

Claims

1. A circular RNA precursor comprising the following elements from 5' to 3' direction in the following order:

- a) a 3' self-splicing intron fragment;
- b) a first residual circularizing element;
- c) a nucleotide sequence of interest;
- d) a second residual circularizing element; and
- e) a 5' self-splicing intron fragment;

wherein the circular RNA precursor allows generation of a circular RNA comprising the first residual circularizing element, the nucleotide sequence of interest, and the second residual circularizing element through the self-splicing of the circular RNA precursor,

wherein the total length of the first residual circularizing element and the second residual circularizing element is about 5 to about 100 nucleotides, preferably about 20 to about 36 nucleotides,

wherein the first residual circularizing element and the second residual circularizing element are configured such that the circular RNA comprising them has reduced immunogenicity or has comparable or increased circularization efficiency relative to a control RNA, such as a control circular RNA comprising the circularizing elements of SEQ ID NO:29 and SEQ ID NO:64 or a control linear RNA comprising the same nucleotide sequence of interest.

2. The circular RNA precursor of claim 1, wherein the self-splicing intron is selected from Group I introns and Group II introns, preferably, the self-splicing intron is selected from IC3 subgroup of Group I introns,

more preferably, the self-splicing intron is selected from a Group I intron of the cyanobacterium *Anabaena*, a Group I intron from a T4 phage or a Group I intron from *Azoarcus* sp. BH72,

more preferably, the self-splicing intron is selected from the Group I intron of the *Anabaena* pre-tRNA-Leu gene, the Group I intron of the td gene of T4 phage, or the Group I intron of the pre-tRNA-Ile gene of *Azoarcus* sp. BH72,

most preferably,, the self-splicing intron is the Group I intron of the *Anabaena* pre-tRNA-Leu gene.

3. The circular RNA precursor of any one of claims 1-2, wherein the 3' self-splicing intron fragment is derived from a 3' terminal portion of a native self-splicing intron starting from an internal split site to the 3' end of the native self-splicing intron, and wherein the 5'

self-splicing intron fragment is derived from a 5' terminal portion of the native self-splicing intron starting from the internal split site to the 5' end of the native self-splicing intron, and, the 3' self-splicing intron fragment and the 5' self-splicing intron fragment in combination retain the self-splicing activity of the native self-splicing intron,

preferably, wherein the internal split site is located within the P6, P2, P5, P8 or P9 region of the Group I intron or the D4 region of the Group II intron, more preferably, the internal split site is located within the P6 region of the Group I intron.

4. The circular RNA precursor of claim 3, wherein the 3' self-splicing intron fragment is a sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100% sequence identity to the 3' terminal portion of a native self-splicing intron, and the 5' self-splicing intron fragment is a sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100% sequence identity to the 5' terminal portion of a native self-splicing intron.

5. The circular RNA precursor of any one of claims 1-4, wherein

i) the self-splicing intron is Group I intron of the *Anabaena* pre-tRNA-Leu gene, and the 3' self-splicing intron fragment comprises or consists of a nucleotide sequence of SEQ ID NO: 1 or a nucleotide sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, 100% identity to SEQ ID NO: 1, and the 5' self-splicing intron fragment comprises or consists of a nucleotide sequence of SEQ ID NO: 2 or a nucleotide sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, 100% identity to SEQ ID NO: 2;

ii) the self-splicing intron is the Group I intron of the *td* gene of T4 phage, and the 3' self-splicing intron fragment comprises or consists of a nucleotide sequence of SEQ ID NO: 5 or a nucleotide sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, 100% identity to SEQ ID NO: 5, and the 5' self-splicing intron fragment comprises or consists of a nucleotide sequence of SEQ ID NO: 6 or a nucleotide sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, 100% identity to SEQ ID NO: 6; or

iii) the self-splicing intron is the Group I intron of the pre-tRNA-Ile gene of *Azoarcus* sp. BH72, and the 3' self-splicing intron fragment comprises or consists of a nucleotide sequence of SEQ ID NO: 3 or a nucleotide sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, 100% identity to SEQ ID NO: 3, and the 5' self-splicing intron fragment comprises or consists of a nucleotide sequence of SEQ ID NO: 4 or a nucleotide sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, 100% identity to SEQ ID NO: 4.

6. The circular RNA precursor of any one of claims 1-5, wherein the first residual circularizing element and the second residual circularizing element are configured to be capable of forming a stem-loop structure, e.g., upon self-splicing for circularization, preferably wherein,

i) the loop of the stem-loop structure comprises the splicing junction;

ii) the predicted free energy of the stem-loop structure is lower than about -1 kal/mol, lower than about -2 kal/mol, lower than about -3 kal/mol, lower than about -4 kal/mol, lower than about -5 kal/mol, lower than about -6 kal/mol, lower than about -7 kal/mol, lower than about -8 kal/mol, lower than about -9 kal/mol, lower than about -10 kal/mol, or lower; and/or

iii) the first residual circularizing element comprises the sequence structure of the following formula: 5'-first loop sequence-first pairing sequence-first non-pairing sequence-3'; and the second residual circularizing element comprises the sequence structure of the following formula: 5'-second non-pairing sequence-second pairing sequence-second loop sequence-3',

wherein the first non-pairing sequence or the second non-pairing sequence may be independently present or absent,

the first pairing sequence and the second pairing sequence can complementarily pair to each other to form the stem of the stem-loop structure, wherein the first loop sequence and the second loop sequence can form the loop of the stem-loop structure, e.g., through self-splicing for circularization.

7. The circular RNA precursor of claim 6,

wherein the first loop sequence

i) comprises or consists of one or more nucleotides which can pair with the P1 region of the corresponding self-splicing intron to form a P10 duplex region during the circularization;

ii) consist of a nucleotide sequence of (N)_n, wherein N represents any nucleotides (A, G, U, or C), n represents an integer from 1-20, for example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20; and/or

iii) comprises or consists of the sequence of AAAA, AA, UUUU, UAAA, CAAAA, or GAAAA, preferably AAAA; and/or

wherein the second loop sequence

i) comprises or consists of one or more nucleotides which can pair with the internal guide sequence (IGS) of the corresponding self-splicing intron to form a P1 duplex region during the circularization; and/or

ii) e comprises or consists of CUU or CUC, preferably CUU,

more preferably, wherein the loop of the stem-loop structure has a sequence of CUUAAAA, CUUUUUU, CUUAA, CUUGAAA, CUUUAAA, CUUCAAA or CUCAAAA, preferably CUUAAAA.

8. The circular RNA precursor of any one of claims 6-7, wherein

i) the stem portion of the stem-loop structure comprises 2-15 or more consecutive matched base pairs, preferably, the stem portion of the stem-loop structure comprises 5, 6 or 7 consecutive matched base pairs;

ii) the stem portion in the stem-loop structure comprises up to 2 base pair mismatches, or the stem portion comprises only 1 base pair mismatch, preferably, the stem portion comprises no base pair mismatches; and/or

iii) the first pairing sequence comprises only Gs and the second pairing sequence comprises only Cs; iv) the first pairing sequence comprises only Cs and the second pairing sequence comprises only Gs; v) the first pairing sequence includes only A and the second pairing sequence includes only U; or the first pairing sequence comprises or consists of the sequence of any one of SEQ ID NO:42-55, and, the second pairing sequence comprises or consists of the sequence of any one of SEQ ID NO:78-93.

9. The circular RNA precursor of any one of claims 1-8, wherein the first residual circularizing element comprises or consists of from 5' to 3' direction a 3' exon region and optionally a first spacer, the second residual circularizing element comprises or consists of from 3' to 5' direction a 5' exon region and optionally a second spacer;

preferably, wherein the 3' exon region is derived from the native 3' exon of the self-splicing intron, the 5' exon region is derived from the native 5' exon of the self-splicing intron, and the 3' exon region and 5' exon region can be recognized and/or spliced by the self-splicing intron or a combination of the 3' self-splicing intron fragment and the 5' self-splicing intron fragment;

more preferably, wherein 3' exon region is a sequence having at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 99%, or at least 99% sequence identity with the native 3' exon or a contiguous fragment of about 1- about 50 nucleotides starting from the 5' terminal nucleotide of the native 3' exon, the 5' exon region is a sequence having at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 99%, or at least 99% sequence identity with the native 5' exon or a contiguous fragment of about 1- about 50 nucleotides starting from the 3' terminal nucleotide of the native 5' exon.

10. The circular RNA precursor of any one of claims 1-9, wherein the first residual

circularizing element comprises or consists of a nucleotide sequence having at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or 100% sequence identity to a nucleotide sequence selected from SEQ ID NOs: 13-55; and/or

the second residual circularizing element comprises or consists of a nucleotide sequence having at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or 100% sequence identity to a nucleotide sequence selected from SEQ ID NOs: 56-93.

11. The circular RNA precursor of any one of claims 1-10, wherein,

1) the first residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 15 and the second residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 57;

2) the first residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 14 and the second residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 58;

3) the first residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 18 and the second residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 59;

4) the first residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 19 and the second residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 60;

5) the first residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 20 and the second residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 61;

6) the first residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 21 and the second residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 62;

7) the first residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 23 and the second residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 56;

8), the first residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 24 and the second residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 56;

9) the first residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 26 and the second residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 56;

10) the first residual circularizing element comprises or consists of a nucleotide

sequence of SEQ ID NO: 27 and the second residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 56;

11) the first residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 28 and the second residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 56;

12) the first residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 13 and the second residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 67;

13) the first residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 13 and the second residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 59;

14) the first residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 13 and the second residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 56;

15) the first residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 17 and the second residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 59; or

16) the first residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 22 and the second residual circularizing element comprises or consists of a nucleotide sequence of SEQ ID NO: 63.

12. The circular RNA precursor of any one of claims 1-11, wherein the circular RNA precursor further comprises a 5' homology arm sequence and a 3' homology arm sequence capable of complementary pairing to each other to form a homology arm double-stranded region,

wherein the 5' homology arm sequence is upstream of the 3' self-splicing intron fragment and the 3' homology arm sequence is downstream of the 5' self-splicing intron fragment, preferably, wherein

i) the homology arm is about 5-50 nucleotides in length, preferably, about 40 nucleotides in length;

ii) the two homology arm sequences may be polyA and polyT, respectively, or polyG and polyC, respectively; and/or

iii) one of the homology arm sequence has the nucleotide sequence of any one of SEQ ID NO: 151-161 or a nucleotide sequence having at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% sequence identity with any one of SEQ ID NO: 151-161, while the other homology arm sequence has the corresponding

complementary sequence.

13. The circular RNA precursor of any one of claims 1-12,

wherein the nucleotide sequence of interest is a non-protein coding sequence, for example, the non-protein-coding sequence is selected from antisense RNA, aptamer, guide RNA, or a non-protein-coding RNA naturally existing in an organism; or

wherein the nucleotide sequence of interest comprises at least one protein-coding sequence and a translation initiation element such as an internal ribosome entry site (IRES) operably linked thereto, preferably, wherein

i) the translation initiation element, such as an IRES, is located upstream to the 5' end of the at least one protein-coding sequence.

ii) the translation initiation element, such as an IRES, is located downstream to the 3' end of the at least one protein-coding sequence;

iii) the protein-coding sequence encodes a protein of eukaryotic, prokaryotic or viral origin, for example, a protein for therapeutic or diagnostic use;

iv) the translation initiation element is an internal ribosome entry site (IRES) which is selected from the following IRES sequences: Taura syndrome virus, blood-sucking bug virus, Tyler's encephalomyelitis virus, simian virus 40, red fire ant virus 1, cereal constrictor virus, reovirus Endothelial hyperplasia virus, Forman poliovirus 1, soybean inchworm virus, Kashmir bee virus, human rhinovirus 2, glass leafhopper virus-1, human immunodeficiency virus type 1, glass leafhopper virus-1, lice P virus, Hepatitis C virus, Hepatitis A virus, Hepatitis GB virus, Foot and mouth disease virus, Human enterovirus 71, Equine rhinovirus, Tea inchworm-like virus, Encephalomyocarditis virus (EMCV), Drosophila C virus, Cruciferous tobacco Virus, cricket paralysis virus, bovine viral diarrhea virus 1, black queen cell virus, aphid lethal paralysis virus, avian encephalomyelitis virus, acute bee paralysis virus, hibiscus yellow ring spot virus, swine fever virus, human FGF2, human SFTPA1, Human AML1/RUNX1, Drosophila Antennae, Human AQP4, Human AT1R, Human BAG-1, Human BCL2, Human BiP, Human c-IAP1, Human c-myc, Human eIF4G, Mouse NDST4L, Human LEF1, Mouse HIF1 α , Human n.myc, mouse Gtx, human p27kipl, human PDGF2/c-sis, human p53, human Pim-1, mouse Rbm3, Drosophila reaper, canine Scamper, Drosophila Ubx, human UNR, mouse UtrA, human VEGF-A, human XIAP, Drosophila hairless, Saccharomyces cerevisiae TFIID, Saccharomyces cerevisiae YAP1, human c-src, human FGF-1, simian picornavirus, turnip crepe disease virus, eIF4G aptamer, Coxsackie Virus B3 (CVB3) or Coxsackie virus A (CVA1/2), preferably, the IRES is CVB3, BRAV-1_L, PV1_L, CAV2_L, BRAV-1, PV1, or CAV2; and/or

v) the IRES comprises a nucleotide sequence set forth in one of SEQ ID NOs: 105-135,

or comprise a nucleotide sequence having at least 75%, e.g., at least 80%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% , or at least 99% sequence identity to one of SEQ ID NOs: 105-135,

preferably, wherein the nucleotide sequence of interest is about 10- about 20000 nucleotides in length.

14. The circular RNA precursor of any one of claims 1-13, wherein the circular RNA precursor does not contain nucleotide chemical modification.

15. A nucleic acid vector for generating a circular RNA molecule, said vector comprises a coding sequence of the circular RNA precursor of any one of claims 1-14, and optionally an RNA polymerase promoter sequence operably linked to the coding sequence of the circular RNA precursor, preferably, the promoter is a T7 RNA polymerase promoter, a T6 viral RNA polymerase promoter, a SP6 viral RNA polymerase promoter, a T3 viral RNA polymerase promoter or a T4 viral RNA polymerase promoter, more preferably a T7 RNA polymerase promoter.

16. A circular RNA, which is prepared from the circular RNA precursor of any one of claims 1-14 or from the nucleic acid vector of claim 15.

17. A circular RNA, which comprises a first residual circularizing element, a nucleotide sequence of interest, and a second residual circularizing element, wherein the first residual circularizing element, the nucleotide sequence of interest, and the second residual circularizing element are defined in any one of claims 1-14.

18. The circular RNA of any one of claims 16-17, wherein the circular RNA does not contain nucleotide chemical modification.

19. Use of the circular RNA precursor any one of claims 1-14 and/or circular RNA of any one of claims 16-18 as an expression vector.

20. A pharmaceutical composition comprising the circular RNA precursor of any one of claims 1-14 and/or the nucleic acid vector of claim 15 and/or circular RNA of any one of claims 16-18, and a pharmaceutically acceptable carrier.

21. A method for preparing a circular RNA, the method comprises:

1) providing a circular RNA precursor of any one of claims 1-14 or obtaining a circular RNA precursor by transcribing from the nucleic acid vector of claim 15;

2) incubating the circular RNA precursor in the presence of a divalent metal cation at a temperature at which RNA circularization occurs; and

3) harvesting the circular RNA obtained in step 2),

optionally the method further comprises a step of recovering or purifying the circular

RNA as produced,

preferably, wherein the divalent metal cation is Mg^{2+} and/or Mn^{2+} ; and/or
the concentration of the divalent metal cation is about 5 mM to about 550 mM.

22. A method for preparing a circular RNA, the method comprises

a) providing a nucleic acid vector comprising a self-splicing intron-based RNA circularizing elements as a transcription template; and

b) incubating the nucleic acid vector in an in vitro transcription system comprising a divalent metal cation and an RNA polymerase, and optionally a monovalent metal cation and/or a monovalent anion, for a first time period during which the linear RNA produced by in vitro transcription is self-circularized under the action of the RNA circularizing elements to produce a circular RNA,

optionally the method further comprises a step of recovering or purifying the circular RNA as produced,

preferably,

the nucleic acid vector is the nucleic acid vector of claim 15,

preferably, wherein

i) the divalent metal cation in the in vitro transcription system is Mg^{2+} ;

ii) the monovalent metal cation is Na^+ or K^+ ;

iii) the monovalent metal anion is Cl^- or CH_3COO^- (OAc);

iv) the concentration of the divalent metal cation in the system during the first time period is from about 5 mM to about 50 mM;

v) the concentration of the monovalent metal cation in the system during the first time period is from about 5 mM to about 100 mM;

vi) the concentration of the monovalent anion in the system during the first time period is from about 5 mM to about 150 mM;

vii) the method does not include a step of isolating and/or purifying the linear RNA produced by the in vitro transcription;

viii) the buffer of the in vitro transcription system is selected from Tris-HCl buffer, or HEPES buffer, or MES buffer, or citrate buffer, or phosphate buffer, preferably, the buffer of the in vitro transcription system is HEPES buffer;

ix) the in vitro transcription system has a pH of about 5- about 8, preferably, the in vitro transcription system has a pH of about 7.5;

x) the RNA polymerase is selected from a T7 RNA polymerase, a T6 viral RNA polymerase, a SP6 viral RNA polymerase, a T3 viral RNA polymerase, or a T4 viral RNA polymerase, preferably, the RNA polymerase is T7 RNA polymerase;

xi) the first time period is about 0.5 hours to about 24 hours, preferably, the first time period is 3 hours; and/or

xii) the incubation for the first time period is carried out at about 16°C to about 60°C, preferably, the incubation for the first time period is carried out at about 37°C.

24. The method of any one of claims 22-23, wherein after incubation for the first time period in step b), the method further comprises step c):

adding an additional amount of metal cation to the in vitro transcription system and incubating for a second time period; or

changing the buffer of the system, adding a metal cation and incubating for a second time period,

preferably, wherein

i) the metal cation added for the incubation of the second time period is a divalent metal cation, such as Mg^{2+} or Mn^{2+} ;

ii) during the incubation of the second time period, the metal cation is added to a final concentration of about 5 mM to about 550 mM;

iii) the buffer of the system is changed to Tris-HCl buffer, or HEPES buffer, or MES buffer, or citrate buffer, or phosphate buffer during the second time period;

iv) the pH of the system in the second time period is 5-8;

v) the second time period is about 5 minutes to about 2 hour; and/or

vi) the incubation for the second time period is carried out at about 25°C to about 75°C.

25. A method for purifying a circular RNA, the method comprises:

a) contacting a mixture comprising circular RNA and uncircularized linear circular RNA precursor with a circular RNA-specific probe under a condition that allows the circular RNA-specific probe to specifically bind to and form a complex with the circular RNA;

b) separating the complex from one or more components in the mixture that are not bound to the circular RNA-specific probe; and

c) releasing the circular RNA from the complex,

wherein the circular RNA is prepared by circularizing a linear circular RNA precursor, preferably wherein the circular RNA is prepared by ligating both ends of a linear circular RNA precursor with an RNA ligase, or, the circular RNA is prepared by the self-splicing ribozyme activity of self-splicing intron-based circularizing elements contained in the linear circular RNA precursor.

26. The method of claim 25, wherein the circular RNA-specific probe

i) is a single-stranded DNA or RNA probe;

ii) specifically hybridizes to a region flanking the circularization junction of the circular

RNA;

iii) is about 10 nucleotides- about 35 nucleotides in length or longer; and/or

iv) is immobilized on a support such as a solid support, e.g., the circular RNA-specific probe is immobilized on the support after binding to the circular RNA or the circular RNA-specific probe is pre-immobilized on the support.

27. The method of any one of claims 25-26, wherein the condition in step a) include denaturing the RNA at between about 60°C and about 95°C for about 2 minutes to about 10 minutes, then gradually reducing the temperature to below about 40°C to allow the circular RNA annealing to the circular RNA-specific probe; and/or

wherein step c) is performed by increasing the temperature to about 60°C to about 95°C (e.g., about 60°C, about 62°C, about 64°C, about 66°C, about 68°C) C, about 70°C, about 75°C, about 80°C, about 85°C, about 90°C, about 95°C) to release the circular RNA.

28. The method of any one of claims 25-27, wherein

i) the condition in step a) include a high salt concentration range of 0.25M-2M, preferably, the salt is NaCl or a guanidine salt;

ii) in step c) the circular RNA is released by elution with an elution buffer, such as a low salt buffer, preferably, the elution buffer is Tris-EDTA buffer (TE buffer) or water; and/or

iii) in step b), the one or more components are removed by washing the complex with a washing buffer.

29. The method of any one of claims 25-28, the method further includes the following steps:

1) contacting the mixture comprising circular RNA and uncircularized linear circular RNA precursor to a linear circular RNA precursor-specific probe in a condition that allows the linear circular RNA precursor-specific probe to specifically bind to and form a complex with the linear circular RNA precursor;

2) removing the complex formed by the linear circular RNA precursor-specific probe with the linear circular RNA precursor from the mixture, and

3) collecting the circular RNA-containing mixture obtained in step 2),

preferably, wherein steps 1)-3) are performed before step a), for example, steps 1)-3) may be performed multiple times before step a), e.g., 2, 3, 4 or more times; or wherein steps 1)-3) are performed concurrently with steps a)-c).

30. A method for purifying circular RNA, the method comprises:

1) contacting a mixture comprising circular RNA and uncircularized linear circular RNA precursor to a linear circular RNA precursor-specific probe under a condition that allows the linear circular RNA precursor-specific probe to specifically bind to and form a

complex with the linear circular RNA precursor; and

2) removing the complex formed by the linear circular RNA precursor-specific probe with the linear circular RNA precursor from the mixture,

3) collecting the circular RNA-containing mixture obtained in step ii), and

optionally, steps 1)-3) are performed multiple times, e.g., 2 times, 3 times, 4 times or more,

preferably, wherein the linear circular RNA precursor-specific probe specifically binds to the linear circular RNA precursor and does not substantially bind to the circular RNA; or wherein the linear circular RNA precursor-specific probe is immobilized on a support, such as a solid support, for example, the linear circular RNA precursor-specific probe is then immobilized on the support after binding to the linear circular RNA precursor, or the linear circular RNA precursor-specific probe is pre-immobilized on the support.

31. The method of any one of claims 25-30, wherein the linear circular RNA precursor comprises the following elements arranged in the following order from the 5' to 3' direction:

- a) a 3' self-splicing intron fragment;
- b) a first residual circularizing element;
- c) a nucleotide sequence of interest;
- d) a second residual circularizing element; and
- e) a 5' self-splicing intron fragment;

wherein the linear circular RNA precursor is capable of removing the 3' self-splicing intron fragment and the 5' self-splicing intron fragment by self-splicing, generating a circular RNA comprising the first residual circularizing element, the nucleotide sequence of interest and the second residual circularizing element,

preferably, wherein

i) the circular RNA-specific probe specifically hybridizes to at least a portion of the first residual circularizing element and a portion of the second residual circularizing element;

ii) the linear precursor RNA-specific probe hybridizes to a portion of the linear circular RNA precursor outside the first residual circularizing element, the nucleotide sequence of interest, and the second residual circularizing element;

iii) the linear precursor RNA-specific probe hybridizes to the 3' self-splicing intron fragment or a portion thereof or a 5' flanking sequence thereof, or the 5' self-splicing intron fragment or a portion thereof or a 3' flanking sequence thereof; and/or

iv) the linear circular RNA precursor contains a sequence selected from SEQ ID NOs:96-101 or a complement sequence thereof, preferably, SEQ ID NO:100 or a complement sequence thereof outside the first residual circularizing element, the nucleotide

sequence of interest, and the second residual circularizing element, to which the linear precursor RNA-specific probe specifically hybridizes.

32. The method of claim 31, the molar ratio of the probe to the RNA molecules in the mixture is from about 1:1 to about 100,000:1.

33. A method for purifying circular RNA, the method comprises:

1) adding a linear RNA-specific tag to the linear RNA in a mixture comprising circular RNA and linear RNA;

2) contacting the mixture comprising circular RNA and linear RNA with a linear RNA probe that specifically binds to the tag under a condition that allows the probe to specifically bind to and form a complex with the linear RNA; and

3) removing the complex formed by the linear RNA probe with the linear RNA from the mixture,

4) collecting the circular RNA-containing mixture obtained in step 3),

optionally, steps 2)-4) are performed multiple times, e.g., 2 times, 3 times, 4 times or more.

34. The method of claim 33, wherein

i) the tag comprises a polyA, polyG, polyU, or polyC sequence;

ii) the tag is about 10-200nt in length or the probe is about 10-200nt in length;

iii) the tag is added to the linear RNA by adding a PolyA/T/C/G polymerase or a ligase to the mixture;

iv) the linear RNA probe specifically binds to the added tag without substantially binding to the circular RNA;

v) the linear RNA probe is a single-stranded DNA probe, or a single-stranded RNA probe; and/or

vi) the linear RNA probe is immobilized on a support such as a solid support, for example, the linear RNA probe is immobilized on the support after binding to the linear RNA, or, the linear RNA probe is pre-immobilized on the support.