

End-joining long nucleic acid polymers

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Supporting Material

Here we present detailed information on the origin of the formation of homo-dimers (A+A and B+B), the electrophoretic shift caused by the binding of STV to molecules A and B, and the influence of the STV concentration during and after the first incubation step. We then investigate the influence of incubation time and concentration of molecule B on the overall efficiency of dimer formation. Finally, we present detailed experimental information on the figures presented in both the main text and those presented here.

Origin of homo-dimer formation: incubation temperature and incomplete STV removal

In the main text we used gel purification to remove the excess unbound STV after the first incubation, combined with a lower temperature during the second incubation. Here we demonstrate why these measures are necessary to prevent the formation of homo-dimers (A+A and B+B).

In figure S1(a), lane 1, we demonstrate what happens when the second incubation step is performed at 37 °C instead of 4 °C and the purification is performed using column purification rather than gel purification. We used the same DNA molecules as in figure 1(b) from the main text: 3.5 kb DNA (A) and 2.2 kb DNA (B). Apart from the bands that were already discussed in figure 1(b) of the main text, we now find two additional bands at 4.4 kb and 7.0 kb, corresponding to the homo-dimers B+B and A+A, respectively. We now

demonstrate that gel purification can prevent formation of the B+B homo-dimers, and a lower temperature during the second incubation can prevent the formation of the A+A homo-dimers.

The B+B homo-dimer forms primarily due to the fact that not all STV is removed by the Microcon column purification (which we also show experimentally in figure S2). If some STV remains after the purification, B+STV complexes will form as soon as the B molecules are added and so allow for the formation of B+B homo-dimers (in addition to A+B hetero-dimers). To demonstrate that this is indeed what happens, we carry out the same protocol in figure S1(b), but this time again using gel purification to ensure complete removal of the unbound STV. The temperature during the second incubation, however, is kept at 37 °C instead of 4 °C. Molecule A (3.5 kb DNA) was incubated with excess STV and then loaded on an agarose gel in lanes 2 and 3. The band containing only the fraction of A bound to STV was extracted from the gel and used in the second incubation. As a control, the same amount of A but without STV was loaded in lane 4 and gel extracted in the same manner. The gel-extracted samples from lanes 2 and 4 were subsequently incubated with molecule B (shown for reference in lane 1) at 37 °C. The resulting products can be seen in lanes 6 and 7, respectively. In lane 7, containing the control in the absence of STV, no dimer formation occurs. In lane 6, we see the band corresponding to the hetero-dimer A+B (at 5.7 kb), and indeed, the band corresponding to the homo-dimer B+B has disappeared: the gel purification completely removes the unbound STV and formation of the homo-dimer B+B is suppressed.

In figure S1(b), lane 6, a small band is still visible at 7 kb, corresponding to the hetero-dimers A+A. Interestingly, this band also appears in lane 5, where we loaded the gel-extracted A+STV complex (from lane 3) on gel after incubation at 37 °C for 45 minutes, but without adding molecule B. Apparently, even when all free STV is removed and molecule B is omitted, the A+A homo-dimer still forms. This implies that the A+STV complex is itself unstable and can

dissociate into a free molecule A and free STV; when this occurs, it is possible that the free molecule A binds another A+STV complex, forming the homo-dimer A+A. This mechanism is schematically shown in figure S1(c). The formation of the homo-dimer A+A is then the result of the continuous dissociation and association of STV from the biotinylated DNA. Indeed, as we argued in the main text, the dissociation rate of the STV-biotin complex is strongly temperature dependent and lowering the temperature from 37 °C to 4 °C can reduce the rate of formation of this homo-dimer by several orders of magnitude. Indeed, as was shown in figure 1(b) of the main text, at 4 °C formation of both the A+A and B+B homo-dimers is completely suppressed.

Small electrophoretic shift due to STV binding

In figure S1(a), lane 1, the bands at approximately 3.5 kb (A) and 2.2 kb (B) and are each split into two separate bands: a lower band, migrating at exactly the same speed as the molecules in the absence of STV (lane 2), and an upper band, migrating slightly slower. Since the latter only appear in the presence of STV, these upper bands must correspond to molecules A and B that are bound to a single STV molecule. We observe that this gel shift, observable due to the fact that the bound STV reduces the electrophoretic mobility of DNA molecules up to at least 3.5 kb in length, can also conveniently function as a rapid assay to determine the amount of biotinylated product following a labeling reaction (under the assumption that all biotinylated molecules bind STV). To demonstrate this we show in figure S1(d) the molecules A (lane 2) and B (lane 3) after a single incubation of 30 min with an excess of STV. Comparing the intensities of the upper and lower bands in lanes 2 and 3 it can thus be concluded that >90% of the A molecules and >65% of the B molecules are biotinylated. For reference, lane 1 contains the molecules A and B in the absence of STV. In this particular example, the difference in the biotinylated fraction of molecules A and B originates from different purities of the PCR primers used to synthesize molecules A and B.

Influence of STV concentration during and after the 1st incubation

We have investigated how the STV concentration during and after the 1st incubation influences the formation of A+B dimers. In figure S2 we show a pulsed field electrophoresis image of an EtBr stained 1% agarose gel in 0.5x TBE. Here, we end-joined two molecules of DNA, A (12.7 kb long) and B (9.6 kb long), using our two-step protocol. For convenience, column purification (Microcon YM-100, Millipore) was used to remove the STV after the first incubation (leading to the formation of B+B homo-dimers). The table below the gel image explains in detail the contents of each lane.

Lane 2 contains the product after binding 12.7 kb DNA to 9.6 kb DNA using the two-step protocol described in the main text. Using markers M1 (lambda EcoRI marker, Promega) and M2 (lambda moncut marker, Promega) as a reference, we can see that bands form at approximately 19 kb (B+B dimer) and 22 kb (A+B dimer). A band at 25 kb (A+A dimer) is faintly visible, which can be attributed to the dissociation of the A+STV complex. As we argued before, the band corresponding to dimer B+B forms because the YM-100 purification does not completely remove all free STV. This is demonstrated directly in lane 3: here we incubated a sample containing only STV in the first step (no DNA present), purified with the YM-100 column, and added molecule B in the second incubation. We clearly see a band corresponding to dimer B+B, implying there must still have been some STV present after the YM-100 purification. In comparison, lane 9 contains the product in the absence of STV in the first incubation, and clearly no dimers form.

The figure also demonstrates that an excess of STV is required to prevent formation of homo-dimers in the first incubation: Lanes 1 and 5 were loaded with molecules A and B, respectively, which were both incubated with a 100-fold molar excess of STV for 30 min, purified over a Microcon YM-100 column, and then immediately loaded on gel. Clearly, formation of dimers is not observed. In contrast, lanes 4 and 8 show the results of incubation of the 9.6 kb

and 12.7 kb DNA molecules respectively with a 1:1 ratio of STV to DNA. In both cases homo-dimer formation occurs.

Homo-dimers also form after following the regular protocol without adding B in the second incubation (lane 8). In this case, the A+STV construct is kept at 37 °C overnight, allowing for the dissociation of STV and thus causing dimer formation. We finally demonstrate that the formed homo-dimers are really the dimers A+A and B+B by using our 2-step protocol to end-join molecules A and B to themselves, see lanes 6 and 7. Here we incubated molecules 9.6 kb (lane 6) and 12.7 kb (lane 7) with themselves using the two-step protocol, forming only homo-dimers A+A and B+B.

Optimization of dimer formation

As discussed in the main text, we have found that there is typically a significant amount (up to about 50%) of molecules A and B in the final product that do not form dimers but still bind STV. In an effort to reduce this fraction and optimize dimer formation, we have investigated two factors: the influence of the duration of the second incubation step and the relative concentrations of the molecules used.

In figure S3 we varied the duration of the second incubation step from 10 minutes to 24 hours. We used the same 2.2 kb DNA (A) and 3.5 kb DNA (B) molecules as used in the main text. In this experiment we used column purification to remove the STV and the incubations were performed at 37 °C, and samples were taken out after the times indicated in the figure and immediately stored at -20 °C. After 24 hours, all samples were simultaneously loaded on a 0.75% agarose gel. As is clear from figure S2, no noticeable change in the relative amount of dimers (both homo-dimers and hetero-dimers) is visible between 10 minutes incubation and 24 hours incubation. We conclude that a longer incubation does not demonstrably reduce the amount of A+STV and B+STV molecules.

Furthermore, we have investigated the effect of DNA concentration on the maximum yield of homo- and hetero-dimers. In figure S4 we varied the concentration of the second molecule B in the second incubation. Here, we used the 3.5 kb DNA molecule (A) with a 100-fold excess of STV at 4 °C in the first incubation. STV was removed by gel purification, after which the sample was divided over 5 different samples and incubated with 5 different amounts of 2.2 kb DNA (B). After the gel extraction the concentration of the first molecule could not be determined precisely, but it can be estimated from the intensity of the lanes on the agarose gel. We estimate this to be about 10-20 ng, because the lanes in marker M contain 25 ng each (except for the lane at 5kb, which contains 75 ng). The molar ratios of B to A stated in the figure result from this estimation. The figure shows that the fraction of molecules A that does not form dimers does not significantly change even when we add approximately a 24-fold excess of the second molecule B. From the fact that neither a longer incubation time nor a higher concentration of molecule B improves the final yield of homo- or hetero-dimers we conclude that the maximum dimer formation is probably limited by the STV itself, as discussed also in the main text.

Detailed experimental information on figures 1-3 and S1-S4

The 3.5 kb and 2.2 kb DNA fragments used in figure 1, lane 2, were incubated as follows: 470 fmol of 3.5 kb DNA was incubated at 37 °C for 30 min with 20 pmol STV in buffer 1 (0.25x TBE at pH 8.3, 2 mM MgCl₂) in a total volume of 20 µl. Subsequently, the STV was removed using gel purification and 65 fmol 2.2 kb DNA was added. The buffer and volume were adjusted to 30 µl of buffer 1, and this was incubated for 45 min at 4 °C, after which the entire sample was loaded on gel. For lanes 3 and 4 we incubated 40 fmol 3.5 kb DNA with 4 pmol STV and 65 fmol 2.2 kb DNA with 6.5 pmol STV, respectively, at 37 °C for 30 min.

The sample loaded in lane 1 in figure 2(a) was prepared by incubating 250 fmol of 20 kb DNA (A) with 25 pmol STV in buffer 1 in a total volume of 40 μ l at 37 $^{\circ}$ C for 30 min. The STV was then removed using a Microcon YM-100 column, after which 250 fmol of 10 kb DNA (B) was added and buffer and volume were adjusted to 100 μ l of buffer 1. After overnight incubation at 4 $^{\circ}$ C, 10 μ l of the sample was loaded on gel.

The sample loaded in lane 2 in figure 3(a) was prepared by incubating 70 fmol of 3.5 kb DNA (A) with 7 pmol STV in buffer 1 for 30 min at 37 $^{\circ}$ C. After removal of the STV using column purification, the 70 fmol of 4.2 kb ssRNA was added, the buffer was adjusted to buffer 1, and the sample was incubated for 30 min at 4 $^{\circ}$ C. For lane 1 the same procedure was followed except that no STV was added. In lane 3 we loaded a sample prepared as follows: 100 fmol of 2.2 kb DNA (A) was incubated with 10 pmol STV in buffer 1 for 30 min at 37 $^{\circ}$ C. After removal of STV using column extraction 65 fmol of 4.2 kb dsRNA was added and this was incubated for another 30 min at 4 $^{\circ}$ C in buffer 1.

For the samples loaded in figure 3(b) lanes 1 and 2 we incubated a total amount of 75 fmol of 4.2 kb dsRNA in buffer 1 for 30 min at 37 $^{\circ}$ C. In lane 2, 7.5 pmol of STV was added before the incubation. After column purification, again 75 fmol of 4.2 kb dsRNA was added to both samples and the samples were incubation for another 30 min at 4 $^{\circ}$ C in buffer 1.

The 3.5 kb and 2.2 kb DNA fragments used in figure S1(a), lane 2, were incubated as follows: 100 fmol of 3.5 kb DNA was incubated at 37 $^{\circ}$ C for 30 min with 30 pmol STV in buffer 2 (0.5x TBE, pH=8.3 and 4 mM $MgCl_2$) in a total volume of 20 μ l. Subsequently, the STV was removed using a Microcon YM-100 column and 100 fmol 2.2 kb DNA was added. The buffer and volume were adjusted to 30 μ l of buffer 2, and this was incubated for 45 min at 37 $^{\circ}$ C, after which the entire sample was loaded on gel. The samples loaded in lanes 2 and 3 in figure S1(b) were prepared by incubating 200 fmol of 3.5 kb DNA (A) with 60 pmol of STV in a total volume of 20 μ l in. Incubation was done at 37 $^{\circ}$ C for 45 min. Lane 4 was loaded with the same amount of molecule A in buffer 2,

but no STV was added. Lane 1 was loaded with 100 fmol of 2.2 kb DNA. The bands in lanes 2, 3 and 4 were gel extracted and one of the samples was incubated with 140 fmol of 2.2 kb DNA (B). Buffer and volume were adjusted to 30 μ l of buffer 2 for all three samples. After incubation for 45 min at 37 °C, the entire samples were again loaded on gel in lanes 5 to 7. In figure S1(d) we used approximately 75 fmol of 2.2 kb DNA and 50 fmol of 3.5 kb DNA. Both molecules were incubated with a 100x excess of STV in buffer 1 at 37 °C for 30 min and loaded in lanes 2 (3.5 kb) and 3 (2.2 kb). In lane 1 the same amount of molecules was loaded in the absence of STV.

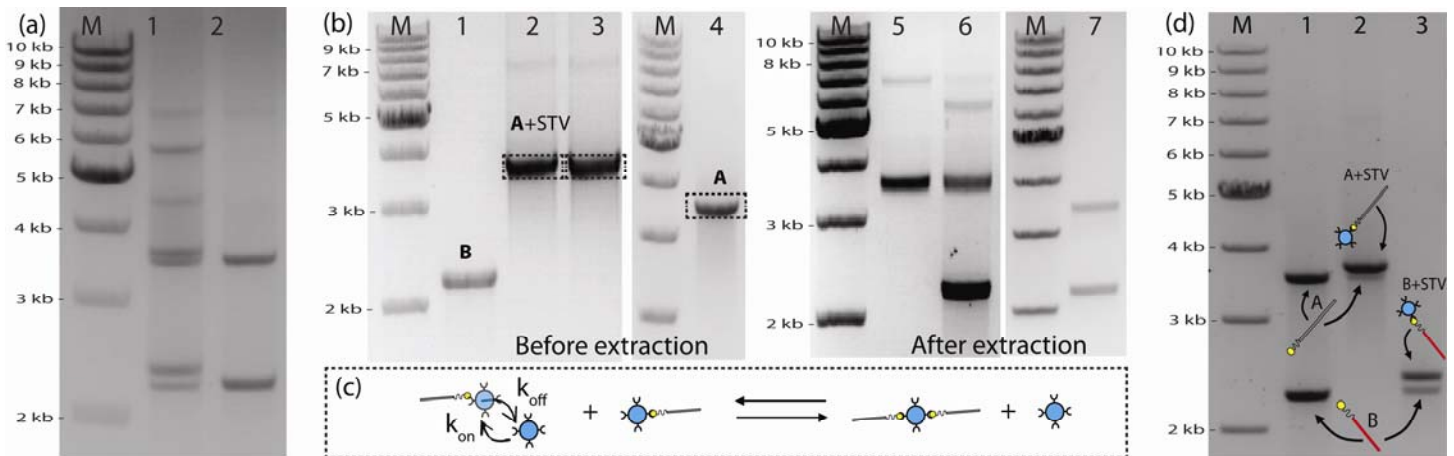
Detailed information on the samples loaded in figure S2 is given in table S1.

In figure S3 we used a single sample prepared as follows: 1 pmol of 2.2 kb DNA (A) was incubated with 30 pmol of STV in buffer 2 for 30 min at 37 °C. STV was then removed using column purification, and 650 fmol of 3.5 kb DNA (B) was added. The buffer and volume was adjusted to 100 μ l of buffer 2 and the sample was incubated at 37 °C. Samples of 7 μ l were taken out after the times indicated in the figure and stored at -20 °C. After the last sample was taken out all samples were loaded simultaneously on an agarose gel.

In figure S4 we used a single sample prepared as follows: 360 fmol of 3.5 kb DNA (A) was incubated with 20 pmol of STV for 30 min at 4 °C in buffer 2. STV was removed using gel purification, and the sample was divided in 5 samples containing equal amounts of the recovered A+STV fragment. To different samples we added 20 fmol, 40 fmol, 80 fmol and 160 fmol of 2.2 kb DNA (B), respectively and the samples were incubated at 4 °C for another 45 min.

FIGURES

Figure S1



Influence of temperature and STV purification (a) 0.8% agarose gel image of the result of our protocol at 37 °C instead of 4 °C and using column purification instead of gel purification. The same A (3.5 kb DNA) and B molecules (2.2 kb DNA) were used as in figure 1(b) in the main text. Apart from the bands discussed earlier, we now find two additional bands, corresponding to the homo-dimers A+A (at 7.0 kb) and B+B (at 4.4 kb).

(b) 0.8% agarose gel image of the result of our protocol at 37 °C, but using gel purification to remove the unbound STV after the first incubation. The B+B homo-dimer no longer appears, whereas the A+A homo-dimer is still present. This indicates that the B+B homo-dimer formation primarily forms because of residual STV after the removal. By using gel purification, all free STV is separated from the sample, and the homo-dimer B+B can no longer form. The A+A band, however, can still form because of dissociation of STV from the A+STV complex, as depicted in (c). This dissociation is more pronounced because of the higher temperature used compared to the normal protocol at 4 °C. Combining both low temperature with gel purification to remove the unbound STV is therefore essential to prevent homo-dimer formation.

(d) Electrophoretic shift due to STV: lane 1 contains A and B in the absence of STV and lanes 2 and 3 contain the molecules A and B, respectively, incubated with an excess of STV. The two upper bands correspond to molecules A and B bound to STV molecules. The relative intensity of the upper compared to the lower band is a measure of how many of the molecules are biotinylated (and can bind STV).

Figure S2

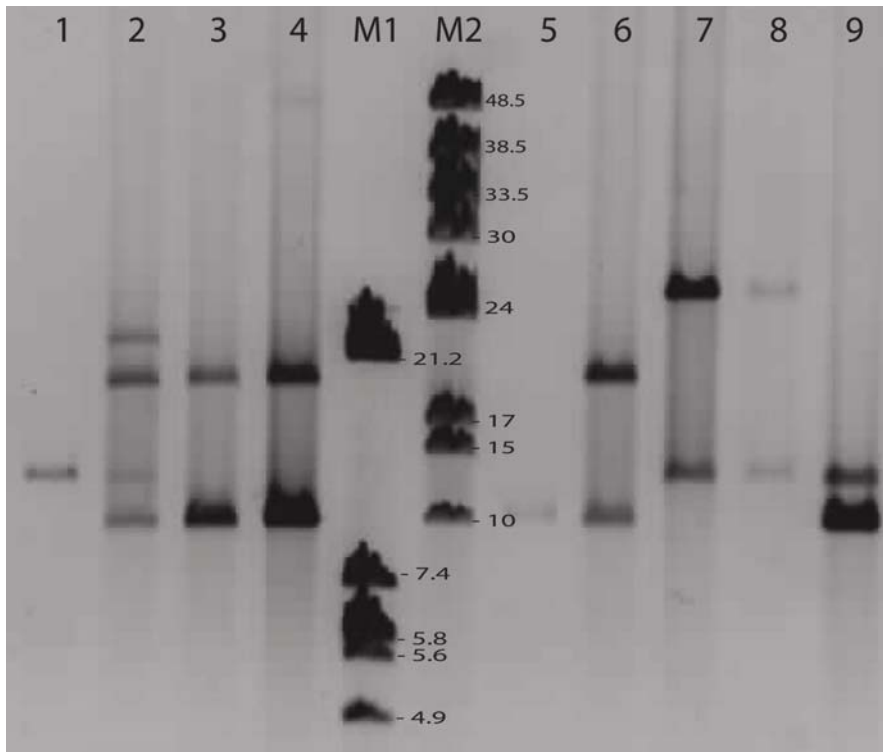


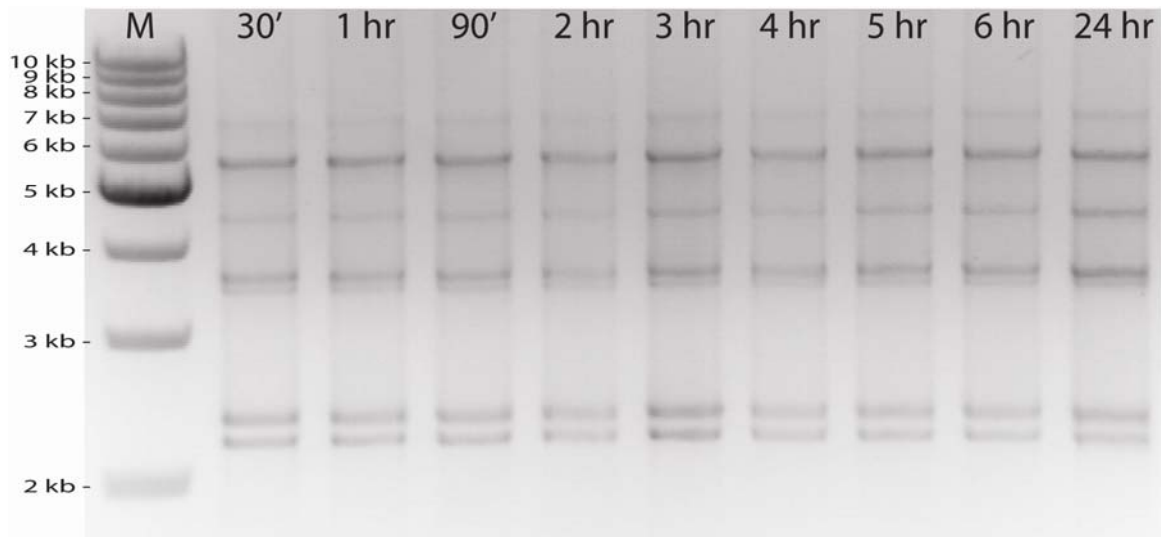
Table S2:

Lane	1st incubation	purification	2nd incubation	remark
1	12.7 kb + 100x STV	YM-100	n.a.	excess STV
2	12.7 kb + 100x STV	YM-100	9.6 kb	normal protocol
3	STV only	YM-100	9.6 kb	incomplete removal of STV
4	-	-	9.6 kb + 1x STV	equimolar STV in step 2
M1	-	-	-	
M2	-	-	-	
5	9.6 kb + 100x STV	YM-100	n.a.	excess STV
6	9.6 kb + 100x STV	YM-100	9.6 kb	protocol for homo-dimers
7	12.7 kb + 100x STV	YM-100	12.7 kb	protocol for homo-dimers
8	12.7 kb + 100x STV	YM-100	-	dissociation of STV
9	12.7 kb	YM-100	9.6 kb	no STV

Influence of concentration and incomplete removal of STV. Pulsed field electrophoresis agarose gel image showing the influence of STV during and after the 1st incubation. See table S2 for details on the contents of each lane.

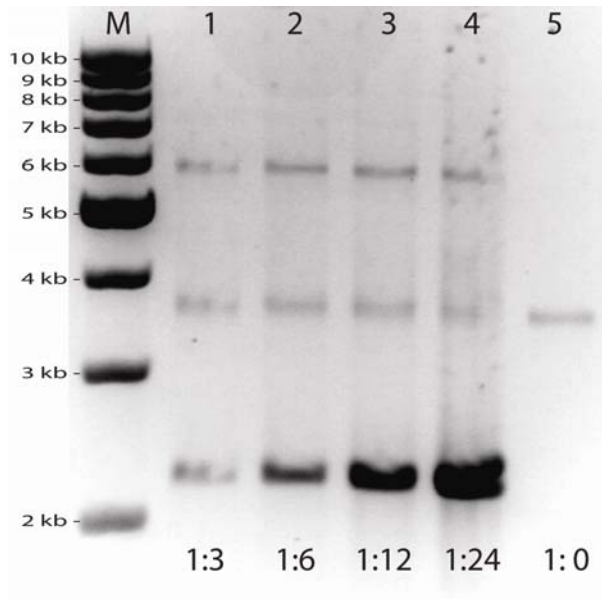
Lanes 1 and 5 show that an excess of STV prevents formation of homo-dimers in the 1st incubation step. In contrast, lane 4 was loaded with a 2:1 ratio of DNA:STV, clearly leading to dimer formation. Lane 8 shows the result of following our normal protocol, but not adding the B molecule. The dissociation of STV from the A+STV complex causes dimer formation after overnight incubation. The incomplete removal of STV by the YM-100 purification is illustrated in lane 3: only STV in buffer was purified over the YM-100 column, after which molecule B was added and loaded in lane 3 after 45 min of incubation. The presence of dimers indicates that not all STV was removed. The regular 2-step protocol is demonstrated in lane 2 for hetero-dimer formation (12.7 kb+9.6 kb), and in lanes 6 and 7 for homo-dimers (9.6kb+9.6 kb and 12.7 kb+12.7 kb respectively). No dimers are formed when the protocol is performed in the absence of STV, see lane 9.

Figure S3



Influence of duration of the second incubation step. Experiment in which the duration of the second incubation step was varied from 30 min to 24 hr. Molecule A (3.5 kb DNA) was incubated at 37 °C with a 30x molar excess of STV for 30 min and loaded on a gel. After gel extraction of the A+STV complex, molecule B (2.2 kb DNA) was added and the sample was incubated at 37 °C. Small samples were taken out after the times indicated above the lanes and immediately stored at -20 °C. After all samples were taken, the molecules were simultaneously loaded on the gel. No significant changes are observed in the relative intensity of the bands at 3.5 kb DNA, indicating that the equilibrium situation is already reached after 30 min.

Figure S4



Influence of relative concentration on dimer formation. Molecule A (3.5 kb) was incubated with different amounts of molecule B (2.2 kb) following our 2-step protocol at 4°C; gel extraction was used to remove all STV after the first incubation. The approximate relative molar concentrations of A:B are indicated below each lane. The intensity of the band at 3.5 kb (A molecules that do not form dimers) does not decrease significantly even when molecule B is added in 24-fold excess.