Supplemental Materials for

Variola Type IB DNA Topoisomerase: DNA Binding and Supercoil Unwinding using Engineered DNA Minicircles

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Plasmid and Primer Sequences

pMC454 Sequence

The sequence in italics was generated by gene synthesis (IDT) and the MCsp sequence is in color.

Color Code for Labeling Sequence Elements:

1. attR Sequence
2. Specific CCCTT Site
3. 2 Nb.BbvCI Sites and Zn-Finger Motif
4. PstI and NheI Restriction Sites
5. 206-mer Primer Sites
6. NcoI and PstI Restriction Sites

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GGGGCGAATTGGGCCCGACGTCGCATGCTCCTCTTAGACTCGAGGAATTCCGTCTGCGAACGCCAGCAAGT
AAGACGTAGCCCAGCGCGTCGGCCGCCATGCCGGCGATAATGGCCTGCTTCTCGCCGAAACGTTT
GGTGGCGGGACCAGTGACGAAGGCTTGAGCGAGGGCGTGCAAGATTCCGAATACCGCAAGCGACA
GGCCGATCATCGTCGCGCTCCAGCGAAAGCGGTCCTCGCCGAAAATGACCCAGAGCGCTGCCGGCA
ACCTGTCCTACGAGTCTGCTGATAAAAGAAGAACAGTGCTATAAGTGGCCGAGCACGATAGTCTGCCC
CGCCACCAGGAAGAGACTGAGTGTTAGGGTTAAGGCTCAAGGCGATCGAAACTGAAGACTGAC
CAGCGTAGTCTGCTGCAG
TACCGCTCTTTGTTTTATATTTATATATATATATATATATATATATATATTATATTATTATTATTATTATTATTTATATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTAT
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GACGCTGCCTTTATCCGTTAACATATCGTCTTGTAGTCACCAACCGGTAAGACAGCAGACTTATCGCC
ACTGGAAGCAGCCACTGTTAAACAGAATACGAGGAGGATGATGTCAGCGGCTCTCAGAGCTCT
TGAAGTGTGCCTTAACACTCGACATCAGAGGAGCTATTTGTGATTCTCGGCCTCTCAGGAG
CCAGTTACCCTTGAAAAGAGAGTGTTTACCTGTCTTTGATCCGCAAAACACCCAGCTGTTACGG
TGTTTTTTTTGTGTTGGCAAGAGCAAGTTTACCCCCGAGAAAAAAGCTCCTCAAGATCTTT
TATTTCTATCGGGTTCGCTGACAGTCTGCAGACATCATGCAGATACT
CACCTGATGACTGAACGGTACGCCAGAGCGAGACTTAGTCGGGTAAACGCTACCAGAGCAGCGAT
GCTAATGCGATGTCGATAGTTACTAACGGGTCATTGTTCGATTAACTGCCGCAGCGATTAACATC
AGGCACCAGTAGCATGACCATAAACAGTAGTGTTACCAGGATGGCGAGCTTAGCAGTATGACGTA
GCCTACGGTAAACCTGTCGTGCCAGCTGCATTAATGAATCAACGTTTAACTTATACCTGAGCATG
CCAATGCCAGCGTCAGACATCACATGCATAGACTCACCTGATGACTCGAATAGGCGTCGGCCGCC
ATGCCGCGATAATGGCCTGCTACTGCCGAACGTTGGTGGCTGACCAGTGACGAAGGCTTGAGCGA
GGGCGTGCAAGATTACCGAATACCGCAAGCGACAGGCCGAACATCGTCGCAACCAGCGAGTTCCG
TCTGCGAACGCCAGCAAGACGTAGCCCAGCGTCGGCCATGTCGCGAACACCGGAAGGAGCTGACT
GTTGAAGGCGAACAAGGGCATCGGACTGAATGTGTTCACAGGTTGCTCCAGGCTATGATAGAAT
G
CTAGC
CTGCAG

Sequence of 661 bp oligonucleotide for construction of pMC1103 and pMC1752

PstI restriction sites are underlined and NheI restriction sites are italicized.

GCCTAGCTGCAGTAACGTATACCTGAGATCCGAAATCGCAGCTCAGATCACATCGAATACATC
CACCTGATGACTGAACCGTACCCAGAGCGAGACTTAGTCTCCGAGTTAAACCCATACAGAGAGAT
GCTAATGCGATGTCGATAGTTACTAACGGGTCATTGTTCGATTAACTGCCGCAGCGATTAACATC
AGGCACCAGTAGCATGACCATAAACAGTAGTGTTACCAGGATGGCGAGCTTAGCAGTATGACGTA
GCCTACGGTAAACCTGTCGTGCCAGCTGCATTAATGAATCAACGTTTAACTTATACCTGAGCATG
CCAATGCCAGCGTCAGACATCACATGCATAGACTCACCTGATGACTCGAATAGGCGTCGGCCGCC
ATGCCGCGATAATGGCCTGCTACTGCCGAACGTTGGTGGCTGACCAGTGACGAAGGCTTGAGCGA
GGGCGTGCAAGATTACCGAATACCGCAAGCGACAGGCCGAACATCGTCGCAACCAGCGAGTTCCG
TCTGCGAACGCCAGCAAGACGTAGCCCAGCGTCGGCCATGTCGCGAACACCGGAAGGAGCTGACT
GTTGAAGGCGAACAAGGGCATCGGACTGAATGTGTTCACAGGTTGCTCCAGGCTATGATAGAAT
GCTAGCTGCAG

Sequence of mutagenesis primer for deletion of the NheI Site from pMC1103
Mutated positions are in bold.

5’-CCAGGCTATGATAGAATGAGGCCTGCAGTAAGCTTTACCTGAGC-3’

**Primer sequences for the formation of nonspecific 206mer**

Forward: GCAGATACTCACCTGATGACTG
Reverse: CACGACAGGTTTACCAGACTG

**Sequence of nonspecific 206mer**

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GCAGATACTCACCTGATGACTGAACGGATCGCCTCAGCGACGATTATTTCGGGTCGACGCTACC
AGCAGCGATGCTAAATGATGTCGATAGTTAATTACCAGATGCTAATGATGTCGATAGTTAACC
ATCGGCCAGTACGCTGGGTGGCTAAACAGTAGTGTCTTACCAGATGCCAGCTTAGCAGTGGTA
AACCTGTGCTG
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Figure S1. Determination of the average superhelical density of 454 bp minicircles MC<sup>sp</sup> and MC<sup>sp*</sup> (see Table 1). Electrophoresis was performed using 5% native polyacrylamide gels run in 1X TBE and in the presence of the indicated amounts of chloroquine (CQ). Gels were run at 3 V/cm for 18 hours at room temperature. The average superhelical density was determined by fitting the bands to a Gaussian distribution in Prism and dividing by the number of helical turns in the DNA (turns = 454bp ÷ 10.5bp/turn) as described<sup>1</sup>. 
Figure S2. Determination of the average superhelical density of 1,752 bp MC$_{sp2}$. (A) Electrophoresis was performed using 2% agarose gels run in presence of CQ and TAE Buffer. Gels were run at 4 V/cm for 17 hours at 4°C. (B) The average superhelical density was determined as described in the legend of Figure S1.
Figure S3. Background corrected fluorescence emission spectra of 50 nM 18U-FAM/18-IABK before (black circles) and after (red squares) addition of 1 µM vTopo. A 10-fold fluorescence increase resulted from the complete cleavage of the substrate DNA by vTopo.
Figure S4. Differences in ethidium bromide (EtBr) staining between relaxed (P) and supercoiled (S) MC\textsuperscript{sp}. (A) Equal concentrations (0.25 to 5 nM) of supercoiled and relaxed MC\textsuperscript{sp} were mixed and loaded onto a 2% agarose gel. Following electrophoresis, the gel was stained with EtBr, destained with distilled H\textsubscript{2}O, and the bands were imaged. (B) The normalized fluorescence intensities (defined relative to the band with the greatest intensity) were determined and plotted against the known MC\textsuperscript{sp} concentration. The experiment was performed in duplicate and the slopes of the relaxed (black squares) and supercoiled (red circles) forms of MC\textsuperscript{sp} were determined. The ratio of the slopes (1.4) was taken as the correction factor in the experimental rate measurements. It is reasonably assumed that MC\textsuperscript{ns} behaves identically to MC\textsuperscript{sp} because it differs by only 2 bp.
**Figure S5.** SYBR green staining in agarose gels is identical for the supercoiled (S) and relaxed (P) forms of MC$_{sp2}$. The relaxed form of MC$_{sp2}$ was generated by reacting supercoiled MC$_{sp2}$ with vTopo under single turnover conditions of 10-fold excess vTopo as described in the Materials and Methods. The multiple product bands result from a thermal distribution of low superhelical density circular forms that persist after strand ligation. Equal concentrations of supercoiled (A) and relaxed (B) MC$_{sp2}$ were loaded onto 3% agarose gels. Following electrophoresis, gels were stained with SYBR green and imaged as described. Gels were performed in duplicate. (C) The staining intensity was determined following subtraction of the
intensity of the contaminating and overlapped nicked band (*) using the Gaussian fitting described in the main text. The substrate and products intensities were plotted against the known MC$^{\text{sp}2}$ concentration. The relaxed (black circles) and supercoiled (red squares) forms of MC$^{\text{sp}2}$ are fitted to the same line. In addition, this control establishes that MC$^{\text{sp}2}$ stains linearly with SYBR Green up to 32 ng. Under our reaction conditions, no more than 24 ng of MC$^{\text{sp}2}$ are loaded into any one lane on the gel.

**Figure S6.** SYBR Green staining in polyacrylamide gels is identical for the supercoiled (S) and relaxed (P) forms of MC$^{\text{sp}}$. The relaxed form of MC$^{\text{sp}}$ was generated by reacting supercoiled MC$^{\text{sp}}$ with vTopo under single turnover conditions of 10-fold excess vTopo as described in the Materials and Methods. (A) Equal concentrations of each topological form of MC$^{\text{sp}}$ were loaded onto 6% acrylamide gels. Following electrophoresis, gels were stained with SYBR Green and imaged. (B) The staining intensity was determined using the Gaussian fitting method described in the main text and plotted against the known DNA concentration. The plots of MC$^{\text{sp}}$ (black
circles), MC^{sp*} (blue triangles), and relaxed MC^{sp} (red squares) were fitted to the same line. The fluorescence increase was linear with MC up to 6 ng. Under our reaction conditions, no more than 5 ng of MC^{sp} were loaded into any one lane on the gel. The experiment was performed in duplicate.

Figure S7. MC^{ns} isolated from competition binding assays remains supercoiled during the course of the experiment. Following the 180 s competition binding experiment (see Materials and Methods), the entire reaction volume was removed from each cuvette and quenched with 150 µL of 2X Quench Buffer. Fifty-five microliters of the quenched reaction were then loaded onto a 2% agarose gel in 1X TAE and run for 45 min. at 100 V. The gel was stained with SYBR Green and imaged. The gel image shows that all concentrations of MC^{ns} used in the competition binding experiment migrate with the same mobility as the control supercoiled MC (leftmost lane). Thus, MC^{ns} was not relaxed by vTopo during the course of the binding reactions.
Figure S8. Resolved substrate ($S^{9-14}$) topoisomers of $MC^{sp2}$ relax with the same rates. (A) 5 nM $MC^{sp2}$ was relaxed with 5 nM vTopo. The substrate topoisomers were resolved by
electrophoresis using 2% agarose gels run in presence of 5 µg/mL CQ. The band intensities of the individual topoisomer bands $s^{-9}$-$s^{-14}$ were quantified using the Gaussian shape fitting method in the Quantity One software and the integrated intensities were fitted to single exponential decays. (B-G) Individual decay time courses for $s^{-9}$-$s^{-14}$. The decay rate constants were identical for each substrate topoisomer between $s^{-9}$ and $s^{-13}$ ($k_{lim} = 0.019 \pm 0.0004$ s$^{-1}$). The small amount of the minor topoisomer $s^{-14}$ made the Gaussian curve fitting difficult. The error bars estimate the upper and lower limits of the $s^{-14}$ band areas and the dashed curve is drawn using the $k_{lim}$ value measured for the other substrate topoisomers. These results support the simplified model where $s^{-9}$-$s^{-14}$ were treated as a substrate pool (S) rather than individual topoisomers.
Dynafit 3 script files for numerical simulations of supercoil unwinding

**MC\textsuperscript{sp}**

[task]

\begin{verbatim}
task = fit
data = progress
\end{verbatim}

[mechanism]

\begin{verbatim}
ES --> ES* : klim
ES1 <-> ES1* : kcl klig
ES1* --> EP : kslp
ES* --> EP : ksp
\end{verbatim}

[constants]

\begin{verbatim}
klim = .02?
kcl = 0.3
klig = 4
kslp = 2?
Kslp = .03?
\end{verbatim}

[concentrations]

\begin{verbatim}
ES = 3.6
ES1 = 0.55
EP = 0
\end{verbatim}

[progress]

\begin{verbatim}
directory ./Breeana/data
extension txt
file MC454IntP | response EP = 1
file MC454IntI | response ES1 = 1
file MC454IntS | response ES = 1
\end{verbatim}

[output]

\begin{verbatim}
directory ./Breeana/output/MC454Int
\end{verbatim}

[end]

**MC\textsuperscript{sp*}**

[task]

\begin{verbatim}
task = fit
data = progress
\end{verbatim}

[mechanism]

\begin{verbatim}
ES --> ES* : klim
ES* --> EI : ksi
EI <-> EI* : kcl klig
EI* --> EP : kip
ES* --> EP : ksp
\end{verbatim}

[constants]

\begin{verbatim}
klim = .02?
kcl = 0.3
klig = 4
kip = .18?
ksi = 6?
\end{verbatim}

[concentrations]

\begin{verbatim}
ES = 4.8
EI = 0
\end{verbatim}
EP = 0

[progress]
directory ./Breeana/data
extension txt
file MC454SCIntP | response EP = 1
file MC454SCIntI | response EI = 1
file MC454SCIntS | response ES = 1

[output]
directory ./Breeana/output/MC454SCInt

[end]

**MC^sp2 with WT vTopo**

[task]
task = fit
data = progress

[mechanism]
ES --> ES* : klim
ES* --> EI1 : ksi1
EI1 <-> EI1* : kcl klig
EI1* --> EP : ki1p
ES* --> EI2 : ksi2
EI2 <-> EI2* : kcl klig
EI2* --> EP : ki2p
ES* --> EP : ksp

[constants]
klim = .017?
kcl = 0.3
klig = 4
ki1p = .05?
ksi1 = 1?
ksi2 = .8?

[concentrations]
ES = 4
EI1 = 0
EI2 = 0
EP = 0

[progress]
directory ./Breeana/data
extension txt
file MC1752P | response EP = 1
file MC1752I123 | response EI1 = 1
file MC1752I45 | response EI2 = 1
file MC1752S | response ES = 1

[output]
directory ./Breeana/output/MC1752IPool

[end]

**MC^sp2 with K271E vTopo**

[task]
task = fit
data = progress

[mechanism]
ES -> ES* : klim
ES* -> EI1 : ksi1
EI1 <-> EI1* : kcl klig
EI1* -> EP : ki1p
ES* -> EI2 : ksi2
EI2 <-> EI2* : kcl klig
EI2* -> EP : ki2p
ES* -> EP : ksp

[constants]
klim = .002?
kcl = 0.0129
klig = .17
ksi1 = 1?
ksi2 = 1?

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already positioned at the specific site (i.e. rate-limiting translocation obscures cleavage in the reaction of substrate topoisomers).

\( k_{\text{lig}} \) – the chemical step of ligation. In the models, this rate is fixed at 4 s\(^{-1}\) for WT vTopo based on previous experiments with small oligonucleotides and the pUC19 plasmid\(^2\). For intermediates with low superhelical density, ligation competes effectively with supercoil unwinding. The ratio between rate of unwinding and ligation determines the number of supercoils released per cleavage event. It also influences the overall rate at which product formation takes place from intermediates (i.e. under conditions of rapid equilibrium cleavage/ligation followed by slow supercoil unwinding).

\( k_{s,p}/k_{s,i} \) – the ratio of the rates of product and intermediate formation arising from cleavage of the substrate pool. Because \( k_{\text{lim}} \) is the rate-limiting step in substrate disappearance, the individual values for the rapid steps \( k_{s,p} \) and \( k_{s,i} \) are not measured (i.e. \( k_{s,p} \) and \( k_{s,i} \gg k_{\text{lim}} \)). However, the amount of intermediates and product, which are generated from a common cleaved substrate pool, accurately reveals the ratio \( k_{s,p}/k_{s,i} \). It is important to note that \( 1/k_{s,i} \) includes (i) the unwinding time to produce an intermediate with \( \Delta n \) fewer supercoils, and (ii) the time required for ligation. Therefore, the lower limit time for \( k_{s,i} \) is \( 1/k_{\text{lig}} = 1/4 \text{ s}^{-1} = 0.25 \text{ s} \).

\( k_{i,p}/k_{\text{lig}} \) - the ratio of product formation from intermediate pools as compared to the ligation rate. As above, we assign a ligation rate of 4 s\(^{-1}\).

**Stepwise methods used in kinetic modeling**

We sought the simplest kinetic model to describe the data. We began with complex models and then performed step-by-step simplification to arrive at the models used. The details of these simplifications and any assumptions that were used are described below.
MC<sup>sp</sup>. For this substrate, two substrate populations $s^{-2.3}$ and $s^{-1}$ existed (Fig. 6B). There was no observable intermediate formation, as the disappearance of $s^{-2.3}$ followed a single exponential decay to product with rate constant $k_{\text{lim}}$ without populating $s^{-1}$. Thus, there is no information of partitioning between intermediates and product with this substrate. Due to its single supercoil, topoisomer $s^{-1}$ could only proceed to product. Since $s^{-1}$ disappeared more slowly than $s^{-2.3}$ and the chemical steps of cleavage and ligation are known to be similar in linear and supercoiled substrates, the slower rate is most reasonably attributed to rapid equilibrium cleavage and ligation followed by slow unwinding of this topoisomer because of its low superhelical density ($k_{s^{-1}\rightarrow p}$). We assigned $k_{\text{cl}}$ and $k_{\text{lig}}$ as above, and report the ratio $k_{s^{-1}\rightarrow p}/k_{\text{lig}}$ in Table 2.

MC<sup>sp*</sup>. For this substrate, a single intermediate ($i^{-1}$) accumulates. The $s^{-4.5.6}$ substrate pool followed a single exponential decay and there is no evidence that $s^{-4.5.6}$ partitions into another substrate band. Thus, the substrate pool was treated in the same way as described above for $s^{-2.3}$ to give $k_{\text{lim}}$. Since the decay of $s^{-4.5.6}$ resulted in both product and the $i^{-1}$ intermediate, the ratios $k_{s^{-1}\rightarrow p}/k_{s^{-1}\rightarrow i}$ and $k_{i^{-1}\rightarrow p}/k_{\text{lig}}$ could be determined. Trial and error simulations demonstrated that these ratios were invariant as long as $k_{s^{-1}\rightarrow p}$, $k_{s^{-1}\rightarrow i}$, and $k_{\text{lig}}$ were much greater than $k_{\text{lim}}$. We assigned $k_{\text{cl}}$ and $k_{\text{lig}}$ as above, and report the ratios $k_{s^{-1}\rightarrow p}/k_{s^{-1}\rightarrow i}$, $k_{s^{-1}\rightarrow p}/k_{\text{lig}}$ in Table 2.

MC<sup>sp2</sup>. For this substrate, five intermediates accumulate and then disappear into product bands. As above, the substrate was treated as a single pool that followed a single exponential decay to product and intermediates (see justification of this simplification in Figure S8). With the accumulation of multiple intermediates, it was also important to confirm whether more highly supercoiled intermediates relaxed into intermediates with lower superhelical densities. Using the same procedure outlined for the substrate topoisomers (Figure S8), we were able to discern two populations of intermediates (I1 and I2) that behaved distinctly. Pool I1 consisted of $i^{-5.6.7}$ with...
each member of the pool appearing and disappearing with similar rates. Pool I2 consisted of $i^{2,4}$ which appeared and disappeared with a similar rates but more slowly than I1.

**K271E mutant vTopo.** The model for K271E and the MC$^{sp2}$ substrate was obtained using the same procedures described above for WT vTopo. The value for $k_{\text{lim}}$ was obtained directly from the rate of disappearance of the substrate pool. However, $i^1$ was classified as a product because it did not disappear with time, and accordingly, it was pooled with the product bands. It is likely that $i^1$ eventually disappears over a longer time, but this was not observable because of the slow cleavage rate of the K271E mutant$^3$. This treatment of the $i^1$ species does affect the data analysis because very few intermediates accumulate with K271E. For K271E, $k_{\text{cl}}$ and $k_{\text{lig}}$ were set 20-fold lower than the WT enzyme based on previous results with linear substrates$^3$. Although it is possible that these calculated values are not precise, the ratio $k_{s\rightarrow p}/k_{s\rightarrow i}$ is completely independent of any assumptions. Therefore, the major conclusion that fewer intermediates accumulate with K271E is also independent of assumptions.

**References**

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