

CELL-FREE PRODUCTION OF RIBONUCLEIC ACID

RELATED APPLICATIONS

[0001] This application claims the benefit under 35 U.S.C. § 119(e) of U.S. provisional application number 62/140,407, filed March 30, 2015, which is incorporated by reference herein in its entirety.

BACKGROUND OF INVENTION

[0002] RNA interference (RNAi) refers to a cellular mechanism that uses the DNA sequence of a gene to turn the gene “off” – a process referred to as “silencing.” In a wide variety of organisms, including animals, plants, and fungi, RNAi is triggered by double-stranded RNA (dsRNA). Double-stranded RNA (*e.g.*, therapeutic dsRNA) has been produced in living cells and *in vitro* using purified, recombinant enzymes and purified nucleotide triphosphates (*see, e.g.*, European Patent No. 1631675 A1 and U.S. Patent Application Publication No. US2014/0271559 A1). However, the large-scale production of dsRNA using such systems is challenging, inefficient, and expensive.

BRIEF SUMMARY OF INVENTION

[0003] Provided herein is a platform for cell-free production (also referred to as biosynthesis) of RNA, including dsRNA. The methods, compositions (*e.g.*, cells and cell lysates), and systems of the present disclosure are based on a process that involves cell-free (*e.g.*, using cell lysate(s)) degradation of polymeric RNA (*e.g.*, mRNA, tRNA and/or rRNA) into monophosphate and diphosphate ribonucleotide monomers. Following RNA degradation, the monophosphate and diphosphate ribonucleotide monomers are converted to ribonucleotide triphosphate, and then the ribonucleotide triphosphates are polymerized to form desired RNA (*e.g.*, dsRNA).

[0004] Thus, the present disclosure provides methods of producing a cell lysate for cell-free production of ribonucleic acid (RNA). The methods may comprise (a) culturing cells to a desired cell density, wherein the cells comprise at least one engineered nucleic acid containing a promoter operably linked to a sequence encoding a nuclease (*e.g.*, S1 Nuclease, NucA, PNPase, RNase II, RNase III, or RNase R) that includes a protease-recognition site and is linked to a periplasmic-targeting sequence, wherein at least one endogenous ribonuclease (*e.g.*, RNase III, RNase I, RNaseR, PNPase, RNase II, and/or RNase T) is genetically inactivated or inactivated via targeted proteolysis in the cells, (b) lysing cells

produced in step (a), thereby producing a first cell lysate, and (c) incubating the first cell lysate under conditions that result in RNA depolymerization and NMP and NDP phosphorylation, thereby producing a first cell lysate containing nucleotide 5'-monophosphates (or a mixture of nucleotide 5'-monophosphates and nucleotide 5'-diphosphates).

[0005] In some embodiments, the methods further comprise (d) culturing cells that comprise (i) at least one engineered nucleic acid containing a promoter operably linked to a sequence encoding a cognate protease that cleaves the protease-recognition site of the nuclease or targeted endogenous RNases, wherein the cognate protease (*e.g.*, human rhinovirus 3C protease) is linked to a periplasmic-targeting sequence, (ii) at least one engineered nucleic acid containing a promoter operably linked to a sequence encoding a nucleotide kinase (*e.g.*, uridylate kinase, cytidylate kinase, guanylate kinase, adenylate kinase, nucleoside phosphate kinase, pyruvate kinase, and polyphosphate kinase), (iii) at least one engineered nucleic acid containing a promoter operably linked to a sequence encoding an RNA polymerase, and (iv) an engineered deoxyribonucleic acid (DNA) template containing a promoter operably linked to a sequence encoding a RNA transcript, wherein at least one endogenous ribonuclease is genetically inactivated or inactivated via targeted proteolysis in the cells; and (e) lysing cultured cells produced in step (d), to produce a second cell lysate.

[0006] In some embodiments, the methods further comprise combining the first cell lysate produced in step (b), the second cell lysate produced in step (e), and polyphosphate and/or glucose, thereby producing a mixture; and incubating the mixture under conditions that result in the production of RNA (*e.g.*, dsRNA).

[0007] In some embodiments, the methods comprise (a) culturing cells to a desired cell density, wherein the cells comprise (i) at least one engineered nucleic acid containing a promoter operably linked to a sequence encoding a nuclease that includes a protease-recognition site and is linked to a periplasmic-targeting sequence, and (ii) at least one engineered nucleic acid containing a promoter operably linked to a sequence encoding a nucleotide kinase, wherein at least one endogenous ribonuclease is genetically inactivated or inactivated via targeted proteolysis in the cells, (b) lysing cells produced in step (a), thereby producing a first cell lysate, and (c) incubating the first cell lysate with polyphosphate and/or glucose under conditions that result in RNA depolymerization, thereby producing a first cell lysate containing a mixture of nucleotide 5'-triphosphates.

[0008] In some embodiments, the methods further comprise (d) culturing cells that comprise (i) at least one engineered nucleic acid containing a promoter operably linked to a

sequence encoding a cognate protease that cleaves the protease-recognition sequence of the nuclease, wherein the cognate protease is linked to a periplasmic-targeting sequence, (ii) at least one engineered nucleic acid containing a promoter operably linked to a sequence encoding an RNA polymerase, and (iii) an engineered deoxyribonucleic acid (DNA) template containing a promoter operably linked to a sequence encoding a RNA transcript that includes complementary domains linked by a hinged domain, wherein at least one endogenous ribonuclease is genetically inactivated or inactivated via targeted proteolysis in the cells, and (e) lysing cultured cells produced in step (d), to produce a second cell lysate.

[0009] In some embodiments, the methods further comprise combining the first cell lysate produced in step (b), the second cell lysate produced in step (e), and polyphosphate and/or glucose, thereby producing a mixture; and incubating the mixture under conditions that result in production of RNA.

[0010] Also provided herein are engineered cells and cell lysates comprising a nuclease that includes a protease-recognition site and is linked to a periplasmic-targeting sequence, a nucleotide kinase, and a mixture of nucleotide 5'-monophosphates and nucleotide 5'-diphosphates.

[0011] Further provided herein are engineered cells and cell lysates comprising an RNA polymerase, and an engineered DNA template encoding an RNA.

[0012] Some aspects of the present disclosure provide cell-free methods of producing ribonucleic acid (RNA), the methods comprising (a) combining a first cell lysate with a second cell lysate, wherein the first cell lysate comprises (i) a nuclease that includes a protease-recognition site, and (ii) nucleotide 5'-monophosphates, and the second cell lysate comprises (iii) a cognate protease that cleaves the protease-recognition site of the nuclease, (iv) nucleotide kinase, (v) an RNA polymerase, and (vi) an engineered deoxyribonucleic acid (DNA) template containing a promoter operably linked to a sequence encoding an RNA of interest, thereby forming a reaction mixture, and (b) incubating the reaction mixture under conditions that result in production of the RNA of interest (*e.g.*, a double-stranded RNA of interest).

[0013] In some embodiments, the methods comprise (a) combining a first cell lysate with a second cell lysate, wherein the first cell lysate comprises (i) a nuclease that includes a protease-recognition site and is linked to a periplasmic-targeting sequence, (ii) nucleotide kinase and polyphosphate and/or glucose, and (iii) nucleotide 5'-triphosphates, and the second cell lysate comprises (iv) a cognate protease that cleaves the protease-recognition site of the nuclease, (v) an RNA polymerase, and (vi) an engineered deoxyribonucleic acid

(DNA) template containing a promoter operably linked to a sequence encoding an RNA of interest, thereby forming a reaction mixture, and (b) incubating the reaction mixture under conditions that result in production of the RNA of interest (*e.g.*, a double-stranded RNA of interest).

[0014] In any one of the embodiments provided herein, a cell lysate or reaction mixture may be free of nuclease activity.

[0015] The details of several embodiments of the invention are set forth in the accompanying Figures and the Detailed Description. Other features, objects, and advantages of the invention will be apparent from the description and from the claims.

BRIEF DESCRIPTION OF DRAWINGS

[0016] *Figure 1* shows a flowchart of an example of a process for double-stranded ribonucleic acid (dsRNA) biosynthesis. In this example, cells of one population express an engineered RNA-specific nuclease, sequestered in the periplasm, and at least one engineered protease-targeted endogenous RNase. Cells of another population express an engineered DNA-dependent RNA polymerase and/or RNA-dependent RNA polymerase, an engineered site-specific protease sequestered to the periplasm, at least one protease-targeted endogenous RNase, and an engineered DNA template encoding a desired RNA.

[0017] *Figure 2* shows a schematic of polyphosphate-catalyzed conversion of nucleotide monophosphate/diphosphate (NMP/NDP) to nucleotide triphosphate (NTP).

[0018] *Figure 3* shows a schematic of the conversion of an engineered DNA template to a dsRNA product.

[0019] *Figure 4* shows a schematic of the conversion of an engineered DNA template to a dsRNA product via an intermediate single-stranded RNA (ssRNA).

DETAILED DESCRIPTION OF INVENTION

[0020] Provided herein, in some aspects, are methods, compositions, and systems for the cell-free production of ribonucleic acid (RNA), such as double-stranded RNA (dsRNA). The methods provided herein, conceptually, involve three main processes: (1) the degradation of intracellular polymeric RNA into nucleotide monomers—a combination of nucleotide monophosphates (NMPs) and nucleotide diphosphates (NDPs), (2) the conversion of the NMPs and NDPs to nucleotide triphosphates (NTPs), which serve as “building blocks” for the formation of polymeric RNA, and (3) the polymerization of the NTPs to produce RNA, including dsRNA. In some embodiments, cells of one population are engineered to produce

the products necessary for steps 1 and 2 of the process, while cells of another population are engineered to produce products (*e.g.*, enzymes and nucleic acid template) necessary for step 3 of the process. Cell lysates produced from each cell population are then combined for the cell-free production of RNA. In other embodiments, cells of one population are engineered to produce the products necessary for step 1 of the process, while cells of another population are engineered to produce products (*e.g.*, enzymes and nucleic acid template) necessary for steps 2 and 3 of the process. Cell lysates produced from each cell population are then combined for the cell-free production of RNA.

[0021] It should be understood that while the RNA production process described herein is described conceptually in “steps,” the steps of the process do not need to be performed separately (*e.g.*, in a separate cell lysate or separate reaction mixture). For example, the products necessary for step 1 of the process may be expressed by one cell population, the products necessary for step 2 of the process may be expressed by another cell population, and the products necessary for step 3 of the process may be expressed by yet another cell population. Alternatively, the products necessary for steps 1 and 2 of the process may be expressed by one cell population, and the products necessary for step 3 of the process may be expressed by another cell population. Further, the products necessary for step 1 of the process may be expressed by one cell population, and the products necessary for steps 2 and 3 of the process may be expressed by another cell population.

[0022] It should also be understood that steps 1, 2, and 3 need not be performed sequentially. For example, a population of cells expressing products for one, or more than one, step may be cultured in parallel with a different population of cells expressing products for one, or more than one, step of the cell-free RNA production process of the present disclosure.

Step 1: Cell-free production of nucleotide 5'-monophosphates (5'-NMPs) and/or nucleotide 5'-diphosphates (5'-NDPs)

[0023] Some aspects of the present disclosure provide methods and compositions for the degradation of intracellular polymeric RNA into nucleotide monomers—a combination of nucleotide monophosphates (NMPs) and nucleotide diphosphates (NDPs). *Escherichia coli* (*E. coli*) cells can contain up to 25% RNA by weight. This RNA exists almost entirely in an oligomeric state in one of three forms: tRNA, rRNA, and mRNA. This RNA can be depolymerized to produce monomeric ribonucleotides that can serve as the building blocks for RNA biosynthesis.

[0024] Chemical means of depolymerization lead to the production of a mixture of 3'-NMPs and 5'-NMPs. 3'-NMPs cannot be used for dsRNA synthesis, and, therefore, chemical depolymerization decreases overall yield of the final RNA product. An alternative to chemical depolymerization of RNA is enzymatic depolymerization using nucleases, as described herein. Non-limiting examples of nucleases that may be used in accordance with the present disclosure are listed in *Table 1*.

Table 1. Example nucleases for RNA depolymerization to 5'-NMPs and 5'-NDPs

Nuclease	Host Organism(s)	EC #
S1 Nuclease	<i>Vigna radiate</i> , <i>Aspergillus oryzae</i> , <i>Apium graveolens</i> , others	3.1.30.1
NucA	<i>Serratia marcescens</i>	3.1.30.2
PNPase	<i>E. coli</i>	2.7.7.8
RNase II	<i>E. coli</i>	3.1.13.1
RNase III	<i>E. coli</i>	3.1.26.3
RNase R	<i>E. coli</i>	3.1.13.-

[0025] Existing processes for the enzymatic depolymerization of RNA to 5'-NMPs (as described in, *e.g.*, EP1587947B1, U.S. Patent No. 3,223,592, and U.S. Patent No. 2,844,514, each of which is incorporated herein by reference) typically involve mixing exogenous nucleases and lysed *Saccharomyces cerevisiae* cells and incubating the mixture to produce monomeric NMPs. These NMPs are typically the end product and are used, for example, as flavoring additives in food products.

[0026] The present disclosure, by contrast, encompasses enzymatic depolymerization whereby an engineered cell (*e.g.*, an engineered *E. coli* cell) expresses, for example, one or more nucleases (*e.g.*, *see* Table 1) in the periplasm of the cell. Periplasmic nuclease expression ensures that cytotoxic RNA depolymerization does not occur during cell growth. Upon cell lysis, the periplasmically-expressed nuclease(s) is/are released from the periplasm and contact cellular polymeric RNA (*e.g.*, an RNA containing two or more contiguous ribonucleotides), thereby depolymerizing the polymeric RNA into 5'-NMPs and/or 5'-NDPs. The resulting 5'-NMPs and/or 5'-NDPs, referred to herein as “monomers,” are used as starting materials for the polymerization of a particular RNA of interest (referred to as an RNA product), such as, for example, a dsRNA, without (or without significant) purification or separation of lysate components. It should be understood, however, that following the production of RNA, as provided herein, the RNA may be purified for use as a therapeutic agent.

[0027] The present disclosure, in some embodiments, contemplates the direct use of the 5'-NMP and/or 5'-NDP monomers in RNA polymerization. However, the presence of active nucleases, under some conditions, may result in depolymerization of a desired dsRNA product. Thus, provided herein is a means to functionally inactivate cellular nuclease(s) (*e.g.*, an overexpressed nuclease), to permit use of the monomers as substrates in subsequent RNA polymerization reactions without the need for purification of the monomers. Functional inactivation of a nuclease following RNA depolymerization may be achieved, in some embodiments, by targeted proteolysis, as described, for example, U.S. Publication No. 2012/0052547 A1, published on March 1, 2012; and International Publication No. WO 2015/021058 A2, published February 12, 2015, each of which is incorporated herein by reference). Other means of targeted/inducible protein inactivation are also contemplated herein.

Step 2: Cell-free conversion of 5'-NMPs and 5'-NDPs to 5'- NTPs

[0028] Some aspects of the present disclosure provide methods and compositions for the conversion of the NMPs and NDPs to nucleotide triphosphates (NTPs), which serve as “building blocks” for the formation of polymeric RNA. Processes involving purified proteins or cell extracts for converting 5'-NMPs and 5'-NDPs to 5'-NTPs are known (*e.g.*, U.S. Patent No. 6,022,713, incorporated herein by reference). Non-limiting examples of NMP kinases that may be used to convert 5'-NMPs to 5'-NDPs in accordance with the present disclosure are listed in *Table 2*. As would be appreciated by one of skill in the art, any enzyme or other agent with the desired kinase activity may be used in the present invention. In some embodiments, adenosine triphosphate (ATP) or guanosine triphosphate (GTP) is the phosphate donor.

Table 2. Example NMP kinases

Enzyme Name	Host Organism	EC #	Reaction
Uridylate kinase	<i>E. coli</i>	2.7.4.22	UMP + ATP → UDP + ADP
Cytidylate kinase	<i>E. coli</i>	2.7.4.25	CMP + ATP → CDP + ADP
Guanylate kinase	<i>E. coli</i>	2.7.4.8	GMP + ATP → GDP + ADP
Adenylate kinase	<i>E. coli</i>	2.7.4.3	AMP + ATP → 2 ADP

[0029] Non-limiting examples of NDP kinases that may be used to convert NDPs to NTPs in accordance with the present disclosure are listed in *Table 3*. The phosphate donor for nucleoside diphosphate kinase (Ndk) is ATP or GTP, the phosphate donor for pyruvate kinase (Pyk) is phosphoenolpyruvate (PEP), and the phosphate donor for polyphosphate

kinase (Ppk) is polyphosphate. *See Figure 2* for an illustration of a polyphosphate-catalyzed process of the present disclosure. As would be appreciated by one of skill in the art, any enzyme or other agent with the desired kinase activity may be used in accordance with the present invention.

Table 3. Example NDP kinases

Enzyme Name	Host Organism	EC #	Reaction
Nucleoside phosphate kinase	<i>E. coli</i>	2.7.4.6	$\text{NDP} + \text{ATP} \rightarrow \text{NTP} + \text{ADP}$
Pyruvate kinase	<i>E. coli</i>	2.7.1.40	$\text{NDP} + \text{PEP} \rightarrow \text{NTP} + \text{pyruvate}$
Polyphosphate kinase	<i>E. coli</i>	2.7.4.1	$\text{NDP} + \text{polyphosphate}_n \rightarrow \text{NTP} + \text{polyphosphate}_{n-1}$

[0030] Cell lysates of the present disclosure are used for cell-free enzymatic conversion of (1) NMPs to NDPs, (2) NDPs to NTPs, and (3) NTPs to dsRNA. In some embodiments, it is advantageous to eliminate/delete or inactivate in cell lysate(s) enzymes that degrade NMPs, NDPs, and/or NTPs. Non-limiting examples of enzymes that may be deleted or inactivated in accordance with the present disclosure are listed in *Table 4*. Thus, in some embodiments, the present disclosure contemplates engineering cells that do not express, or that express an inactive or inactivatable form of, at least one (*e.g.*, 1, 2, 3, or 4) of the enzymes listed in *Table 4*.

Table 4. Examples of enzymes that degrade NMPs, NDPs, and NTPs

Enzyme Name	Host Organism	EC #	Reaction
Nucleoside monophosphatase (aka 5'-nucleotidase)	<i>E. coli</i>	3.1.3.5	$\text{NMP} + \text{H}_2\text{O} \rightarrow \text{nucleoside} + \text{P}_i$
Nucleoside diphosphatase	<i>E. coli</i>	3.6.1.6	$\text{NDP} + \text{H}_2\text{O} \rightarrow \text{NMP} + \text{P}_i + \text{H}^+$
Nucleoside triphosphatase	<i>E. coli</i>	3.6.1.1 5	$\text{NTP} + \text{H}_2\text{O} \rightarrow \text{NDP} + \text{P}_i + \text{H}^+$
Nucleoside triphosphate phosphohydrolase	<i>E. coli</i>	3.6.1.1 9	$\text{NTP} + \text{H}_2\text{O} \rightarrow \text{NMP} + \text{PP}_i + \text{H}^+$

[0031] Enzymatic conversion of (1) NMPs to NDPs, (2) NDPs to NTPs, and (3) NTPs to dsRNA requires, in some instances, a high-energy phosphate donor feedstock (*e.g.*, polyphosphate). In some embodiments, it is advantageous to eliminate/delete or inactivate in cell lysate(s) enzymes that degrade polyphosphate. A non-limiting example of such an enzyme is *E. coli* exopolyphosphatase (EC 3.6.1.1.1; $\text{polyphosphate}_n + \text{H}_2\text{O} \rightarrow \text{polyphosphate}_{n-1} + \text{P}_i$). Thus, in some embodiments, the present disclosure contemplates

engineering cells that do not express, or that express an inactive or inactivatable form of, exopolyphosphatase. The present disclosure also contemplates the inactivation of other phosphatase enzymes, for example, through protease targeting.

[0032] In some embodiments, the activity of an enzyme (*e.g.*, a nuclease) is eliminated from a cell by deletion of the cognate gene from the genome of the host (referred to as a gene “knockout”), provided the activity is non-essential for cell viability. In some embodiments, the activity of an enzyme is inhibited or reduced in a controllable manner using, for example, targeted proteolysis, as described, for example, U.S. Publication No. 2012/0052547 A1, published on March 1, 2012; and International Publication No. WO 2015/021058 A2, published February 12, 2015, each of which is incorporated herein by reference). Other means of targeted enzyme inactivation are also contemplated herein.

Step 3: Cell-free production of ribonucleic acid (RNA) and/or double-stranded RNA (dsRNA)

[0033] Some aspects of the present disclosure provide methods and compositions for the polymerization of NTPs to produce RNA, including dsRNA. Enzymatically-derived 5'-NTPs are polymerized into RNA using an RNA polymerase that produces an RNA transcript. Non-limiting examples of RNA polymerases that may be used in accordance with the present disclosure are listed in *Table 5*.

Table 5. Examples of polymerases for the conversion of NTPs to RNA

Enzyme Name	Host Organism	Function
T7 RNA Polymerase	T7 Phage	DNA-dependent RNA polymerase
Φ6 RdRP	Phage Φ6	RNA-dependent RNA polymerase

Production of dsRNA using a DNA-dependent RNA polymerase.

[0034] Some aspects of the present disclosure are directed to the cell-free production of double-stranded RNA (dsRNA). In some embodiments, a dsRNA molecule is encoded by a DNA template that contains a promoter operably linked to a sequence encoding RNA (*e.g.*, an RNA transcript). In some embodiments, the RNA contains two complementary domains linked via a hinge domain. With reference to the example shown in *Figure 3*, upon binding of complementary domains (domain 1 and domain 3) of single-stranded RNA (ssRNA) template to each other, the hinge domain (domain 2) forms a loop-like structure.

[0035] Two nucleic acid domains (*e.g.*, discrete nucleotide sequences) are “complementary” to one another if they base-pair, or bind, to each other to form a double-stranded nucleic acid molecule via Watson-Crick interactions (also referred to as hybridization). As used herein, “binding” refers to an association between at least two molecules or two regions of the same molecule due to, for example, electrostatic, hydrophobic, ionic, and/or hydrogen-bond interactions under physiological conditions. In some embodiments, a complementary domain has a length of 4 to 1000 nucleotides, or more. For example, a complementary domain may have a length of 4 to 10, 4 to 20, 4 to 30, 4 to 50, 4 to 60, 4 to 70, 4 to 80, 4 to 90, 4 to 100, 4 to 200, 4 to 300, 4 to 400, or 4 to 500, or 4 to 1000 nucleotides. In some embodiments, a complementary domain has a length of 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides. In some embodiments, a complementary domain has a length of 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 nucleotides.

[0036] In some embodiments, one of the two complementary domains is target-specific, referred to here as the (+) complementary domain. That is, a complementary domain may be designed to be complementary (*e.g.*, identical) to a target nucleic acid of interest. Thus, in some embodiments, a (+) complementary domain is designed to be complementary to a target nucleic acid, and a (-) complementary domain is designed to be complementary to the (+) complementary domain.

[0037] A “hinge domain” in the context of the nucleic acids of the present disclosure refers to the single-stranded region that separates and is between “complementary domains.” Typically, a hinge domain is non-specific, meaning that it is not designed to bind to another nucleic acid, such as a target nucleic acid. A hinge domain forms a loop-like structure upon binding of the complementary domains to form a double-stranded region. In some embodiments, a hinge domain has a length of 4 to 500 nucleotides, or more. For example, a hinge domain may have a length of 4 to 10, 4 to 20, 4 to 30, 4 to 50, 4 to 60, 4 to 70, 4 to 80, 4 to 90, 4 to 100, 4 to 200, 4 to 300, 4 to 400, or 4 to 500 nucleotides. In some embodiments, a hinge domain has a length of 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 nucleotides.

[0038] It should be understood that a “double-stranded RNA” of the present disclosure encompasses wholly double-stranded molecules, which do not contain a single-stranded region (*e.g.*, a loop or overhang), as well as partially double-stranded molecules, which contain a double-stranded region and a single-stranded region (*e.g.*, a loop or overhang). The dsRNA product depicted at the bottom of *Figure 3* is considered a partially double-stranded

molecule, while the dsRNA product depicted at the bottom of *Figure 4* is considered a wholly double-stranded molecule.

[0039] Cell-free production of an RNA, including dsRNA, may be catalyzed, in some embodiments, by a highly processive DNA-dependent T7 RNA polymerase encoded from the T1 gene, although other DNA-dependent RNA polymerases may be used in accordance with the present disclosure. Thus, in some embodiments, an engineered DNA template includes a T7-specific promoter operably linked to a nucleotide sequence encoding an RNA molecule of interest. Typically, a promoter is located immediately 5' of the coding sequence. In some embodiments, engineered DNA template includes a T7-specific transcriptional terminator. A transcriptional terminator is typically located immediately 3' of the coding sequence. As shown in *Figure 3*, sequence encoding domain 1, domain 2, and domain 3 is flanked at each end by a transcription promoter and a transcriptional terminator. Similarly, as shown in *Figure 4*, sequence encoding domain 1 is flanked at each end by a transcription promoter and a transcriptional terminator.

[0040] In some embodiments, a DNA template (*e.g.*, containing a 5' promoter and a 3' terminator) is located on an expression vector containing an endonuclease restriction site outside of the coding region. In such embodiments, a cell may be engineered to express a cognate restriction endonuclease that cleaves the endonuclease restrictions site of the vector, resulting in linearization of the DNA template. Linearization permits, for example, “run-off transcription” of the desired RNA molecule, improving overall yield. In some embodiments, the cognate restriction endonuclease is *I-SceI*. Other restriction endonucleases are known and may be used in accordance with the present disclosure. Non-limiting examples include *EcoRI*, *EcoRII*, *BamHI*, *HindIII*, *TaqI*, *NotI*, *HinFI*, *Sau3AI*, *PvuII*, *SmaI*, *HaeIII*, *HgaI*, *AluI*, *EcoRV*, *EcoP15I*, *KpnI*, *PstI*, *SacI*, *Sall*, *ScaI*, *SpeI*, *SphI*, *StuI*, and *XbaI*, *FokI*, *AscI*, *AsiAI*, *NotI*, *FseI*, *PacI*, *SdaI*, *SgfL*, *SfiI*, *PmeI*.

Production of dsRNA using an RNA-dependent RNA polymerase.

[0041] In some embodiments, a dsRNA molecule is encoded by a DNA template that contains a promoter operably linked to a sequence encoding a messenger RNA (mRNA) that contains a single target domain (*e.g.*, a domain that is complementary to a target nucleic acid of interest). With reference to the example shown in *Figure 4*, following production of a single-stranded RNA (ssRNA), an RNA-dependent RNA polymerase (RdRP) may be used to synthesize a dsRNA of interest. In some embodiments, an RdRP isolated from phage $\phi 6$ is

used to synthesize dsRNA. Phage $\phi 6$ is a double stranded RNA virus that infects members of the genus *Pseudomonas*. Phage $\phi 6$ is one of the most well-studied dsRNA viruses. This phage encodes an RdRP that is capable of synthesizing RNA using an RNA template, yielding a dsRNA molecule. The $\phi 6$ RdRP is capable of polymerizing RNA absent a primer molecule, thus the polymerase requires only template RNA (Wright, S. *et al*, 2012. *Journal of Virology*. Mar;86(5):2837-49; Van Dijk, AA., *et al*, 2004. *J Gen Virol*. May;85(Pt 5), incorporated herein by reference). Other viral RdRPs are contemplated herein.

[0042] In some embodiments, the DNA template encoding the RNA containing a single target domain is transcribed using a DNA-dependent RNA polymerase, such as, for example, the T7 RNA polymerase, and then the resulting RNA transcript serves as a template for an RNA-dependent RNA polymerase, such as, for example, the phage $\phi 6$ RdRP, to synthesize a complementary RNA molecule, yielding a dsRNA (*e.g.*, *Figure 4*).

Inhibition of host RNA polymerase

[0043] Lysis and depolymerization of cellular ribonucleic acids as described above typically yields a pool of 5'-ribonucleotides. Competition between the native RNA polymerase and the heterologous T7 RNA polymerase and/or $\phi 6$ RdRP for these pools of ribonucleotides may, in some instances, decrease yield of the desired RNA product. Nonetheless, RNA polymerase is essential for the viability of intact cells. Thus, provided herein are at least two methods for disrupting native RNA polymerase function. For example, targeted proteolysis, as described elsewhere herein, may be used to disrupt activity of at least one of the key components of the native RNA polymerase, leading to sufficient disruption of RNA polymerase activity. As another example of disrupting native RNA polymerase function, the T7 phage protein Gp2 may be expressed. Gp2 is the second T7 phage protein that is translated following infection of *E. coli* by T7 phage. This protein disrupts host RNA polymerase function thus eliminating competition between native RNA polymerase and the phage polymerase (Bae, B. *et al*, 2013. *Proc Natl Acad Sci U S A*. Dec 3;110(49):19772-7).

Improved product yields through inactivation of host ribonucleases

[0044] One aspect to the cell-free production of RNA (*e.g.*, dsRNA) is the inhibition/prevention of product degradation during and after synthesis. In all living organisms, the ability to cleave and process RNA molecules is essential to viability.

Numerous native *E. coli* enzymes, referred to as ribonucleases, or RNases, catalyze the depolymerization of RNA molecules with varying affinities. Some of these RNases, including RNase D, responsible for tRNA maturation, are highly substrate specific, while others, including RNase I, are capable of depolymerizing a wide range of RNA polymers. Non-limiting examples of RNases, as well as their cognate genes, proposed activities, and example sequences are shown in Table 6.

[0045] In some embodiments, cell are engineered such that 50% to 100% (*e.g.*, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%) of endogenous ribonuclease (RNase) activity that would depolymerize or otherwise degrade the RNA product of interest is inactivated or deleted in cell lysate(s) produced from the engineered cells. This may be achieved by any means known in the art. In some embodiments, this is achieved by a chromosomal deletion of an endogenous gene encoding an RNase, provided the RNase is not essential for cell viability. Examples of genes that may be deleted from the genome of cells are listed in *Table 6*. In some embodiments, if the RNase is essential for cell viability, the cells may be engineered to contain endogenous RNases engineered to be sensitive to targeted proteolytic degradation (*e.g.*, expressed from their native/endogenous promoters). Examples of RNases/nucleases that may be protease-targeted, as provided herein, are listed in *Table 7*. In some embodiments, a combination of the foregoing approaches is used. Alternatively, or in addition, nuclease inhibitors may be used (*e.g.*, added to a cell lysate or reaction mixture). It should be noted that simultaneous deletion of *pnp* and *rnb* is lethal to a cell. Thus, the present disclosure also contemplates protease-targeting of one or both of the encoded enzymes.

Table 6. Examples of activities whose disruption could result in improved dsRNA yields in the dsRNA production strain

Enzyme	Gene	Function
RNase III	<i>rnc</i>	Cleaves dsRNA, rRNA and some mRNA
RNase I	<i>rna</i>	General ribonuclease, broad substrate specificity. Localizes to periplasm.
RNaseR	<i>rnr</i>	Cleaves some dsRNA, poly-A mRNA, mRNA and rRNA
PNPase	<i>pnp</i>	General mRNA degradation, tRNA maturation and degradation.
RNase II	<i>rnb</i>	Exonuclease. Plays a role in tRNA processing
RNase T	<i>rnt</i>	Processing of tRNAs, rRNA and other stable RNAs. Capable of degrading ssDNA and ssRNA.

Table 7. Examples of ribonucleases that could be inactivated via targeted proteolytic degradation

Enzyme	Gene	Function
RNase R	<i>rnr</i>	Cleaves some dsRNA, poly-A mRNA, mRNA and rRNA
RNase E	<i>rne</i>	Processes rRNA, tRNA and other RNAs. Associates with the “degradasome”
PNPase	<i>pnp</i>	General mRNA degradation, tRNA maturation and degradation.
RNase II	<i>rnb</i>	Exonuclease. Plays a role in tRNA processing

[0046] The *E. coli* RNase III enzyme preferentially cleaves dsRNA as well as some single-stranded mRNA molecules (Robertson, HD and J.J. Dunn. 1974. *J Biol Chem.* Apr 25;250(8):3050-6.; Lybecker, M. et al. 2014. The double-stranded transcriptome of *Escherichia coli*. *Proc Natl Acad Sci U S A.* Feb 25;111(8):3134-9, each of which is incorporated herein by reference). The presence of this enzyme in a cell-free system may limit the ability to accumulate high concentrations of dsRNA, as the product would readily be cleaved. The gene encoding RNase III, *rnc*, is not essential for cell viability, thus the present disclosure, in some embodiments, contemplates the chromosomal deletion/mutation of *rnc* in engineered cells, prior to cell-free production of an RNA of interest, or protease-targeting of RNase III (Takiff, H.E. et al. 1989. *J Bacteriol.* May;171(5):2581-90, each of which is incorporated herein by reference).

[0047] Similarly, the broad substrate exoribonuclease RNase I is not essential for the viability of *E. coli*. RNase I, which localizes to the periplasmic space in intact *E. coli* cells, catalyzes the depolymerization of a wide range of RNA molecules including rRNA, mRNA and tRNA (Neu, H.C. and L.A. Heppel. 1964. *J Biol Chem.* Nov;239:3893-900). Under physiological conditions the periplasmic localization of this enzyme means that the enzyme has little impact on RNA stability within the cell. However, the mixing of periplasm and cytoplasm in the cell-free methods of the present disclosure permits RNase I access to cellular RNA. Its presence may significantly reduce product output through the degradation of the ssRNA intermediate. Thus, the present disclosure, in some embodiments, contemplates the chromosomal deletion/mutation of *rna*, the gene encoding RNase I, or the protease-targeting of RNase I (Kaplan, R. and D. Apirion. 1974. *J Biol Chem.* Jan 10;249(1):149-51, incorporated herein by reference).

[0048] Two other broad-substrate exoribonucleases, RNase R and RNase T, catalyze the depolymerization of dsRNA, rRNA, tRNA, and mRNA as well as small unstructured RNA molecules (Cheng and Deutscher. 2002; Cheng and Deutscher. 2005; Vincent and Deutscher. 2009; Viswanathan *et al.*, 1998). The genes that encode RNase R and RNase T, *rnr* and *rnt*,

respectively, are not essential for cell viability. Thus, the present disclosure, in some embodiments, contemplates the chromosomal deletion/mutation of *rnr* and/or *rnt*, or the protease-targeting of RNase R and/or RNase.

[0049] A central component for RNA turnover within living *E. coli* cells, specifically mRNA, is a group of enzymes referred to as the degradosome (Bandyra, K. and B.F. Luisi, 2013. *RNA Biol.* Apr;10(4):627-35; A.J. Carpousis, 2002. *Biochem Soc Trans.* Apr;30(2):150-5, each of which is incorporated herein by reference). This degradosome contains of two ribonucleases, RNase E and PNPase, a RNA helicase, and several other proteins with roles in RNA degradation. RNase E functions as an endoribonuclease that regulates rRNA maturation as well as mRNA turnover. RNase E, without being bound by theory, may work in concert with exoribonucleases, such as PNPase and RNase II, to constantly turn over cellular mRNA pools. In this model, RNase E first cleaves the target RNA molecule internally, and the smaller RNA fragments are subsequently depolymerized to monomers. Disruption of the gene encoding RNase E, *rne*, is lethal in *E. coli* (Goldblum, K. and D. Apririon, 1981. *J Bacteriol.* Apr;146(1):128-32, incorporated herein by reference). Thus, the present disclosure, in some embodiments, contemplates the protease-targeting of RNase R and/or RNase. In some embodiments, the present disclosure contemplates protease-targeting of only one of PNPase and RNase II.

[0050] As described above, *E. coli* PNPases, encoded by the *pnp* gene associates with the degradosome wherein it serves as an exoribonuclease with 3' → 5' processivity (A.J. Carpousis, 2002). PNPase plays a role in mRNA degradation as well as tRNA maturation and turnover. Similarly, RNase II, encoded by the *rnb* gene, depolymerizes both mRNA and tRNA in a 3' → 5' direction. While neither of the two genes is essential, disruption of both simultaneously is synthetically lethal. Thus, the present disclosure, in some embodiments, contemplates the protease-targeting of PNPase and RNase II.

Engineered Cells and Nucleic Acids

[0051] “Engineered cells” of the present disclosure are cells that comprise at least one engineered (*e.g.*, recombinant or synthetic) nucleic acid, or are otherwise modified such that they are structurally and/or functionally distinct from their naturally-occurring counterparts. In some embodiments, an engineered cell comprises at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 engineered nucleic acids. In some embodiments, an engineered cell comprises 2 to 5, 2 to 10, or 2 to 20 engineered nucleic acids. In some embodiments, an engineered cell comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10,

11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 engineered nucleic acids. It should be understood that a cell that contains an engineered nucleic acid is considered an “engineered cell.”

[0052] In some embodiments, a culture of “cells” or “engineered cells” contains a homogenous population or a heterogeneous population of cells. For example, a culture of cells may contain more than one type of cell, each type of cell expressing at least one engineered nucleic acid of the present disclosure.

[0053] The term “nucleic acid” refers to at least two nucleotides covalently linked together, and in some instances, may contain phosphodiester bonds (*e.g.*, a phosphodiester “backbone”). Nucleic acids (*e.g.*, components, or portions, of nucleic acids) may be naturally occurring or engineered. “Naturally occurring” nucleic acids are present in a cell that exists in nature in the absence of human intervention. “Engineered nucleic acids” include recombinant nucleic acids and synthetic nucleic acids. A “recombinant nucleic acid” refers to a molecule that is constructed by joining nucleic acid molecules (*e.g.*, from the same species or from different species) and, typically, can replicate in a living cell. A “synthetic nucleic acid” refers to a molecule that is chemically or by other means synthesized or amplified, including those that are chemically or otherwise modified but can base pair with naturally occurring nucleic acid molecules. Recombinant and synthetic nucleic acids also include those molecules that result from the replication of either of the foregoing. It should be understood that engineered nucleic acids may contain portions of nucleic acids that are naturally occurring, but as a whole, engineered nucleic acids do not occur naturally and require human intervention. In some embodiments, a nucleic acid encoding a product of the present disclosure is a recombinant nucleic acid or a synthetic nucleic acid. In other embodiments, a nucleic acid encoding a product is naturally occurring.

[0054] An engineered nucleic acid encoding a product (*e.g.*, a protein or nucleic acid), as provided herein, is operably linked to a “promoter,” which is a control region of a nucleic acid at which initiation and rate of transcription of the remainder of a nucleic acid are controlled. A promoter drives expression or drives transcription of the nucleic acid that it regulates.

[0055] A promoter may be one naturally associated with a gene or sequence, as may be obtained by isolating the 5′ non-coding sequences located upstream of the coding segment of a given gene or sequence. Such a promoter can be referred to as “endogenous.”

[0056] In some embodiments, a coding nucleic acid sequence may be positioned under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with the encoded sequence in its natural environment. Such promoters

may include promoters of other genes; promoters isolated from any other cell; and synthetic promoters or enhancers that are not “naturally occurring” such as, for example, those that contain different elements of different transcriptional regulatory regions and/or mutations that alter expression through methods of genetic engineering that are known in the art. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including polymerase chain reaction (PCR).

[0057] A promoter is considered to be “operably linked” when it is in a correct functional location and orientation in relation to the nucleic acid it regulates to control (“drive”) transcriptional initiation and/or expression of that nucleic acid.

[0058] Engineered nucleic acids of the present disclosure may contain a constitutive promoter or an inducible promoter. A “constitutive promoter” refers to a promoter that is constantly active in a cell. An “inducible promoter” refers to a promoter that initiates or enhances transcriptional activity when in the presence of, influenced by, or contacted by an inducer or inducing agent. Inducible promoters for use in accordance with the present disclosure include any inducible promoter described herein or known to one of ordinary skill in the art. Examples of inducible promoters include, without limitation, chemically/biochemically-regulated and physically-regulated promoters such as alcohol-regulated promoters, tetracycline-regulated promoters, steroid-regulated promoters, metal-regulated promoters, pathogenesis-regulated promoters, temperature/heat-inducible, and light-regulated promoters.

[0059] An inducer or inducing agent may be endogenous or a normally exogenous condition (*e.g.*, light), compound (*e.g.*, chemical or non-chemical compound) or protein that contacts an inducible promoter in such a way as to be active in regulating transcriptional activity from the inducible promoter. Thus, a “signal that regulates transcription” of a nucleic acid refers to an inducer signal that acts on an inducible promoter. A signal that regulates transcription may activate or inactivate transcription, depending on the regulatory system used. Activation of transcription may involve directly acting on a promoter to drive transcription or indirectly acting on a promoter by inactivation a repressor that is preventing the promoter from driving transcription. Conversely, deactivation of transcription may involve directly acting on a promoter to prevent transcription or indirectly acting on a promoter by activating a repressor that then acts on the promoter.

[0060] Engineered nucleic acids of the present disclosure may contain a transcriptional terminator. A “transcriptional terminator” is a nucleic acid sequence that causes transcription

to stop. A terminator may be unidirectional or bidirectional. It is comprised of a DNA sequence involved in specific termination of an RNA transcript by an RNA polymerase. A terminator sequence prevents transcriptional activation of downstream nucleic acid sequences by upstream promoters. Thus, in certain embodiments, a terminator that ends the production of an RNA transcript is contemplated. The most commonly used type of terminator is a forward terminator. When placed downstream of a nucleic acid sequence that is usually transcribed, a forward transcriptional terminator will cause transcription to abort. In some embodiments, bidirectional transcriptional terminators are provided, which usually cause transcription to terminate on both the forward and reverse strand. In some embodiments, reverse transcriptional terminators are provided, which usually terminate transcription on the reverse strand only. In prokaryotic systems, terminators usually fall into two categories (1) rho-independent terminators and (2) rho-dependent terminators. Rho-independent terminators are generally composed of palindromic sequence that forms a stem loop rich in G-C base pairs followed by a string of uracil bases.

[0061] Terminators for use in accordance with the present disclosure include any terminator of transcription described herein or known to one of ordinary skill in the art. Non-limiting examples of terminators include the termination sequences of genes such as, for example, the bovine growth hormone terminator, and viral termination sequences such as, for example, the T0 terminator, the TE terminator, Lambda T1 and the T1T2 terminator found in bacterial systems. In some embodiments, the termination signal may be a sequence that cannot be transcribed or translated, such as those resulting from a sequence truncation.

[0062] Enzymes of the present disclosure may be encoded by nucleic acids that are located genomically (referred to as a “genomically-located nucleic acid”) or are located episomally (referred to as an “episomally-located nucleic acid”). A nucleic acid that is located genomically in a cell is a nucleic acid that is located in the genome of the cell. A nucleic acid that is located episomally in a cell is a nucleic acid that is located on an autonomously-replicating episome in the cell, such as a plasmid. Genomically-located nucleic acids and episomally-located nucleic acids may be endogenous (*e.g.*, originating from within the cell) to the cell or exogenous to the cell (*e.g.*, originating from outside the cell). Typically, exogenous nucleic acids are engineered nucleic acids (*e.g.*, recombinant or synthetic).

[0063] Engineered nucleic acids may be introduced into host cells using any means known in the art, including, without limitation, transformation, transfection (*e.g.*, chemical (*e.g.*, calcium phosphate, cationic polymers, or liposomes) or non-chemical (*e.g.*,

electroporation, sonoporation, impalefection, optical transfection, hydro dynamic)), and transduction (*e.g.*, viral transduction).

[0064] Engineered cells, in some embodiments, express selectable markers. Selectable markers are typically used to select engineered cells that have taken up and expressed an engineered nucleic acid following transfection of the cell (or following other procedure used to introduce foreign nucleic acid into the cell). Thus, a nucleic acid encoding product may also encode a selectable marker. Examples of selectable markers include, without limitation, genes encoding proteins that increase or decrease either resistance or sensitivity to antibiotics (*e.g.*, ampicillin resistance genes, kanamycin resistance genes, neomycin resistance genes, tetracycline resistance genes and chloramphenicol resistance genes) or other compounds. Other selectable markers may be used in accordance with the present disclosure.

[0065] An engineered cell “expresses” a product if the product, encoded by a nucleic acid (*e.g.*, an engineered nucleic acid), is produced in the cell. It is well known in the art that gene expression refers to the process by which genetic instructions in the form of a nucleic acid are used to synthesize a product, such as a protein (*e.g.*, an enzyme).

[0066] In some embodiments, proteins (*e.g.*, nucleases) of the present disclosure may be engineered to contain a protease-recognition sequence and/or a periplasmic-targeting sequence, as provided herein.

[0067] Enzymes or other proteins encoded by a naturally-occurring nucleic acid may be referred to as “endogenous enzymes” or “endogenous proteins.”

[0068] Engineered cells may be prokaryotic cells or eukaryotic cells. In some embodiments, engineered cells are bacterial cells, yeast cells, insect cells, mammalian cells, or other types of cells.

[0069] Engineered bacterial cells of the present disclosure include, without limitation, engineered *Escherichia spp.*, *Streptomyces spp.*, *Zymonas spp.*, *Acetobacter spp.*, *Citrobacter spp.*, *Synechocystis spp.*, *Rhizobium spp.*, *Clostridium spp.*, *Corynebacterium spp.*, *Streptococcus spp.*, *Xanthomonas spp.*, *Lactobacillus spp.*, *Lactococcus spp.*, *Bacillus spp.*, *Alcaligenes spp.*, *Pseudomonas spp.*, *Aeromonas spp.*, *Azotobacter spp.*, *Comamonas spp.*, *Mycobacterium spp.*, *Rhodococcus spp.*, *Gluconobacter spp.*, *Ralstonia spp.*, *Acidithiobacillus spp.*, *Microbacterium spp.*, *Geobacter spp.*, *Geobacillus spp.*, *Arthrobacter spp.*, *Flavobacterium spp.*, *Serratia spp.*, *Saccharopolyspora spp.*, *Thermus spp.*, *Stenotrophomonas spp.*, *Chromobacterium spp.*, *Sinorhizobium spp.*, *Saccharopolyspora spp.*, *Agrobacterium spp.*, and *Pantoea spp.*

[0070] Engineered yeast cells of the present disclosure include, without limitation, engineered *Saccharomyces spp.*, *Schizosaccharomyces*, *Hansenula*, *Candida*, *Kluyveromyces*, *Yarrowia* and *Pichia*.

[0071] In some embodiments, engineered cells of the present disclosure are engineered *Escherichia coli* cells, *Bacillus subtilis* cells, *Pseudomonas putida* cells, *Saccharomyces cerevisiae* cells, or *Lactobacillus brevis* cells. In some embodiments, engineered cells of the present disclosure are engineered *Escherichia coli* cells.

Cell Culture

[0072] Typically, engineered cells are cultured. “Culturing” refers to the process by which cells are grown under controlled conditions, typically outside of their natural environment. For example, engineered cells, such as engineered bacterial cells, may be grown as a cell suspension in liquid nutrient broth, also referred to as liquid “culture medium.”

[0073] Examples of commonly used bacterial *Escherichia coli* growth media include, without limitation, LB (Luria Bertani) Miller broth (1% NaCl): 1% peptone, 0.5% yeast extract, and 1% NaCl; LB (Luria Bertani) Lennox Broth (0.5% NaCl): 1% peptone, 0.5% yeast extract, and 0.5% NaCl; SOB medium (Super Optimal Broth): 2% peptone, 0.5% Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄; SOC medium (Super Optimal broth with Catabolic repressor): SOB + 20 mM glucose; 2x YT broth (2x Yeast extract and Tryptone): 1.6% peptone, 1% yeast extract, and 0.5% NaCl; TB (Terrific Broth) medium: 1.2% peptone, 2.4% yeast extract, 72 mM K₂HPO₄, 17 mM KH₂PO₄ and 0.4% glycerol; and SB (Super Broth) medium: 3.2% peptone, 2% yeast extract, and 0.5% NaCl.

[0074] Examples of high density bacterial *Escherichia coli* growth media include DNAGro™ medium, ProGro™ medium, AutoX™ medium, DetoX™ medium, InduX™ medium, and SecPro™ medium.

[0075] In some embodiments, engineered cells are cultured under conditions that result in expression of products (*e.g.*, proteins and/or nucleic acids). Such culture conditions may depend on the particular product being expressed and the desired amount of the product.

[0076] In some embodiments, engineered cells are cultured at a temperature of 30 °C to 40 °C. For example, engineered cells may be cultured at a temperature of 30 °C, 31 °C, 32 °C, 33 °C, 34 °C, 35 °C, 36 °C, 37 °C, 38 °C, 39 °C or 40 °C. Typically, engineered cells, such as engineered bacterial cells, are cultured at a temperature of 37 °C.

[0077] In some embodiments, engineered cells are cultured for a period of time of 12 hours to 72 hours, or more. For example, engineered cells may be cultured for a period of time of 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, or 72 hours. Typically, engineered cells, such as engineered bacterial cells, are cultured for a period of time of 12 to 24 hours. In some embodiments, engineered cells are cultured for 12 to 24 hours at a temperature of 37 °C.

[0078] In some embodiments, engineered cells are cultured (*e.g.*, in liquid cell culture medium) to an optical density, measured at a wavelength of 600 nm (OD600), of 5 to 25. In some embodiments, engineered cells are cultured to an OD600 of 5, 10, 15, 20, or 25.

[0079] In some embodiments, engineered cells are cultured to a density of 1×10^4 to 1×10^8 viable cells/ml cell culture medium. In some embodiments, engineered cells are cultured to a density of 1×10^4 , 2×10^4 , 3×10^4 , 4×10^4 , 5×10^4 , 6×10^4 , 7×10^4 , 8×10^4 , 9×10^4 , 1×10^5 , 2×10^5 , 3×10^5 , 4×10^5 , 5×10^5 , 6×10^5 , 7×10^5 , 8×10^5 , 9×10^5 , 1×10^6 , 2×10^6 , 3×10^6 , 4×10^6 , 5×10^6 , 6×10^6 , 7×10^6 , 8×10^6 , 9×10^6 , 1×10^7 , 2×10^7 , 3×10^7 , 4×10^7 , 5×10^7 , 6×10^7 , 7×10^7 , 8×10^7 , 9×10^7 , or 1×10^8 viable cells/ml. In some embodiments, engineered cells are cultured to a density of 2×10^5 to 3×10^7 viable cells/ml.

[0080] In some embodiments, engineered cells are cultured in a bioreactor. A bioreactor refers simply to a container in which cells are cultured, such as a culture flask, a dish, or a bag that may be single-use (disposable), autoclavable, or sterilizable. The bioreactor may be made of glass, or it may be polymer-based, or it may be made of other materials.

[0081] Examples of bioreactors include, without limitation, stirred tank (*e.g.*, well mixed) bioreactors and tubular (*e.g.*, plug flow) bioreactors, airlift bioreactors, membrane stirred tanks, spin filter stirred tanks, vibromixers, fluidized bed reactors, and membrane bioreactors. The mode of operating the bioreactor may be a batch or continuous processes and will depend on the engineered cells being cultured. A bioreactor is continuous when the feed and product streams are continuously being fed and withdrawn from the system. A batch bioreactor may have a continuous recirculating flow, but no continuous feeding of nutrient or product harvest. For intermittent-harvest and fedbatch (or batch fed) cultures, cells are inoculated at a lower viable cell density in a medium that is similar in composition to a batch medium. Cells are allowed to grow exponentially with essentially no external manipulation until nutrients are somewhat depleted and cells are approaching stationary growth phase. At this point, for an intermittent harvest batch-fed process, a portion of the cells and product may be harvested, and the removed culture medium is replenished with fresh medium. This process may be repeated several times. For production of recombinant proteins and antibodies, a fedbatch process may be used. While cells are growing exponentially, but

nutrients are becoming depleted, concentrated feed medium (*e.g.*, 10-15 times concentrated basal medium) is added either continuously or intermittently to supply additional nutrients, allowing for further increase in cell concentration and the length of the production phase.

Fresh medium may be added proportionally to cell concentration without removal of culture medium (broth). To accommodate the addition of medium, a fedbatch culture is started in a volume much lower than the full capacity of the bioreactor (*e.g.*, approximately 40% to 50% of the maximum volume).

[0082] Some methods of the present disclosure are directed to large-scale production of dsRNA. For large-scale production methods, engineered cells may be grown in liquid culture medium in a volume of 5 liters (L) to 50 L, or more. In some embodiments, engineered cells may be grown in liquid culture medium in a volume of greater than (or equal to) 10 L. In some embodiments, engineered cells are grown in liquid culture medium in a volume of 5 L, 10 L, 15 L, 20 L, 25 L, 30 L, 35 L, 40 L, 45 L, or 50 L, or more. In some embodiments, engineered cells may be grown in liquid culture medium in a volume of 5 L to 10 L, 5 L to 15 L, 5 L to 20 L, 5 L to 25 L, 5 L to 30 L, 5 L to 35 L, 5 L to 40 L, 5 L to 45 L, 10 L to 15 L, 10 L to 20 L, 10 L to 25 L, 20 L to 30 L, 10 L to 35 L, 10 L to 40 L, 10 L to 45 L, 10 L to 50 L, 15 L to 20 L, 15 L to 25 L, 15 L to 30 L, 15 L to 35 L, 15 L to 40 L, 15 L to 45 L, or 15 to 50 L.

Cell Lysates

[0083] Typically, culturing of engineered cells is followed by lysing the cells. “Lysing” refers to the process by which cells are broken down, for example, by viral, enzymatic, mechanical, or osmotic mechanisms. A “cell lysate” refers to a fluid containing the contents of lysed cells (*e.g.*, lysed engineered cells), including, for example, organelles, membrane lipids, proteins, and nucleic acids. Cell lysates of the present disclosure may be produced by lysing any population of engineered cells, as provided herein.

[0084] Methods of cell lysis, referred to as “lysing,” are known in the art, any of which may be used in accordance with the present disclosure. Such cell lysis methods include, without limitation, physical lysis and chemical (*e.g.*, detergent-based) lysis.

[0085] Cell lysis can disturb carefully controlled cellular environments, resulting in protein degradation and modification by unregulated endogenous proteases and phosphatases. Thus, in some embodiments, protease inhibitors and/or phosphatase inhibitors may be added to lysis reagents, or these activities may be removed by gene inactivation or protease targeting.

[0086] Cell lysates, in some embodiments, may be combined with at least one nutrient. For example, cell lysates may be combined with Na_2HPO_4 , KH_2PO_4 , NH_4Cl , NaCl , MgSO_4 , CaCl_2 . Examples of other nutrients include, without limitation, magnesium sulfate, magnesium chloride, magnesium orotate, magnesium citrate, potassium phosphate monobasic, potassium phosphate dibasic, potassium phosphate tribasic, sodium phosphate monobasic, sodium phosphate dibasic, sodium phosphate tribasic, ammonium phosphate monobasic, ammonium phosphate dibasic, ammonium sulfate, ammonium chloride, ammonium hydroxide,

[0087] Cell lysates, in some embodiments, may be combined with at least one cofactor. For example, cell lysates may be combined with adenosine diphosphate (ADP), adenosine triphosphate (ATP), nicotinamide adenine dinucleotide (NAD^+), or other non-protein chemical compounds required for activity of an enzyme (*e.g.*, inorganic ions and coenzymes).

[0088] In some embodiments, cell lysates are incubated under conditions that result in RNA depolymerization. In some embodiments, cell lysates are incubated under conditions that result in production of dsRNA.

[0089] Methods of the present disclosure include incubating a (at least one) cell lysate under conditions that result in RNA depolymerization and/or production of dsRNA. A cell lysate may be incubated at temperature of 4 °C to 45 °C, or higher. For example, engineered cells may be incubated at a temperature of 4 °C, 5 °C, 6 °C, 7 °C, 8 °C, 9 °C, 10 °C, 11 °C, 12 °C, 13 °C, 14 °C, 15 °C, 16 °C, 17 °C, 18 °C, 19 °C, 20 °C, 21 °C, 22 °C, 23 °C, 24 °C, 25 °C, 26 °C, 27 °C, 28 °C, 29 °C, 30 °C, 31 °C, 32 °C, 33 °C, 34 °C, 35 °C, 36 °C, 37 °C, 38 °C, 39 °C, 40 °C, 41 °C, 42 °C, 43 °C, 44 °C, or 45 °C. In some embodiments, a cell lysate is incubated at a temperature of 4-6 °C, 25 °C, or 37 °C. In some embodiments, a cell lysate is incubated at a temperature of 15 °C to 45 °C.

[0090] In some embodiments, a cell lysate is incubated for a period of time of 30 minutes (min) to 48 hours (hr), or more. For example, engineered cells may be cultured for a period of time of 30 min, 45 min, 1 hr, 2 hrs, 3 hrs, 4 hrs, 5 hrs, 6 hrs, 7 hrs, 8 hrs, 9 hrs, 10 hrs, 11 hrs, 12 hrs, 18 hrs, 24 hrs, 30 hrs, 36 hrs, 42 hours, or 48 hours. In some embodiments, a cell lysate is incubated for a period of time of 2 hr to 48 hr. In some embodiments, a cell lysate is incubated for 24 hours at a temperature of 37 °C.

[0091] The volume of cell lysate used for a single reaction may vary. In some embodiments, the volume of a cell lysate is 1 to 150 m^3 . For example, the volume of a cell lysate may be 1 m^3 , 5 m^3 , 10 m^3 , 15 m^3 , 20 m^3 , 25 m^3 , 30 m^3 , 35 m^3 , 40 m^3 , 45 m^3 , 50 m^3 , 55 m^3 , 60 m^3 , 65 m^3 , 70 m^3 , 75 m^3 , 80 m^3 , 85 m^3 , 90 m^3 , 95 m^3 , 100 m^3 , 105 m^3 , 110 m^3 , 115

m³, 120 m³, 125 m³, 130 m³, 135 m³, 140 m³, 145 m³, or 150 m³. In some embodiments, the volume of a cell lysate is 25 m³ to 150 m³, 50 m³ to 150 m³, or 100 m³ to 150 m³.

[0092] In some embodiments, a cell lysate or reaction mixture free of nuclease (*e.g.*, ribonuclease) activity. A cell lysate or reaction mixture is considered “free of nuclease activity” if the cell lysate or reaction mixture is free of nucleases or if the amount of nuclease activity in the cell lysate or reaction is not detectable by standard assays used to detect protein (*e.g.*, enzyme) activity. Non-limiting examples of enzymes assays include both continuous assays (*e.g.*, spectrophotometric, fluorometric, calorimetric, chemiluminescent, light scattering, and microscale thermophoresis) and discontinuous assays (*e.g.*, radiometric and chromatographic). Other enzymes assays are contemplated herein.

Protease Targeting

[0093] Engineered cells of the present disclosure may express (*e.g.*, endogenously express) nuclease(s) necessary for RNA depolymerization that may, in turn, degrade a desired RNA product. To prevent, or reduce, degradation of RNA produced by the methods provided herein, endogenous and/or engineered nucleases can be modified to include a site-specific protease-recognition sequence in their protein sequence such that the nuclease may be “targeted” and cleaved for inactivation during RNA production (see, *e.g.*, U.S. Publication No. 2012/0052547 A1, published on March 1, 2012; and International Publication No. WO 2015/021058 A2, published February 12, 2015, each of which is incorporated herein by reference). Nucleases that contain site-specific protease-recognition sequence are referred to as “protease-targeted nucleases.” Cleavage of a protease-targeted nuclease results upon contact of the nuclease with a cognate site-specific protease in a cell lysate. Thus, engineered cells of a particular population may comprise an engineered nucleic acid encoding a protease-targeted nuclease, or other protease-targeted ribonuclease(s). Protease targeting of other enzymes is also contemplated herein.

[0094] As described in more detail below, protease-targeted nucleases (*e.g.*, nucleases, or other ribonucleases, such as those described in *Table 1*, *Table 6* and *Table 7*) of the present disclosure may also contain a periplasmic-targeting sequence. Sequestration of the protease-targeted nuclease to the periplasm during the cell growth phase prevents the nuclease from degrading cellular RNA required for cell growth.

[0095] In some embodiments, cells of the present disclosure contain an engineered nucleic acid encoding a cognate protease. In some embodiments, a cognate protease contains a periplasmic-targeting sequence, as discussed below. In some embodiments, cells of one

population (*e.g.*, of a first culture) contain an engineered nucleic acid encoding a protease-targeted nuclease, and cells of a different population (*e.g.*, of a second culture) contain an engineered nucleic acid encoding a cognate protease that cleaves the protease-recognition sequence of the nuclease.

[0096] Examples of proteases that may be used in accordance with the present disclosure include, without limitation, alanine carboxypeptidase, *Armillaria mellea*, astacin, bacterial leucyl aminopeptidase, cancer procoagulant, cathepsin B, clostripain, cytosol alanyl aminopeptidase, elastase, endoproteinase Brg-C, enterokinase, gastricsin, gelatinase, Gly-X carboxypeptidase, glycyl endopeptidase, human rhinovirus 3C protease, hypodermin C, Iga-specific serine endopeptidase, leucyl aminopeptidase, leucyl endopeptidase, lysC, lysosomal pro-X carboxypeptidase, lysyl aminopeptidase, methionyl aminopeptidase, myxobacter, nardilysin, pancreatic endopeptidase E, picornain 2B, picornain 3C, proendopeptidase, prolyl aminopeptidase, proprotein convertase I, proprotein convertase II, russellysin, saccharopepsin, semenogelase, T-plasminogen activator, thrombin, tissue kallikrein, tobacco etch virus (TEV), togavirin, tryptophanyl aminopeptidase, U-plasminogen activator, V8, venombin B, venombin BB and Xaa-pro aminopeptidase.

Periplasmic Targeting

[0097] In some embodiments, a nuclease (*e.g.*, a ribonuclease) or other enzyme (*e.g.* protease) of an engineered cell contains a periplasmic-targeting sequence responsible for sequestering the nuclease or other enzyme to the periplasm of the cell. A “periplasmic-targeting sequence” is an amino acid sequence that targets to the periplasm of a cell the protein to which it is linked. A protein that is linked to a periplasmic-targeting sequence will be sequestered in the periplasm of the cell in which the protein is expressed.

[0098] Periplasmic-targeting sequences may be derived from the N-terminus of bacterial secretory protein, for example. The sequences vary in length from about 15 to about 70 amino acids. The primary amino acid sequences of periplasmic-targeting sequences vary, but generally have a common structure, including the following components: (i) the N-terminal part has a variable length and generally carries a net positive charge; (ii) following is a central hydrophobic core of about 6 to about 15 amino acids; and (iii) the final component includes four to six amino acids which define the cleavage site for signal peptidases.

[0099] Periplasmic-targeting sequences of the present disclosure, in some embodiments, may be derived from a protein that is secreted in a Gram negative bacterium. The secreted protein may be encoded by the bacterium, or by a bacteriophage that infects the bacterium.

Examples of Gram negative bacterial sources of secreted proteins include, without limitation, members of the genera *Escherichia*, *Pseudomonas*, *Klebsiella*, *Salmonella*, *Caulobacter*, *Methylobacter*, *Acetobacter*, *Achromobacter*, *Acinetobacter*, *Aeromonas*, *Agrobacterium*, *Alcaligenes*, *Azotobacter*, *Burkholderia*, *Citrobacter*, *Comamonas*, *Enterobacter*, *Erwinia*, *Rhizobium*, *Vibrio*, and *Xanthomonas*.

[00100] Examples of periplasmic-targeting sequences for use in accordance with the present disclosure include, without limitation, sequences selected from the group consisting of:

MKIKTGARILALSALTTMMFSASALA (SEQ ID NO: 1);
 MKQSTIALALLPLLFTPVTKA (SEQ ID NO: 2);
 MMITLRKLPLAVAVAAGVMSAQAMA (SEQ ID NO: 3);
 MNKKVLTLSAVMASMLFGAAHA (SEQ ID NO: 4);
 MKYLLPTAAAGLLLLAAQPAMA (SEQ ID NO: 5);
 MKKIWLALAGLVLAFSASA (SEQ ID NO: 6);
 MMTKIKLLMLIIFYLIISASAAHA (SEQ ID NO: 7);
 MKQALRVAFGFLILWASVLHA (SEQ ID NO: 8);
 MRVLLFLLLSLFMLPAFS (SEQ ID NO: 9); and
 MANNDLFQASRRRFLAQLGGLTVAGMLGPSLLTPRRATA (SEQ ID NO: 10).

Examples

[00101] Engineered cells of a first population express a protease-targeted nuclease, including, but not limited, to those listed in *Table 1*, which is localized to the periplasm. Engineered cells of a second population express at least one NMP and/or NDP kinase, a DNA-dependent RNA polymerase and/or a RNA-dependent RNA polymerase, and a site-specific protease localized to the periplasm. Additionally, cells of the second population contain a DNA template encoding a dsRNA of interest, wherein the DNA template is flanked by genetic elements (*e.g.*, 5' promoter, 3' terminator) that regulate transcription of the DNA template. Cells of each population are engineered to contain inactivated or protease-targeted RNases, including, but not limited to, those listed in *Table 6* and *Table 7*, or chromosomal deletions of genes encoding such RNases.

[00102] Cells of the first population are grown to a high cell density using conventional fermentation means with glucose and/or other sugars or carbon-based molecules serving as the carbon and energy source. Upon reaching an appropriate cell density, expression of the nuclease is induced through either the addition of an inducer molecule or through other

means. Protein induction is allowed to continue until a sufficient level of protein expression is reached. Following growth and induction, the biomass is concentrated, where necessary. The biomass is subsequently lysed, allowing access of the nuclease to the cellular oligomeric RNA molecules. This material is incubated, preferentially at 37 °C, until appropriate RNA depolymerization has occurred (*e.g.*, 2 hours to 24 hours).

[00103] Cells of the second population are grown, induced and concentrated in a manner similar to the cells of the first population. The resulting biomass is lysed and mixed with the lysate from cells of the first population. Mixing of the cellular lysates allows for proteolytic inactivation of the nuclease expressed in cells of the first population as well as the various targeted RNases from both cells populations. The monomeric NMPs and/or NDPs yielded from the nuclease-mediated depolymerization of oligomeric RNA in cells of the first population are subsequently converted to NTPs through the activity of at least one of the nucleotide kinases listed in *Table 2* and *Table 3*. Polyphosphate is added to the reaction to generate the required ATP for phosphorylation of the NMPs and NDPs via polyphosphate kinase. The resulting NTPs are oligomerized via the action of either the DNA-dependent RNA polymerase and/or RNA-dependent RNA polymerase using the aforementioned extrachromosomal DNA molecule as template yielding the desired, sequence-specific dsRNA molecule.

Other Embodiments

[00104] In the claims articles such as “a,” “an,” and “the” may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include “or” between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The invention includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The invention includes embodiments in which more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process.

[00105] Furthermore, the invention encompasses all variations, combinations, and permutations in which one or more limitations, elements, clauses, and descriptive terms from one or more of the listed claims is introduced into another claim. For example, any claim that is dependent on another claim can be modified to include one or more limitations found in any other claim that is dependent on the same base claim. Where elements are presented as

lists, *e.g.*, in Markush group format, each subgroup of the elements is also disclosed, and any element(s) can be removed from the group. It should be understood that, in general, where the invention, or aspects of the invention, is/are referred to as comprising particular elements and/or features, certain embodiments of the invention or aspects of the invention consist, or consist essentially of, such elements and/or features. For purposes of simplicity, those embodiments have not been specifically set forth in haec verba herein. It is also noted that the terms “comprising” and “containing” are intended to be open and permits the inclusion of additional elements or steps. Where ranges are given, endpoints are included. Furthermore, unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or sub-range within the stated ranges in different embodiments of the invention, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise.

[00106] This application refers to various issued patents, published patent applications, journal articles, and other publications, all of which are incorporated herein by reference. If there is a conflict between any of the incorporated references and the instant specification, the specification shall control. In addition, any particular embodiment of the present invention that falls within the prior art may be explicitly excluded from any one or more of the claims. Because such embodiments are deemed to be known to one of ordinary skill in the art, they may be excluded even if the exclusion is not set forth explicitly herein. Any particular embodiment of the invention can be excluded from any claim, for any reason, whether or not related to the existence of prior art.

[00107] Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation many equivalents to the specific embodiments described herein. The scope of the present embodiments described herein is not intended to be limited to the above Description, but rather is as set forth in the appended claims. Those of ordinary skill in the art will appreciate that various changes and modifications to this description may be made without departing from the spirit or scope of the present invention, as defined in the following claims.

CLAIMS

What is claimed is:

1. A method of producing a cell lysate for cell-free production of ribonucleic acid (RNA), the method comprising:
 - (a) culturing cells comprising a periplasmic space to a desired cell density, wherein the cells comprise at least one engineered nucleic acid containing a promoter operably linked to a sequence encoding a nuclease that includes a protease-recognition site and is linked to a periplasmic-targeting sequence, wherein at least one endogenous ribonuclease is genetically inactivated or inactivated via targeted proteolysis in the cells;
 - (b) lysing cells produced in step (a), thereby producing a first cell lysate, and
 - (c) incubating the first cell lysate under conditions that result in RNA depolymerization, thereby producing a first cell lysate containing nucleotide 5'-monophosphates.
2. The method of claim 1, wherein the promoter operably linked to a sequence encoding a nuclease is inducible.
3. The method of claim 1 or 2 further comprising:
 - (d) culturing cells that comprise (i) at least one engineered nucleic acid containing a promoter operably linked to a sequence encoding a cognate protease that cleaves the protease-recognition site of the nuclease, wherein the cognate protease is linked to a periplasmic-targeting sequence, (ii) at least one engineered nucleic acid containing a promoter operably linked to a sequence encoding a nucleotide kinase, (iii) at least one engineered nucleic acid containing a promoter operably linked to a sequence encoding an RNA polymerase, and (iv) an engineered deoxyribonucleic acid (DNA) template containing a promoter operably linked to a sequence encoding an RNA, wherein at least one endogenous ribonuclease is genetically inactivated or inactivated via targeted proteolysis in the cells; and
 - (e) lysing cultured cells produced in step (d), to produce a second cell lysate.
4. The method of claim 3, wherein at least one of the promoters of (d)(i), (d)(ii), or (d)(iii) is inducible.

5. The method of any one of claims 1-4, wherein the nuclease is selected from the group consisting of S1 Nuclease, NucA, PNPase, RNase II, RNase III, and RNase R.
6. The method of any one of claims 1-5, wherein the cells contain at least one protease-targeted endogenous ribonuclease and/or at least one chromosomal deletion in a gene encoding an endogenous ribonuclease.
7. The method of claim 6, wherein at least one endogenous ribonuclease is selected from the group consisting of RNase III, RNase I, RNaseR, PNPase, RNase II, RNase T, RNase E and combinations thereof.
8. The method of any one of claims 3-7, wherein the nucleotide kinase is a nucleotide monophosphate kinase.
9. The method of claim 8, wherein the nucleotide monophosphate kinase is selected from the group consisting of uridylate kinase, cytidylate kinase, guanylate kinase, and adenylylate kinase.
10. The method of any one of claims 3-7, wherein the nucleotide kinase is a nucleotide diphosphate kinase.
11. The method of claim 10, wherein the nucleotide diphosphate kinase is selected from the group consisting of nucleoside phosphate kinase, pyruvate kinase, and polyphosphate kinase.
12. The method of claim 1, wherein at least one endogenous enzyme that degrades polyphosphate is genetically inactivated in the cells.
13. The method of claim 12, wherein at least one of the endogenous enzyme(s) that degrades polyphosphate is selected from the group consisting of nucleoside monophosphatase (aka 5'-nucleotidase), nucleoside diphosphatase, nucleoside triphosphatase, nucleoside triphosphate phosphohyrolase, and exopolyphosphatase.

14. The method of claim 12 or 13, wherein the cells contain at least one protease-targeted enzyme that degrades polyphosphate and/or at least one chromosomal deletion in a gene encoding enzyme that degrades polyphosphate.
15. The method of any one of claims 3-4 or 8-11, wherein the RNA polymerase is a DNA-dependent RNA polymerase.
16. The method of claim 15, wherein the DNA-dependent RNA polymerase is T7 RNA polymerase.
17. The method of claim 15 or 16, wherein the sequence of the engineered DNA template encodes an mRNA containing complementary domains linked by a hinged domain.
18. The method of claim 17, wherein the promoter is a T7 promoter, and wherein a T7 transcriptional terminator is located at the 3' end of the sequence encoding the mRNA containing complementary domains linked by a hinged domain.
19. The method of any one of claims 3-14, wherein the cells of step (d) comprise at least two engineered nucleic acids, each containing a promoter operably linked to a sequence encoding an RNA polymerase, wherein one of the engineered nucleic acids encodes a DNA-dependent RNA polymerase, and one of the engineered nucleic acids encodes an RNA-dependent RNA polymerase.
20. The method of claim 19, wherein the DNA-dependent RNA polymerase is T7 RNA polymerase, and the RNA-dependent RNA polymerase is $\Phi 6$ RdRP.
21. The method of claim 20, wherein the sequence of the engineered DNA template contains a promoter operably linked to a sequence encoding an mRNA containing a single target domain.
22. The method of claim 21, wherein the promoter is a T7 promoter, and a T7 transcriptional terminator is located at the 3' end of the sequence encoding the mRNA containing a single target domain.

23. The method of any one of claims 18-22, wherein the engineered DNA template is located on an expression vector containing an endonuclease cleavage site.
24. The method of claim 23, wherein the endonuclease cleavage site is an *I-SceI* endonuclease cleavage site.
25. The method of claim 23 or 24, wherein the cells of step (d) further comprise a nucleic acid encoding an endonuclease that cleaves the endonuclease cleavage site.
26. The method of any one of claims 1-25, wherein the cells contain at least one protease-targeted endogenous RNA polymerase and/or at least one chromosomal deletion in a gene encoding an endogenous RNA polymerase.
27. The method of any one of claims 1-25, wherein the cells of step (a) and/or step (d) further comprise an engineered nucleic acid containing an inducible promoter operably linked to a sequence encoding a T7 phage Gp2 protein.
28. The method of any one of claims 1-27, wherein the cells of step (a) and/or step (d) are cultured in the presence of a sugar.
29. The method of claim 28, wherein the sugar is glucose.
30. The method of any one of claims 3-29 further comprising:
combining the first cell lysate produced in step (b), the second cell lysate produced in step (e), and polyphosphate, thereby producing a mixture; and
incubating the mixture under conditions that result in production of RNA.
31. The method of any one of claims 1-30, wherein the RNA is double-stranded RNA (dsRNA).
32. A method of producing a cell lysate for cell-free production of ribonucleic acid (RNA), the method comprising:
(a) culturing cells comprising a periplasmic space to a desired cell density, wherein the cells comprise (i) at least one engineered nucleic acid containing a promoter

operably linked to a sequence encoding a nuclease that includes a protease-recognition site and is linked to a periplasmic-targeting sequence, and (ii) at least one engineered nucleic acid containing a promoter operably linked to a sequence encoding a nucleotide kinase, wherein at least one endogenous ribonuclease is genetically inactivated or inactivated via targeted proteolysis in the cells;

(b) lysing cells produced in step (a), thereby producing a first cell lysate, and

(c) incubating the first cell lysate in the presence of polyphosphate under conditions that result in RNA depolymerization, thereby producing a first cell lysate containing a mixture of nucleotide 5'-triphosphates.

33. The method of claim 32 further comprising:

(d) culturing cells comprising a periplasmic space that comprise (i) at least one engineered nucleic acid containing a promoter operably linked to a sequence encoding a cognate protease that cleaves the protease-recognition sequence of the nuclease, wherein the cognate protease is linked to a periplasmic-targeting sequence, (ii) at least one engineered nucleic acid containing a promoter operably linked to a sequence encoding an RNA polymerase, and (iii) an engineered deoxyribonucleic acid (DNA) template containing a promoter operably linked to a sequence encoding a messenger RNA (mRNA) that includes complementary domains linked by a hinged domain, wherein at least one endogenous ribonuclease is genetically inactivated or inactivated via targeted proteolysis in the cells; and

(e) lysing cultured cells produced in step (d), to produce a second cell lysate.

34. The method of claim 33 further comprising:

combining the first cell lysate produced in step (b), the second cell lysate produced in step (e), and optionally polyphosphate, thereby producing a mixture; and incubating the mixture under conditions that result in production of RNA.

35. The method of any one of claims 32-34, wherein the RNA is double-stranded RNA (dsRNA).

36. A cell lysate comprising a nuclease with a protease-recognition site and a periplasmic-targeting sequence, and a cognate protease with a periplasmic-targeting sequence.

37. A cell-free method of producing ribonucleic acid (RNA), the method comprising:
- (a) combining a first cell lysate with a second cell lysate, wherein
the first cell lysate comprises (i) a nuclease that includes a protease-recognition site, and (ii) nucleotide 5'-monophosphates, and
the second cell lysate comprises (iii) a cognate protease that cleaves the protease-recognition site of the nuclease, (iv) nucleotide kinase, (v) an RNA polymerase, and (vi) an engineered deoxyribonucleic acid (DNA) template containing a promoter operably linked to a sequence encoding an RNA of interest, thereby forming a reaction mixture; and
 - (b) incubating the reaction mixture under conditions wherein the nuclease of the first cell lysate is cleaved by the cognate protease and said conditions result in the production of the RNA of interest.
38. The method of claim 37, wherein the RNA of interest is a double-stranded RNA.
39. A cell-free method of producing ribonucleic acid (RNA), the method comprising:
- (a) combining a first cell lysate with a second cell lysate, wherein
the first cell lysate comprises (i) a nuclease that includes a protease-recognition site, (ii) nucleotide kinase, and (iii) nucleotide 5'-triphosphates, and
the second cell lysate comprises (iv) a cognate protease that cleaves the protease-recognition site of the nuclease, (v) an RNA polymerase, and (vi) an engineered deoxyribonucleic acid (DNA) template containing a promoter operably linked to a sequence encoding an RNA of interest, thereby forming a reaction mixture; and
 - (b) incubating the reaction mixture under conditions wherein the nuclease of the first cell lysate is cleaved by the cognate protease and said conditions result in the production of the RNA of interest.
40. The method of claim 39, wherein the RNA of interest is a double-stranded RNA.
41. The method of any one of claims 37-40, wherein the first cell lysate and/or the second cell lysate are free of nuclease activity.