SUPPLEMENTAL INFORMATION

Table S1, related to Figure 3. Sequence alignment of the starting enzyme and 31 cloned individuals obtained after the 10th round of in vitro evolution.

```
              10        20        30        40        50
 5´  .........|.........|.........|.........|.........|..  3´
wt   GGAAGUUGUGCUCGAUUGUUACGUAAGUAACAGUUUGAAUGGGUUGAAGUUU
37*  .........UA..AC.................C....G..............
10   .........UA..AC.................C....G..............
18   .........UA..AC.................C....G..............
34   .........UA..AC.................C....G.........-....
35   .........UA..AC.................C....G.........-....
 3   .........UA..AC.................C.A..G.........-....
 4   .........UA..AC.................C.A..G.........-....
 8   .........UA..AC.................C.A..G.........-....
11   .........UA..AC.................U....G..............
16   .........UA..AC.................U....G..............
19   .........UA..AC.................U.C..G..............
45   .........UA..AC..............-..U....G..............
12   .........UA..AC..........G......U....G..............
13   .........UA..AC....A............U....G.....C........
21   .........UA..AC...A..........U..U.A..G.........-....
 2   .........UA..A.G..................A..G.........-....
41   .........UA..A.G..................A..G.........-....
25   .........UA..A.G..................A..G..............
48   .........UA..A...................-A..G.........-....
22   .........UA..A.C..-....-.....-...-A..G.........-....
 9   .........UA..A..................-.C..G..............
28   .........UA..A..........G.......-.C..G.........-....
30   .........UA....A................-.A..G.........-....
29   .........UA....A................-.A..G.........-..C.
39   .........UA....A..........A.....-.A..G.........-....
40   ...G......A..A.G................-.A..G.........-....
23   ....-.....A..A.G..........A.....C-A..G.....CC.......
 1   ........AU.A.U.C............G...C.G..G.........-....
46   ........A..A.U.C................C.G..GC....GG.......
26   ..G.....A..A.U............A.....U.G..GC........-....
15   ........A..A.U-.................C....GC......-......
```

Sequence of the wild-type enzyme (E1) is shown, with underlined nucleotides indicating positions that were mutagenized in the starting population. Clone numbers are indicated at the left. Clone 37 (asterisk) corresponds to the F1 enzyme. 48 clones were obtained, of which 31 were sequenced.

Figure S1, related to Figure 2. Self-replication activity of individual clones obtained after the 6th (white), 8th (gray), and 10th (black) rounds of in vitro evolution. The activity of the E1 enzyme (wt) and of the population as a whole after rounds 6 and 8 are indicated by dashed lines. Reaction conditions: 1 μM each substrate, 25 mM $MgCl_2$, pH 8.5, 42 °C.

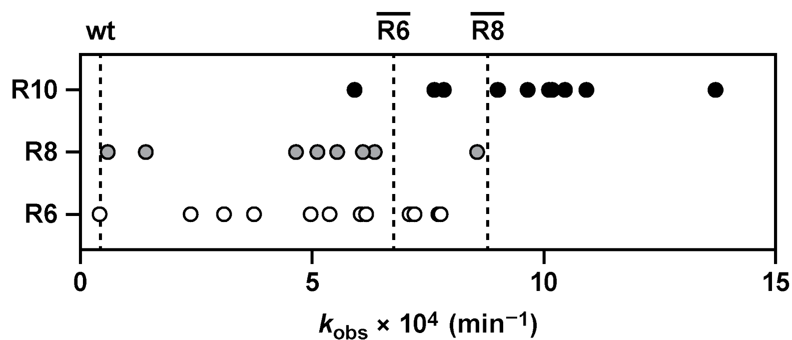

Figure S2, related to Figure 4. Temperature dependence of the replicating E1 and F1 enzymes. Both the exponential growth rate (left axis) and doubling time (right axis) are indicated. Self-replication of F1 (green) employed 10 μM of each substrate. Cross-replication of F1 (blue) and F1´ (red) and of E1 (black) and E1´ (white) employed 5 μM of each substrate. Reaction conditions: 0.05 μM starting enzyme, 25 mM $MgCl_2$, pH 8.5.

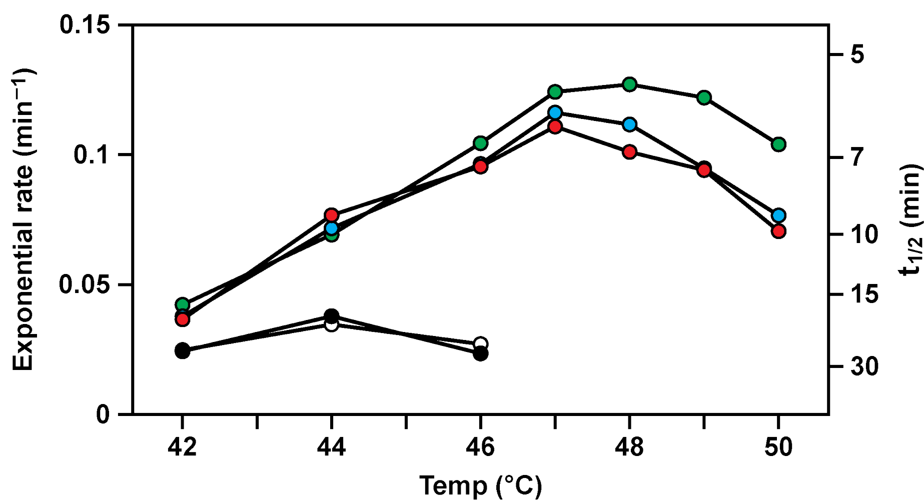

Figure S3, related to Figure 5. Substrate concentration dependence of the replicating E1 and F1 enzymes. Both the exponential growth rate (left axis) and doubling time (right axis) are indicated. Self-replication of E1 (gray) and F1 (black) were carried out at 44 and 48 °C, respectively. Reaction conditions: 0.05 μM starting enzyme, 25 mM $MgCl_2$, pH 8.5, 42 °C.

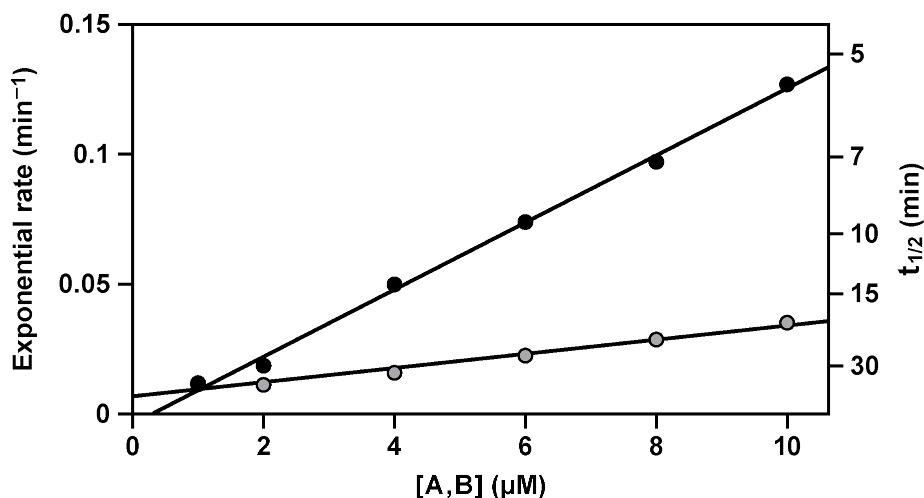

Figure S4, related to Figure 6. Catalytic activity of the cross-replicating F1 (black) and F1´ (gray) enzymes in the component ligation reactions, conducted under enzyme-excess, single-turnover conditions. Values for k_{obs} were obtained for various concentrations of enzyme based on the initial rate of reaction, then fit to the Michaelis–Menten equation: $k_{obs} = k_{cat}[E] / (K_m + [E])$. Reaction conditions: ≤3 nM A (or A´), 100 μM B (or B´), 25 mM $MgCl_2$, pH 8.5, 42 °C.

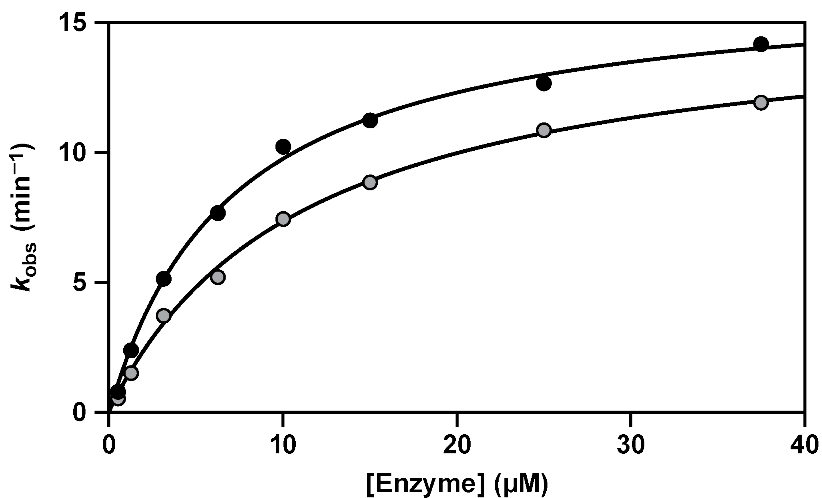

Table S2, related to Figure 1. Sequences of synthetic oligonucleotides used in this study.

Description	Sequence (5´→3´)
Library of DNA templates	GGCTAATACGACTCACTATAGAGACCGCAACTTGAGAAA-GTTGTGCTCGATTGTTACGTAAGTAACAGTTTGAATGGGT-TGAAGTTTGAGACCCTCGTGATGTCCAGTCGC
Primer for 2nd-strand synthesis for A-format reactions	GCGACTGGACATCACGAG
Guide RNA for M1 cleavage	GGAGUAAGUUGCGGUCUCACCAUC
Substrate for A-format reactions	biotin-d(ATCGCTTGATCGCT)-r(GGUUGAAGAAU)
1st half-template for substrate for B-format reactions	GGCTAATACGACTCACTATTGAGACCGCAACTTA•GCCC-GGACTCGGGTTCCAGTACTA
2nd half-template for substrate for B-format reactions	GTGGTGCCCGGACTCGGTAGTACTGGAAC
Substrate for B-format reactions	GAGACCGCAACUUAA-biotin
Primer for 2nd-strand synthesis for B-format reactions	TAAGTTGCGGTCTC
Forward 1st PCR primer for A-format	ATCGCTTGATCGCTGGTTGAAGAAT
Reverse PCR primer for A-format[a]	GCGACTGGACATCACGAG*GGTCTCAAAC*
Forward 2nd PCR primer for A-format	GGCTAATACGACTCACTATAGAGACCGCAACTTGAG
Forward PCR primer for B-format[b]	GGCTAATACGACTCACTATAGGAGCGAGAAAGTTG
Reverse PCR primer for B-format	TAAGTTGCGGTCTC•ATTCTTCAACCTTCCGGTCTCAAA*C*
Forward PCR primer for converting B-format to A-format	GGCTAATACGACTCACTATAGAGACCGCAACTTGAGAAA-GTTG
Reverse 1st PCR primer for converting A-format to self-replication format	GAATATTCATGCGGTCTCAAA
Forward 2nd PCR primer for converting A-format to self-replication format	GGCTAATACGACTCACTATA
Reverse 2nd PCR primer for converting A-format to self-replication format[c]	TAAGTTGCGGTCTCATACT

[a] Also used as reverse PCR primer for converting B-format to A-format.

[b] Also used as forward 1st PCR primer for converting A-format to self-replication format and as forward PCR primer for converting B-format to self-replication format.

[c] Also used as reverse PCR primer for converting B-format to self-replication format.

T7 RNA polymerase promoter sequence in red; mutagenized nucleotides underlined; region of hybridization in blue; dot indicates corresponding site for cleavage by M1 RNA; nucleotides in italics were not present in the reverse PCR primer during the first four rounds of in vitro evolution.