suite 1002, Waltham, MA 02451 (US).

(72) **Inventors: SAKURAI-SANGRIA, Atsuko**; C/o Rvac Medicines (us), Inc., 880 Winter Street, Waltham, MA 02451 (US). **TAN, Yang**; C/o Kudo Biotechnology, Inc., 117 Kendrick Street, Needham, MA 02494 (US). **GREGORY, Sonia**; C/o Rvac Medicines (us), Inc., 880 Winter Street, Needham, MA 02451 (US). **PALA, Meltem, Corbacioglu**; C/o Kudo Biotechnology, Inc., 117 Kendrick Street, Needham, MA 02494 (US).

(74) **Agent: LU, Yu** et al.; Mccarter & English, LLP, 265 Franklin Street, Boston, MA 02110 (US).

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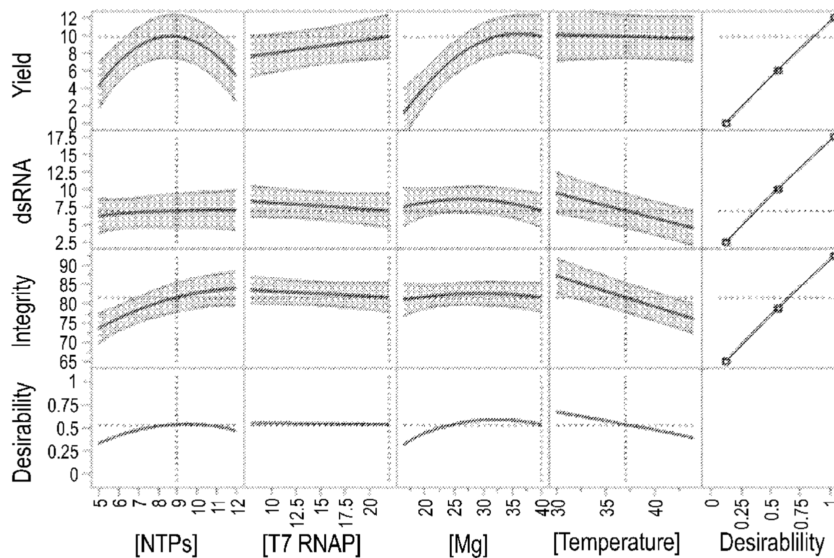
(54) **Title:** METHOD OF MRNA MANUFACTURE

FIG. 1



(57) **Abstract:** The invention described herein provides optimized in vitro transcription (IVT) processes for efficiently producing mRNA, which may be useful for developing a robust manufacturing process to ensure a successful GMP campaign of mRNA-based pharmaceutical composition.

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METHOD OF mRNA MANUFACTURE

REFERENCE TO RELATED APPLICATION

This application claims priority to International Patent Application No. PCT/CN2023/109412, filed on July 26, 2023, the entire contents of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

mRNA is produced by *in vitro* transcription (IVT) which contains multiple parameters that can affect its outcome. Optimizing the IVT reaction is critical for developing a robust manufacturing process to ensure a successful GMP campaign.

SUMMARY OF THE INVENTION

The invention described herein pertains to optimized *in vitro* transcription (IVT) based on a design of experiment (DoE) approach on two mRNA products (*i.e.*, KD-001 and KD-002). The IVT conditions for KD-001 was selected based on the mRNA integrity, yield, and dsRNA content, and the process performance was confirmed by pilot scale runs before the GMP run.

By leveraging the KD-001 process development knowledge, a simplified DoE study was performed for KD-002 which identified a more economical condition that requires less T7 RNA polymerase (RNAP) in IVT. Optionally, a T7 RNAP with reduced by-product (*e.g.*, dsRNA) is used to further enhance RNA quality.

Thus one aspect of the invention provides a method of producing RNA via *in vitro* transcription (IVT), the method comprising carrying out the *in vitro* transcription at 37±0.5°C, under a condition in which the molar ratio of Mg^{2+} concentration and total NTP concentration is about 0.75 to 1.

In certain embodiments, the concentration for each of ATP, UTP, CTP, and GTP is independently about 7.5-12.5 mM (*e.g.*, about 10 mM for each of the four NTPs).

In certain embodiments, the Mg^{2+} concentration is about 28-45 mM (*e.g.*, about 30-40 mM, about 32 mM, or about 40 mM).

In certain embodiments, the condition comprises 5-25 kU/mL of RNA Polymerase (RNAP) (*e.g.*, 8 kU/mL, 15 kU/mL, or 22 kU/mL).

In certain embodiments, the RNAP is a T7 RNAP (specific for a T7 promoter).

In certain embodiments, the condition comprises: about 10 mM for each of the four NTPs (*e.g.*, 10 mM ATP, 10 mM UTP, 10 mM CTP, and 10 mM GTP), about 8 kU/mL of T7 RNAP, about 32-40 mM Mg^{2+}, and about 37°C.

In certain embodiments, the method further comprises removing DNA template for the IVT with DNase (such as DNase I), and/or removing protein / enzymes for IVT with proteinase (such as Proteinase K).

In certain embodiments, the method further comprises purifying RNA product of the IVT (such as via spin-column-based RNA purification or lithium chloride precipitation).

In certain embodiments, the IVT is carried out in a volume of about 20-100 mL, such as about 20 mL, about 30 mL, about 50 mL, about 75 mL, or about 100 mL.

In certain embodiments, the yield of the IVT is about 5-12 mg/mL, about 6-10 mg/mL, about 7-10 mg/mL, about 8-10 mg/mL, about 9-10 mg/mL, or about 10 mg/mL.

In certain embodiments, the integrity of the RNA is at least about 80%, 85%, 88%, 90%, 92%, 94%, 95%, 96%, 97% or more.

In certain embodiments, dsRNA content of the IVT product is less than about 600 pg/μg RNA, less than about 500 pg/μg RNA, less than about 400 pg/μg RNA, less than about 300 pg/μg RNA, less than about 200 pg/μg RNA, less than about 100 pg/μg RNA, less than about 80 pg/μg RNA, less than about 60 pg/μg RNA, less than about 40 pg/μg RNA, less than about 20 pg/μg RNA, or less than about 10 pg/μg RNA.

In certain embodiments, the RNAP is a variant T7 RNAP having one or more residue changes compared to a wild-type T7 RNAP.

In certain embodiments, the variant T7 RNAP (a) is at least 97 %, at least 98 % or at least 99 % identical to SEQ ID NO: 1, and (b) comprises 1, 2, 3, 4, or 5 mutations (*e.g.*, a substitution or deletion) compared to SEQ ID NO: 1 at residues D130, N171, K172, R173, Y178, R298, Y385, K387, D388, and/or F880.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the effects of each of the 4 factors in Example 1 on yield, integrity, and dsRNA content.

FIG. 2 shows comparison of the IVT conditions for KD-001/RVM-V001.

FIGs. 3A and 3B show batch analysis for pilot scale and GMP run materials for KD-001//RVM-V001, specifically fragment analysis of pilot scale material (FIG. 3A) and GMP material (FIG. 3B).

FIG. 4 shows comparison of the IVT conditions for KD-002/RVM-V002.

DETAILED DESCRIPTION OF THE INVENTION

There is a need for consistent, high-quality mRNAs to develop mRNA vaccines or therapeutics, such as patient-specific cancer vaccines or protein replacement therapies for rare diseases. Improved *in vitro* transcribed (IVT) mRNA synthesis would lead to simplified cell-free manufacturing and reduced risk of insertional mutagenesis.

The invention described herein provides *in vitro* transcription (IVT) methods for RNA production, which can be useful for, *e.g.*, large scale GMP (Good Manufacturing Practice) level RNA production. The RNA so produced can be used in a variety of utilities, including mRNA for vaccine production or for *in vitro* translation. For example, the mRNA produced by the subject IVT methods may be useful as patient-specific cancer vaccines, or as agents useful to treat certain rare diseases by way of protein replacement therapy.

The terms "*in vitro* transcription" or "RNA *in vitro* transcription," as used herein, relate to a process wherein RNA is synthesized in a cell-free system (*in vitro*). DNA, particularly plasmid DNA, can be used as template for the generation of RNA transcripts using RNA polymerase. That is, RNA may be obtained by DNA-dependent *in vitro* transcription of an appropriate DNA template, which in some embodiment may include a linearized plasmid DNA template.

RNA transcription from the DNA template may be initiated from a promoter on the DNA template. In certain embodiments, the promoter for controlling *in vitro* transcription is a promoter for a DNA-dependent RNA polymerase. Particular examples of DNA-dependent RNA polymerases include T7, T3, and SP6 RNA polymerases, either wild type or modified or mutated forms.

In certain embodiments, a DNA template for *in vitro* RNA transcription may be obtained by cloning of a nucleic acid, in particular cDNA corresponding to the respective RNA to be *in vitro* transcribed, and introducing it into an appropriate vector for *in vitro* transcription (IVT vector), for example, into a plasmid DNA.

In certain embodiments, as templates for *in vitro* transcription, an IVT vector may have the following structure: a 5' RNA polymerase promoter enabling RNA transcription, followed by a gene of interest (GOI) which is flanked either 3' and/or 5' by untranslated regions (UTR), and a 3' polyadenyl cassette containing A nucleotides. Prior to *in vitro* transcription, a circular plasmid is linearized downstream of the polyadenyl cassette by a (*e.g.*, type II) restriction endonuclease. The polyadenyl cassette thus corresponds to the later poly (A) sequence in the transcript.

High stability and translation efficiency of an RNA useful in a therapeutic, such as in a cancer vaccine, are required to produce an effective immune response. Both can be improved by structural modifications of the 5'-CAP, the 3' poly (A)-tail as well as the 5' and 3' untranslated regions (UTRs). Sequence elements within the UTRs affect translational efficiency (mainly 5'-UTR) and RNA stability (mainly 3'-UTR).

The term "3'-untranslated region (3'-UTR)" relates to a region which is located at the 3' end of a gene, downstream of the termination codon of a protein-encoding region, and which is transcribed but is not translated into an amino acid sequence, or to the corresponding region in an RNA molecule. The 3'-untranslated region typically extends from the termination codon for a translation product to the poly (A) sequence which is usually attached after the transcription process. The 3'-untranslated regions of mammalian mRNA typically have a homology region known as the AAUAAA hexanucleotide sequence. This sequence is presumably the poly (A) attachment signal and is frequently located from 10 to 30 bases upstream of the poly (A) attachment site. The poly (A) sequence is important for the nuclear export, translation, and stability of mRNA. The sequence is shortened over time, and, when it is short enough, the mRNA is enzymatically degraded.

The terms "polyadenyl sequence," "poly (A) sequence" or "poly (A) tail" refer to a sequence of adenyl residues which is typically located at the 3' end of an RNA molecule. The invention provides for such a sequence to be attached during RNA transcription by way of a DNA template on the basis of repeated thymidyl residues in the strand complementary to the coding strand, whereas said sequence is normally not encoded in the DNA but is attached to the free 3' end of the RNA by a template-independent RNA polymerase after transcription in the nucleus. In one embodiment, a poly (A) sequence has at least 20, preferably at least 40, preferably at least 80, preferably at least 100 and preferably up to 500, preferably up to 400, preferably up to 300, preferably up to 200, and in particular up to 150, A nucleotides,

preferably consecutive A nucleotides, and in particular about or fewer than 120 A nucleotides, about 100 nucleotides, or about 80 nucleotides.

Examples of 5’ cap options include: Cap0; Cap1 (Co-Capped or enzymatic); Cap2 (Co-Capped); and Uncapped. Further cap analogs have been described previously (U.S. Pat. No. 7,074,596, WO 2008/016473, WO 2008/157688, WO 2009/149253, WO 2011/015347, and WO 2013/059475, all incorporated herein by reference). The synthesis of N7-(4-chlorophenoxyethyl) substituted dinucleotide cap analogs has also been described (Kore *et al.*, 2013. Bioorg. Med. Chem. 21(15):4570-4, incorporated herein by reference).

Example of Poly(A) tail options include: 100A Tail; Custom tail, *e.g.*, ≤ 120A; or No Tail.

In one embodiment, the DNA template is linearized with a suitable restriction enzyme, before it is transcribed *in vitro*. The cDNA may be obtained by reverse transcription of mRNA or chemical synthesis. Moreover, the DNA template for *in vitro* RNA synthesis may also be obtained by gene synthesis.

Methods for *in vitro* transcription are known in the art (see, for example, Geall *et al.* (2013) Semin. Immunol. 25(2): 152-159; Brunelle *et al.* (2013) Methods Enzymol. 530:101-14, both incorporated herein by reference). Reagents used in said method typically include:

1) a linearized DNA template with a promoter sequence that has a high binding affinity for its respective RNA polymerase such as bacteriophage-encoded RNA polymerases;
2) ribonucleoside triphosphates (NTPs), typically NTPs for the four bases (adenine, cytosine, guanine and uracil);
3) optionally a cap analog as defined below (*e.g.*, $m^7G(5')ppp(5')G$ (m^7G)), or no cap;
4) a DNA-dependent RNA polymerase capable of binding to the promoter sequence within the linearized DNA template (*e.g.*, T7, T3 or SP6 RNA polymerase);
5) optionally a ribonuclease (RNase) inhibitor to inactivate any contaminating RNase;
6) optionally a pyrophosphatase to degrade pyrophosphate, which may inhibit transcription;

7) $MgCl_2$, which supplies Mg^{2+} ions as a co-factor for the RNA polymerase;

8) a buffer to maintain a suitable pH value, which can also contain antioxidants (*e.g.*, DTT), amines such as betaine and/or polyamines such as spermidine at optimal concentrations.

Thus the invention described herein provides a method of producing RNA via *in vitro* transcription (IVT), the method comprising carrying out the *in vitro* transcription at 37±0.5°C, under a condition in which the molar ratio of Mg^{2+} concentration and total NTP concentration is about 0.75 to 1.

In certain embodiments, the molar ratio is about 0.8-0.95. In certain embodiments, the molar ratio is about 0.8-0.9. In certain embodiments, the molar ratio is about 0.8. In certain embodiments, the molar ratio is about 0.85. In certain embodiments, the molar ratio is about 0.9. In certain embodiments, the molar ratio is about 0.95. In certain embodiments, the molar ratio is about 1.0. In certain embodiments, the molar ratio is about 1.05.

As used herein, the term "about" refers to values within ±5% of the value it modifies. For example, "about 1" includes any value between 0.95-1.05 (inclusive).

The invention is partly based on the discovery that the ratio of Mg^{2+} concentration and total NTP concentration affects IVT, in that a ratio much large (*e.g.*, >8) or smaller (*e.g.*, <1.65) than the claimed 0.75-1 range may be detrimental to IVT efficiency and/or yield.

In some embodiments, the *in vitro* transcription is carried out at an incubation temperature in the range of 30 to 50°C, such as 37±0.5°C. In some embodiments, the *in vitro* transcription is carried out between 20-240 min, such as about 60 min.

In certain embodiments, the RNA is siRNA, gRNA (guide RNA), or other RNA or precursors thereof.

In certain embodiments, the RNA is mRNA. In certain embodiments, the mRNA has a length between about 10 nts – about 14,000 nts, between about 100 – about 10,000 nts, between about 200 – about 8,000 nts, between about 500 - about 5,000 nts, or between about 1,000 to about 3,000 nts.

In certain embodiments, the mRNA comprises a 5' cap. In certain embodiments, the 5' cap is a cap analog incorporated into the mRNA during the IVT.

In certain embodiments, the cap analog is a dinucleotide cap, a trinucleotide cap, or a tetranucleotide cap. In some embodiments, the cap analog is a trinucleotide cap.

In some embodiments, the trinucleotide cap is selected from the group consisting of GAA, GAC, GAG, GAU, GCA, GCC, GCG, GCU, GGA, GGC, GGG, GGU, GUA, GUC, GUG, and GUU.

In some embodiments, the trinucleotide cap is selected from the group consisting of $m^7GpppApA$, $m^7GpppApC$, $m^7GpppApG$, $m^7GpppApU$, $m^7GpppCpA$, $m^7GpppCpC$, $m^7GpppCpG$, $m^7GpppCpU$, $m^7GpppGpA$, $m^7GpppGpC$, $m^7GpppGpG$, $m^7GpppGpU$, $m^7GpppUpA$, $m^7GpppUpC$, $m^7GpppUpG$, and $m^7GpppUpU$.

In some embodiments, the trinucleotide cap is selected from the group consisting of $m^7G_{3'OMe}pppApA$, $m^7G_{3'OMe}pppApC$, $m^7G_{3'OMe}pppApG$, $m^7G_{3'OMe}pppApU$, $m^7G_{3'OMe}pppCpA$, $m^7G_{3'OMe}pppCpC$, $m^7G_{3'OMe}pppCpG$, $m^7G_{3'OMe}pppCpU$, $m^7G_{3'OMe}pppGpA$, $m^7G_{3'OMe}pppGpC$, $m^7G_{3'OMe}pppGpG$, $m^7G_{3'OMe}pppGpU$, $m^7G_{3'OMe}pppUpA$, $m^7G_{3'OMe}pppUpC$, $m^7G_{3'OMe}pppUpG$, and $m^7G_{3'OMe}pppUpU$.

In some embodiments, the trinucleotide cap is selected from the group consisting of $m^7G_{3'OMe}pppA_{2'OMe}pA$, $m^7G_{3'OMe}pppA_{2'OMe}pC$, $m^7G_{3'OMe}pppA_{2'OMe}pG$, $m^7G_{3'OMe}pppA_{2'OMe}pU$, $OMepppC_{2'OMe}pA$, $m^7G_{3'OMe}pppC_{2'OMe}pC$, $m^7G_{3'OMe}pppC_{2'OMe}pG$, $m^7G_{3'OMe}pppC_{2'OMe}pU$, $m^7G_{3'OMe}pppG_{2'OMe}pA$, $m^7G_{3'OMe}pppG_{2'OMe}pC$, $m^7G_{3'OMe}pppG_{2'OMe}pG$, $m^7G_{3'OMe}pppG_{2'OMe}pU$, $m^7G_{3'OMe}pppU_{2'OMe}pA$, $m^7G_{3'OMe}pppU_{2'OMe}pC$, and $m^7G_{3'OMe}pppU_{2'OMe}pG$, and $m^7G_{3'OMe}pppU_{2'OMe}pU$.

In some embodiments, the trinucleotide cap is selected from the group consisting of $m^7GpppA_{2'OMe}pA$, $m^7GpppA_{2'OMe}pC$, $m^7GpppA_{2'OMe}pG$, $m^7GpppA_{2'OMe}pU$, $m^7GpppC_{2'OMe}pA$, $m^7GpppC_{2'OMe}pC$, $m^7GpppC_{2'OMe}pG$, $m^7GpppC_{2'OMe}pU$, $m^7GpppG_{2'OMe}pA$, $m^7GpppG_{2'OMe}pC$, $m^7GpppG_{2'OMe}pG$, $m^7GpppG_{2'OMe}pU$, $m^7GpppU_{2'OMe}pA$, $m^7GpppU_{2'OMe}pC$, $m^7GpppU_{2'OMe}pG$, and $m^7GpppU_{2'OMe}pU$.

In some embodiments, the trinucleotide cap is $m^7GpppA_{2'OMe}pG$.

As used herein, "total NTP concentration" refers to the concentration of all of the nucleoside triphosphates (NTPs) in the IVT reaction mixture. Typically, the NTPs include at least ATP, UTP, CTP, and GTP (or analogs or derivatives thereof, such as modified NTPs), but it can omit at least one of the typical 4 types of NTPs if the template and the final RNA product lacks the NTP(s) that is / are missing. For example, a polyA sequence only requires the one of the NTPs (*i.e.*, ATP) to be present in the NTP mixture.

In certain embodiments, the different types of NTPs in the NTP mixture all have roughly the same concentration.

In certain embodiments, at least two different types of NTPs in the NTP mixture have different concentrations. For example, if a template for RNA synthesis is GC rich, the concentration for GTP and CTP may be enriched accordingly, *e.g.*, to be roughly proportional to the GC content of the template. In other embodiments, all NTPs in the NTP mixture have roughly the same concentration even though the template is heavily biased towards a particular nucleotide (*e.g.*, GC rich or AT rich).

In certain embodiments, the concentration for each of the nucleoside triphosphates (*e.g.*, ATP, UTP, CTP, and GTP) is independently about 7.5-12.5 mM. In certain embodiments, each of ATP, UTP, CTP, and GTP has about the same concentration, *e.g.*, about 10 mM for each of the four NTPs ATP, UTP, CTP, and GTP.

In certain embodiments, the nucleoside triphosphates (NTPs) consist of unmodified ATP, UTP, CTP, and GTP. In some embodiments, the nucleoside triphosphate may be selected from the group consisting of unmodified ATP, GTP, CTP, and UTP.

In certain embodiments, the one or more of the nucleoside triphosphates (NTPs) is, comprises, or is replaced with modified nucleoside triphosphate (including analogs thereof). In some embodiments, the nucleoside triphosphate may be selected from the group consisting of modified nucleoside triphosphate, including, but not limited to, m1A (N1-methyl adenosine), m6A (N6-methyl adenosine), m5C (5-methyl cytidine), 5moU (5-methoxyuridine), psi or Ψ (pseudouridine), m1Ψ, or a labeled NTP, which label may be a biotin, a fluorescent substance, a digoxin, a radioactive element, or the like.

In certain embodiments, the examples of modified NTPs that may be used in the methods of the invention include: N1-Me-Ψ; 5moU; 5meC & Ψ; Cy5-UTP or Thio-CTP. Further suitable modified nucleotides have been described previously in WO 2013/052523 (incorporated herein by reference).

In certain embodiments, all UTPs in the NTP mixture are replaced by one or more modified UTP, such as Ψ (pseudouridine) or m1Ψ. In certain embodiments, 50%, 60%, 70%, 80%, 90%, or 100% of the UTPs in the NTP mixture are replaced by Ψ (pseudouridine). In certain embodiments, 50%, 60%, 70%, 80%, 90%, or 100% of the UTPs in the NTP mixture are replaced by m1Ψ.

In certain embodiments, the Mg^{2+} concentration is about 28-45 mM (*e.g.*, about 30-40 mM, about 32 mM, or about 40 mM). In certain embodiments, the Mg^{2+} concentration is

about 32 mM. In certain embodiments, the Mg^{2+} concentration is about 35 or 36 mM. In certain embodiments, the Mg^{2+} concentration is about 40 mM.

In certain embodiments, the Mg^{2+} is provided as $MgCl_2$. In certain embodiments, the counterion of Mg^{2+} is Cl^-.

In certain embodiments, the condition comprises 5-25 kU/mL of RNA Polymerase (RNAP) (*e.g.*, 8 kU/mL, 15 kU/mL, or 22 kU/mL). In certain embodiments, the condition comprises about 8 kU/mL of RNAP. In certain embodiments, the condition comprises about 10 kU/mL of RNAP. In certain embodiments, the condition comprises about 12 kU/mL of RNAP. In certain embodiments, the condition comprises about 14 kU/mL of RNAP. In certain embodiments, the condition comprises about 16 kU/mL of RNAP. In certain embodiments, the condition comprises about 18 kU/mL of RNAP. In certain embodiments, the condition comprises about 20 kU/mL of RNAP. In certain embodiments, the condition comprises about 22 kU/mL of RNAP. In certain embodiments, the condition comprises about 25 kU/mL of RNAP. In certain embodiments, the condition comprises about 30 kU/mL of RNAP.

As used herein, 1 unit (U) of RNAP activity is defined as the amount of enzyme required to incorporate 1 nmol of [^{3}H] ATP into acid-insoluble precipitates within 1 hour at 37°C, pH 8.0.

In certain embodiments, the RNAP is a T7 RNAP, *e.g.*, one that is specific for a bacterial phage T7 promoter.

In certain embodiments, the T7 RNAP is from a commercial source, such as the T7 Turbo RNA Polymerase from Vazyme (Nanjing, PRC). Suitable T7 RNAP include T7 Turbo RNA Polymerase (200 U/μL, GMP Grade, Cat. No. GMP4120PB).

According to the manufacturer, the T7 Turbo RNA polymerase (T7 RNAP) is a protein encoded by the bacteriophage T7 DNA expressed in the recombinant *E. coli*, Clone number D05. It is a DNA-dependent 5'→ 3' RNA polymerase that highly specifically recognizes T7 promoter sequences. It uses single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) containing T7 promoter sequences as the template and NTPs as the substrate to synthesize RNA complementary to the ssDNA or dsDNA template strand downstream of the promoter. Compared with wild-type T7 RNA Polymerase, T7 Turbo RNA Polymerase effectively reduces the content of dsRNA, a by-product produced during transcription. While its co-transcription reaction product also has a higher capping rate.

Also according to the manufacture, the T7 Turbo RNA Polymerase is a GMP grade recombinant T7 RNA Polymerase mutant. Through strict control of its production process, process-related impurities such as host proteins, exogenous DNA, RNase, as well as microbial and bacterial endotoxins are kept at minimum. Ampicillin and any animal-derived raw materials and excipients are not used or added in the entire production process. GMP-compliant production and quality management standards are adopted, ensuring traceability of the production process and raw materials and excipients, and meeting the requirements for raw materials and excipients in mRNA vaccine production and other related fields.

In certain embodiments, the T7 RNAP is the T7 RNA Polymerase from Vazyme (Nanjing, PRC). Suitable T7 RNAP include T7 RNA Polymerase (200 U/μL, GMP Grade, Cat. No. GMP4101PB).

In certain embodiments, the T7 RNAP is a wild-type T7 RNAP having the amino acid sequence of SEQ ID NO: 1.

MNTINIAKNDFSDIELAAIPFNTLADHYGERLAREQLALEHESYEMGEARFRKMFERQ
LKAGEVADNAAAKPLITTLLPKMIARINDWFEEVKAKRGKRPTAFQFLQEIKPEAVAYITIKT
TLACLTSADNTTVQAVASAIGRAIEDEARFGRIRDLEAKHFKKNVEEQLNKRVGHVYKKAF
MQVVEADMLSKGLLGGEAWSSWHKEDSIHVGVRCIEMLIESTGMVSLHRQNAGVVGQDS
ETIELAPEYAEAIATRAGALAGISPMFQPCVVPPKPWTGITGGGYWANGRRPLALVRTHSKK
ALMRYEDVYMPEVYKAINIAQNTAWKINKKVLAVANVITKWKHCPVEDIPAIEREELPMKP
EDIDMNPEALTAWKRAAAAVYRKDKARKSRRISLEFMLEQANKFANHKAIWFPYNMDWR
GRVYAVSMFNPQGNDMTKGLLTLAKGKPIGKEGYYWLKIHGANCAGVDKVPFPERIKFIEE
NHENIMACAKSPLENTWWAEQDSPFCFLAFCFEYAGVQHHGLSYNCSLPLAFDGSCSGIQH
FSAMLRDEVGGRAVNLLPSETVQDIYGIVAKKVNEILQADAINGTDNEVVTVTDENTGEISE
KVKLGTKALAGQWLAYGVTRSVTKRSVMTLAYGSKEFGFRQQVLEDTIQPAIDSGKGLMF
TQPNQAAGYMAKLIWESVSVTVVAAVEAMNWLKSAAKLLAAEVKDKKTGEILRKRCAVH
WVTPDGFPVWQEYKKPIQTRLNLMFLGQFRLQPTINTNKDSEIDAHKQESGIAPNFVHSQD
GSHLRKTVVWAHEKYGIESFALIHDSFGTIPADAANLFKAVRETMVDTYESCDVLADFYDQ
FADQLHESQLDKMPALPAKGNLNLRDILESDFAFA（SEQ ID NO：1）

In certain embodiments, the RNAP is a variant T7 RNAP having one or more residue changes compared to a wild-type T7 RNAP (SEQ ID NO: 1).

In certain embodiments, the variant T7 RNAP (a) is at least 97 %, at least 98 % or at least 99 % identical to SEQ ID NO: 1, and (b) comprises 1, 2, 3, 4, or 5 mutations (*e.g.*, a substitution or deletion) compared to SEQ ID NO: 1 at residues D130, N171, K172, R173, Y178, R298, Y385, K387, D388, and/or F880.

In certain embodiments, the variant, relative to SEQ ID NO: 1, comprises: (1) a mutation at position K172, and further comprising a mutation of any one, two, three or four

amino acids at a position selected from the group consisting of D130, R173, Y178, R298, Y385, K387, D388 or F880, the type of mutation being a substitution or deletion; or (2) a mutation at position K173 and further comprising a mutation at any one, two or three amino acids selected from positions D130, Y178, R298, K387 or D388 of the type of substitution or deletion; or (3) a mutation of any two or three amino acids at positions R298, Y385, K387 or D388, said mutation being a substitution.

In certain embodiments, the variant comprises a mutant group selected from any one of the following: K172 + R173, D130 + K172, K172 + K387, K172 + F880, K172 + D388, K172 + R298, D130 + R173, R173 + Y178, R173 + D388, R173 + R298, K387 + R298, Y385 + R298, D388 + R298, Y385 + K387, Y385 + D388, K387 + D388, K172 + R173 + Y385, K172 + R173 + D388, K172 + R173 + K387, K172 + R173 + F880, K172 + R173 + Y178, D130 + K172 + R173, D130 + K172 + Y178, D130 + K172 + K387, D130 + R173 + D388, K172 + Y178 + D388, D130 + K172 + D388, K172 + K387 + R298, Y385 + K387 + D388, K172 + R173 + Y385 + F880, K172 + R173 + D388 + F880, K172 + R173 + Y178 + D388, D130 + K172 + R173 + D388, D130 + K172 + R173 + Y178, D130 + R173 + Y178 + K387, D130 + K172 + Y178 + D388, or D130 + K172 + R173 + Y178 + D388.

In certain embodiments, 1) the mutation type at the N171 position is N171G; 2) the mutation type at position K172 is selected from DEL172, K172A, K172G, K172E, K172D, K172H, K172Y, K172S, K172W, K172P, K172I, K172M, K172V, K172F, K172T, K172C, K172N or K172L; 3) the mutation type at the R173 position is selected from DEL173, R173A, R173C, R173G, R173E, R173D, R173H, R173Y, R173S, R173W, R173P, R173N or R173Q; 4) the mutation type at Y178 position is Y178H; 5) the mutation type at position R298 is R298A; 6) mutation type at Y385 position is selected from Y385A, Y385D or Y385E; 7) the mutation type at the K387 position is selected from K387Q, K387Y, K387S or K387A; 8) mutation type at D388 position is selected from D388A, D388G or D388L; and 9) the mutation type at the F880 position is selected from F880A, F880G or F880W.

In certain embodiments, 1) the mutation type at position D130 is D130E; 2) the mutation type at position K172 may be selected from DEL172, K172A or K172G; 3) the type of mutation at the R173 position may be selected from DEL173, R173A, R173G or R173C; 4) the mutation type at Y178 position can be selected from Y178H or Y178P; 5) the mutation type at position R298 is R298A; 6) the mutation type at the Y385 position is Y385A; 7) the mutation type at the K387 position can be selected from K387S, K387Y or K387G; 8) the

mutation type at the D388 position can be selected from D388Y, D388A or D388G; and 9) the type of mutation at the F880 position may be selected from F880A or F880Y.

In certain embodiments, the amino acid sequence of the variant is any one of SEQ ID NO: 2-141 in WO2024131998A2 (incorporated herein by reference).

In some embodiments, the variant T7 RNA polymerase comprises at least one modification (or mutation) selected from the group consisting of: P266L, Q744L, Q744P, Y639F, H784A, E593G, Y639V, V685A, H784G, S430P, N433T, S633P, F849I and F880Y.

In some embodiments, the at least one modification includes Y639F and H784A. In some embodiments, the at least one modification includes E593G, Y639V, V685A and H784G. In some embodiments, the at least one modification includes S430P, N433T, S633P, F849I and F880Y. In some embodiments, the at least one modification includes S430P, N433T, S633P, F849I , F880Y and P266L. In some embodiments, the at least one modification includes S430P, N433T, S633P, F849I , F880Y, Y639F and H784A. In some embodiments, the at least one modification includes S430P, N433T, S633P, F849I , F880Y, P266L, Y639F and H784A. In some embodiments, the at least one modification includes S430P, N433T, S633P, F849I , F880Y, E593G, Y639V, V685A and H784G. In some embodiments, the at least one modification includes S430P, N433T, S633P, F849I , F880Y, P266L, E593G, Y639V, V685A and H784G.

In some embodiments, the at least one modification of the T7 RNA polymerase facilitates initiation-elongation transition. In some embodiments, the at least one modification increases promoter clearance. In some embodiments, the at least one modification increases stability and/or activity of the polymerase. In some embodiments, the at least one modification increases thermos stability of the polymerase.

In certain embodiments, the condition comprises: about 10 mM for each of the four NTPs (*e.g.*, 10 mM ATP, 10 mM UTP, 10 mM CTP, and 10 mM GTP), about 8-22 kU/mL of T7 RNAP, about 32-40 mM Mg^{2+}, and about 37°C.

In certain embodiments, the condition comprises: about 10 mM for each of the four NTPs (*e.g.*, 10 mM ATP, 10 mM UTP, 10 mM CTP, and 10 mM GTP), about 8 kU/mL of T7 RNAP, about 40 mM Mg^{2+}, and about 37°C.

In certain embodiments, the condition comprises: about 10 mM for each of the four NTPs (*e.g.*, 10 mM ATP, 10 mM UTP, 10 mM CTP, and 10 mM GTP), about 22 kU/mL of T7 RNAP, about 32 mM Mg^{2+}, and about 37°C.

In certain embodiments, the condition further comprises pyrophosphatase, such as a yeast inorganic pyrophosphatase. In certain embodiments, the pyrophosphatase is from a commercial source, such as Pyrophosphatase, Inorganic (yeast, 0.1 U/μL, GMP Grade, Cat. No. GMP4103PC).

In certain embodiments, the condition further comprises an RNase inhibitor. In certain embodiments, the RNase inhibitor is from a commercial source, such as the Murine RNase Inhibitor from Vazyme (40 U/μL, GMP Grade, Cat. No. GMP4102PA), which is a recombinant *E. coli*-expressed murine RNase inhibitor that binds RNase A, B, and C 1:1 in a non-competitive manner to inhibit the activity of all three enzymes and protect RNA from degradation.

In certain embodiments, the condition further comprises an IVT buffer. In certain embodiments, the buffer is RNase-free ddH$_2$O, which may be prepared using DEPC. In certain embodiments, the IVT buffer is from a commercial source, such as the 10× Transcription Buffer (GMP Grade) from Vazyme (Cat. No. GMP4101R), which is a recombinant *E. coli*-expressed murine RNase inhibitor that binds RNase A, B, and C 1:1 in a non-competitive manner to inhibit the activity of all three enzymes and protect RNA from degradation.

In certain embodiments, the condition further comprises a DNA template for transcribing the RNA. In certain embodiments, the DNA template comprises an operably linked promoter, such as a T7 promoter, that facilitates the IVT.

In certain embodiments, the method further comprises removing DNA template for the IVT, after the IVT, with DNase. In certain embodiments, the DNase is DNase I. In certain embodiments, the DNase I is from a commercial source, such as DNase I (1 U/μl, GMP Grade) from Vazyme (Nanjing, PRC, Cat. No. GMP4104PC).

In certain embodiments, the method further comprises removing protein / enzymes for IVT, after the IVT, with proteinase. In certain embodiments, the proteinase is Proteinase K.

In certain embodiments, the method further comprises purifying RNA product of the IVT. In certain embodiments, RNA purification comprises spin-column-based RNA purification. In certain embodiments, RNA purification comprises lithium chloride precipitation.

In certain embodiments, the IVT is carried out in a volume of about 20-100 mL. In certain embodiments, the IVT is carried out in a volume of about 20 mL. In certain

embodiments, the IVT is carried out in a volume of about 30 mL. In certain embodiments, the IVT is carried out in a volume of about 50 mL. In certain embodiments, the IVT is carried out in a volume of about 75 mL. In certain embodiments, the IVT is carried out in a volume of about 100 mL. In certain embodiments, the IVT is carried out in a volume of about 150 mL. In certain embodiments, the IVT is carried out in a volume of about 200 mL.

In certain embodiments, the yield of the IVT is about 5-12 mg/mL. In certain embodiments, the yield of the IVT is about 6-10 mg/mL. In certain embodiments, the yield of the IVT is about 8-10 mg/mL. In certain embodiments, the yield of the IVT is about 9-10 mg/mL. In certain embodiments, the yield of the IVT is about 10 mg/mL.

In certain embodiments, the integrity of the RNA is at least about 80%, 85%, 88%, 90%, 92%, 94%, 95%, 96%, 97% or more.

In certain embodiments, dsRNA (as impurity or by-product of IVT) content of the IVT product is less than about 600 pg/μg RNA. In certain embodiments, dsRNA content of the IVT product is less than about 500 pg/μg RNA. In certain embodiments, dsRNA content of the IVT product is less than about 400 pg/μg RNA. In certain embodiments, dsRNA content of the IVT product is less than about 300 pg/μg RNA. In certain embodiments, dsRNA content of the IVT product is less than about 200 pg/μg RNA. In certain embodiments, dsRNA content of the IVT product is less than about 100 pg/μg RNA. In certain embodiments, dsRNA content of the IVT product is less than about 80 pg/μg RNA. In certain embodiments, dsRNA content of the IVT product is less than about 60 pg/μg RNA. In certain embodiments, dsRNA content of the IVT product is less than about 40 pg/μg RNA. In certain embodiments, dsRNA content of the IVT product is less than about 20 pg/μg RNA. In certain embodiments, dsRNA content of the IVT product is less than about 10 pg/μg RNA.

It should be understood that any one embodiment of the invention, including embodiment described only in the examples and claims, can be combined with any one or more other embodiments of the invention described herein, unless such combination is expressly disclaimed or is improper.

With the general aspects of the invention described above, the following examples are provided for illustrative purpose only, and are by no means limiting.

EXAMPLES

Example 1 Initial DoE for KD-001 (RVM-V001)

RVM-V001 drug substance is a single-stranded, chemically capped and polyadenylated mRNA, which is around 4300 nucleotides (nt) in length. The DoE was performed with the following four factors to maximize the IVT yield and mRNA integrity, while lowering dsRNA content.

Factor	Level
NTPs (mM)	5 - 12
T7 RNAP (U/µL)	8 - 22
Mg (mM)	16.5 - 40
Temperature (°C)	30 - 44

Response	Method
Yield (mg/mL)	A260
mRNA integrity (%)	Fragment analyzer
dsRNA content (ng/µg RNA)	Dot blot

More specifically, initial DoE study was designed to examine the effect of the reaction temperature, concentrations of NTP, T7 RNA polymerase (T7 RNAP), and magnesium (Mg) on the RVM-V001 mRNA yield and quality. IVT reactions were performed using a linearized RM-001 pDNA as a template. The IVT reagents were obtained from New England Biolabs (NEB), Sigma, Thermo Fisher, and TriLink Biotechnologies.

Following the IVT reaction, the samples were treated with DNaseI, then mRNA was purified by a spin-column-based RNA purification kit or lithium chloride precipitation. mRNA yield was measured by A260 absorption, mRNA integrity was measured by fragment analyzer, and dsRNA content was measured by J2 antibody-based dot blot.

The results of these experiments were summarized in FIG. 1. The black lines show the interactions of each factor and response. The red dotted lines indicate where the actual conditions and results data points were. For example, the top left square shows that 9 mM NTPs were used, and the yield was about 10 mg/mL.

The data in FIG. 1 indicate that Mg had significant impact on yield. In addition, higher temperature resulted in low dsRNA but negatively impacted mRNA integrity.

Example 2 Refine the DoE to optimize IVT conditions

To further optimize the IVT condition, the second DoE was designed with three factors with narrower range. The reaction temperature was set to 37°C based on the initial DoE outcome. IVT reactions and mRNA analysis were performed as described in FIG. 1. A comparison of IVT yield and mRNA integrity from 10 test conditions are shown in FIG. 2 and below.

Factor	**Level**
NTPs (mM)	5 - 10
Mg (mg)	16.5 - 40
T7 RNAP (U/μL)	8 - 22

Response	**Method**
Yield (mg/mL)	A260
mRNA integrity (%)	Fragment analyzer
dsRNA content (ng/μg RNA)	ELISA

The specific conditions used here for FIGs. 2 & 4 are summarized below. The temperature was 37°C for all 10 conditions.

Condition	NTP (mM)	T7 RNAP (kU/mL)	Mg (mM)
2	10	8	16.5
4	10	22	16.5
5	5	8	40
7	5	22	40
1	5	8	16.5
3	5	22	16.5
9	7.5	15	28.25
6	10	8	40
8	10	22	40
10	10	22	32

Conditions #6, #8, and #10 were identified as high yield, high quality condition. Since there was no significant difference in dsRNA content (data not shown), condition #10 was selected for the RVM-V001 GMP campaign.

Example 3 Pilot scale runs and GMP batch production

To confirm the performance of the identified IVT condition prior to the GMP run, two pilot scale runs were performed using a scale-down process with downstream purification. Three batches of KD-001/RVM-V001 were produced with different scales using the identified IVT condition.

Specifically, IVT was carried out at a 20 mL reaction volume, the reaction was treated with DNaseI to digest the template DNA, then with proteinase K to degrade the enzymes. In-process samples were taken at this step to determine the IVT yield by lithium chloride purification followed by A260 measurement. After the proteinase K treatment, the mRNA was purified using a combination of column chromatography and tangential flow filtration. The final bulk drug substance was analyzed for the predetermined quality attributes including mRNA integrity by fragment analyzer and dsRNA content by dot blot (data shown in FIG. 3). The GMP batch was produced at a 100 mL IVT scale with the same purification process as

the pilot scale run. The results demonstrated that three batches were consistent in mRNA quality and yield.

All the batches demonstrated consistent process performance (FIG. 3) and met all the predetermined release specifications (not shown).

Batch	IVT Scale	IVT yield	Integrity	dsRNA content
Pilot #1	20 mL	9.9 mg/mL	91 %	Met release criteria
Pilot #2	20 mL	9.5 mg/mL	93 %	Met release criteria
GMP	100 mL	10.4 mg/mL	97 %	Met release criteria

Example 4 Cost reduction by optimizing IVT and substituting T7 RNAP for a lower-cost alternative

Following the successful KD-001 GMP campaign, KD-002/RVM-V004 (2000 nt mRNA) IVT optimization was performed. By leveraging the KD-001 DoE data shown in FIG. 2, a small number of IVT conditions were tested for KD-002. Condition #6 was selected since it requires 60 % less T7 RNAP than #8 without impacting mRNA integrity. The results were shown in FIG. 4.

More specifically, the IVT condition used for RVM-V001 was tested for another mRNA product encoding a shorter gene of interest, RVM-V004. Although the IVT performance was comparable to RVM-V001 (data not shown), a mini DoE study was performed using the same design used in FIG. 2. Conditions #2 and #4 were removed since the yield was low with RVM-V001. Condition #6 produced the high quality mRNA with high yield, with minimal T7 RNAP input among the conditions tested.

Furthermore, to further reduce the raw material cost, lower-cost alternative T7 RNAPs from several suppliers were tested to evaluate the enzyme performance. Supplier A is the current supplier. Suppliers B and C were identified as possible substitutes based on the acceptable yield and high integrity. However, dsRNA content was higher in mRNA produced with supplier B’s T7 RNAP. Meanwhile, the quality of mRNA produced using supplier C’s enzyme was comparable to the control group.

Supplier	IVT yield	Integrity	dsRNA	Reagent cost normalized by yield
A	**7.9 mg/mL**	**91 %**	**+**	**100% (benchmark)**
B	5.4 mg/mL	91 %	++	74 %
C	**5.7 mg/mL**	**91 %**	**+**	**66 %**
D	6.7 mg/mL	76 %	++	78 %
E	6.3 mg/mL	75 %	+++	80 %

Overall, the data presented herein show that:

- Using a DoE approach, the IVT conditions were optimized to produce high yield, high quality mRNA with low dsRNA content. The process was successfully scaled up for GMP production.
- Based on the DoE results and accumulated process knowledge, the IVT condition for another mRNA product was efficiently optimized.
- T7 RNAP is one of the most expensive raw material required for IVT. The data here shows that its production cost can be reduced by optimizing IVT and identifying a lower-cost alternative with comparable performance.

In summary, using the DoE approach, Mg concentration was identified as an important factor to produce high quality mRNA drug substance, and IVT condition was optimized for RVM-V001 and V004.

Example 5 Cost reduction by optimizing IVT and substituting T7 RNAP for a lower-cost alternative

Two conditions used above – Conditions 10 and 6, hereinafter called IVT A and IVT B, respectively - were further used for IVT performance in this example, with different constructs.

Using the KD-001/RVM-V001 (4300 NT) as a benchmark, KD-003, KD-004, and KD-005 mRNA (with approximate lengths of about 3800 NT, 1050 NT, and 1800 NT, respectively) were produced using IVT A and IVT B conditions as described above.

The KD-003 mRNA had high yield and integrity, and its dsRNA content was lower

than the benchmark KD-001 mRNA. KD-004 and KD-005 constructs also demonstrated high integrity, but showed lower yield which may be promoter specific.

Construct	IVT condition	IVT yield	Integrity	dsRNA content by ELISA
KD-001	IVT A	8.6 mg/mL	90.2 %	Not tested
KD-001	IVT B	5.2 mg/mL	88.0 %	774 pg/µg RNA
KD-003	IVT A	8.5 mg/mL	86.9 %	523 pg/µg RNA
KD-003	IVT B	8.0 mg/mL	85.4 %	471 pg/µg RNA
KD-004	IVT A	1.4 mg/mL	90.4 %	Not tested
KD-004	IVT B	2.2 mg/mL	96.7 %	Not tested
KD-005	IVT A	1.5 mg/mL	95.2 %	Not tested
KD-005	IVT B	3.1 mg/mL	95.8 %	Not tested

The IVT B condition was further used with a mutant T7 RNAP from supplier Vazyme (T7 Turbo RNA Polymerase (200 U/µL, GMP Grade, Cat. No. GMP4120PB)). As a control, the IVT was also performed using the supplier's recommended condition. IVT B condition demonstrated better performance in IVT yield compared to the supplier's recommended condition. The mRNA integrity was in the similar range of 80-90% as observed with wild type T7 RNAP. Of note, dsRNA impurity level was significantly reduced using the mutant T7 RNAP in IVT B condition (compare data in Table above).

Construct	IVT condition	IVT yield	Integrity	dsRNA content by ELISA
KD-001	IVT B	6.6 mg/mL	87.3 %	53 pg/µg RNA (run 1) 39 pg/µg RNA (run 1)
KD-001	Supplier's condition	0.1 mg/mL	78.4 %	Not tested
KD-003	IVT B	7.5 mg/mL	86.7 %	10.7 pg/µg RNA
KD-003	Supplier's condition	0.6 mg/mL	88.4 %	Not tested

CLAIMS

1. A method of producing RNA via *in vitro* transcription (IVT), the method comprising carrying out the *in vitro* transcription at 37±0.5°C, under a condition in which the molar ratio of Mg^{2+} concentration and total NTP concentration is about 0.75 to 1.

2. The method of claim 1, wherein the concentration for each of ATP, UTP, CTP, and GTP is independently about 7.5-12.5 mM (*e.g.*, about 10 mM for each of the four NTPs).

3. The method of claim 1 or 2, wherein the Mg^{2+} concentration is about 28-45 mM (*e.g.*, about 30-40 mM, about 32 mM, or about 40 mM).

4. The method of any one of claims 1-3, wherein the condition comprises 5-25 kU/mL of RNA Polymerase (RNAP) (*e.g.*, 8 kU/mL, 15 kU/mL, or 22 kU/mL).

5. The method of claim 4, wherein the RNAP is a T7 RNAP (specific for a T7 promoter).

6. The method of any one of claims 1-5, wherein the condition comprises: about 10 mM for each of the four NTPs (*e.g.*, 10 mM ATP, 10 mM UTP, 10 mM CTP, and 10 mM GTP), about 8 kU/mL of T7 RNAP, about 32-40 mM Mg^{2+}, and about 37°C.

7. The method of any one of claims 1-6, wherein the method further comprises removing DNA template for the IVT with DNase (such as DNase I), and/or removing protein / enzymes for IVT with proteinase (such as Proteinase K).

8. The method of any one of claims 1-7, wherein the method further comprises purifying RNA product of the IVT (such as via spin-column-based RNA purification or lithium chloride precipitation).

9. The method of any one of claims 1-8, wherein the IVT is carried out in a volume of about 20-100 mL, such as about 20 mL, about 30 mL, about 50 mL, about 75 mL, or about 100 mL.

10. The method of any one of claims 1-9, wherein the yield of the IVT is about 5-12 mg/mL, about 6-10 mg/mL, about 7-10 mg/mL, about 8-10 mg/mL, about 9-10 mg/mL, or about 10 mg/mL.

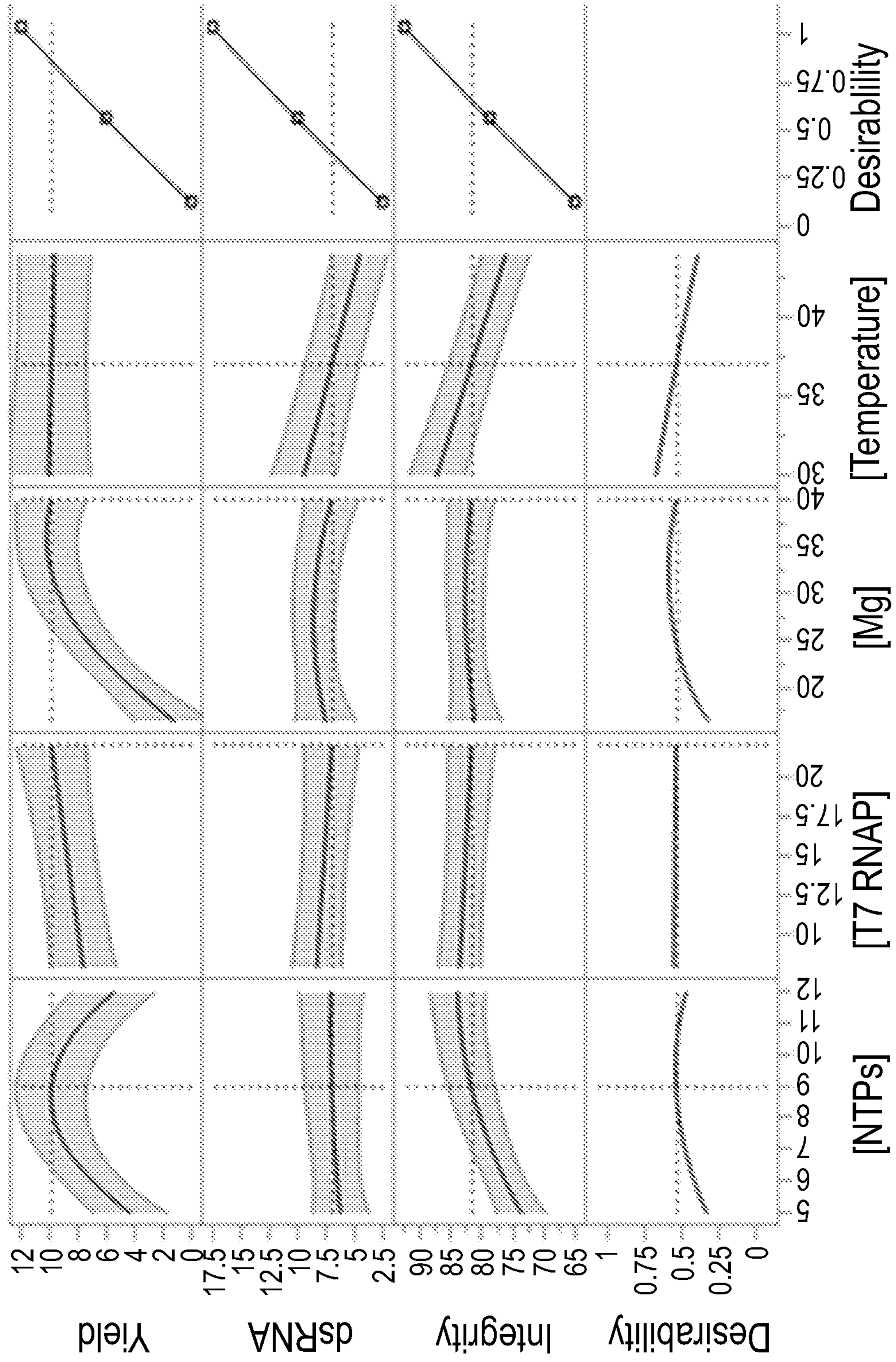
11. The method of any one of claims 1-10, wherein the integrity of the RNA is at least about 80%, 85%, 88%, 90%, 92%, 94%, 95%, 96%, 97% or more.

12. The method of any one of claims 1-10, wherein dsRNA content of the IVT product is less than about 600 pg/µg RNA, less than about 500 pg/µg RNA, less than about 400 pg/µg RNA, less than about 300 pg/µg RNA, less than about 200 pg/µg RNA, less than about 100 pg/µg RNA, less than about 80 pg/µg RNA, less than about 60 pg/µg RNA, less than about 40 pg/µg RNA, less than about 20 pg/µg RNA, or less than about 10 pg/µg RNA.

13. The method of claim 12, wherein the RNAP is a variant T7 RNAP having one or more residue changes compared to a wild-type T7 RNAP.

14. The method of claim 13, wherein the variant T7 RNAP (a) is at least 97 %, at least 98 % or at least 99 % identical to SEQ ID NO: 1, and (b) comprises 1, 2, 3, 4, or 5 mutations (*e.g.*, a substitution or deletion) compared to SEQ ID NO: 1 at residues D130, N171, K172, R173, Y178, R298, Y385, K387, D388, and/or F880.

FIG. 1



SUBSTITUTE SHEET (RULE 26)

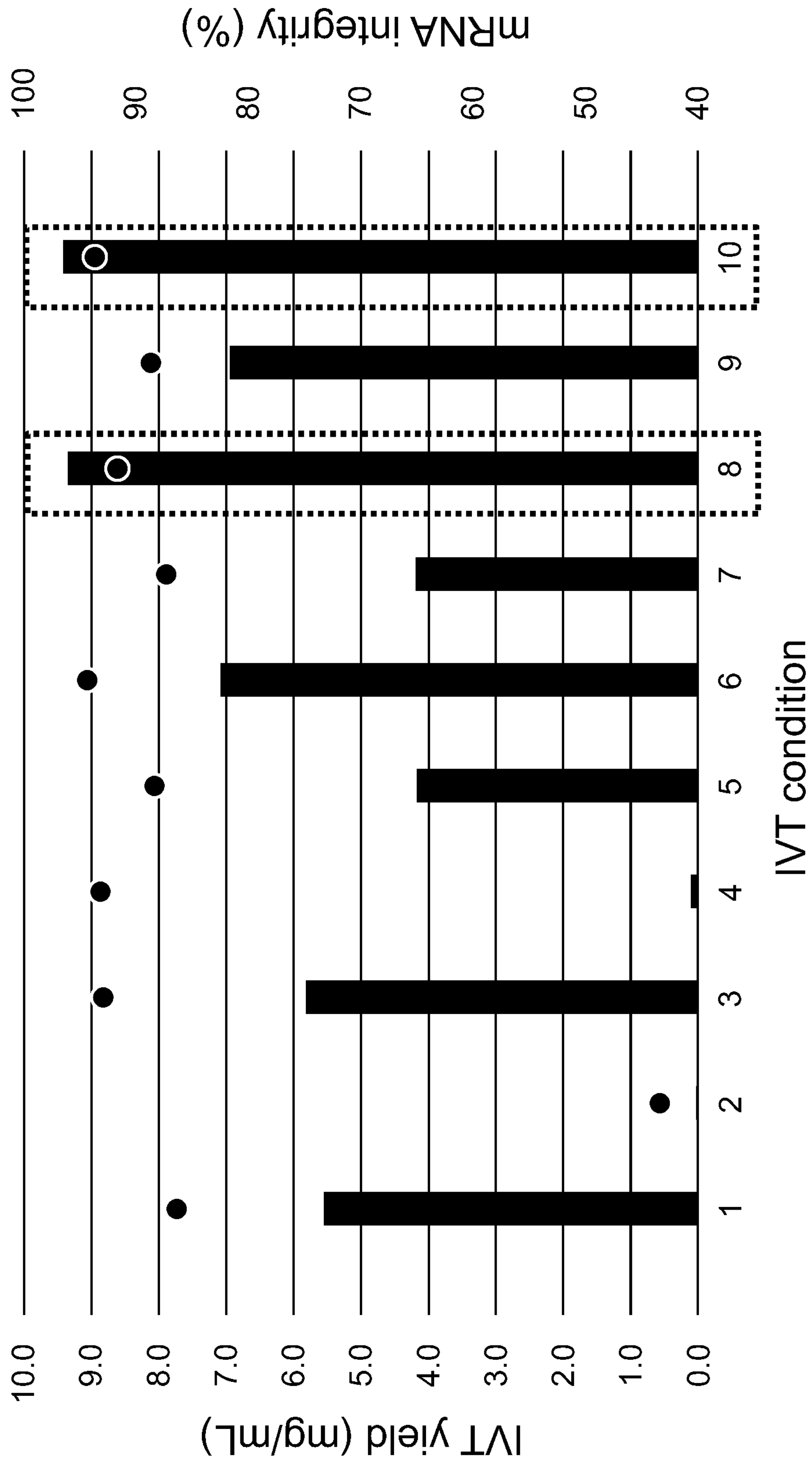
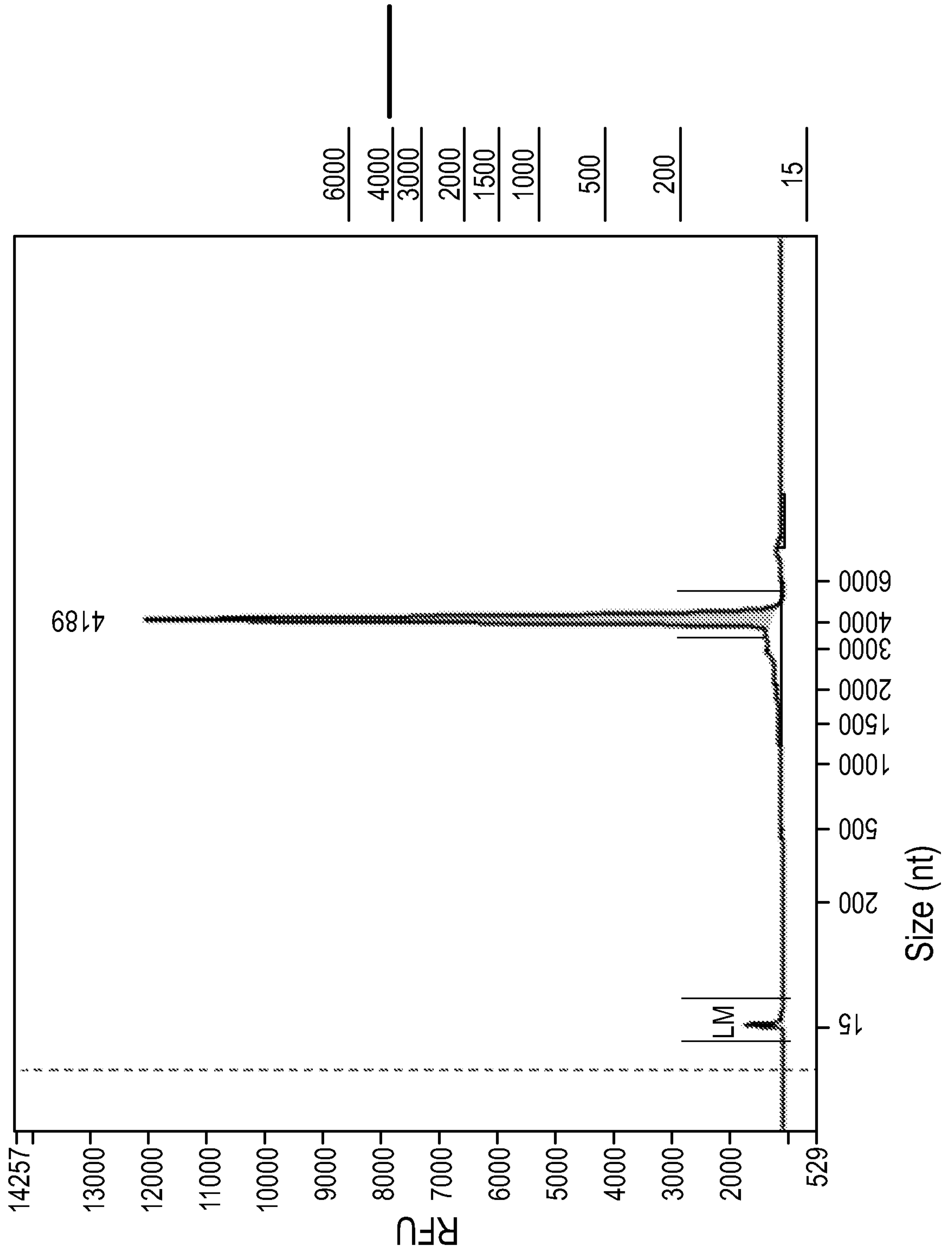


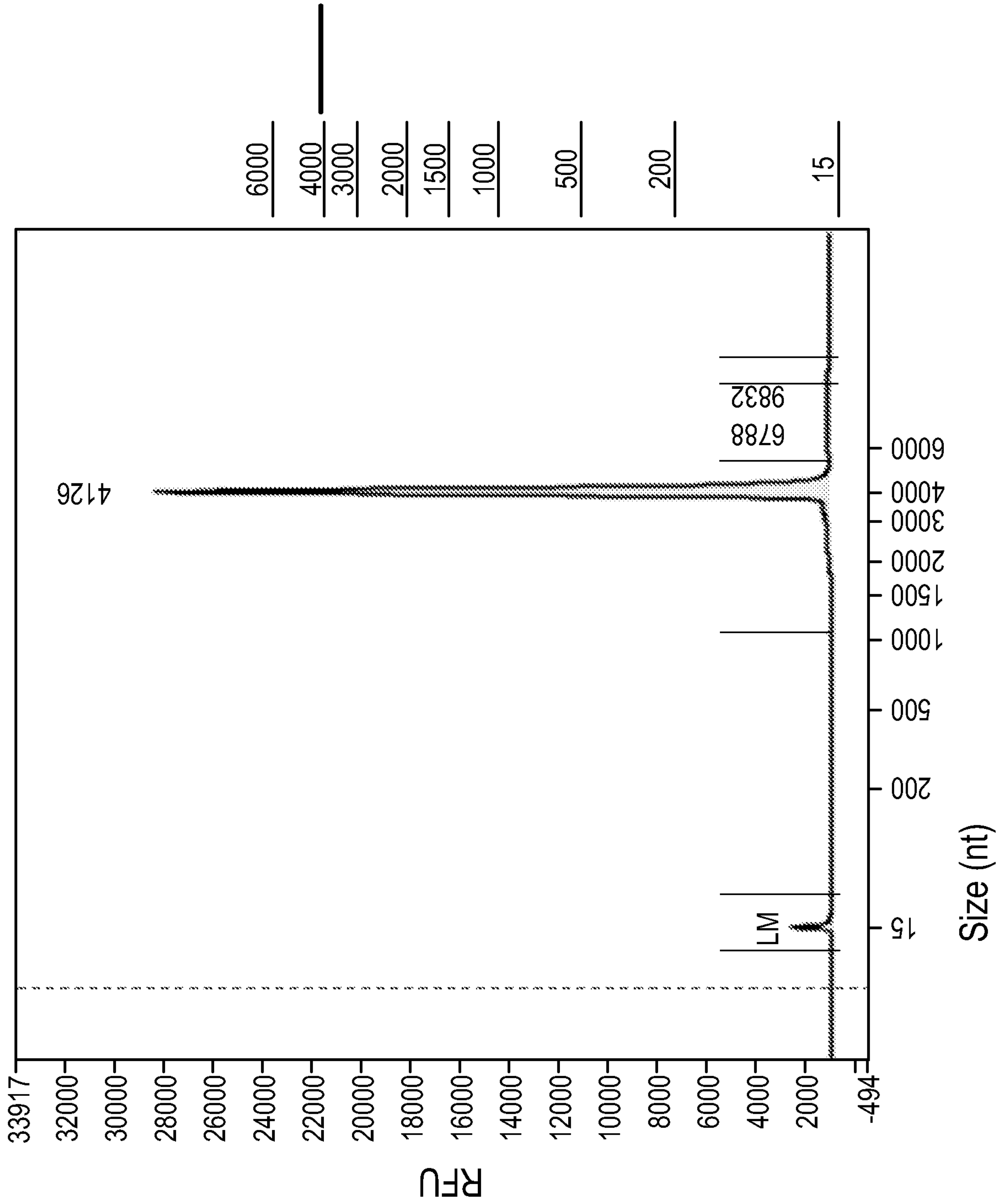
FIG. 2

FIG. 3A



SUBSTITUTE SHEET (RULE 26)

FIG. 3B



SUBSTITUTE SHEET (RULE 26)

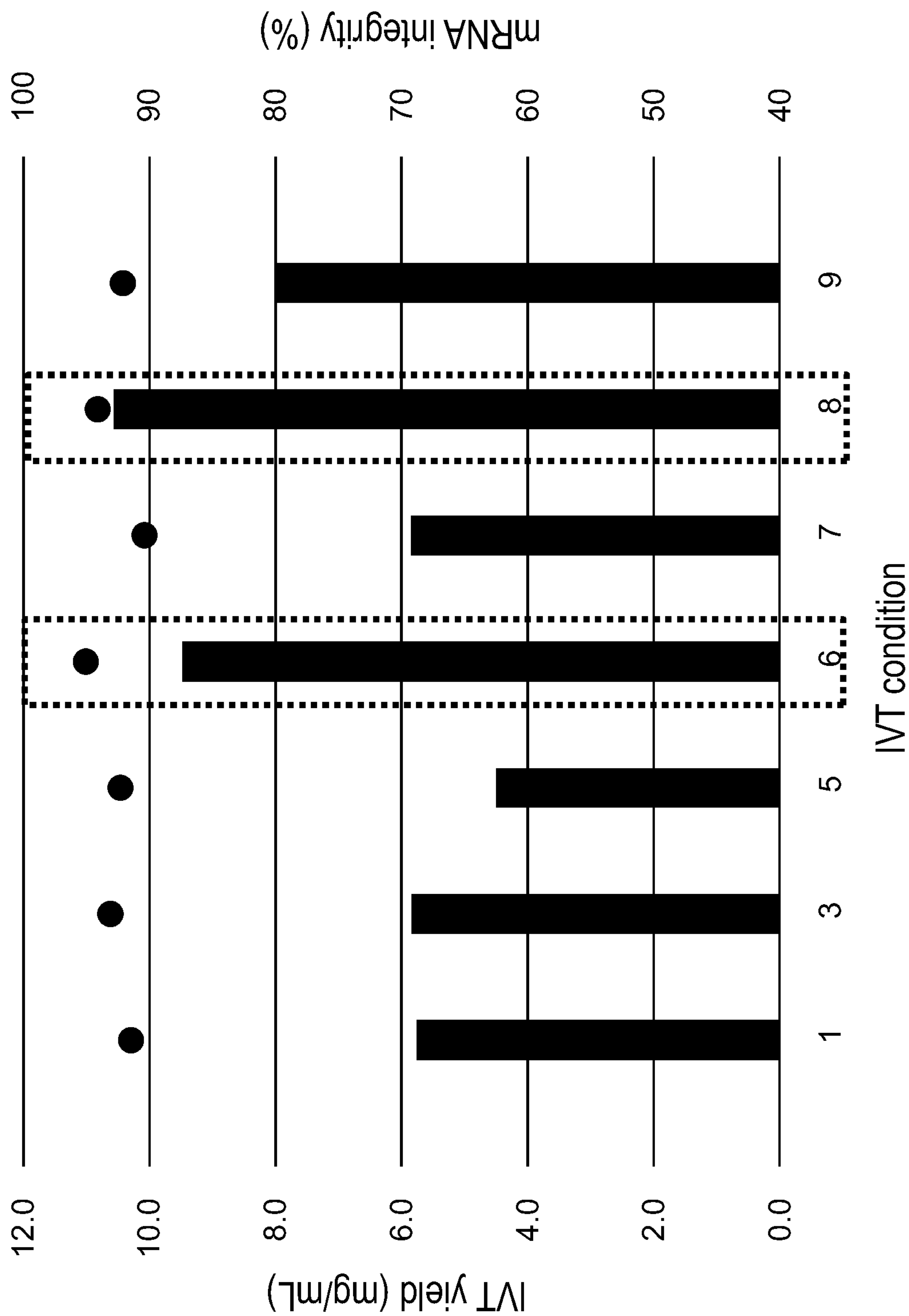


FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/039670

A. CLASSIFICATION OF SUBJECT MATTER

IPC: ***C12P 19/34*** (2024.01); ***C12Q 1/68*** (2024.01); ***C12N 9/12*** (2024.01)

CPC: ***C12P 19/34; C12Q 1/6844; C12N 9/1247***

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2019/0247417 A1 (MODERNATX INC.) 15 August 2019 (15.08.2019) entire document	1-3
A	US 2019/0010485 A1 (CUREVAC AG) 10 January 2019 (10.01.2019) entire document	1-3
A	WO 2023/031773 A1 (GLAXOSMITHKLINE BIOLOGICALS SA) 09 March 2023 (09.03.2023) entire document	1-3
A	YOUNG et al., Modeling and optimization of a batch process for in vitro RNA production, Biotechnology and bioengineering, Vol. 56, No. 2. 20 October 1997, Pgs. 210-220, [retrieved on 18 November 2024]. Retrieved from the internet: <URL:https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/10.1002/(SICI)1097-0290(19971020)56:2%3C210::AID-BIT10%3E3.0.CO;2-K>. entire document	1-3

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "D" document cited by the applicant in the international application
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 November 2024 (19.11.2024)	**03 December 2024 (03.12.2024)**

Name and mailing address of the ISA/US	Authorized officer
COMMISSIONER FOR PATENTS **MAIL STOP PCT, ATTN: ISA/US** **P.O. Box 1450** **Alexandria, VA 22313-1450** **UNITED STATES OF AMERICA**	**TAINA MATOS**
Facsimile No. **571-273-8300**	Telephone No. **571-272-4300**

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
   a. ☐ forming part of the international application as filed.
   b. ☑ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)),
      ☑ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/039670

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☑ Claims Nos.: **4-14**
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).