

COMPOSITIONS AND METHODS FOR PURIFYING POLYRIBONUCLEOTIDES

Background

Polyribonucleotides are useful for a variety of therapeutic and engineering applications. Thus,
5 new compositions and methods for separating and purifying polyribonucleotides are useful.

Summary

In one example, the disclosure features a method of separating a polyribonucleotide having a
target region from a plurality of polyribonucleotides. In this method, a sample that includes the plurality of
10 polyribonucleotides is provided. A subset of the plurality of polyribonucleotides in the sample have the
target region. The method further includes contacting the sample with an oligonucleotide that hybridizes
to the target region and separating the polyribonucleotide having the target region that is hybridized to the
oligonucleotide from the plurality of polyribonucleotides in the sample.

In another example, the disclosure features a method of separating a linear polyribonucleotide
15 having a target region from a plurality of polyribonucleotides that includes a mixture of linear
polyribonucleotides and circular polyribonucleotides. In this method, a sample that includes the plurality
of polyribonucleotides is provided. A subset of the plurality of linear polyribonucleotides in the sample
have the target region. The method further includes contacting the sample with an oligonucleotide that
hybridizes to the target region and separating the linear polyribonucleotide having the target region that is
20 hybridized to the oligonucleotide from the plurality of polyribonucleotides in the sample. In one aspect, the
present disclosure refers to a method of separating a linear polyribonucleotide comprising a target region
from a plurality of polyribonucleotides comprising a mixture of linear polyribonucleotides and circular
polyribonucleotides, the method comprising: (a) providing a sample comprising the plurality of
polyribonucleotides, wherein a subset of the plurality of polyribonucleotides comprise the linear
25 polyribonucleotide comprising the target region; (b) contacting the sample with an oligonucleotide that
hybridizes to the target region; and (c) separating the linear polyribonucleotide comprising the target
region that is hybridized to the oligonucleotide from the plurality of polyribonucleotides in the sample. In
some examples, polyribonucleotide having the target region lacks a polyA sequence (*i.e.*, a polyA tail). In
some examples, the target region is not located at a 3' or 5' terminus of the polyribonucleotide having the
30 target region. The circular polyribonucleotides or a subset thereof may lack the target region.

In another example, the disclosure features a method of separating a polyribonucleotide having a
target region from a plurality of polyribonucleotides. The method includes providing a sample that
includes the plurality of polyribonucleotides, wherein a subset of the plurality of polyribonucleotides have
the target region. The target region is not located at a 3' or 5' terminus of the polyribonucleotide having
the target region (*i.e.*, the target region is located internally on the polyribonucleotide). The method
35 further includes contacting the sample with an oligonucleotide that hybridizes to the target region and
separating the polyribonucleotide having the target region that is hybridized to the oligonucleotide from
the plurality of polyribonucleotides in the sample. In one aspect, the

present disclosure refers to a method of separating a polyribonucleotide comprising a target region from a plurality of polyribonucleotides, the method comprising: (a) providing a sample comprising the plurality of polyribonucleotides, wherein a subset of the plurality of polyribonucleotides comprise the target region, wherein the target region is not located at a 3' or 5' terminus of the polyribonucleotide comprising the target region; (b) contacting the sample with an oligonucleotide that hybridizes to the target region; and (c) separating the polyribonucleotide comprising the target region that is hybridized to the oligonucleotide from the plurality of polyribonucleotides in the sample.

In another example, the disclosure features a method of separating a linear polyribonucleotide having a target region from a plurality of circular polyribonucleotides having the target region. The method includes providing a sample that includes the plurality of polyribonucleotides. The method further includes contacting the sample with an oligonucleotide. The oligonucleotide hybridizes to the target region of the linear polyribonucleotide with a first binding affinity, and the oligonucleotide hybridizes to the target region of the circular polynucleotide with a second binding affinity that is different from the first binding affinity and separating the linear polyribonucleotide having the target region that is hybridized to the oligonucleotide from the plurality of circular polyribonucleotides in the sample. In one aspect, the present disclosure refers to a method of separating a linear polyribonucleotide comprising a target region from a plurality of circular polyribonucleotides comprising the target region, the method comprising: (a) providing a sample comprising the plurality of polyribonucleotides; (b) contacting the sample with an oligonucleotide, wherein the oligonucleotide hybridizes to the target region of the linear polyribonucleotide with a first binding affinity, and wherein the oligonucleotide hybridizes to the target region of the circular polynucleotide with a second binding affinity that is different from the first binding affinity; and (c) separating the linear polyribonucleotide comprising the target region that is hybridized to the oligonucleotide from the plurality of circular polyribonucleotides in the sample. In some examples, the first binding affinity is less than the second binding affinity, and the oligonucleotide preferentially binds the linear polyribonucleotide.

In another example, the disclosure features a method of separating a polyribonucleotide in a first conformation having a target region from a plurality of polyribonucleotides in a second conformation having the target region. The method includes providing a sample that includes the plurality of polyribonucleotides. The method further includes contacting the sample with an oligonucleotide. The oligonucleotide hybridizes to the target region of the polynucleotide in the first conformation with a first binding affinity, and the oligonucleotide hybridizes to the target region of the polynucleotide in the second conformation with a second binding affinity that is different from the first binding affinity and separating the polyribonucleotide in the first conformation having the target region that is hybridized to the oligonucleotide from the plurality of polyribonucleotides in the second conformation in the sample. In one aspect, the present disclosure refers to a method of separating a polyribonucleotide in a first conformation comprising a target region from a plurality of polyribonucleotides in a second conformation comprising the target region, the method comprising: (a) providing a sample comprising the plurality of polyribonucleotides; (b) contacting the sample with an oligonucleotide, wherein the oligonucleotide hybridizes to the target region of the polynucleotide in the first conformation with a first binding affinity, and wherein the oligonucleotide hybridizes to the target region of the polynucleotide in the second conformation with a second binding affinity that is different from the first binding affinity; and (c) separating the polyribonucleotide in the first conformation comprising the target region that is hybridized to the oligonucleotide from the plurality of polyribonucleotides in the second conformation in the sample. In some examples, the first binding affinity is less than the second binding affinity, and the oligonucleotide preferentially binds the polyribonucleotide in the first conformation.

In some examples of any of the aspects described herein, separating includes immobilizing the oligonucleotide.

In some examples of any of the methods described herein, the target region is not located at a 5' or 3' terminus of the polyribonucleotide having the target region. For example, the target region may be internally located on the polyribonucleotide. In some examples, the target region is not located at a 3' terminus of the polyribonucleotide.

In some examples of any of the methods described herein, the target region is located at a 5' or 3' terminus of the polyribonucleotide, and the target region does not contain a polyA sequence. In some examples, the target region is located at a 3' terminus of the polyribonucleotide and does not contain a polyA sequence.

In some examples of any of the methods described herein, the target region does not contain a polyA sequence.

In some examples of any of the above aspect, the oligonucleotide is conjugated to a particle. The particle may be, for example, a magnetic particle or a bead. The bead may be, *e.g.*, a crosslinked agarose, *e.g.*, a SEPHAROSE® bead.

In some examples of any of the above aspects, the oligonucleotide is conjugated to a first capture agent.

In some examples, the oligonucleotide is conjugated to the first capture agent or the particle by way of a chemical linker. The chemical linker may include, *e.g.*, triethylene glycol. In some examples, the chemical linker is conjugated to a 3' end or a 5' end of the oligonucleotide.

In some examples, the first capture agent includes an antigen (*e.g.*, biotin).

In some examples, the method further includes contacting the sample with a second capture that binds to the first capture agent. The second capture agent may include, for example, an antibody or antigen-binding fragment thereof (*e.g.*, streptavidin).

In some examples, the second capture agent is conjugated to a particle. The particle may be, for example, a magnetic particle or a bead. The bead may be, *e.g.*, a crosslinked agarose, *e.g.*, a SEPHAROSE® bead. In some examples, the second capture agent is conjugated to the particle with a chemical linker. The chemical linker may include, *e.g.*, triethylene glycol.

In some examples, the polyribonucleotide having the target region includes an intron or portion thereof (*e.g.*, a half-intron). The target region may include an intron or portion thereof. In some

examples, the intron or portion thereof is a catalytic intron (*e.g.*, a Group I catalytic intron or a Group II catalytic intron) or a portion thereof. In some examples the intron or portion thereof has a length of at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, or at least 100 nucleotides. In some examples the intron or portion thereof has a length of 20 to 500, 20 to 400, 20 to 300, 20 to 200, 20 to 100, 50 to 500, 50 to 400, 50 to 300, 50 to 200, 100 to 500, 100 to 400, 100 to 300, or 100 to 200 nucleotides.

In some examples, the target region includes a half-intron (*e.g.*, a 5' or 3' portion of a catalytic intron). In some examples, the target region includes a 5' half-intron (*e.g.*, a 5' half-intron corresponding to a 5' portion of a catalytic intron). In some examples, the target region includes a 3' half-intron (*e.g.*, a 3' half-intron corresponding to a 3' portion of a catalytic intron). In some examples, the target region includes a 5' half of a Group I catalytic intron. In some examples, the 5' half of the Group I catalytic intron is from a 5' portion of a cyanobacterium *Anabaena* pre-tRNA-Leu gene, a *Tetrahymena* pre-rRNA, a T4 phage td gene, or a variant thereof. In some examples, the target region includes a 3' half of a Group I catalytic intron. In some examples, the 3' half of the Group I catalytic intron is selected from a 3' portion from a cyanobacterium *Anabaena* pre-tRNA-Leu gene, a *Tetrahymena* pre-rRNA, a T4 phage td gene, or a variant thereof.

The target region may be located 5' or 3' to the intron or portion thereof or may include a portion of the region located 5' or 3' to the intron or portion thereof.

In some examples, the method includes separating a spliced polyribonucleotide from a non-spliced or partially spliced polyribonucleotide. In some examples, the spliced polyribonucleotide is a circular polyribonucleotide. In some examples, the spliced polyribonucleotide is a linear polyribonucleotide. In some examples, the spliced polyribonucleotide lacks an intron or portion thereof (*e.g.*, the spliced polyribonucleotide lacks a catalytic intron, such as a Group I catalytic intron, or a portion thereof).

In some examples, the method enriches an amount of the spliced polyribonucleotide by at least 10% (*e.g.*, by at least 20%, 30%, 40%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 99%, or more) relative to the sample.

In some examples, the polyribonucleotide having the intron or portion thereof is a linear polyribonucleotide.

In some examples, the oligonucleotide has at least 5 nucleotides (*e.g.*, at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, or more nucleotides) in length. In some examples, the oligonucleotide is, *e.g.*, from 5-100, 5-95, 10-90, 10-80, 12-60, 15-50, 15-40, 15-30, 18-30, 20-25, or 20-22 nucleotides in length. In some examples, the oligonucleotide is 23 nucleotides in length.

In some examples, the oligonucleotide may have a melting temperature (T_m) of, for example, from about 45 °C to about 75 °C, *e.g.*, about 46 °C, 47 °C, 48 °C, 49 °C, 50 °C, 51 °C, 52 °C, 53 °C, 54 °C, 55 °C, 56 °C, 57 °C, 58 °C, 59 °C, 60 °C, 61 °C, 62 °C, 63 °C, 64 °C, 65 °C, 66 °C, 67 °C, 68 °C, 69 °C, 70 °C, 71 °C, 72 °C, 73 °C, 74 °C, or 75 °C. In some examples, the oligonucleotide has a T_m of about 45 °C to about 65 °C.

In some examples, the method further includes the step of washing the captured polyribonucleotide having the target region (*e.g.*, after the contacting and/or after separating step) one or

more (*e.g.*, two, three, four, five, or more) times. In some examples, the method further includes washing the first and/or second capture agent one or more (*e.g.*, two, three, four, five, or more) times.

In some examples, the method further includes the step of performing a first elution step to release the captured polyribonucleotide having a target region. The first elution step may include, *e.g.*,
5 adding a first buffer and/or heating the sample, *e.g.*, to at least 50 °C, 55 °C, 60 °C, 65 °C, 70 °C, 75 °C, 80 °C, or higher.

In some examples, the method further includes performing a second elution step. The second elution step may include adding a second buffer and/or heating the sample, *e.g.*, to at least 50 °C, 55 °C, 60 °C, 65 °C, 70 °C, 75 °C, 80 °C, or higher. In some examples, the second buffer includes a denaturing
10 agent, *e.g.*, formamide or urea. The second buffer may include, *e.g.*, from about 40% to about 60% formamide (*e.g.*, about 40%, 45%, 50%, 55%, or 60% formamide).

In some examples, the method includes incubating the sample with the oligonucleotide (*e.g.*, conjugated to the first capture agent) for at least ten (*e.g.*, at least 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, or more) minutes.

15 In some examples, the method includes collecting a portion of the sample that is not bound by the oligonucleotide. In some examples, the method includes collecting a portion of the sample that is not bound by the capture agent (*e.g.*, the first and/or second capture agent).

In some examples, the method includes providing a plurality of oligonucleotides, wherein each oligonucleotide hybridizes to a distinct target region. In some examples, each oligonucleotide is
20 conjugated to a first capture agent.

In some examples, the method includes providing an oligonucleotide that hybridizes to multiple target regions. In some examples, the method includes providing a single oligonucleotide that hybridizes to a 3' half intron and a 5' half intron.

In some examples, the method includes providing a first oligonucleotide that hybridizes to a 3'
25 half-intron and a second oligonucleotide that hybridizes to a 5' half-intron. In some examples, the method includes providing a plurality of oligonucleotides that each hybridize to a distinct region on the 3' half-intron and/or the 5' half-intron.

In some examples, the oligonucleotide has at least 80% (*e.g.*, at least 85%, 90%, 95%, 97%, 99%, or 100%) complementarity an equal length portion of the target region.

30 In some examples, the method includes providing the oligonucleotide at a molar ratio of 10:1 to 1:10 (*e.g.*, 10:1, 5:1, 2:1, 1:2, 1:5, or 1:10) to the polyribonucleotide.

In another aspect, the disclosure features a population of polyribonucleotides produced by the method of any of the examples described herein. The population may include, *e.g.*, a circular polyribonucleotide lacking a target region and the circular polyribonucleotide includes at least 1% (*e.g.*, at
35 least 5%, *e.g.*, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, 60%, 70%, 80%, 90%, 95%, 97%, 99%, or more) (mol/mol) of the total polyribonucleotides in the composition.

In some examples, the population has less than 50% (*e.g.*, less than 40%, 30%, 20%, 10%, 5%, 4%, 3%, 2%, or 1%) (mol/mol) linear polyribonucleotides.

In another example, the disclosure features a composition that includes a mixture of
40 polyribonucleotides. A first subset of the mixture includes a circular polyribonucleotide lacking a target region, and a second subset of the plurality of the polyribonucleotides includes a linear polyribonucleotide

having a target region. The first subset includes at least 1%, (*e.g.*, at least 5%, *e.g.*, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, 60%, 70%, 80%, 90%, 95%, 97%, 99%, or more) (mol/mol) of the total polyribonucleotides in the composition. In one aspect, the present disclosure refers to a composition comprising a mixture of polyribonucleotides, wherein a first subset of the mixture
5 comprises a circular polyribonucleotide lacking a target region, and a second subset of the plurality of the polyribonucleotides comprises a linear polyribonucleotide comprising the target region, wherein the first subset comprises at least 40% (mol/mol) of the total polyribonucleotides in the composition.

In another example, the disclosure features a composition that includes a polyribonucleotide having a target region and an oligonucleotide configured to hybridize to the target region, wherein the
10 oligonucleotide is conjugated to a first capture agent (*e.g.*, an antigen, such as biotin). In one aspect, the present disclosure refers to a composition comprising a polyribonucleotide comprising a target region and an oligonucleotide configured to hybridize to the target region, wherein the oligonucleotide is conjugated to a first capture agent. The composition may further include, *e.g.*, a polyribonucleotide lacking the target region. The polyribonucleotide lacking the target region may be, *e.g.*, a circular polyribonucleotide. The
15 composition may further include a second capture agent (*e.g.*, an antibody or antigen binding fragment thereof, such as streptavidin) configured to bind the first capture agent. The second capture agent may be conjugated to a particle.

In some examples, the composition is produced by a method as described herein.

In some examples of any of the compositions as described herein, the linear polyribonucleotide
20 includes an intron or portion thereof. The target region may include the intron or portion thereof. The target region may be located 5' or 3' to the intron or portion thereof.

In some examples of any of the above aspects, the polyribonucleotide and/or oligonucleotide may be modified.

In another example, the disclosure features a pharmaceutical composition that includes a
25 composition as described herein, *e.g.*, produced by a method as described above, and a diluent, carrier, or excipient.

Definitions

To facilitate the understanding of this disclosure, a number of terms are defined below. Terms
30 defined herein have meanings as commonly understood by a person of ordinary skill in the areas relevant to the disclosure. Terms such as "a", "an," and "the" are not intended to refer to only a singular entity but include the general class of which a specific example may be used for illustration. The term "or" is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternative are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or". The
35 terminology herein is used to describe specific embodiments, but their usage is not to be taken as limiting, except as outlined in the claims.

As used herein, any values provided in a range of values include both the upper and lower bounds, and any values contained within the upper and lower bounds.

As used herein, the term "about" refers to a value that is within $\pm 10\%$ of a recited value.

As used herein, the term "carrier" is a compound, composition, reagent, or molecule that
40 facilitates the transport or delivery of a composition (*e.g.*, a circular polyribonucleotide) into a cell by a covalent modification of the circular polyribonucleotide, *via* a partially or completely encapsulating agent, or a combination thereof. Non-limiting examples of carriers include carbohydrate carriers (*e.g.*, an anhydride-modified phytoglycogen or glycogen-type material), nanoparticles (*e.g.*, a nanoparticle that
45 encapsulates or is covalently linked binds to the circular polyribonucleotide), liposomes, fusosomes, *ex vivo* differentiated reticulocytes, exosomes, protein carriers (*e.g.*, a protein covalently linked to the circular polyribonucleotide), or cationic carriers (*e.g.*, a cationic lipopolymer or transfection reagent).