

Figure 8 is a representation of the sequence of an AAV2 ITR together with representations of possible conformations of single stranded nucleic acids of the invention with ITR-style structures;

Figure 9 is a representation of the template used in Example 1; and

Figure 10 is a gel photograph showing the results of Example 1.

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Summary

In an aspect of the present disclosure, there is provided a nucleic acid template for the cell-free, *in vitro* manufacture of single stranded nucleic acid constructs with sequestered ends, comprising a sequence encoding the following elements in a single stranded nucleic acid from 5' to 3':

- 10 i) a first processing motif, adjacent to
 ii) a first conformational motif,
 iii) a sequence of interest,
 iv) a second conformational motif, adjacent to
 v) a second processing motif,

15 wherein a processing motif includes self-complementary sequences capable of forming a base-paired section including a recognition site for an endonuclease and an associated cleavage site, and wherein a conformational motif includes at least one sequence capable of forming intramolecular hydrogen bonds and assuming a conformation which sequesters an end of the single stranded construct.

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In another aspect of the present disclosure, there is provided a method of manufacturing single stranded nucleic acid molecules with sequestered ends, comprising:

- 25 (a) amplification of a circular template using a polymerase capable of rolling circle amplification, wherein said template comprises a sequence encoding the following elements in a single stranded nucleic acid:

- 30 i) a first processing motif, adjacent to
 ii) a first conformational motif,
 iii) a sequence of interest,
 iv) a second conformational motif, adjacent to
 v) a second processing motif,

35 wherein a processing motif includes self-complementary sequences capable of forming a base-paired section including a recognition site for an endonuclease containing a cleavage site, and wherein the conformational motif includes at least one sequence capable of forming intramolecular hydrogen bonds and assuming a conformation which sequesters the end of the single stranded nucleic acid construct, said amplification producing a single stranded nucleic acid concatemer, and

- 40 (b) processing said single stranded nucleic acid concatemer using one or more endonucleases which recognise the cleavage sites in one or more of said processing motifs.

single stranded nucleic acid concatemer with two or more repeats of a sequence unit, said sequence unit comprising the following elements:

- i) a first processing motif, adjacent to
- ii) a first conformational motif,
- iii) a sequence of interest,
- iv) a second conformational motif, adjacent to
- v) a second processing motif,

wherein a processing motif includes self-complementary sequences capable of forming a base-paired section including a recognition site for an endonuclease and an associated a cleavage site, and wherein the conformational motif includes at least one sequence capable of forming intramolecular hydrogen bonds.

In a further aspect of the present disclosure, there is provided a single stranded linear nucleic acid molecule with sequestered ends wherein at least one sequestered end forms a G quadruplex.

In another aspect of the present disclosure, there is provided a single stranded linear nucleic acid molecule with sequestered ends wherein at least one sequestered end forms an ITR structure with a double stranded D section.

The single stranded nucleic acid molecule of the present disclosure has sequestered ends. The single stranded nucleic acid molecule of the present disclosure is a linear single strand of nucleic acid and therefore has a terminal nucleotide at each end. The terminal nucleic acid residues are not free, i.e. not exposed as in a purely linear single stranded nucleic acid molecule which has not assumed any further conformation. The ends of the nucleic acid are therefore secured or tucked away within the construct and are not immediately accessible to enzymes such as single strand nucleases and the like. The ends of the single stranded nucleic acid may be sequestered by including the terminal nucleotide within a conformation which acts to protect the ends. The terminal nucleotide at each end of the linear ssDNA is therefore kept apart or away from any agents which may act upon it in order to start to degrade the nucleic acid molecule. In general, enzymes locate the terminal nucleotides and from this residue start to chew up the single stranded nucleic acid.

The single stranded nucleic acid molecule may be prepared from a template nucleic acid. The design of this template nucleic acid is unique.

Accordingly, the present disclosure provides:

A nucleic acid template for the cell-free, *in vitro* manufacture of single stranded nucleic acid molecules with sequestered ends, comprising a sequence encoding the following elements:

- i) a first processing motif, adjacent to
- ii) a first conformational motif,
- iii) a sequence of interest,
- iv) a second conformational motif, adjacent to

v) a second processing motif,

wherein a processing motif includes sequences capable of forming a base-paired section including a recognition site for an endonuclease and an associated cleavage site, and wherein the
5 conformational motif includes at least one sequence capable of forming intramolecular hydrogen bonds.

Thus, the template of the present disclosure encodes the single stranded nucleic acid as described herein. The single stranded nucleic acid is linear. The linear single stranded nucleotide has
10 sequestered ends.

Alternatively described, the combination of a processing motif and a conformational motif adjacent to each other in either the forward orientation (processing motif then conformational motif) or the

reverse orientation (conformational motif then processing motif) can be used. These are the formatting elements.

Thus, the template may comprise the following sequences encoding the following elements in the order described:

- i) a forward formatting element;
- ii) a sequence of interest;
- iii) a reverse formatting element.

The template of the present disclosure may be amplified using any suitable polymerase enzyme, in order to manufacture the single stranded nucleic acid product.

The single stranded nucleic acid product is linear and has sequestered ends.

The template may be double or single stranded. One strand of the template is complementary to the desired linear single stranded nucleic acid product with sequestered ends, and therefore directs the production of the same. The template directs the construction of the product when contacted with a polymerase enzyme, and thus the template is replicated or amplified. The terms amplified or replicated may be used interchangeably in the art.

The template of the present disclosure may be contacted with a polymerase capable of rolling circle amplification (RCA). The template of the present disclosure may be amplified using a polymerase capable of catalysing rolling circle amplification (RCA). RCA is an isothermal enzymatic process where long single stranded DNA or RNA is synthesised using a circular DNA template and special DNA or RNA polymerases. The RCA product is a concatemer containing tens to hundreds or thousands of tandem repeats that are complementary to the circular template. Thus, the contacting of the template with a polymerase may result in an "amplification" of the template, producing a complementary single strand of nucleic acid.

Therefore, any template described herein may be amplified using a polymerase capable of rolling circle amplification or replication. This results in the production of a long single-stranded concatemeric nucleic acid molecule. Due to the presence of the formatting elements (comprising a processing motif adjacent to a conformational motif, in either the forward or reverse orientation), the concatemer can be simply processed by the addition of the requisite endonucleases. Cleavage within the processing motifs by the endonucleases releases the sequence of interest, flanked on either side by conformational motifs. As released, these conformational motifs act to sequester the ends of the single stranded nucleic acid by forming a hydrogen-bonded section which secures the terminal nucleotide. The conformational motif in the single stranded nucleic acid molecules do, therefore, assume a conformation using hydrogen bonding which sequesters the terminal nucleotide. The terminal nucleotide may be secured by being included within or embraced within the conformation assumed with or without intramolecular base-pairing or hydrogen bonding. Alternatively, the terminal nucleotide may be secured by intramolecular base-pairing or hydrogen bonding, such that the conformational motif increases the stability of these intramolecular interactions.

Thus, the terminal residues of the linear single stranded nucleic acid product are formed by the action of the endonuclease on the processing motif, the terminal residue is the residue at the end of the molecule once the endonuclease has cleaved the longer intermediate product. Thus, the formatting element may be described as comprising a processing motif adjacent to the conformational motif, wherein the cleavage site generates the terminal residue which is sequestered by the conformational motif. The processing motif and conformational motif can be described as adjacent, adjoining or contiguous. Alternatively described, there is no extraneous or intervening nucleic acid sequence between the processing motif and the conformational motif. The action of the endonuclease generates the terminal residue, which is subsequently sequestered.

Accordingly, the present disclosure provides:

A method of manufacturing single stranded nucleic acid molecules with sequestered ends, comprising:

(a) amplification of a circular template using a polymerase capable of rolling circle amplification, wherein said template comprises a sequence encoding the following elements:

- i) a first processing motif, adjacent to
- ii) a first conformational motif,
- iii) a sequence of interest,
- iv) a second conformational motif, adjacent to
- v) a second processing motif,

wherein a processing motif includes sequences capable of forming a base-paired section including a recognition site for an endonuclease and an associated cleavage site, and wherein the conformational motif includes at least one sequence capable of forming intramolecular hydrogen bonds,

the amplification producing a nucleic acid concatemer, and

(b) processing said nucleic acid concatemer using one or more endonucleases which recognise the cleavage sites in one or more of said processing motifs.

The single stranded nucleic acid produced is linear, with sequestered ends.

Alternatively put, the present disclosure comprises:

A method of manufacturing single stranded nucleic acid molecules with sequestered ends, comprising:

(a) the amplification of a circular template using a polymerase capable of rolling circle amplification, wherein said template comprises a sequence encoding the following elements:

- i) a forward formatting element,
- iii) a sequence of interest,
- iv) a reverse formatting element,

5 wherein a forward formatting element comprises a processing motif adjacent to a conformational motif, and a reverse formatting element comprises a conformational motif adjacent to a processing motif; a processing motif includes sequences capable of forming a base-paired section including a recognition site for an endonuclease and an associated cleavage site, wherein the conformational motif includes at least one sequence capable of forming intramolecular hydrogen bonds,

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the amplification producing a nucleic acid concatemer, and

(b) processing said nucleic acid concatemer using one or more endonucleases which recognise the cleavage sites in one or more of said processing motifs.

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The single stranded nucleic acid molecules of the present disclosure are linear, with sequestered ends.

20 The processing steps results in single stranded nucleic acid constructs with sequestered ends. The ends are sequestered since in the processed format, the conformational motifs are able to form or assume their desired conformation, which is stabilised by intramolecular hydrogen bonding. The end of the single stranded nucleic acid molecule is sequestered by the conformation assumed by the conformational motif. The terminal nucleotide may be secured by being included within a conformation, making it sterically difficult for it to be approached by exonucleases, or included in intramolecular bonding within the conformation motif, the entirety of which makes the terminal nucleotide more stable to exonucleases. Since the molecule has two ends and two conformational motifs, each works to assume a conformation embracing the relevant end or terminal nucleotide. The molecule has two ends, with two terminal residues, since the nucleic acid is linear.

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30 The concatemer is an intermediate product during the manufacture of the single stranded nucleic acid molecules of the present disclosure, but may have some utility of its own, of its own due to its composition as a multimeric linked chain of sequences of interest which may serve to increase the local concentration or potency of said sequences in applications where that may be an advantage, for example in bio-sensing and the like. Affinity binding is one possible application.

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Accordingly, the present disclosure provides:

A single stranded oligonucleotide concatemer with two or more repeats of a sequence unit, said sequence unit comprising the following elements:

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- i) a first processing motif, adjacent to
- ii) a first conformational motif,
- iii) a sequence of interest,
- iv) a second conformational motif, adjacent to
- 45 v) a second processing motif,

wherein a processing motif includes sequences capable of forming a base-paired section including a recognition site for an endonuclease and an associated cleavage site, and wherein the conformational motif includes at least one sequence capable of forming intramolecular hydrogen bonds.

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Alternatively put, the present disclosure provides:

A single stranded nucleic acid concatemer with two or more repeats of a sequence unit, said sequence unit comprising the following elements:

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- i) a first processing motif, adjacent to
- ii) a first conformational motif,
- iii) a sequence of interest,
- iv) a second conformational motif, adjacent to
- v) a second processing motif,

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wherein a processing motif includes sequences capable of forming a base-paired section including a recognition site for an endonuclease and an associated cleavage site, and wherein the conformational motif includes at least one sequence capable of forming intramolecular hydrogen bonds.

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The single stranded nucleic acid molecules of the present disclosure are linear, with sequestered ends.

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Alternatively, if the processing motif and conformational motif are taken together as a processing element, the present disclosure provides:

A single stranded oligonucleotide concatemer with two or more repeats of a sequence unit, said sequence unit comprising the following elements:

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- i) a forward formatting element,
- iii) a sequence of interest,
- iv) a reverse formatting element,

wherein a forward formatting element comprises a processing motif adjacent to a conformational motif, and a reverse formatting element comprises a conformational motif adjacent to a processing motif; a processing motif includes sequences capable of forming a base-paired section including a recognition site for an endonuclease and an associated cleavage site, wherein the conformational motif includes at least one sequence capable of forming intramolecular hydrogen bonds.

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The single stranded nucleic acid molecules of the present disclosure are linear, with sequestered ends.

The terminal nucleotide of the conformational motif, or indeed the terminal nucleotide of the single stranded nucleic acid construct is usually the nucleotide which was adjacent to the processing motif and "released" from the concatemeric nucleic acid by the action of the endonuclease. It is this terminal nucleotide that forms the end of the single stranded nucleic acid construct, and is duly sequestered in order to delay degradation.

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Claims

1. A nucleic acid template for the cell-free, *in vitro* manufacture of single stranded nucleic acid constructs with sequestered ends, comprising a sequence encoding the following elements in a single stranded nucleic acid from 5' to 3':

- i) a first processing motif, adjacent to
- ii) a first conformational motif,
- iii) a sequence of interest,
- iv) a second conformational motif, adjacent to
- v) a second processing motif,

wherein a processing motif includes self-complementary sequences capable of forming a base-paired section including a recognition site for an endonuclease and an associated cleavage site, and wherein a conformational motif includes at least one sequence capable of forming intramolecular hydrogen bonds and assuming a conformation which sequesters an end of the single stranded construct.

2. A nucleic acid template as claimed in claim 1 wherein the cleavage site within a processing motif is adjacent to a conformational motif.

3. A nucleic acid template as claimed in any preceding claim wherein the cleavage site for the endonuclease within a processing motif is at the terminal base pair of the base-paired section.

4. A nucleic acid template as claimed in any preceding claim wherein the conformational motif sequesters an end of the single stranded construct by inclusion within the assumed conformation.

5. A nucleic acid template as claimed in any preceding claim wherein the conformational motif sequesters an end of the single stranded construct by intramolecular base pairing or hydrogen bonding.

6. A nucleic acid template as claimed in any preceding claim wherein the single stranded nucleic acid construct includes any one or more of the following:

- i) an aptamer;
- ii) a nucleic acid enzyme;
- iii) a guide sequence for gene editing;

7. A nucleic acid template as claimed in any preceding claim wherein said intramolecular hydrogen bonds form between the nucleotide bases in the sequence of the conformational motif.

8. A nucleic acid template as claimed in any preceding claim wherein the intramolecular hydrogen bonds involve Watson-Crick base pairs, Hoogsteen base-pairs or non-canonical base-pairing.

9. A nucleic acid template as claimed in any preceding claim wherein a conformational motif may be a sequence which is capable of assuming one or a combination of two or more of the following conformations:

- i) quadruplex;
- ii) hairpin;
- iii) cruciform;
- iv) stem loop; and/or
- v) pseudoknot.

10. A nucleic acid template as claimed in any preceding claim wherein the sequestered end involves the intramolecular base pairing of the terminal nucleotide.

11. A nucleic acid template as claimed in any preceding claim wherein the sequestered end involves including the terminal nucleotide in an ITR structure with a double stranded D region.

12. A method of manufacturing single stranded nucleic acid molecules with sequestered ends, comprising:

(a) amplification of a circular template using a polymerase capable of rolling circle amplification, wherein said template comprises a sequence encoding the following elements in a single stranded nucleic acid:

- i) a first processing motif, adjacent to
- ii) a first conformational motif,
- iii) a sequence of interest,
- iv) a second conformational motif, adjacent to
- v) a second processing motif,

wherein a processing motif includes self-complementary sequences capable of forming a base-paired section including a recognition site for an endonuclease containing a cleavage site, and wherein the conformational motif includes at least one sequence capable of forming intramolecular hydrogen bonds and assuming a conformation which sequesters the end of the single stranded nucleic acid construct, said amplification producing a single stranded nucleic acid concatemer, and

(b) processing said single stranded nucleic acid concatemer using one or more endonucleases which recognise the cleavage sites in one or more of said processing motifs.

13. A method of manufacturing single stranded nucleic acid molecules with sequestered ends as claimed in claim 12 wherein the template is as described in any one of claims 1 to 11.

14. A single stranded nucleic acid concatemer with two or more repeats of a sequence unit, said sequence unit comprising the following elements:

- i) a first processing motif, adjacent to
- ii) a first conformational motif,
- iii) a sequence of interest,
- iv) a second conformational motif, adjacent to
- v) a second processing motif,

wherein a processing motif includes self-complementary sequences capable of forming a base-paired section including a recognition site for an endonuclease and an associated a cleavage site, and wherein the conformational motif includes at least one sequence capable of forming intramolecular hydrogen bonds.

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15. A single stranded linear nucleic acid molecule with sequestered ends wherein at least one sequestered end forms a G quadruplex.

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16. A single stranded linear nucleic acid molecule with sequestered ends wherein at least one sequestered end forms an ITR structure with a double stranded D section.