

Claims

1. A substrate library, comprising a plurality of DNA substrates, wherein each substrate within the library contains a putative target sequence that is 5' of an identifier DNA sequence capable of uniquely identifying said putative target sequence, which is 5' of a sequence that is identical to a reverse PCR primer, and wherein the double stranded DNA substrates within the library differ from one another only by the putative target sequences and identifier DNA sequences.
2. A substrate library according to claim 1, wherein the DNA substrates are double stranded DNA substrates.
3. A substrate library according to claim 2, wherein each substrate within the library additionally contains a sequence complementary to a forward primer, where this sequence is located 5' to the putative target sequence.
4. A substrate library according to claim 1, wherein the DNA substrates are single stranded substrates.
5. A substrate library according to any one of claims 1 to 4, wherein the sequence of each putative target sequence is not identical to the sequence of the related identifier sequence.
6. A substrate library according to any one of claims 1 to 5, wherein each of the putative target sequences in the substrate library is the same length as the other putative target sequences in the substrate library, and wherein each of the identifier DNA sequences in the substrate library is the same length as the other identifier DNA sequences in the substrate library.
7. A substrate library according to any one of claims 1 to 6, wherein the putative target sequences present in the library as a whole includes the characterised target sequence of an endonuclease and all possible single variants of this characterised target sequence.
8. A substrate library according to claim 7, wherein the endonuclease is an RNA guided nuclease, a meganuclease, a TALEN or a zinc finger nuclease.
9. A substrate library according to any preceding claim wherein each DNA substrate has an affinity tag at its 5' end.
10. A method for preparing a substrate library as defined in any one of claims 2 to 3 and 5 to 9, comprising a step of PCR amplification of a plurality of putative target sequences flanked with a) a sequence complementary to a library forward primer and b) a sequence identical to a portion of a library reverse primer sequence, with said library forward primer and library reverse primer, wherein the library reverse primer is a heterogeneous mixture of DNA sequences containing distinct identifier sequences located 5' of a sequence common to all sequences that is complementary to the reverse primer, and wherein the number of distinct identifier sequences is in molar excess of the number of putative target sequences.
11. A method for identifying a DNA target sequence of an endonuclease, comprising the following steps:

- a) contacting a substrate library as defined in claim 1 with an endonuclease under suitable conditions to permit cleavage;
- b) ligating the endonuclease treated library with a DNA sequence including a sequence complementary to a "cleavage" PCR primer;
- c) PCR amplification of the cleaved substrate with cleavage and reverse PCR primers; and
- d) sequencing of the amplified PCR product;

wherein a DNA target sequence in the cleaved product is identified via the sequence of the identifier sequence.

12. A method according to claim 11, wherein the substrate library is as defined in claim 3, and wherein step c) further comprises PCR amplification of the uncleaved substrate with the forward and reverse PCR primers.

13. A method according to claim 11 or claim 12, wherein the substrate library is as defined in claim 9 and wherein the affinity tag is used to attach the product of step (b) to the solid phase, followed by a step of eluting cleaved substrate.

14. A method according to claim 13, wherein the substrate library is as defined in claim 4, and wherein, following the step of eluting cleaved substrate and before step (d), there are the following steps:

- i) cleavage of the affinity tag and elution of uncleaved substrate;
- ii) ligation of the uncleaved substrate to a double stranded DNA sequence containing in the 5' to 3' direction a sequence complementary to an uncut forward primer sequence; and
- iii) PCR amplification of the cleaved substrate with the uncut forward primer and reverse PCR primer.

15. A method according to any one of claims 11 to 14, wherein the endonuclease is selected from the group consisting of an RNA guided nuclease, a meganuclease, a TALEN and a zinc finger nuclease.

16. A method according to claim 15, wherein the nuclease is a naturally occurring or engineered meganuclease.

17. A method for engineering endonucleases, comprising:

- a) conducting the method of claim 11 with a first endonuclease and at least two other endonucleases that differ from the first endonuclease by a single amino acid change at different positions within the endonuclease amino acid sequence using the same substrate library;
- b) comparing the efficiency of cleavage of each endonuclease tested in step a) at a particular substrate;
- c) identifying at least two amino acid changes at different positions that improve the efficiency of cleavage;

d) producing a variant endonuclease containing the at least two amino acid changes identified in step c).

18. A method according claim 17, wherein each endonuclease is selected from the group consisting of an RNA guided nuclease, a meganuclease, a TALEN and a zinc finger nuclease.

19. A variant endonuclease obtained by the method of claim 17 or claim 18.

20. A variant endonuclease according to claim 19 for use in gene editing.