CLAIMS

1. A method of generating a library of variants of a sequence of interest, said method comprising the steps of:

   (a) providing a template DNA molecule comprising said sequence of interest;

   (b) providing a pair of oligonucleotides wherein said oligonucleotides hybridize to opposite strands of said sequence of interest, wherein one of said oligonucleotides is protected, the other oligonucleotide is non-protected, and said oligonucleotides flank said sequence of interest;

   (c) performing an amplification reaction using error-prone polymerase incorporation on said template DNA molecule using said oligonucleotides, thereby generating a population of dsDNA variants, wherein said dsDNA variants each do not comprise an Eco29KI restriction site;

   (d) incubating said population of dsDNA variants with an enzyme capable of selectively degrading the non-protected strand over the protected strand of said dsDNA variants, thereby producing a population of ssDNA variants;

   (e) hybridizing said population of ssDNA variants to ssDNA intermediaries, wherein said ssDNA intermediaries comprise a sequence substantially identical to said sequence of interest or a fragment thereof, generating heteroduplex DNA, wherein said ssDNA intermediaries are uracilated ssDNA intermediaries or methylated ssDNA intermediaries, and wherein said ssDNA intermediaries each comprises one or more Eco29KI restriction sites; and

   (f) transforming said heteroduplex DNA into cells, wherein said cells comprise an enzyme capable of selectively degrading said one or more Eco29KI restriction sites, thereby generating a library of variants of said sequence of interest.

2. The method of claim 1, wherein said template DNA molecule is

   (i) a linear dsDNA molecule,
   (ii) a circular dsDNA molecule, or
   (iii) a circular ssDNA molecule, optionally wherein said template DNA molecule is an uracilated or methylated circular ssDNA molecule;

   optionally wherein the sequence of said template DNA molecule is substantially identical to the sequence of said ssDNA intermediaries.

3. The method of claim 1, wherein said sequence of interest is greater than 100 basepairs, optionally wherein said sequence of interest is between:

    (i) 100 and 2000 basepairs,
    (ii) 300 and 1500 basepairs, and/or
    (iii) 700 and 1200 basepairs.

4. The method of claim 1, wherein said protected oligonucleotide is an oligonucleotide that comprises three or more 5' phosphorothioates and said non-protected oligonucleotide is an oligonucleotide that does not comprise three or more 5' phosphorothioates, optionally wherein:

    (i) said protected oligonucleotide comprises three, four, or five 5' phosphorothioates,
    (ii) said enzyme capable of selectively degrading the non-protected strand over the protected strand is T7 exonuclease, and/or
    (iii) said enzyme capable of selectively degrading the non-protected strand over the protected strand is lambda exonuclease.

5. The method of claim 1, wherein said protected oligonucleotide is an oligonucleotide that comprises a 5' phosphate and said non-protected oligonucleotide is an oligonucleotide that does not comprise a 5' phosphate, optionally wherein said enzyme capable of selectively degrading the non-protected strand over the protected strand is lambda exonuclease.

6. The method of claim 1, wherein 50% of dsDNA variants of said population of dsDNA variants have less than 99.5% identity with said sequence of interest.

7. The method of claim 1, further comprising:

    (i) purifying said population of dsDNA variants prior to incubation with said enzyme capable of selectively degrading the non-protected strand over the protected strand,
    (ii) purifying said population of ssDNA variants prior to hybridization to said ssDNA intermediaries, and/or
    (iii) purifying said heteroduplex DNA prior to transformation into said cells.

8. The method of claim 1, wherein said ssDNA intermediaries are phagemids or vectors comprising sequence fragments substantially identical to the sequence of a phagemid.

9. The method of claim 1, wherein said hybridizing of said population of ssDNA variants to said ssDNA intermediaries comprises co-incubation of said population of ssDNA variants and said ssDNA intermediaries at a denaturing temperature followed by gradual cooling to an annealing temperature, optionally wherein:

    (i) said denaturing temperature is about 90° C.,
    (ii) said annealing temperature is about 55° C., and/or
    (iii) said gradual cooling occurs at a rate of about –1° C. per minute.

10. The method of claim 1, wherein said cells are eukaryotic cells, mammalian cells, insect cells, yeast cells, or bacterial cells.

11. The method of claim 1, wherein:

    (i) said ssDNA intermediaries comprise modified nucleobases or nucleobases other than adenine, guanine, cytosine, and thymine;
    (ii) said ssDNA intermediaries are uracilated ssDNA intermediaries and said cells are Ung+ bacterial cells, preferably wherein said cells are TG1 *E. coli* cells; and/or
    (iii) said ssDNA intermediaries are methylated ssDNA intermediaries, and said cells are Mcr+ bacterial cells.

12. The method of claim 1, wherein step (e) further comprises extending said ssDNA variants hybridized to said ssDNA intermediaries by incubation of said heteroduplex DNA with DNA polymerase, optionally wherein:

    (i) said incubation of said heteroduplex DNA with DNA polymerase comprises incubation of said heteroduplex DNA with a DNA polymerase and a DNA ligase;
    (ii) said ssDNA intermediaries are methylated ssDNA intermediaries and wherein step (e) further comprises the steps of denaturing said heteroduplex DNA and incubating the denatured product with an enzyme that selectively degrades methylated DNA, preferably wherein said methylated ssDNA intermediaries comprise methylated adenine nucleobases, and wherein said enzyme that selectively degrades methylated DNA is DpnI; or

(iii) said ssDNA variants are methylated and said ssDNA intermediaries are not methylated, and wherein step (e) further comprises the steps of denaturing said heteroduplex DNA and incubating the denatured product with an enzyme that selectively degrades non-methylated DNA, preferably wherein said methylated ssDNA variants comprise methylated cytosine or guanine nucleobases, and wherein said enzyme that selectively degrades non-methylated DNA is a restriction enzyme that recognizes the DNA sequence 5'-GATC-3'.

13. The method of claim 1, wherein said transforming of said heteroduplex DNA into said cells comprises the independent transformation of two or more, 10 or more, 20 or more, or 50 or more aliquots of said cells.

14. The method of claim 1, wherein said sequence of interest encodes:

(i) an antibody or a domain or fragment thereof,
(ii) a polymerase or a domain or fragment thereof, or
(iii) an enzyme or a domain or fragment thereof.

15. The method of claim 1, wherein steps 1(a) to 1(d) are performed in parallel from each of two or more different sequences of interest to produce two or more populations of ssDNA variants, wherein said oligonucleotides, said amplification reaction, and said enzyme may differ in the application of any or all of steps 1(a) to 1(d) to each of said two or more different sequences of interest, and said populations of ssDNA variants are admixed prior to or during step 1(e), optionally wherein:

(i) at least two of said two or more different sequences of interest are amplified from a single template DNA molecule, preferably wherein all of said two or more different sequences of interest are amplified from a single template DNA molecule;

(ii) at least two of said two or more different sequences of interest are amplified from different template DNA molecules, preferably wherein each of said two or more different sequences of interest is amplified from a different template DNA molecule;

(iii) one or more of said steps performed in parallel are performed in a single reaction for at least two of said two or more different sequences of interest, preferably wherein one or more of said steps performed in parallel are

performed in a single reaction for all of said two or more different sequences of interest;

(iv) one or more of said steps performed in parallel are carried out separately for at least two of said two or more different sequences of interest, preferably wherein one or more of said steps performed in parallel are carried out separately for each of said two or more different sequences of interest; and/or

(v) said ssDNA intermediaries comprise a sequence substantially identical to each of said two or more different sequences of interest, or fragments thereof, and wherein step 1(e) encompasses the hybridization of each of said two or more populations of ssDNA variants to said ssDNA intermediaries, preferably wherein (a) said two or more populations of ssDNA variants are hybridized to said ssDNA intermediaries simultaneously, or (b) said two or more populations of ssDNA variants are hybridized to said ssDNA intermediaries sequentially.