

CLAIMS

1. A cloning-free and endonuclease-free method to generate a dumbbell-shaped vector that includes a hairpin-structured expression cassette comprising:

- i) providing a preparation comprising a single stranded nucleic acid template comprising a target nucleic acid molecule comprising two complementary sequence segments wherein each segment comprises a transcription promoter sequence and further comprises a transcriptional terminator and wherein said two complementary sequence segments are separated by a third sequence segment;
- ii) providing a preparation wherein said single stranded nucleic acid template additionally comprises a stem;
- iii) contacting said single stranded nucleic acid template with a first oligonucleotide primer comprising a 3'-hydroxyl group that is complementary to at least part of the 3' terminal nucleotide sequence of said single stranded nucleic acid template and further comprising a 5' nucleotide sequence not complementary to the target nucleic acid molecule wherein said oligonucleotide primer comprises the sequence coding for the 3' arm of a hairpin-structured RNA and further contacting said single stranded nucleic acid template with a first blocking oligonucleotide that is complementary to at least part of the 5' terminal nucleotide sequence of said single stranded nucleic acid template;
- iv) providing polymerase chain reaction components to primer extend the 3' annealed first oligonucleotide primer;
- v) contacting said extended oligonucleotide primer with a second oligonucleotide primer comprising a 3'-hydroxyl group that is complementary to at least part of the 3' terminal nucleotide sequence of said extended oligonucleotide primer and further comprising a 5' nucleotide sequence not

complementary to the target nucleic acid molecule wherein said oligonucleotide primer comprises the sequence coding for the 3' arm of a hairpin-structured RNA and further contacting said single stranded nucleic acid template with a second blocking oligonucleotide that is complementary to at least part of the 5' terminal nucleotide sequence of said extended oligonucleotide primer;

- vi) polymerase chain amplify the template to synthesize a pool of template DNA and annealing said templates to create double stranded nucleic acid comprising a sequence coding for the 3' arm of a hairpin-structured RNA at the 5' nucleotide sequences of the plus strand and the minus strand and comprising a sequence coding for the 5' arm of a hairpin-structured RNA at the 3' nucleotide sequences of the plus strand and the minus strand;
- vii) heat denature said double stranded nucleic acid and then cool down to allow intra-molecular refolding of the resulting plus strand and minus strand DNA to create preformed oligomeric stem-loop structures comprised of plus or minus strand DNA;
- viii) contacting said preformed oligomeric loops structures of plus strand and minus strand DNA with a single strand-specific DNA ligase to link the terminal 5'-phosphorylated 5' overhang to the terminal 3'-OH group of the 3' overhang of the same DNA strand in an intramolecular ligation to create covalently closed plus strand-derived and minus strand-derived dumbbell-shaped vector DNA comprising hairpin-structured template DNA for the transcription of hairpin-structured RNA;
- ix) contacting said dumbbell-shaped vector DNA with a DNA exonuclease to remove all primers and non-covalently closed DNA that harbours 5' and 3' ends.

2. A cloning-free and restriction endonuclease-free method to generate a dumbbell-shaped vector that includes an expression cassette comprising:

- i) providing a preparation comprising a single stranded nucleic acid template comprising a target nucleic acid molecule comprising two complementary sequence segments wherein each segment comprises a transcription promoter sequence and further comprises a transcriptional terminator and wherein said two complementary sequence segments are separated by a third sequence segment;
- ii) providing a preparation wherein said single stranded nucleic acid template additionally comprises a stem;
- iii) contacting said single stranded nucleic acid template with a first oligonucleotide primer comprising a 3'-hydroxyl group that is complementary to at least part of the 3' terminal nucleotide sequence of said single stranded nucleic acid template and further comprising a 5' nucleotide sequence not complementary to the target nucleic acid molecule and further contacting said single-stranded nucleic acid with a first set of blocking oligonucleotides comprising one, two, three or more oligonucleotides that are complementary to at least part of the 5' terminal nucleotide sequence of said single stranded nucleic acid template;
- iv) providing polymerase chain reaction components to primer extend the 3' annealed first oligonucleotide primer;
- v) contacting said extended oligonucleotide primer with a second oligonucleotide primer comprising a 3'-hydroxyl group that is complementary to at least part of the 3' terminal nucleotide sequence of said extended oligonucleotide primer and further comprising a 5' nucleotide sequence not complementary to the target nucleic acid molecule and further contacting said single stranded nucleic acid template with a second set of blocking oligonucleotides comprising one, two, three or more oligonucleotides that are complementary to at least part of the 5' terminal nucleotide sequence of said extended oligonucleotide primer;

- vi) polymerase chain amplify the template to synthesize a pool of extended oligonucleotide primers (and annealing said templates to create double stranded nucleic acid comprising a sequence coding for the 3' arm of a hairpin-structured RNA at the 5' nucleotide sequences of the plus strand and the minus strand and comprising a sequence coding for the 5' arm of a hairpin-structured RNA at the 3' nucleotide sequences of the plus strand and the minus strand;)
- vii) contacting said pool of extended oligonucleotide primers with a third oligonucleotide primer comprising a 3' hydroxyl group that is complementary to at least part of the 3' terminal nucleotide sequence of the oligonucleotide primer extended by said first oligonucleotide primer and further comprising a 5' nucleotide sequence not complementary to said extended oligonucleotide primer and further contacting said pool of extended oligonucleotide primers with a fourth oligonucleotide primer comprising a 3' hydroxyl group that is complementary to at least part of the 3' terminal nucleotide sequence of the oligonucleotide primer extended by said second oligonucleotide primer and further comprising a 5' nucleotide sequence not complementary to said extended oligonucleotide primer and further contacting said extended oligonucleotide primers with said first and second set of blocking oligonucleotides;
- viii) repeating steps iv) and vii) using a fifth and sixth, seventh and eight or more pairs of oligonucleotide primers wherein the last set of primers comprises 3' hydroxyl groups and 5' phosphate groups;
- ix) polymerase chain amplify the template to synthesize a pool of extended oligonucleotide primers and annealing said templates to create double stranded nucleic acid comprising a sequence coding for the 3' arm of a hairpin-structured RNA at the 5' nucleotide sequences of the plus strand and the minus strand and comprising a sequence coding for the 5' arm of a hairpin-structured RNA at the 3' nucleotide sequences of the plus strand and the minus strand;

- x) heat denature said double stranded nucleic acid and then cool down to allow intra-molecular refolding of the resulting plus strand and minus strand DNA to create preformed oligomeric stem-loop structures comprised of plus or minus strand DNA;
 - xi) contacting said preformed oligomeric loops structures of plus strand and minus strand DNA with a single strand-specific DNA ligase to link the terminal 5'-phosphorylated 5' overhang to the terminal 3'-OH group of the 3' overhang of the same DNA strand in an intramolecular ligation to create covalently closed plus strand-derived and minus strand-derived dumbbell-shaped vector DNA comprising hairpin-structured template DNA for the transcription of hairpin-structured RNA;
 - xii) contacting said dumbbell-shaped vector DNA with a DNA exonuclease to remove all primers and non-covalently closed DNA that harbours 5' and 3' ends.
3. A cloning-free and restriction endonuclease-free method to generate a dumbbell-shaped dual expression vector comprising two expression cassettes wherein said vector comprises:
- i) providing a preparation comprising a first single stranded nucleic acid template comprising a target nucleic acid molecule comprising the reverse complement of a first transcriptional promoter at the 5' terminal sequence and the sequence of a second transcriptional terminator at the 3' terminal sequence;
 - ii) contacting said first single stranded nucleic acid template with an first oligonucleotide primer comprising a 5'-phosphate and a 3'-hydroxyl group that is complementary to at least part of the 3' terminal nucleotide sequence of said single stranded nucleic acid template and further comprising a 5' nucleotide sequence not complementary to the target nucleic acid molecule wherein said oligonucleotide primer comprises a first sequence of interest to be transcribed

and further comprising the reverse complement sequence of a first transcriptional terminator and further comprising a modified nucleotide sequence that prevents extension of the 5' nucleotide sequence not complementary to the target nucleic acid molecule;

- iii) providing polymerase chain reaction components and primer extending the 3' annealed oligonucleotide primer to form a second template;
- iv) contacting said second template with a second oligonucleotide primer comprising a 5'-phosphate and a 3'-hydroxyl group that is complementary to at least part of the 3' terminal nucleotide sequence of said second template and further comprising a 5' nucleotide sequence not complementary to the second template wherein said oligonucleotide primer comprises a second sequence of interest to be transcribed and further comprising the reverse complement sequence of a second transcriptional terminator and further comprising a modified nucleotide sequence that prevents extension of the 5' nucleotide sequence not complementary to the second template;
- v) providing polymerase chain reaction components and primer extending the 3' annealed oligonucleotide primer to form a double stranded nucleic acid;
- vi) polymerase chain amplify the double stranded nucleic acid to synthesize a pool of template DNA and annealing said templates to create double stranded nucleic acid comprising a 5' nucleotide sequence not complementary to the target nucleic acid molecule; and
- vii) contacting the annealed template nucleic acid with a 5 DNA ligase to link the terminal 5'-phosphate of the non-complementary 5' nucleotide sequence to the 3'-OH of said amplified template nucleic acid to create a terminal loop structure.

4. The method according to any of the claims 1 to 3 wherein said transcription promoter is derived from an RNA polymerase III promoter.

5. The method according to any of the claim 4 wherein said RNA polymerase III promoter is

- i) a U6 promoter; or
- ii) a H1 promoter; or
- iii) a minimal H1 promoter that includes a an inverted polymerase III transcriptional terminator.

6. The method according to any of the claims 1 to 5 wherein said transcription promoter is derived from an RNA polymerase II promoter.

7. The method according to claim 6 wherein said RNA polymerase II promoter is a CMV promoter.

8. The method according to any one of claims 1 to 7 wherein said transcription terminator nucleotide sequence is a RNA polymerase II or RNA polymerase III termination sequence.

9. The method according to claim 8 wherein said RNA polymerase III termination sequence comprises one or more motifs comprising the nucleotide sequence TTTT.

10. The method according to any of the claims 1 to 9 wherein said stem is derived from

- i) an artificial sequence; or
- ii) a microRNA stem; or
- iii) a microRNA-30 stem.

11. The method according to any of the claims 1 to 10 wherein said third sequence segment is an oligomeric DNA sequence derived from

- i) an oligomeric DNA sequence; or
- ii) a tetrameric DNA sequence; or
- iii) comprises the sequence TTTT.

12. The method according to any of the claims 1 to 11 wherein said vector comprises a nucleic acid sequence to be transcribed.

13. The method according to claim 12 wherein said nucleic acid sequence to be transcribed is a therapeutic nucleic acid molecule.

14. The method according to claim 13 wherein said therapeutic molecule is selected from the group: a siRNA or shRNA, a miRNA or pre-miRNA, an antisense RNA, an antisense miRNA, an aptamer, trans-splicing RNA, a guide RNA, a single-guide RNA, crRNA or tracrRNA, a mRNA or pre-mRNA.

15. The method according to claim 12 wherein said nucleic acid sequence to be transcribed encodes a therapeutic protein or peptide.

16. The method according to claim 13 wherein said therapeutic protein or peptide is selected from the group: Cas9, Cas9n, hSpCas9, hSpCas9n, HSVtk, a cell death trigger protein, cholera toxin, diphtheria toxin, alpha toxin, anthrax 5 toxin, exotoxin, pertussis toxin, shiga toxin, shiga-like toxin Fas, TNF, caspases, initiator caspases, caspase 2, 8, 9, 10, 11, 12, and effector caspases, caspase 3, 6, 7 and purine nucleoside phosphorylase.

17. The method according to any of the claims 1 to 16 wherein said first and said second oligonucleotide primers comprise a 5' phosphate.

18. The method according to any of the claims 1 to 17 wherein said hairpin-structured RNA sequence is a small hairpin RNA or a microRNA precursor.

19. The method according to any of the claims 1 to 18 wherein said blocking oligonucleotides are complementary to the complete 5' terminal nucleotide sequence half of said single stranded nucleic acid template or said extended oligonucleotide primer.

20. The method according to claim 19 wherein said blocking oligonucleotides comprise the nucleotide sequences set forth in SEQ ID NO: 9 and SEQ ID NO: 10.

21. The method according to any of the claims 1 to 20 wherein said heat denaturing comprises an incubation at 96°C for 5 min.
22. The method according to any of the claims 1 to 21 wherein said cooling down comprises a gradual cooling down from 96°C to room temperature.
23. The method according to any of the claims 1 to 22 wherein said DNA ligase is a single strand-specific or double strand-specific DNA ligase.
24. The method according to any of the claims 1 to 23 wherein said single strand-specific DNA ligase is the CircLigase.
25. The method according to any of the claims 1 to 24 wherein said exonuclease is the T7 DNA polymerase.
26. The method according to any of the claims 1 to 25 wherein said first and second oligonucleotide primers comprise the reverse complement sequence CAT of a translational start codon in their 5' nucleotide sequence not complementary to the target nucleic acid molecule.
27. The method according to claims 1 to 26 wherein the primers of said last set of primers comprise the reverse complement sequence CTA, TTA or TCA of a translational stop codon TAG, TAA or TGA in their 5' nucleotide sequence not complementary to said extended oligonucleotide primer.
28. The method according to any of the claims 1 to 27 wherein the primers of said last set of primers comprise the reverse complement sequence of a transcriptional terminator in their 5' nucleotide sequence not complementary to said extended oligonucleotide primer.
29. The method according to claims 3 to 28 wherein said oligonucleotide primer comprises a nucleotide sequence that is non-complementary with said target nucleic acid molecule but includes a region of internal complementarity over part of its length that forms a stem loop structure.

30. The method according to claims 3 to 29 wherein said oligonucleotide primer includes a palindromic nucleotide sequence over part of its length.

31. The method according to claims 3 to 30 wherein said oligonucleotide primer modification is the inclusion of a site that is not recognised as template for base-pairing during primer extension by the DNA polymerase in said primer.

32. The method according to claims 3 to 31 wherein said oligonucleotide primer modification is the inclusion of an abasic site in said primer.

33. The method according to claims 3 to 32 wherein said abasic site is an apurinic / apyrimidinic site.

34. The method according to claims 3 to 33 wherein said apurinic / apyrimidinic sites comprise a tetrahydrofuran.

35. The method according to claims 3 to 34 wherein said abasic site comprises at least one or at least three apurinic / apyrimidinic sites.

36. The method according to claims 3 to 35 wherein said abasic site separates the region complementary to the 3' terminal nucleotide sequence of said single stranded nucleic acid template and the 5' nucleotide sequence not complementary to the target nucleic acid molecule.

37. The method according to claims 3 to 36 wherein said oligonucleotide primer comprises a non-complementary nucleotide sequence comprising a transcriptional terminator.

38. A method for the transfection of primary cells isolated from a human subject comprising:

- i) providing an isolated sample comprising cells to be transfected;

- ii) forming a preparation comprising said isolated cell sample and contacting said sample with a dumbbell-shaped vector prepared using the method of any one of claims 1 to 37;
 - iii) providing transformation conditions that enable introduction of said dumbbell-shaped vector into said primary cell sample and sustained expression of a nucleic acid molecule included in said vector.
39. An *ex vivo* method to treat a patient suffering from a disease that would benefit from gene therapy comprising the steps:
- i) obtaining a sample from said subject comprising cells to be transfected;
 - ii) forming a cell culture preparation comprising a dumbbell shaped vector prepared using the method of any one of claims 1 to 37 and providing conditions to transfect said vector into said cells; and
 - iii) administering the transfected cells to said subject.
40. The nucleic acid sequence as set forth in SEQ ID NO: 23.
41. A DNA vector comprising the nucleotide sequence as set forth in SEQ ID NO: 23.