XRCC4:DNA Ligase IV Can Ligate Incompatible DNA Ends and Can Ligate Across Gaps

SUPPLEMENTARY TEXT

SUPPLEMENTARY MATERIALS AND METHODS

Oligonucleotides

All of the nonbiotinylated oligonucleotides used in this study were synthesized by Operon Biotechnologies, Inc. (Huntsville, AL). Biotinylated oligonucleotides were synthesized by the Microchemical Core Facility (Norris Cancer Center, USC). We further purified all of the oligonucleotides using 12% or 15% denaturing polyacrylamide gel electrophoresis (PAGE), and then determined the concentration spectrophotometrically. DNA sequencing of joined products confirmed the lack of errors in the oligonucleotide sequences. Possible contaminants in the oligonucleotide synthesis reagents or in the starting column for the oligonucleotide syntheses are, according to the manufacturer (Glen Research, Ann Arbor, MI) below the level that could account for our observed joined products.

Oligonucleotides were labeled at the 5'-end with [γ-^{32}P]ATP (3000 Ci/mmol) (PerkinElmer Life Sciences, Boston, MA) and T4 polynucleotide kinase (New England Biolabs, Beverly, MA) according to the manufacturer's instructions. Unincorporated radioisotope was removed by using G-25 Sephadex (Amersham Biosciences, Inc., Piscataway, NJ) spin-column chromatography. For the nonbiotinylated oligonucleotides, to make the double-stranded DNA substrate, labeled oligonucleotides were mixed with an equal amount of unlabeled complementary oligonucleotide in a buffer containing 10 mM Tris-hydrochloride, pH 8.0, 1 mM EDTA, pH 8.0, and 100 mM NaCl. The mixture was heated at 100°C for 5 min, allowed to cool to room temperature for 3h, and then incubated at 4°C overnight. For the biotinylated oligonucleotides, it is important to note that labeled oligonucleotides were mixed with half the molar amount of the

complementary biotinylated oligonucleotide (JG193) in a buffer containing 10 mM Tris-hydrochloride, pH 8.0, 1 mM EDTA, pH 8.0, and 100 mM NaCl. The mixture was heated at 100°C for 5 min, allowed to cool to room temperature for 3h, and incubated at 4°C overnight.

The sequences of the oligonucleotides used in this study are as follows:

JG55: 5'-GTT AAG TAT CTG CAT CTT ACT TGA CGG ATG CAA TCG TCA CGT GCT AGA CTA CTG GTC AAG CGG ATC GGG CTC GAG C-3'

JG67: 5'-GTT AAG TAT CTG CAT CTT ACT TGA CGG ATG CAA TCG TCA CGT GCT AGA CTA CTG GTC AAG CGG ATC GGG CTC GAG-3'

JG68: 5'- CGA GCC CGA TCC GCT TGA CCA GTA GTC TAG CAC GTG ACG ATT GCA TCC GTC AAG TAA GAT GCA GAT ACT TAA CAG-3'

JG161: 5'-GTT AAG TAT CTG CAT CTT ACT TGA CGG ATG CAA TCG TCA CGT GCT AGA CTA CTG GTC AAG CGG ATC GGG CTC GAG GGG-3'

JG162: 5'-CGA GCC CGA TCC GCT TGA CCA GTA GTC TAG CAC GTG ACG ATT GCA TCC GTC AAG TAA GAT GCA GAT ACT TAA CCC CC-3'

JG163: 5'-GTT AAG TAT CTG CAT CTT ACT TGA CGG ATG CAA TCG TCA CGT GCT AGA CTA CTG GTC AAG CGG ATC GGG CTC GAC C-3'

JG166: 5'-CGA GCC CGA TCC GCT TGA CCA GTA GTC TAG CAC GTG ACG ATT GCA TCC GTC AAG TAA GAT GCA GAT ACT TAA CAG G-3'

JG186: 5'-CGA GCC CGA TCC GCT TGA CCA GTA GTC TAG CAC GTG ACG ATT GCA TCC GTC AAG TAA GAT GCA GAT ACT TAA CA-3'

JG191: 5'-GTT AAG TAT CTG CAT CTT ACT TGA CGG ATG CAA TCG TCA CGT GCT AGA CTA CTG GTC AAG CGG ATC GGG CTC GTC-3'

JG192: 5'-CGA GCC CGA TCC GCT TGA CCA GTA GTC TAG CAC GTG ACG ATT GCA TCC GTC AAG TAA GAT GCA GAT ACT TAA CTC-3'

JG193: 5'-CGA GCC CGA TCC GCT TGA CCA GTA GTC TAG CAC GTG ACG ATT GCA TCC GTC AAG TAA GAT GCA GAT ACT TAA CX-3' (X=biotin)

JG194: 5'-GTT AAG TAT CTG CAT CTT ACT TGA CGG ATG CAA TCG TCA CGT GCT AGA CTA CTG GTC AAG CGG ATC GGG CTC GAG T-3'

HL3: 5'-CCT CTG AGG GCG AGC CCG AT-3'

YM42: 5'-AGG CTG TGT TAA GTA TCT GCA TTT TTT CGG ATC GGG CTC GCC CTC AGA GG-3'
JG171: 5'-GTT AAG TAT GCA TCT CTG CGA TGC ATG TCA CTC AGA CTA TGG TCA GCG ATC GGC TCG ACC-3'
JG172: 5'-CGA GCC GAT CGC TGA CCA TAG TCT GAG TGA CAT GCA TCG CAG AGA TGC ATA CTT AAC A-3'
JG173: 5'-GTT AAC TCG CAT GTA GTG TGC CTA CTT GCT CAA GCT GAC AGC TGT GAC CAG CGC TCG A-3'
JG174: 5'-CGA GCG CTG GTC ACA GCT GTC AGC TTG AGC AAG TAG GCA CAC TAC ATG CGA GTT AAC AGG-3'
JG185: 5'-GTT AAG TAT CTG CAT CTT ACT TGA CGG ATG CAA TCG TCA CGT GCT AGA CTA CTG GTC AAG CGG ATC GGG CTC GA-3'

Protein purification

The purification of Ku and DNA-PKcs have been described (Ma et al., 2002). XRCC4:DNA ligase IV complex was purified from baculovirus-insect cell system as described (NickMcElhinny et al., 2000). Native DNA polymerase mu was expressed and purified from E. coli, as described previously and in more details below (Tippin et al., 2004). We made some modifications to eliminate potential exonuclease contamination as described in detail below. Polymerase lambda was purified as a recombinant protein from E. coli as described (Shimazaki et al., 2002). DNA ligase I was a gift of Dr. A. Tomkinson (U. Maryland, Baltimore, MD). DNA ligase III was a gift of Dr. Ulf Grawunder (U. Basel, Switzerland).

Expression and purification of native DNA polymerase

Native DNA polymerase mu expression and purification were described previously (Tippin et al., 2004). We added some modifications to eliminate potential exonuclease contamination. Briefly, pol mu cDNA was subcloned into vector pET41b (Novagen) and the recombinant plasmid was used to express pol mu in strain BL21(DE3)R1L Codon Plus (Stratagene). Cells were grown at 30°C in LB medium supplemented with kanamycin (5 ug/mL) and chloramphenicol (30 ug/mL) to OD600 of 0.6. Protein expression was induced with isopropyl-1-thio-β-

D-galactopyranoside (1 mM) for 3 additional hours. Harvested cells were resuspended in lysis buffer (50 mM Tris-Cl (pH 7.5), 1 M NaCl, 2 mM DTT, and 10% sucrose) and lysed with lysozyme (2 mg/mL) with stirring for 1h at 4°C. Soluble protein was recovered by centrifugation (12,000 rpm 30 min in a SS-34 rotor at 4°C). The remaining steps were all carried out at 4°C. Ammonium sulfate was added to 40% saturation, and the protein was recovered by centrifugation (12,000 rpm 30 min in a SS-34 rotor at 4°C). We then resuspended the pellet in PC buffer (50 mM Tris-Cl (pH 7.5), 10% glycerol, 1 mM EDTA, 2 mM DTT) supplemented with 500 mM NaCl and dialyzed against the same buffer overnight. The dialyzed sample was diluted in PC buffer to 125 mM NaCl, and immediately loaded onto a Whatman P11 phosphocellulose column. The column was washed for 20 column volumes with PC buffer supplemented with 125 mM NaCl, and the protein was eluted with a 125 mM to 500 mM NaCl gradient over 10 column volumes. Pol mu containing fractions were pooled and concentrated using a 60% ammonium sulfate cut, and then resuspended and dialyzed in PC buffer supplemented with 250 mM NaCl, and loaded onto a Sephacryl 200 column (Amersham Biosciences) equilibrated with the same buffer. The purest peak fractions were then loaded onto a Mono S column (Amersham Biosciences). The column was washed for 15 column volumes with PC buffer supplemented with 100 mM NaCl, and the protein was eluted with a 100 mM to 1 M NaCl gradient over 20 column volumes. The eluted fractions were aliquoted and stored at -80°C.

Template-independent polymerization on substrates in free solution

The test for terminal deoxynucleotidyl transferase activity in free-solution was performed in a 10 uL reaction. 20 nM of the DNA substrates were first incubated with or without protein Ku in 1X ligation reaction buffer (25 mM Tris-hydrochloride, pH 7.5, 75 mM NaCl, 72.5 mM KCl, 2 mM DTT, 0.025% Triton X-100, and 100 uM EDTA) supplemented with 10% PEG (MW>8,000kD), 50 ug/mL BSA, and 5% glycerol at room temperature for 15 min. Addition was initiated by adding 100 μM of each dNTP, 10 mM $MgCl_2$ with different combinations of proteins as indicated. Reactions were then incubated at 37°C for 1 hour. After

incubation, reactions were stopped, deproteinized by organic extraction, and analyzed on 11% denaturing PAGE gel. Gels were dried, exposed in a PhosphorImager cassette, and the screen was scanned in a Molecular Dynamics PhosphorImager.

Template-independent polymerization on immobilized DNA substrates

10 pmol of biotinylated DNA substrates with an annealing ratio of 1:2 as described above were mixed with 50 ul of streptavidin-agarose suspended in 10 mL of 1X ligation reaction buffer. The mixture was incubated at room temperature on a rotator for 3 hour. After incubation, agarose beads were collected by low-speed centrifugation and washed more than 4 times with 5 mL of 1X ligation reaction buffer until the radiolabel level in the supernatant from the centrifugation was close to the background level. Then 50 uL agarose beads were mixed with 50 uL of 1X ligation reaction buffer**.** The polymerization test using bead-bound substrate was performed in a 20uL reaction. Biotinylated DNA substrates (20 nM) were first incubated with or without protein Ku in 1X ligation reaction buffer supplemented with 10% PEG (MW>8,000kD), 50 ug/mL BSA, and 5% glycerol at room temperature for 15 min. Addition was initiated by adding dNTP at 500 uM or 5 mM and 10 mM $MgCl_2$ with different combinations of proteins as indicated. Reactions were then incubated at 37°C for 1 hour. After incubation, reactions were heated to 100°C for 5 min to denature proteins (and specifically to disrupt the biotin-streptavidin association), deproteinized with organic extraction, and analyzed by 11% denaturing PAGE gel. Gels were dried, exposed in a PhosphorImager cassette, and the screen was scanned in a Molecular Dynamics PhosphorImager.

DNA ligation assay

The DNA ligation assay was performed in a 10 uL reaction. DNA substrates (20 nM) were first incubated with or without protein Ku in 1X ligation reaction buffer supplemented with 10% PEG (MW>8,000kD), 50 ug/mL BSA, and 5% glycerol at room temperature for 15 min. Ligation was initiated by adding 100 uM of each dNTP, 10 mM $MgCl_2$ with different combinations of proteins as indicated. Reactions were then incubated at 37°C for 30 min. After incubation,

reactions were stopped, deproteinized with organic extraction, and analyzed by 8% denaturing PAGE gel. Gels were dried, exposed in a PhosphorImager cassette, and the screen was scanned in a Molecular Dynamics PhosphorImager.

Sequencing of junctions of the ligation products

Dried gels were further exposed to Kodak films. After overnight exposure, films were developed and then dimer ligation products (single-stranded because they were being purified from a denaturing gel) were cut out of the gels by aligning the gel with the film. Gel pieces were then soaked in appropriate amount (~30uL) of TE (10 mM Tris-hydrochloride, pH 8.0, 1 mM EDTA, pH 8.0) overnight. Junctions were then amplified from the dimer ligation products by polymerase chain reaction (PCR). PCR conditions were as follows: 95°C 3 min; 35 cycles of 94°C 45s, 53°C 1.25 min, 72°C 30s; 72°C 2 min. The sequences for the PCR primers were: JG187 (5'-TGCTAGACTACTGGTCAAGC-3'), JG188 (5'-TGCATCCGTCAAGTAAGATG-3'). The size of PCR products was further confirmed by 10% native PAGE gel and cloned into the Top TA cloning vector pCR2.1 (Invitrogen, Calsbad, CA) according to the manufacturer's instructions. Plasmid was then transformed into E. coli, and individual clones were sequenced on a Li-Cor sequencer (Li-Cor, Lincoln, NE) following the manufacturer's instructions.

For Supplementary Figure 6C, reactions were re-run on an 8% native PAGE gel, then the dimer ligation products (double-stranded because they were being purified from a native gel) were cut out of the gels using the same method as above. Dimer ligation products extracted from the gel pieces with TE were then directly TA-cloned and sequenced without any PCR step.

Template-dependent primer extension assay

The primer extension assay was performed in a 10 uL reaction. Radioactively labeled DNA substrate (25 nM) was incubated with the indicated amount of polymerase in 1X primer extension buffer (25 mM Tris-Cl, pH 8.0, 50 mM KCl, 5 mM MgCl2, 1 mM DTT) supplemented with 50 ug/mL of BSA and 50 uM of dNTP mix (50 uM each) at 37°C for 60 min. After incubation, reactions

were directly analyzed by 10% denaturing PAGE gel. Gels were dried, exposed in a PhosphorImager cassette, and the screen was scanned in a Molecular Dynamics PhosphorImager.

SUPPLEMENTARY RESULTS

The head to tail orientation of joining of the substrate in Figures 3 and 4 is favored over the head-to-head orientation. This is likely because a 5'P is located at each side in a head-to-head orientation, and we find that this is disfavored (Suppl. Fig. 5), perhaps due to charge repulsion between the two 5' phosphorylated ends.

REFERENCES

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Tippin, B., Kobayashi, S., Bertram, J.G. and Goodman, M.F. (2004) To slip or skip, visualizing frameshift mutation dynamics for error-prone DNA polymerases. *J. Biol. Chem.*, **279**, 5360-5368.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Template-independent polymerase activity of polymerase mu provides short terminal microhomology for ligation.

(A) Two 73 bp substrates with 3' overhangs were used to test for the ligation by XRCC4: DNA ligase IV. An asterisk indicates the position of the radioisotope label.

(B) This figure is from the same denaturing PAGE gel as Figure 1B. In Figure 1B, we focused on the substrate addition part, while here we focused on the

ligation products, which are much higher on the gel. There is a crack on the top of the gel.

(C) Dimer products from the selected lanes were cut out of the gel, extracted, and then PCR amplified, TA-cloned and sequenced. The junction sequences for the ligatable strand are shown. Though 9 of 10 sequences from lane 8 contained a CC junctional addition, 1 sequence contained a CT addition. Deamination of dCTP to form dUTP presumably accounts for this one T.

Supplementary Figure 2. Template-independent polymerase activity of polymerase mu on immobilized DNA substrates.

(A) Streptavidin agarose beads were used to immobilize two 73bp DNA substrates with 3' overhangs. B designates the 3' biotin group of the substrate. An asterisk indicates the position of the radioisotope label.

(B) This image is simply a darker exposure of Figure 2B to illustrate the multiple cycles of addition using dCTP and dTTP by polymerase mu. No ligation products are seen even on this darker exposure, which documents that the bead immobilization precludes any end-to-end interaction.

Supplementary Figure 3. Ribonucleotide addition by polymerase mu on immobilized DNA substrates.

(A) Diagram of immobilized substrate.

(B) The same substrates as in Figure 2A were used to test for pol mu template-independent polymerase activity with ribonucleotides. In each reaction, 20nM substrate was incubated with the protein(s) indicated above each lane in a 20uL reaction for 1h at 37°C. After incubation, reactions were heated at 100°C for 5 min to disrupt the biotin-streptavidin interaction, and then deproteinized and analyzed by 11% denaturing PAGE. Protein concentration: pol mu, 1.25uM. 500uM of each dNTP/NTP was added to the reaction. No Ku or XRCC4:DNA ligase IV was added in the reaction.

Supplementary Figure 4. Template-dependent primer extension activity of pol mu and pol lambda.

(A) A double-stranded DNA substrate with a 5' overhang was designed to test the template-dependent primer extension of Pol X family polymerases. An asterisk indicates the position of the radioisotope label.

(B) In each reaction, 25 nM substrate was incubated with the polymerase in a 10 uL reaction for 1h at 37°C. After incubation, reactions were directly analyzed by 10% denaturing PAGE. Protein concentrations varied as indicated. 50 uM of dNTP mix (50 uM each) was included to each reaction.

Supplementary Figure 5. Phosphate repulsion can decrease the ligation efficiency.

(A) The same substrate as in Figure 3A was tested for the phosphate effect on ligation efficiency. The left substrate is exactly the same as in Figure 3A in which the bottom strand has a 5' OH end. The bottom strand of the right substrate was also phosphorylated on the 5' end. Two alternative joining products are proposed below each substrate. An asterisk indicates the position of the radioisotope label.

(B) In each reaction, 20nM substrate was incubated with the protein(s) indicated above each lane in a 10uL reaction for 30min at 37°C. After incubation, reactions were deproteinized and analyzed by 8% denaturing PAGE. Protein concentrations: Ku, 25nM; X4-LIV, 50nM; pol mu or lambda, 25nM. Each reaction contains all the four dNTPs (100uM each). 100uM of ATP was also added in each reaction. "M" indicates 50bp DNA ladder.

Supplementary Figure 6. XRCC4:DNA ligase IV and Ku can ligate over a gap.

(A) Two 57 bp substrates with 3' overhangs were tested for ligation in the same reaction. One substrate (JG171/172) has 5' OH on both strands. The top strand from the other substrate (JG*173/174) was radioactively labeled on the 5' end. We note there are at least four joining pathways for the dimer product. However, the proposed joining pathway under the substrate should be favored due to homology on the end for ligation. The backbone (central) sequences (the portion drawn as a top and bottom strand line) for the left duplex is different from the backbone sequence for the right duplex.

(B) In each reaction, 10nM cold substrate and 10nM hot substrate were incubated with the protein(s) indicated above in a 10uL reaction for 30 min at 37°C. After incubation, reactions were deproteinized and analyzed by 8% denaturing PAGE. Protein concentrations: Ku, 25nM; X4-LIV, 50nM; pol mu or lambda, 25nM. Each reaction contains all the four dNTPs (25uM each). 100uM of ATP was also added in each reaction.

(C) The same reactions as in (B) were run on an 8% native PAGE. Then dimer product from lane 4 (a reaction that included only XRCC4:DNA ligase IV and Ku) was cut out of the gel, extracted, and then directly TA-cloned and sequenced. The junction sequences for the ligatable strand were provided.

Supplementary Figure 7. Time course of ligation of a nick, a 1nt-gap, and a fully incompatible DNA end substrate by XRCC4:DNA ligase IV and Ku.

(A) JG*161/162 is a substrate with a nick on the ligatable strand. JG*163/166 is a substrate with a 1 nt-gap on the ligatable strand and with a two base pair terminal microhomology for ligation. JG*163/186 is a substrate with fully incompatible ends. A star indicates the position of the radioisotope label.

(B) In each reaction, 20nM substrate was incubated with the protein(s) indicated above the gel in a 10uL reaction for the indicated time at 37^{o}C. After incubation, reactions were deproteinized and analyzed by 8% denaturing PAGE. Protein concentrations: Ku, 25nM; X4-LIV, 50nM. “M” indicates 50bp DNA ladder.

(C) Ratio of ligation products versus total reaction substrate was quantified and provided.

Supplementary Figure 8. XRCC4:DNA ligase IV and Ku can ligate fully incompatible DNA ends.

(A) A substrate without any homology for ligation was tested for the direct ligation by XRCC4:DNA ligase IV and Ku. Two alternative joining pathways are proposed under the substrate. An asterisk indicates the position of the radioisotope label.

(B) In each reaction, 20nM substrate was incubated with the protein(s) indicated above each lane in a 10uL reaction for 30min at 37°C. After incubation, reactions were deproteinized and analyzed by 8% denaturing PAGE. Protein

concentrations: Ku, 25nM; X4-LIV, 50nM; pol mu or lambda, 25nM. Each reaction contains all the four dNTPs (25uM each). 100uM of ATP was also added in each reaction.

(C) Dimer products from the selected lanes were cut out of the gel, extracted, and then PCR amplified, TA-cloned and sequenced. The junction sequences for the ligatable strand are listed.

Supplementary Figure 9. Ligation for some substrates requires both polymerase and specific nucleotides.

(A) The diagram illustrates the two strands of a duplex substrate with 2 nt 3' overhangs. The asterisk indicates the radiolabel at one 5' end.

(B) This figure is from the same denaturing PAGE gel as Suppl. Fig. 1B. In Suppl. Fig. 1B, we focused on the substrate addition part, while here we focused on the ligation product portion of the gel.

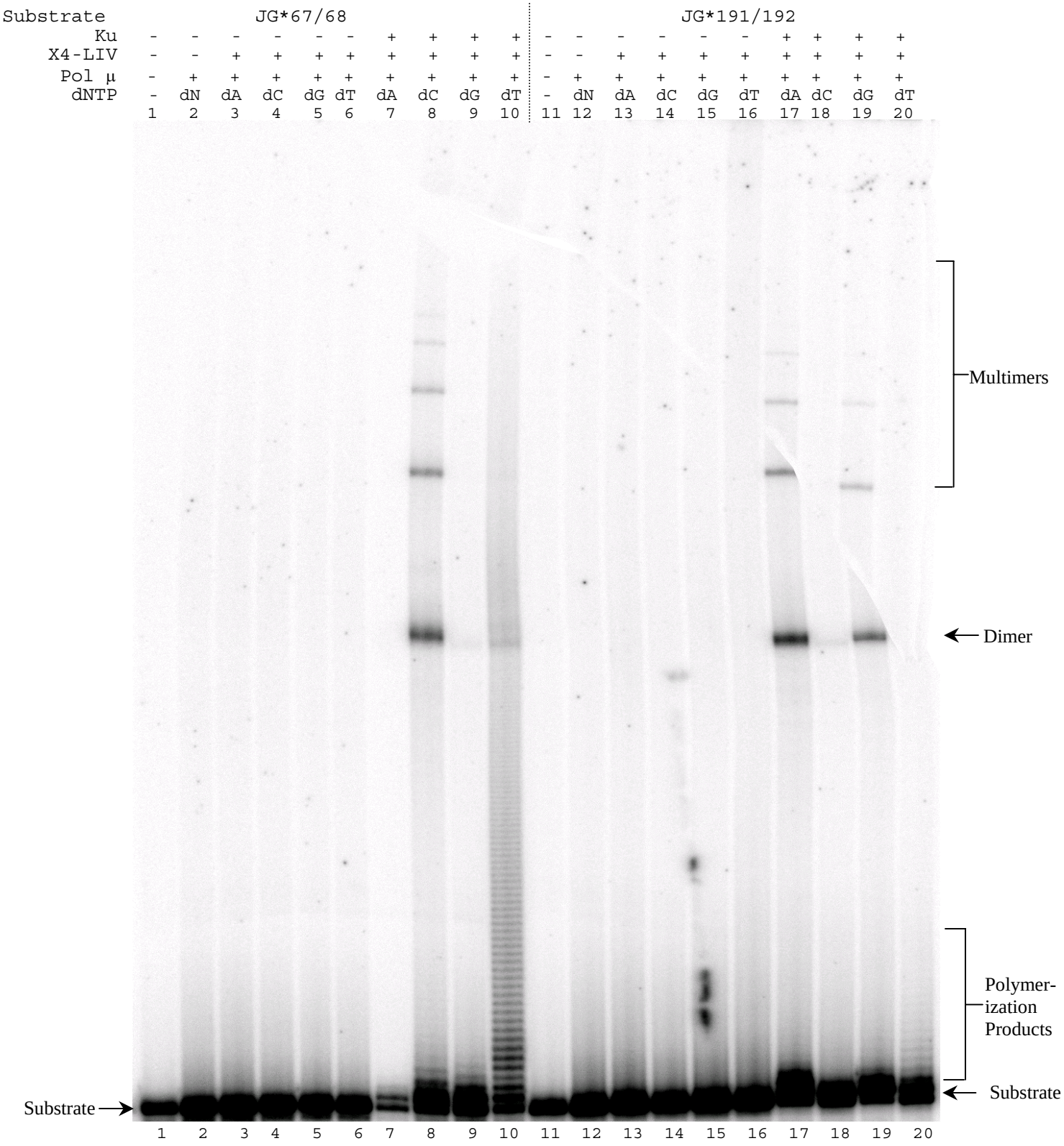
Supplementary Figure 10. DNA-PKcs effect on substrate ligation with polymerase presence.

(A) A 73bp substrate with 3' –TC overhangs was tested for the effect of DNA-PKcs on the substrate ligation. This substrate could not be ligated without the presence of polymerase mu. A star indicates the position of the radioisotope label.

(B) In each reaction, 20nM substrate was incubated with the protein(s) indicated above the gel in a 10uL reaction for 60 min at 37^{o}C. After incubation, reactions were deproteinized and analyzed by 8% denaturing PAGE. Protein concentrations: Ku, 25nM; DNA-PKcs, 25nM; X4-LIV, 50nM; pol mu, 25nM. 100uM of each dNTP was also added to each reaction. DNA-PKcs was pre-incubated with the DNA substrate and Ku at 20°C for 15 min prior to the ligation.

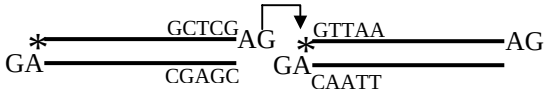
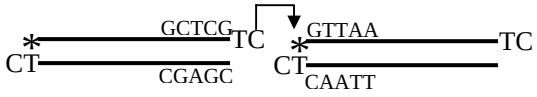
A.

B.



Suppl. Fig. 1A,B

C.

JG*67/68 			JG*191/192 		
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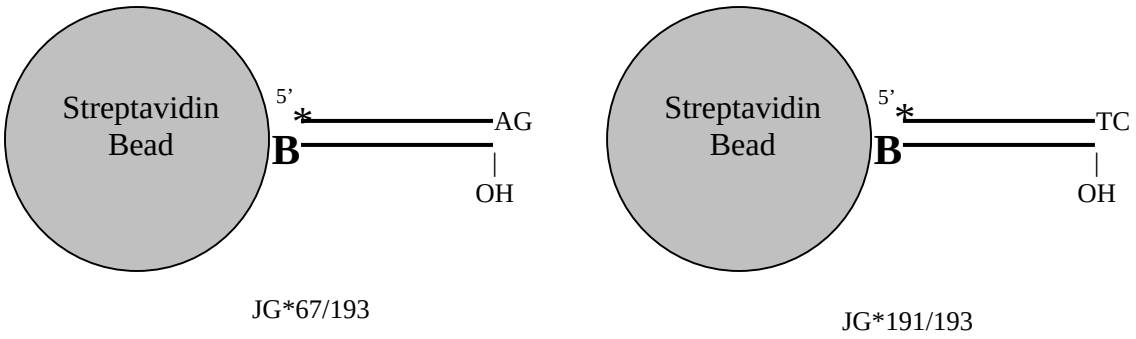
10	a.) GCTCGAG***T***GTTAA	4	19	a.) GCTCGTC***GG***GTTAA	3

Suppl. Fig. 1C

A.



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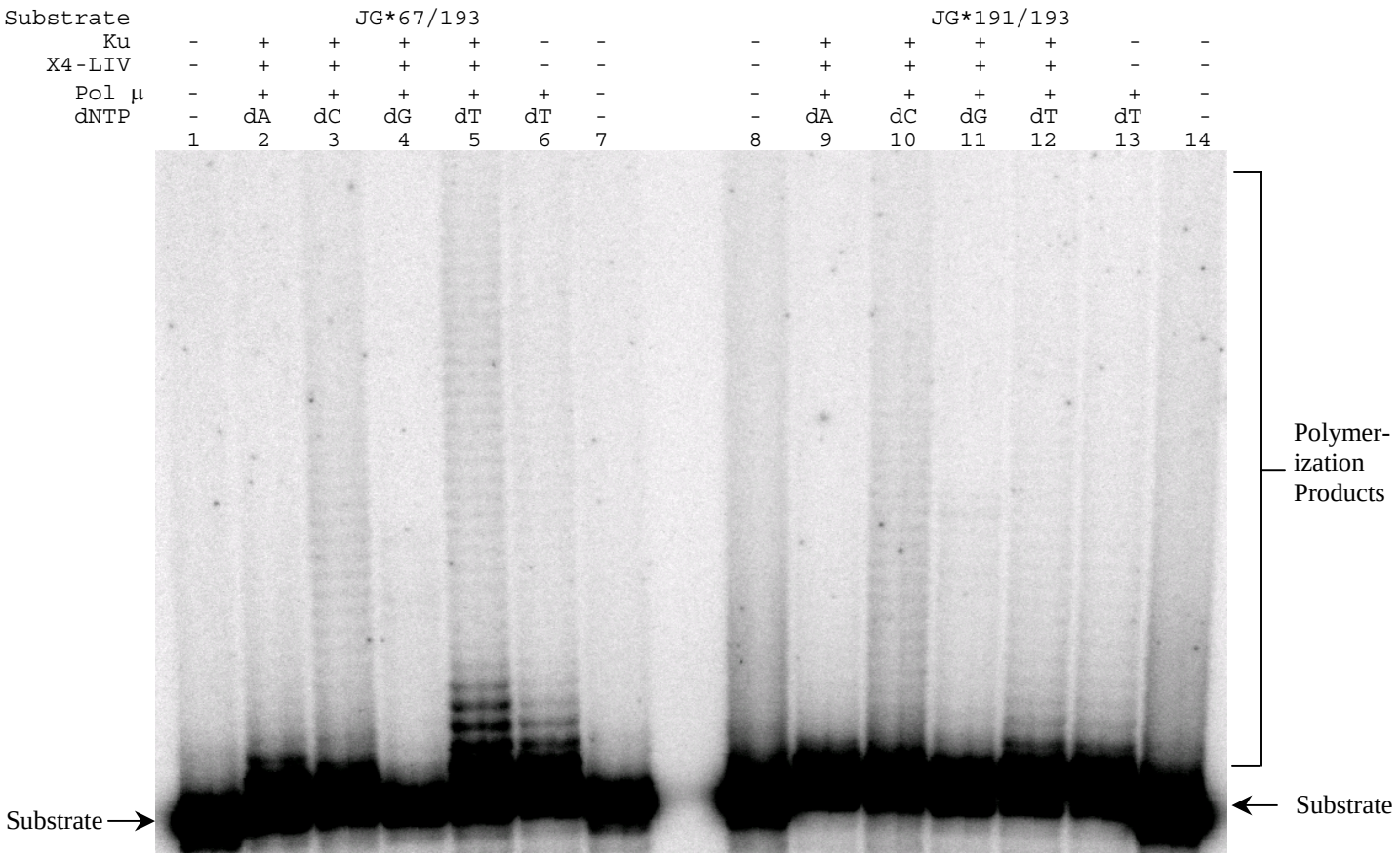
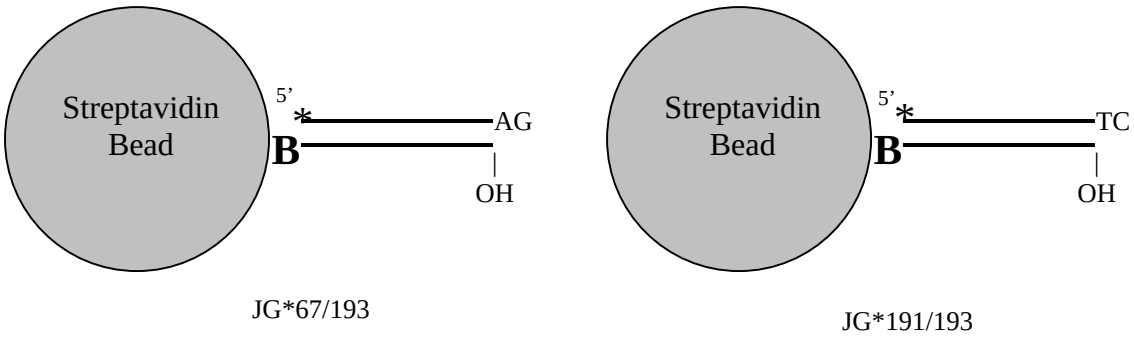


Suppl. Fig. 2

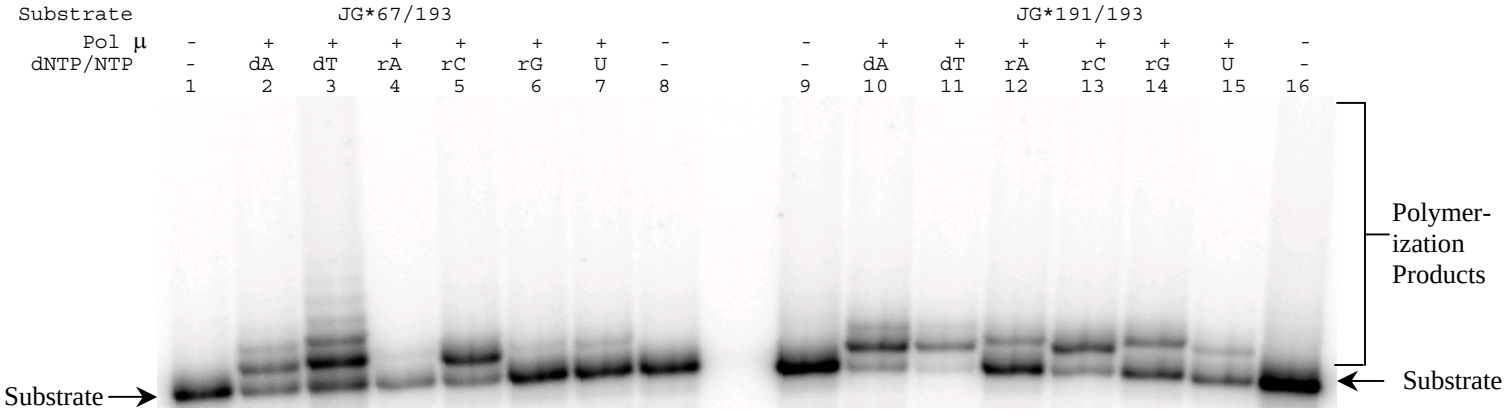
A.



JG*67/193

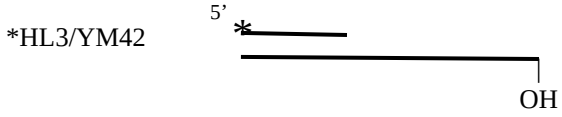
JG*191/193

B.



Suppl. Fig. 3

A.



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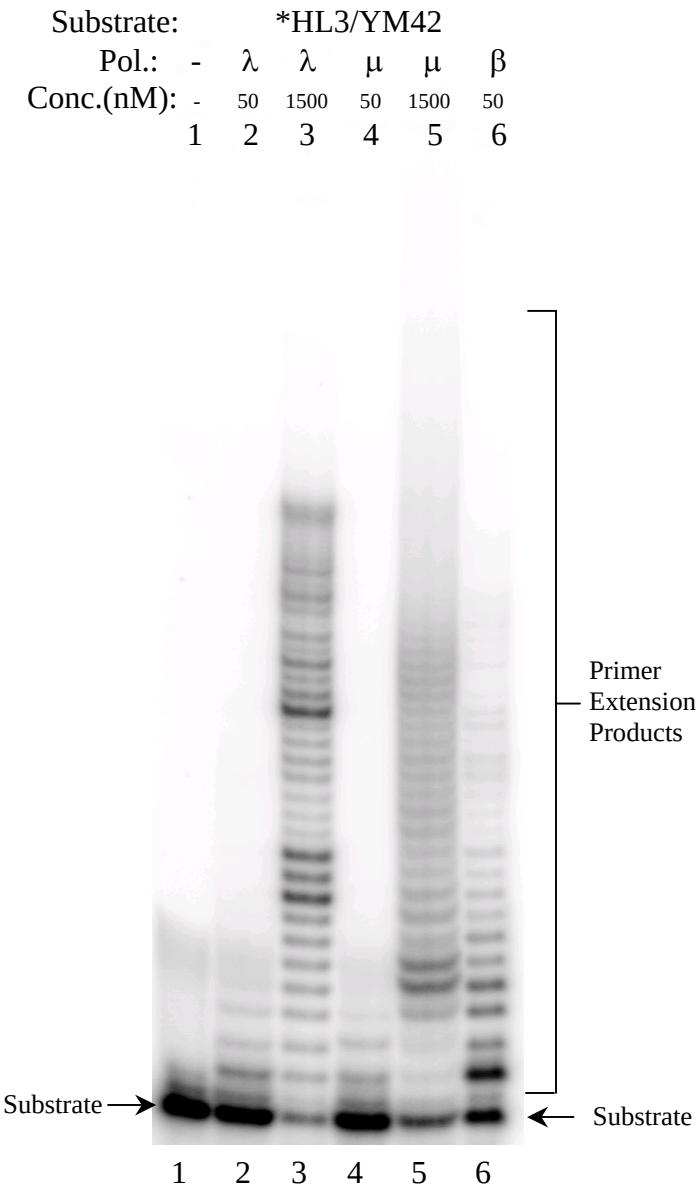
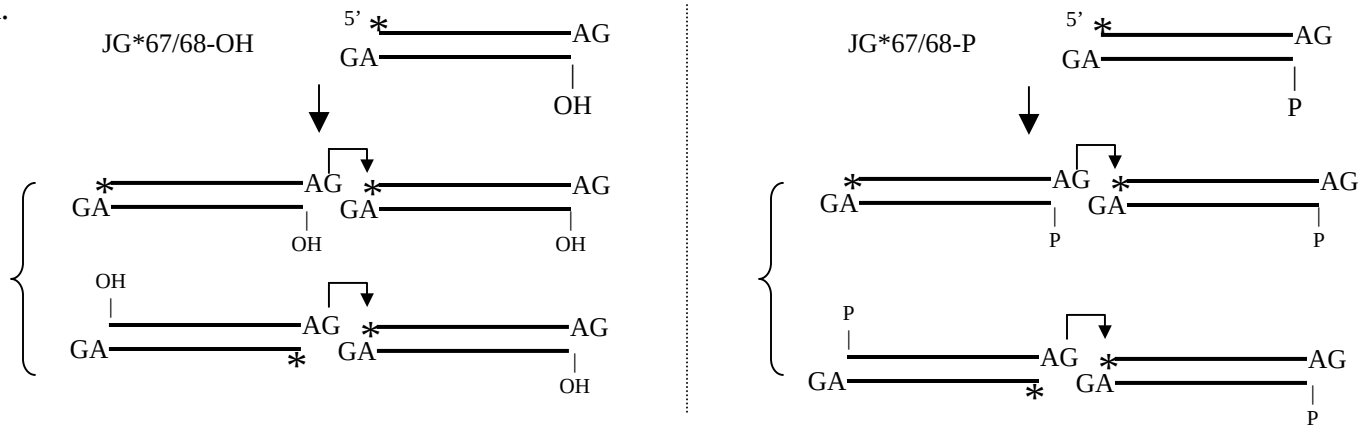


Suppl. Fig. 4

A.



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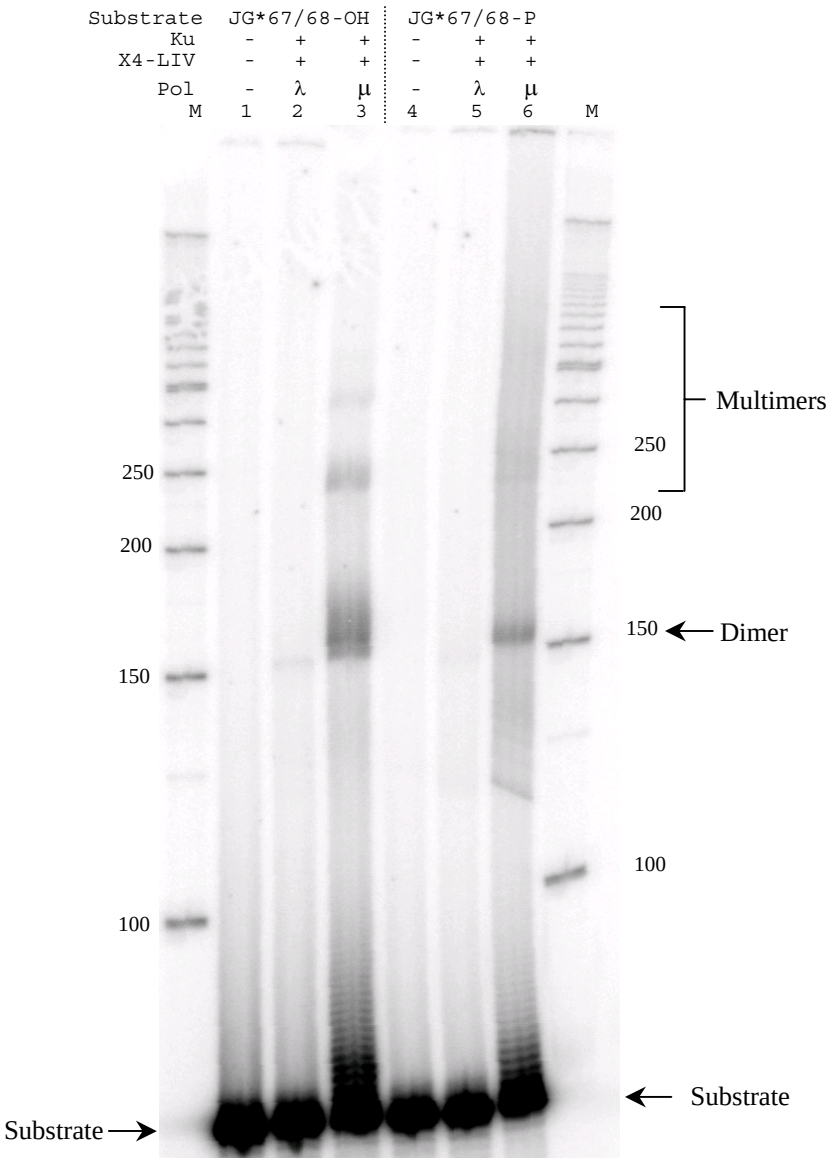
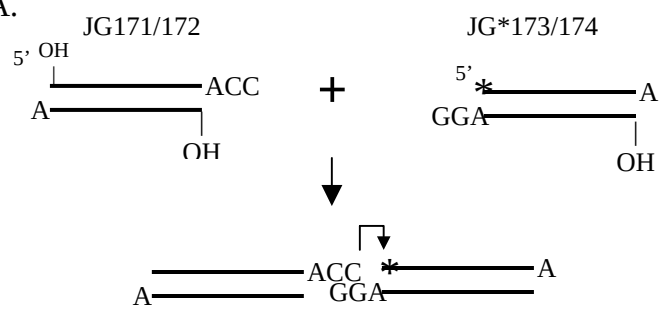


Suppl. Fig. 5

A.



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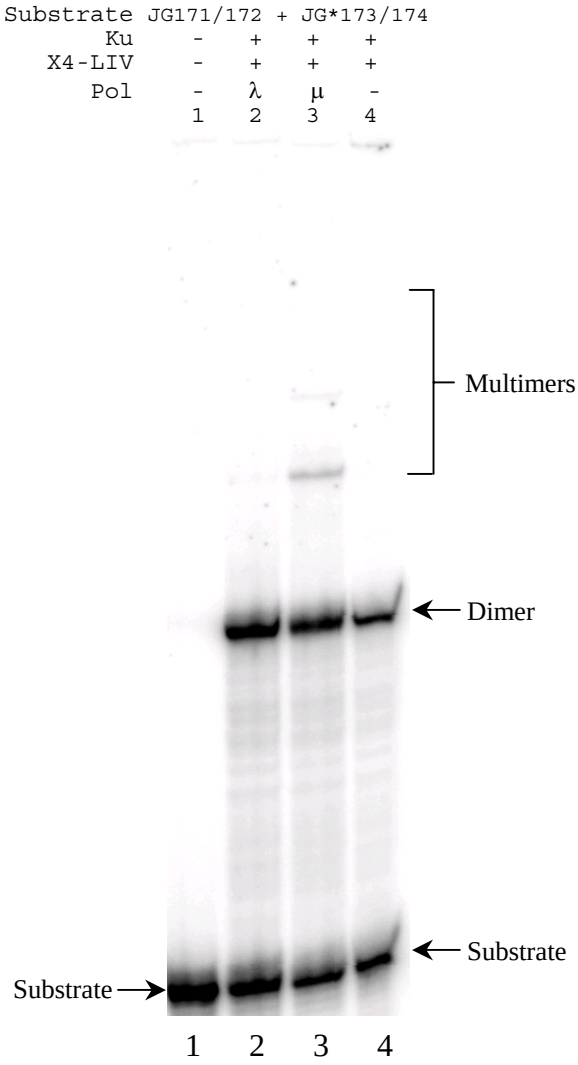
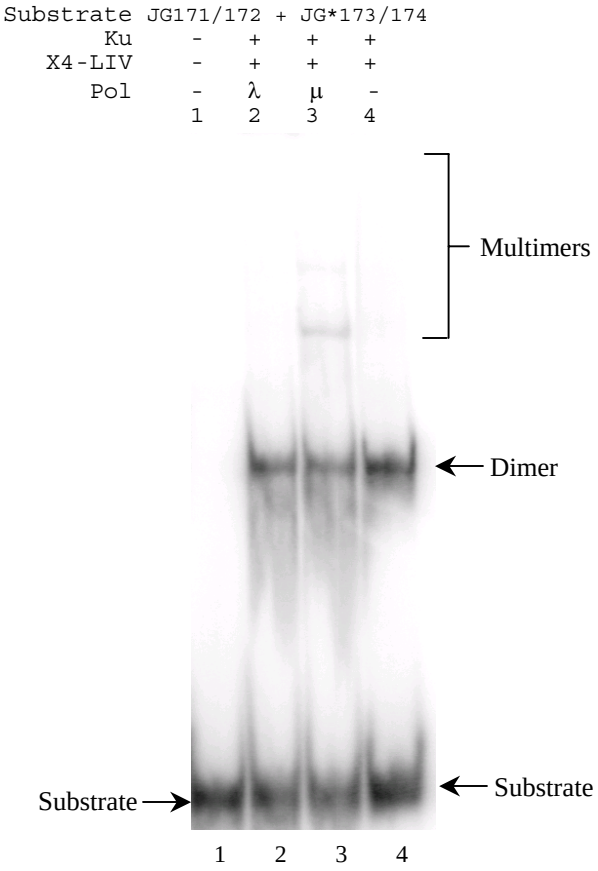
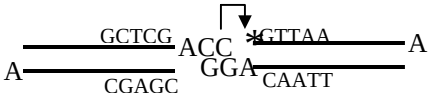


C.



JG171/172 + JG*173/174

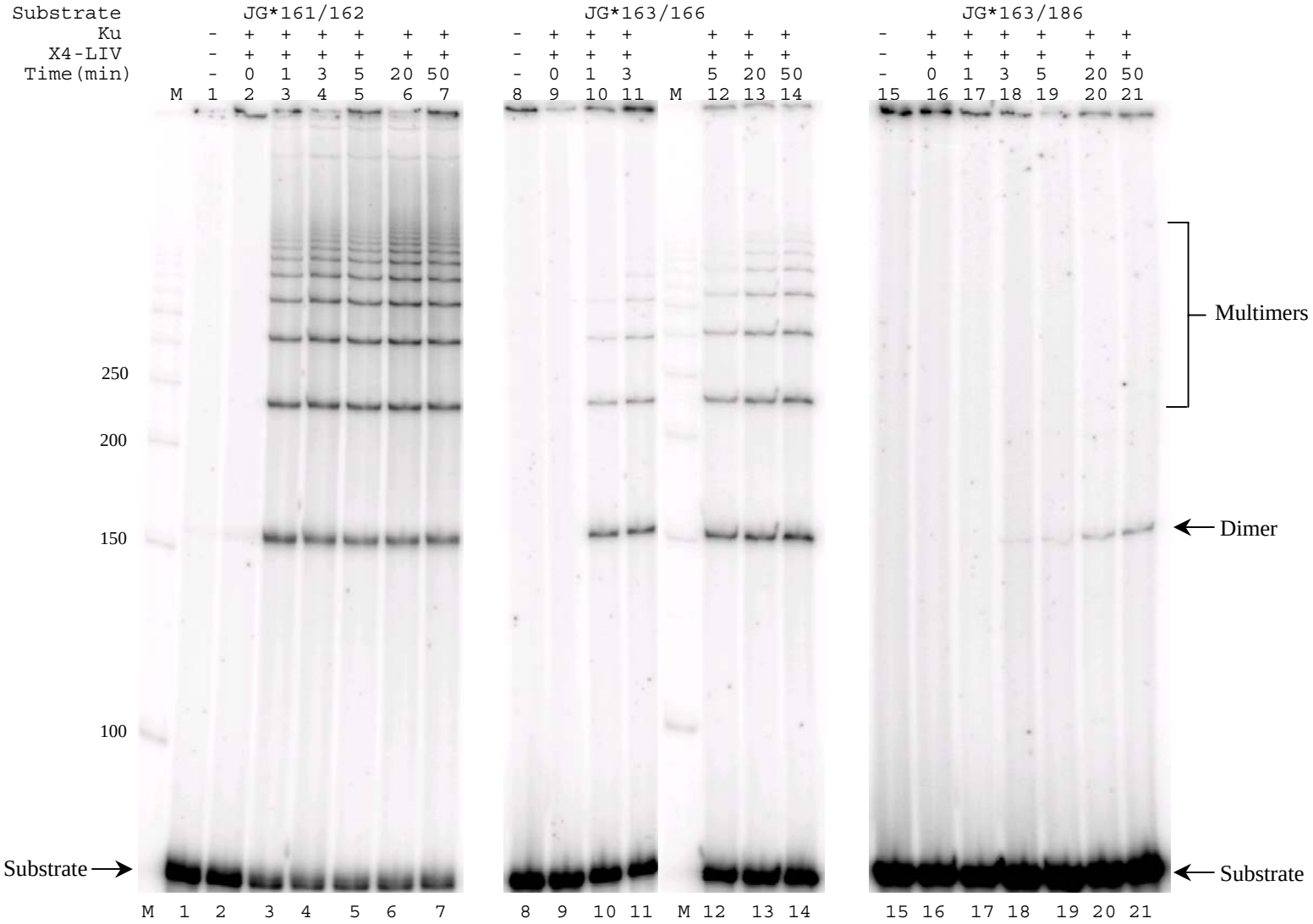


Lane No.	Junction Sequence	No. Molecules Sequenced
4	a.) GCTCGACCGTTAA	14

Suppl. Fig. 6

A.

B.



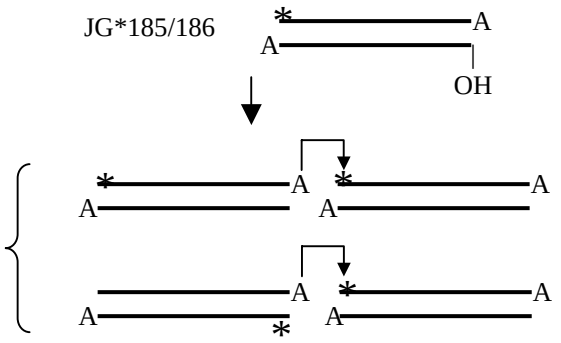
Suppl. Fig. 7A,B.

C.

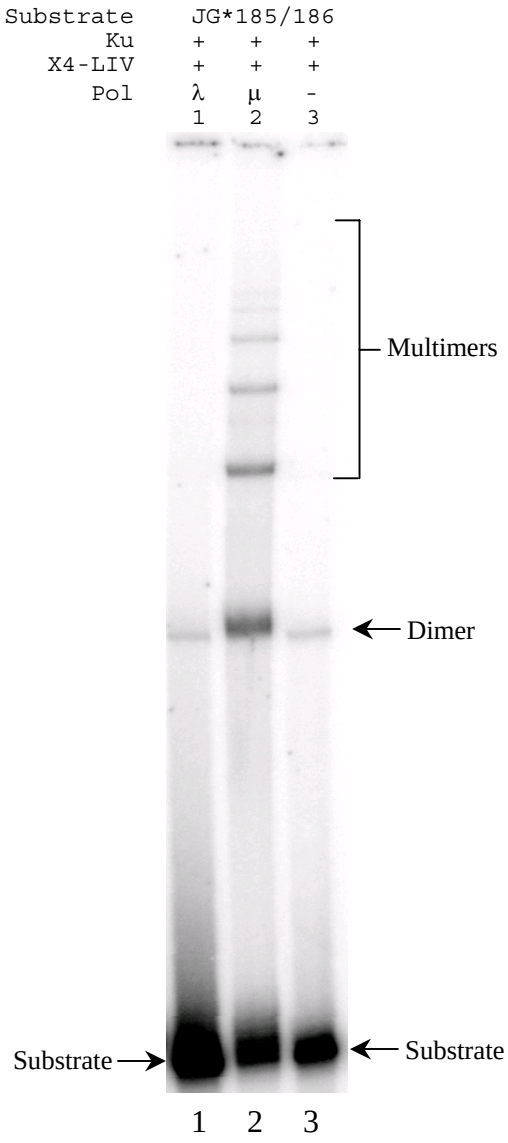
JG*161/162			JG*163/166			JG*163/186		
Lane No.	Time (min)	Product conversion	Lane No.	Time (min)	Product conversion	Lane No.	Time (min)	Product conversion
2	0	0	9	0	0	16	0	0
3	1	46%	10	1	6%	17	1	0
4	3	59%	11	3	9%	18	3	0.04%
5	5	60%	12	5	11%	19	5	0.05%
6	20	67%	13	20	13%	20	20	0.26%
7	50	63%	14	50	15%	21	50	0.45%

Suppl. Fig. 7C.

A.



B.



C.

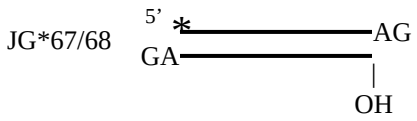
JG*185/186

GCTCG A GTTAA
A A
CGAGC CAATT

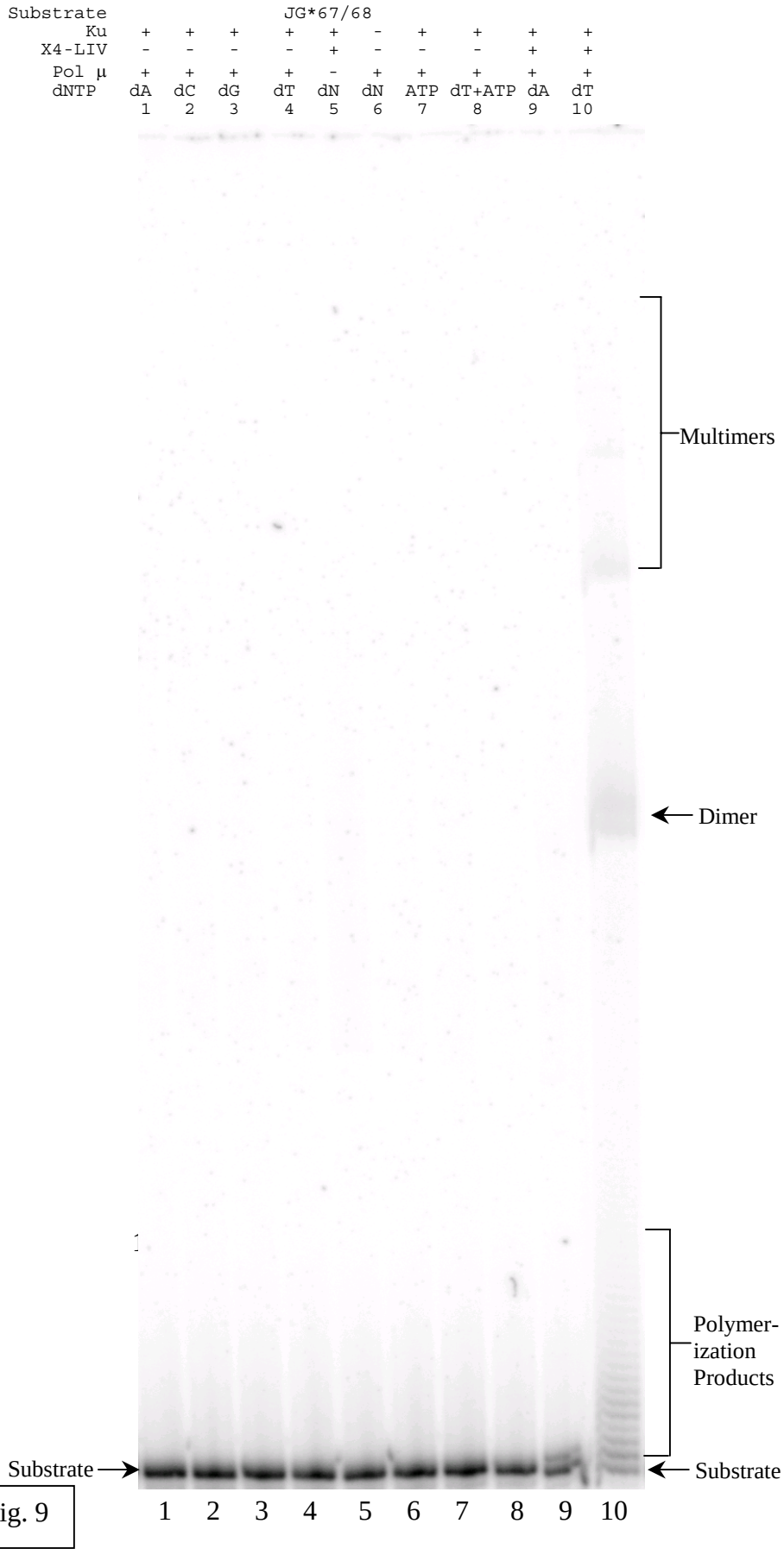
Lane No.	Junction Sequence	No. Molecules Sequenced
2	a.) GCTCGA***T***GTTAA b.) GCTCGAGTTAA	6 1
3	a.) GCTCGAGTTAA	13

Suppl. Fig. 8

A.

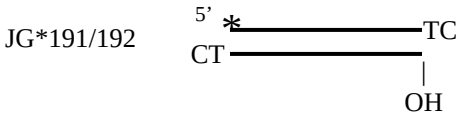


B.

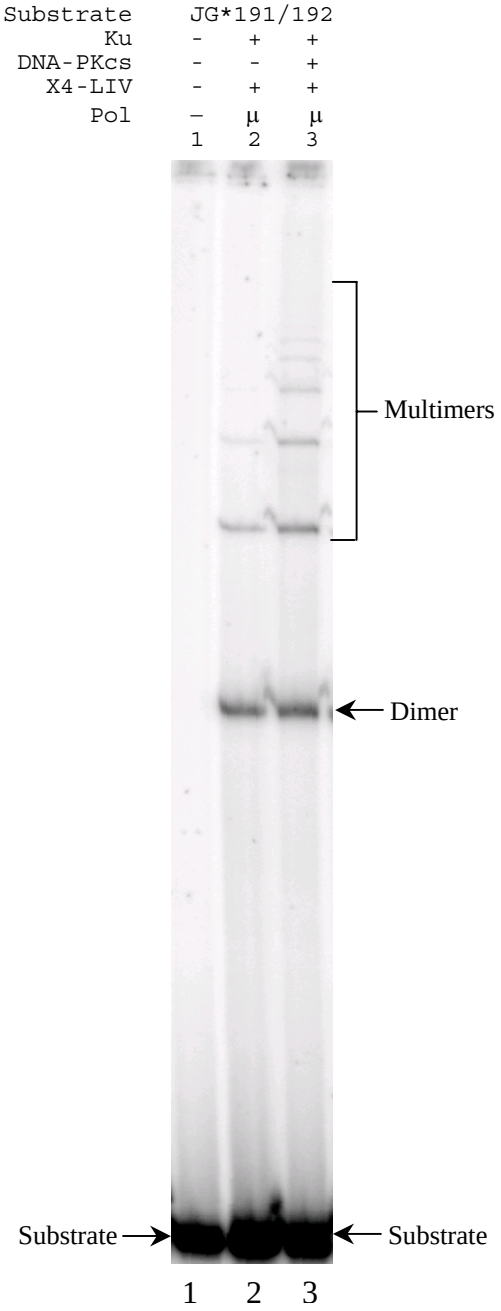


Suppl. Fig. 9

A.



B.



Suppl. Fig. 10