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 adenylate 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 1-14, 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 Φ6 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 produced by the method of any one of claims 1-35, wherein the cell lysate comprises 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.