Supplemental Material for Wang et al.

List

Supplemental Materials and Methods

Oligonucleotide-based DNA substrates

All the oligonucleotides listed in Supplemental Table S1 were purchased from Integrated DNA Technologies (IDT) and purified by denaturing polyacrylamide gels containing 7 M urea in TAE buffer (40 mM Tris-acetate, pH 8.3, 1 mM EDTA) and electro-elution from gel slices into the same buffer. 3' DNA end labeling was done with [α-^{32}P]cordycepin 5'-triphosphate (PerkinElmer) and terminal transferase (Roche), while 5' end labeling was accomplished using [γ-^{32}P]ATP (PerkinElmer) and T4 polynucleotide kinase (New England Biolabs). The unincorporated radioactive nucleotide was removed using a Micro Bio-Spin 6 column (Bio-Rad) according to the manufacturer's instructions.

Substrates were constructed by hybridizing the indicated oligonucleotides (see Supplemental Table S1 for the complete list). The 70-bp substrate was prepared using 70-bp-F and 70-bp-R3; the 70-bp substrate that harbors biotin moieties at both 3' and 5' termini was made using 70-bp-F2 and 70-bp-R2; the 100-bp substrate was prepared using 100-bp-F and 100-bp-R2; the 70-bp dsDNA with 5-nt 3' overhangs was generated using 5-dA-F and 5-dA-R; the 70-bp dsDNA with 10-nt 3' overhangs was prepared with 10-dT-F and 10-dT-R; the 70-bp dsDNA with 30-nt 3' overhangs was prepared with 30-dT-F2 and 30-dT-R2; the 95-bp dsDNA substrate with 5-nt 3' overhangs and a terminal biotin moiety was prepared using 100-bp-F4 and 100-bp-R3; the 95-bp dsDNA with 5-nt 3' overhangs and an internal biotin moiety was generated using 100-bp-F5 and 100-bp-R3; and the 95-bp dsDNA that harbors 5-nt 3' overhangs and a nick was prepared using oligonucleotides 55-nt, 40-nt-5-dT and 100-bp-R2. The 95-bp dsDNA substrate with 5-nt 3' overhangs and a terminal EcoRI binding site was prepared using 1xE-F1 and 1xE-R1; and the 95-bp dsDNA with 5-nt 3' overhangs and an internal EcoRI binding site was generated using 1xE-F2 and 1xE-R2. The hairpin substrate consisting of 50-bp dsDNA, 20-nt loop, and a 5-nt 3' overhang was made using oligonucleotide HP3. After the strand annealing step, reaction

mixtures were resolved in a native polyacrylamide gel in TAE buffer and recovered from gel slices by electro-elution into the same buffer.

Plasmid-length DNA substrates

To prepare the 3-kb dsDNA substrate, pBluescript II SK (-) plasmid DNA was digested with EcoRV (New England Biolabs), followed by purification using the QIAquick gel extraction kit (Qiagen). Then, the DNA was 3'-labeled with [α-^{32}P]cordycepin 5'-triphosphate and terminal transferase as above. For 5' end labeling, the DNA was first dephosphorylated by shrimp alkaline phosphatase (New England Biolabs), purified by the QIAquick gel extraction kit, and then labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase as above. The radiolabeled substrates were purified using the QIAquick gel extraction kit before use.

Expression and purification of wild-type and mutant MRX complexes

Mre11, Rad50 and Xrs2 were overexpressed with the use of GAL-PGK-Mre11, GAL-PGK-Rad50 and GAL-PGK-Xrs2 plasmids in the protease-deficient yeast strain YRP654 and were purified to near homogeneity as described previously (Chen et al. 2001; Trujillo and Sung 2001; Trujillo et al. 2003). Protein concentration was determined by the BCA method (Thermo Scientific) with bovine serum albumin as the standard. To assemble the MRX complex, purified Mre11 (200 μg), Rad50 (300 μg), and Xrs2 (150 μg) were mixed in 400 μl of T buffer (25 mM Tris-HCl, pH 7.5, 10% glycerol, 0.5 mM EDTA, 1 mM DTT and 0.01% Igepal) with 300 mM KCl and incubated on ice for 5 h, followed by gel filtration on a 22-ml Sephacryl-S400 column (GE healthcare). Peak fractions containing the assembled MRX complex were pooled, concentrated to ~1 mg/ml in an Amicon Ultra 30K micro-concentrator (Millipore), and stored in small aliquots at -80°C. The same GAL-PGK protein expression vector was used for the expression and purification of Mre11 and Rad50 mutant proteins. Assembly of mutant MRX complexes followed the procedure developed for the wild-type counterpart.

Expression and purification of Sae2

The plasmid pFB-MBP-Sae2-10xHis for Sae2 expression in insect cells was obtained from Petr Cejka (Cannavo and Cejka 2014). Bacmid and baculovirus were prepared in *Escherichia coli* DH10Bac and *Spodoptera frugiperda* Sf9 cells, respectively, according to the manufacturer's protocols (Bac-to-Bac baculovirus expression system, Invitrogen). *Trichoplusia ni* High Five cells ($1x10^6$ cells/ml) were infected with high-titer P3 baculovirus. Cells were harvested after 46 h and stored at -80°C. All the subsequent steps were carried out at 0-4°C. The cell pellet (~16 g from 1 L of culture) was suspended in 50 ml of T buffer with 300 mM KCl and protease inhibitors (aprotinin, chymostatin, leupeptin and pepstatin A at 5 µg/ml each, 1 mM phenylmethylsulfonyl fluoride). After sonication for 1 min, the crude cell lysate was clarified by ultracentrifugation at 100,000xg for 1 h. The supernatant was incubated with 4 ml of amylose resin (New England Biolabs) for 1 h with constant mixing. After washing the resin with 250 ml of T buffer containing 1 M KCl, bound proteins were eluted with 4 ml of T buffer containing 300 mM KCl and 10 mM maltose for 15 min with gentle agitation. The protein eluate was mixed gently with 2 ml of Ni-NTA agarose (Qiagen) for 1 h, followed by washing of the resin with 250 ml of T buffer containing 1 M KCl and 20 mM imidazole. Sae2 was eluted with 2 ml of T buffer containing 300 mM KCl and 200 mM imidazole for 15 min. The protein pool was diluted with 4 ml of T buffer and further fractionated in a 1-ml Mono Q column (GE healthcare) with a 40 ml gradient of 100-400 mM KCl in T buffer. The Sae2 peak fractions were pooled and concentrated in an Amicon Ultra 30K micro-concentrator and subject to gel filtration on a 24-ml Superdex 200 column (GE healthcare) in T buffer containing 300 mM KCl. The Sae2 peak fractions were pooled, concentrated to ~500 µg/ml and stored in small aliquots at -80°C. The typical yield of Sae2 was ~250 µg from 1 L of cell culture.

Expression and purification of EcoRI-E111Q

The coding sequence for EcoRI-E111Q was amplified by PCR from plasmid pTXBERI-3FL (a kind gift from Eric Greene) (Finkelstein et al. 2010), and cloned into the NcoI and XhoI sites of pET28b to generate plasmid pET28b-EcoRI-E111Q. The plasmid was introduced into *E. coli* Rosetta cells. When the cell culture reached OD_{600} of 0.6, 0.1 mM IPTG was added to induce protein expression. After a 14-h incubation at 16°C, cells were harvested and stored at -80°C. The cell pellet (~17 g from 3 L of culture) was suspended in 50 ml of T buffer with 300 mM KCl and protease inhibitors, and the clarified cell extract was prepared as described above for Sae2. 4 ml of Ni-NTA agarose (Qiagen) and 20 mM imidazole were added to the supernatant, followed by 1 h incubation with gentle agitation. After washing the resin with 250 ml of T buffer containing 1 M KCl and 20 mM imidazole, bound proteins were eluted with 6 ml of T buffer containing 300 mM KCl and 200 mM imidazole. The eluate was diluted with an equal volume of T buffer and further fractionated in a 1-ml SP Sepharose column (GE healthcare) with a 10 ml gradient of 150-850 mM KCl in T buffer. The EcoRI-E111Q peak fractions were pooled and subjected to gel filtration on a 24-ml Superdex 200 column (GE healthcare) in T buffer containing 300 mM KCl. The peak fractions were pooled (~200 µg/ml) and stored in small aliquots at -80°C. The yield of EcoRI-E111Q was ~600 µg from 3 L of culture.

Assembly of the nucleosomal substrate

The DNA used in substrate construction was obtained by PCR using the pBS-1x601-2 plasmid, comprising the 601 nucleosome-positioning sequence (Lowary and Widom 1998) in pBluescript II SK (-), as template. The PCR product was digested with EcoRI and then labeled with ^{32}P by filling in the 4-nt overhang with dTTP, [α-^{32}P]dATP (PerkinElmer) and the Klenow fragment of *E. coli* DNA polymerase I (New England Biolabs) according to the manufacturer's instructions. The unincorporated radioactive nucleotide was removed using a Micro Bio-Spin 6 column as above. The site-specific nucleosome was generated using the purified histone octamer and 3'-labeled DNA substrate as described previously, but with slight modifications (Dyer et al. 2004). Briefly,

the reactions (120 µl each) were assembled by mixing 300 nM radiolabeled DNA substrate with different molar ratios (1.6, 2.0 and 2.4) of histone octamer to DNA in reconstitution buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 mM DTT) containing 2 M NaCl. Step salt dialysis was performed in a dialysis tubing (7 kDa cutoff, Thermo Scientific) against reconstitution buffer with decreasing concentrations (2, 1.6, 1.2, 0.9, 0.6, 0.4, 0.2 and 0.1 M) of NaCl over a period of 24 h. The reconstituted nucleosomal substrate was analyzed by native polyacrylamide gel electrophoresis in TBE buffer (44.5 mM Tris-borate, pH 8.3, 1 mM EDTA).

Supplemental References

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Supplemental Figure Legends

Supplemental Figure S1 (related to Figure 1). Reconstitution of endonucleolytic cleavage reaction with purified Ku, MRX, and Sae2.

(A) Purified Ku, Sae2, MRX and MRX complexes harboring the Mre11 (H125L, D126V) nuclease-dead mutant (MRX-nd) or Rad50 K40A, K40E, or K40R protein were analyzed by SDS-PAGE and Coomassie blue staining. (B) Binding of the 70-bp or 100-bp DNA substrate (5 nM) by Ku was analyzed by electrophoretic mobility shift of the DNA. (C) The 70-bp 3'-labeled substrate (1 nM) was incubated with MRX (40 nM), Sae2 (480 nM), and Ku (8 nM). (D) The endonucleolytic cleavage products from reactions (see Supplemental Fig. S1C and Fig. 1A) containing the Ku-bound 70-bp or 100-bp substrate and MRX-Sae2 were analyzed in a DNA sequencing gel. Product cluster 1 and cluster 2 correspond to cleavage sites approximately 35 nt and 55 nt away from the Ku-occluded 5'-terminated DNA end, respectively. (E) Reactions containing either Sae2 or CtIP (240 nM) were carried out as in Figure 1A. (F) The nuclease activity of MRX-Sae2 was examined using 3' or 5'-labeled 3-kb dsDNA (0.25 nM) as the substrate. The experiment used 20 nM MRX, 240 nM Sae2, and 300 nM Ku. (G) Control experiment showing that MRX harboring the Mre11 (H125L, D126V) nuclease-dead mutant is devoid of the ability to nucleolytically process the Ku-bound substrate with or without Sae2. The reaction conditions were as those used in (F). (H) MRX complexes that harbor Rad50 K40A, K40E, or K40R ATPase-deficient mutant are devoid of the ability to endonucleolytically process the Ku-bound substrate with or without Sae2. The reaction conditions were as those used in (C). (I) Nuclease assay was performed as in (C) with ATP, ADP, ATPγS or AMP-PNP.

Supplemental Figure S2 (related to Figure 1). Reconstitution of endonucleolytic cleavage reaction with RPA, MRX, and Sae2.

(A) Time-course analysis of endonuclease activity of MRX-Sae2 on 70-bp dsDNA containing 5-nt overhangs in the absence or presence of Ku. The results from three independent experiments were quantified and graphed. The error bars represent SD. (B) Binding of the 70-bp dsDNA (10 nM) with either 5-nt or 10-nt 3' overhangs, or the hairpin substrate (10 nM) consisting of 50-bp dsDNA, 20-nt loop, and a 5-nt 3' overhang by RPA. (C) Binding of the 70-bp dsDNA (10 nM) with 30-nt 3' overhangs by RPA. (D) The 70-bp dsDNA (1 nM) that harbors 30-nt 3' overhangs was tested with MRX (40 nM), Sae2 (480 nM), and RPA (32 nM). (E) Time-course analysis was carried out as in (A) except with hairpin substrate containing a 20-nt loop, in the absence or presence of RPA.

Supplemental Figure S3 (related to Figure 2). Nucleosomal and other substrates with internal protein obstacles.

(A) Purified histone octamer (consisting of H2A, H2B, H3, and H4) was analyzed by SDS-PAGE and Coomassie blue staining. (B) The nucleosomal substrate harbors a 147-bp nucleosome-positioning sequence with 15-bp and 70-bp flanking sequences. The nucleosomal substrate generated using the indicated molar ratio of histone octamer to DNA was analyzed by polyacrylamide gel electrophoresis. (C) MRX complexes that harbor either the Mre11 nuclease-dead (nd) mutant or a Rad50 ATPase-deficient mutant (K40A, K40E, or K40R) are devoid of the ability to endonucleolytically process the nucleosomal substrate with or without Sae2. The reaction conditions were as those used in Figure 2B. (D) The two 95-bp substrates (2 nM each) with 5-nt 3' overhangs and harboring a streptavidin-biotin complex either at the DNA end or internally were tested with MRX (40 nM) and Sae2 (480 nM). (E) Binding of the 95-bp dsDNA (5 nM) with 5-nt overhangs and harboring an EcoRI recognition site either at the DNA end or internally by the indicated concentration of EcoRI-E111Q. The asterisk denotes the ^{32}P label in the substrate in all the figure parts.

Supplemental Figure S4 (related to Figure 3). Exonuclease activity of MRX at a nick.

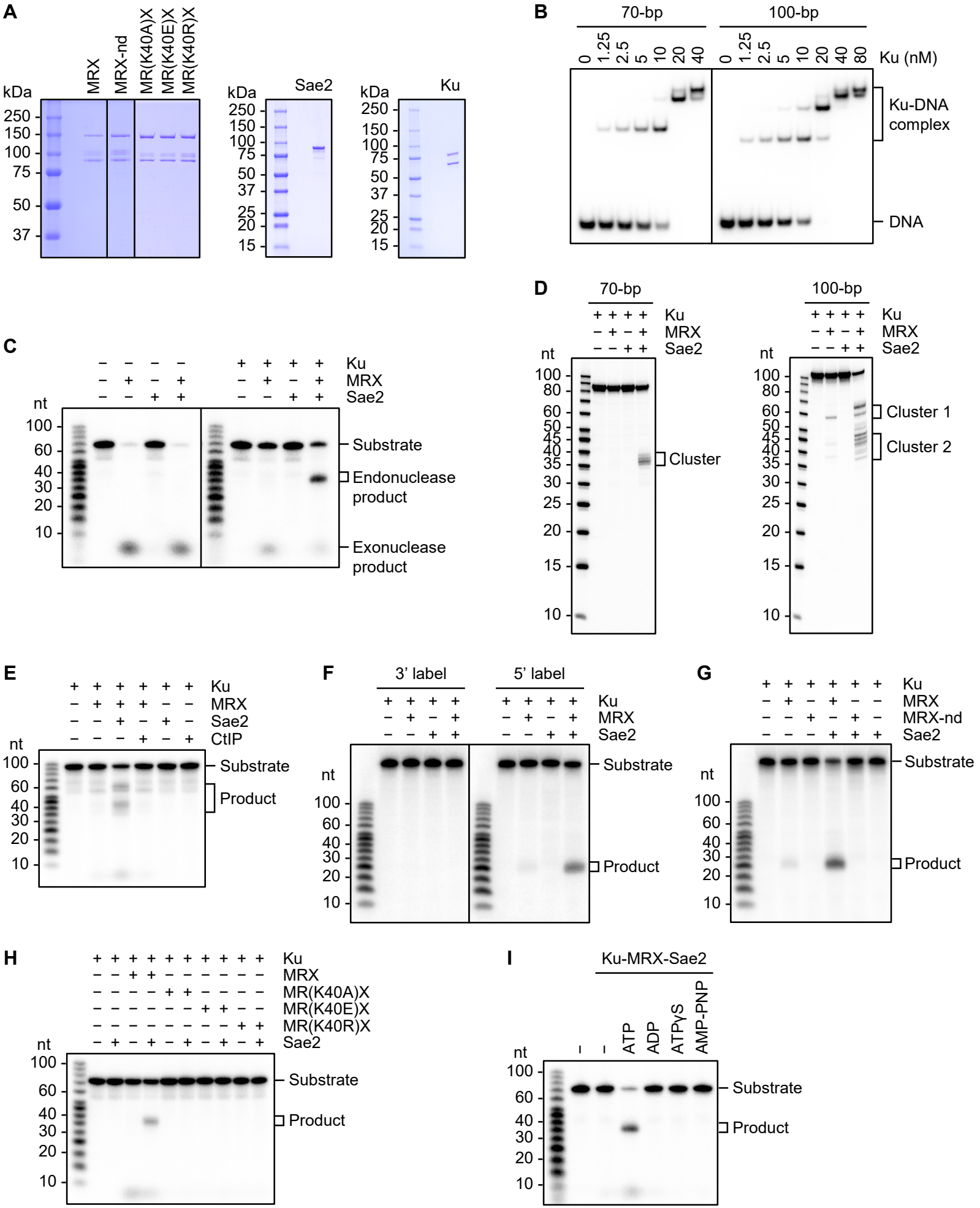
(A) The 100-bp 5'-labeled substrate (1 nM) was tested with MRX (40 nM), Sae2 (480 nM), and Ku (16 nM). The asterisk denotes the ^{32}P label in the substrate. (B) Reactions containing MRX (0.8 nM) and CtIP (30, 60, 120 and 240 nM) were carried out as in Figure 3D. (C) The substrate used in Figure 3D was tested with Mre11 (0.8 or 3.2 nM) and Sae2 (30, 60, 120 and 240 nM). In (B) and (C), the results from three independent experiments were quantified and graphed. The error bars represent SD.

Supplemental Table S1 (related to Supplemental Materials and Methods). DNA sequence of oligonucleotides used in this study.

Oligonucleotide	DNA sequence (5' to 3')
70-bp-F	GTAAGTGCCGCGGTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCG GGCGCGTACTCCACCTCATGCATC
70-bp-R3	GATGCATGAGGTGGAGTACGCGCCCGGGGAGCCCAAGGGCACGCCC TGGCACCCGCACCGCGGCACTTAC
70-bp-F2	G**T**AAGTGCCGCGGTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCG GGCGCGTACTCCACCTCATGCA**T**C (**T**, in bold, is biotin-labeled)
70-bp-R2	GA**T**GCATGAGGTGGAGTACGCGCCCGGGGAGCCCAAGGGCACGCCC TGGCACCCGCACCGCGGCACT**T**AC (**T**, in bold, is biotin-labeled)
100-bp-F	GTAAGTGCCGCGGTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCG GGCGCGTACTCCACCTCATAATCTTCTGCCATGGTCGTAGCAGCCTCC TGCATC
100-bp-R2	GATGCAGGAGGCTGCTACGACCATGGCAGAAGATTATGAGGTGGAGT ACGCGCCCGGGGAGCCCAAGGGCACGCCCTGGCACCCGCACCGCGG CACTTAC

5-dA-F	GTAAGTGCCGCGGTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCG GGCGCGTACTCCACCTCATGCATCAAAAA
5-dA-R	GATGCATGAGGTGGAGTACGCGCCCGGGGAGCCCAAGGGCACGCCC TGGCACCCGCACCGCGGCACTTACAAAAA
10-dT-F	GTAAGTGCCGCGGTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCG GGCGCGTACTCCACCTCATGCATCTTTTTTTTTT
10-dT-R	GATGCATGAGGTGGAGTACGCGCCCGGGGAGCCCAAGGGCACGCCC TGGCACCCGCACCGCGGCACTTACTTTTTTTTTT
30-dT-F2	GTAAGTGCCGCGGTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCG GGCGCGTACTCCACCTCATGCATCTTTTTTTTTTTTTTTTTTTTTTTTTT TTT
30-dT-R2	GATGCATGAGGTGGAGTACGCGCCCGGGGAGCCCAAGGGCACGCCC TGGCACCCGCACCGCGGCACTTACTTTTTTTTTTTTTTTTTTTTTTTTTT TTT
100-bp-F4	G**T**AAGTGCCGCGGTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCG GGCGCGTACTCCACCTCATAATCTTCTGCCATGGTCGTAGCAGCCTCC GTTTTT (**T**, in bold, is biotin-labeled)
100-bp-F5	GTAAGTGCCGCGGTGCGGGTGCCAGGGCGTGCCCTTGGGC**T**CCCCG GGCGCGTACTCCACCTCATAATCTTCTGCCATGGTCGTAGCAGCCTCC GTTTTT (**T**, in bold, is biotin-labeled)
100-bp-R3	CGGAGGCTGCTACGACCATGGCAGAAGATTATGAGGTGGAGTACGCG CCCGGGGAGCCCAAGGGCACGCCCTGGCACCCGCACCGCGGCACTT ACTTTTT
55-nt	TGCCGCGGTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGC

	GTACTCCAC
40-nt-5-dT	CTCATAATCTTCTGCCATGGTCGTAGCAGCCTCCTGCATCTTTTT
1xE-F1	GTA**GAATTC**GCGGTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCG GGCGCGTACTCCACCTCATAATCTTCTGCCATGGTCGTAGCAGCCTCC GTTTTT (**GAATTC**, in bold, is EcoRI binding site)
1xE-R1	CGGAGGCTGCTACGACCATGGCAGAAGATTATGAGGTGGAGTACGCG CCCGGGGAGCCCAAGGGCACGCCCTGGCACCCGCACCGC**GAATTC**T ACTTTTT (**GAATTC**, in bold, is EcoRI binding site)
1xE-F2	GTAAGTGCCGCGGTGCGGGTGCCAGGGCGTGCCCTTGGGC**GAATTC** GGCGCGTACTCCACCTCATAATCTTCTGCCATGGTCGTAGCAGCCTCC GTTTTT (**GAATTC**, in bold, is EcoRI binding site)
1xE-R2	CGGAGGCTGCTACGACCATGGCAGAAGATTATGAGGTGGAGTACGCG CC**GAATTC**GCCCAAGGGCACGCCCTGGCACCCGCACCGCGGCACTTA CTTTTT (**GAATTC**, in bold, is EcoRI binding site)
HP3	TGGGTCAACGTGGGCAAAGATGTCCTAGCAATGTAATCGTCTATGACG TTCCCCCCCCCCCCCCCCCCCCAACGTCATAGACGATTACATTGCTAG GACATCTTTGCCCACGTTGACCCAAAAAA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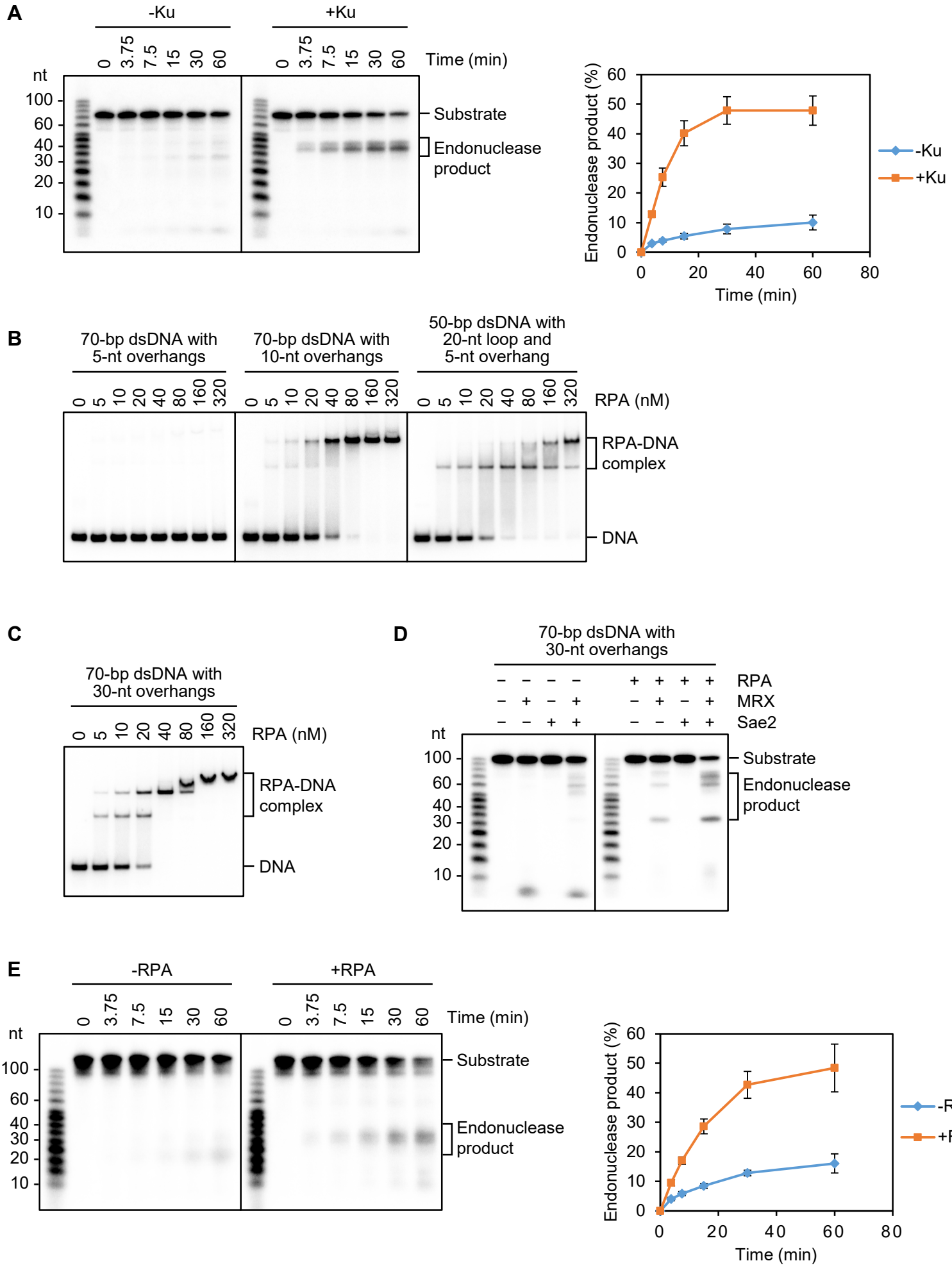
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Substrate
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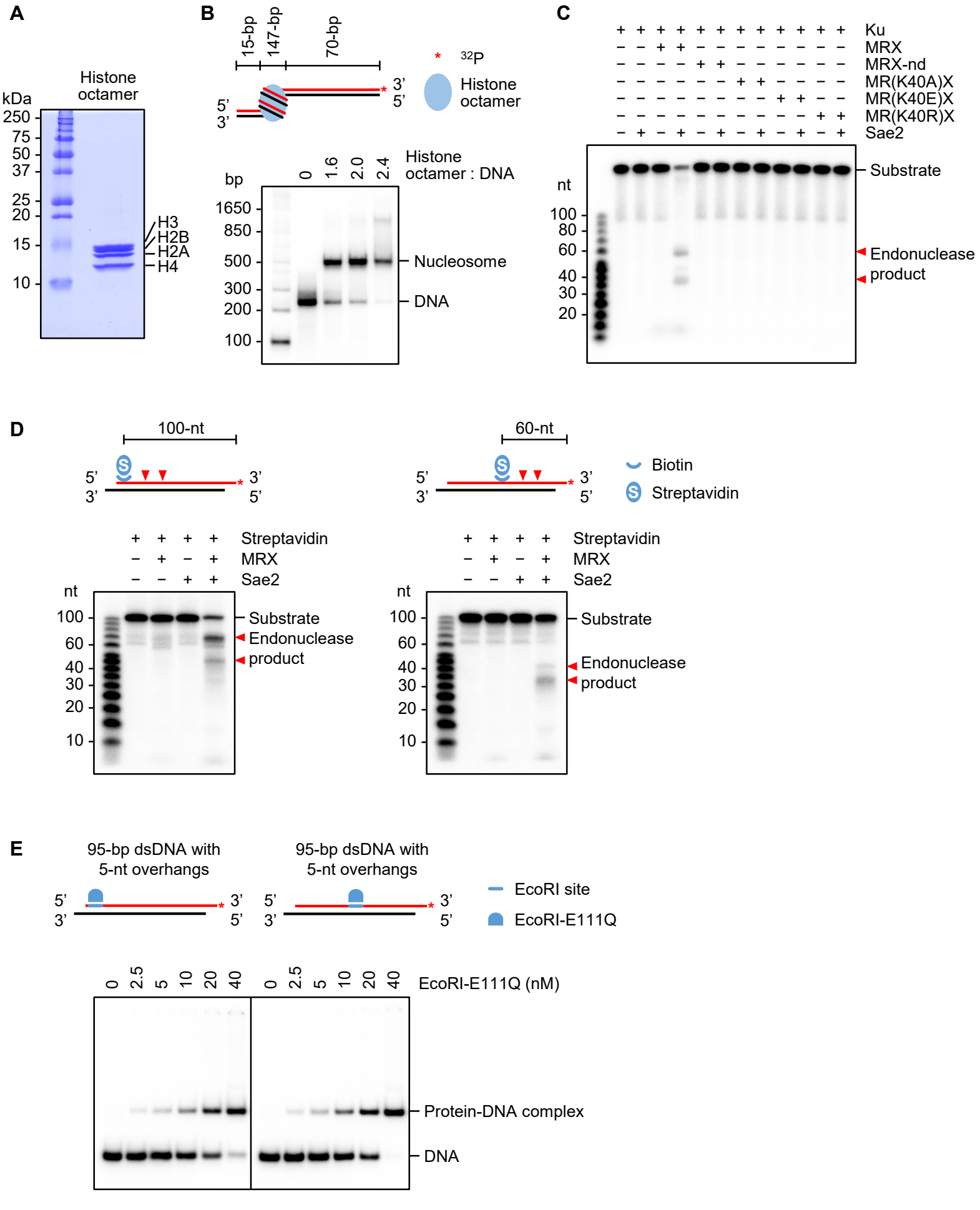
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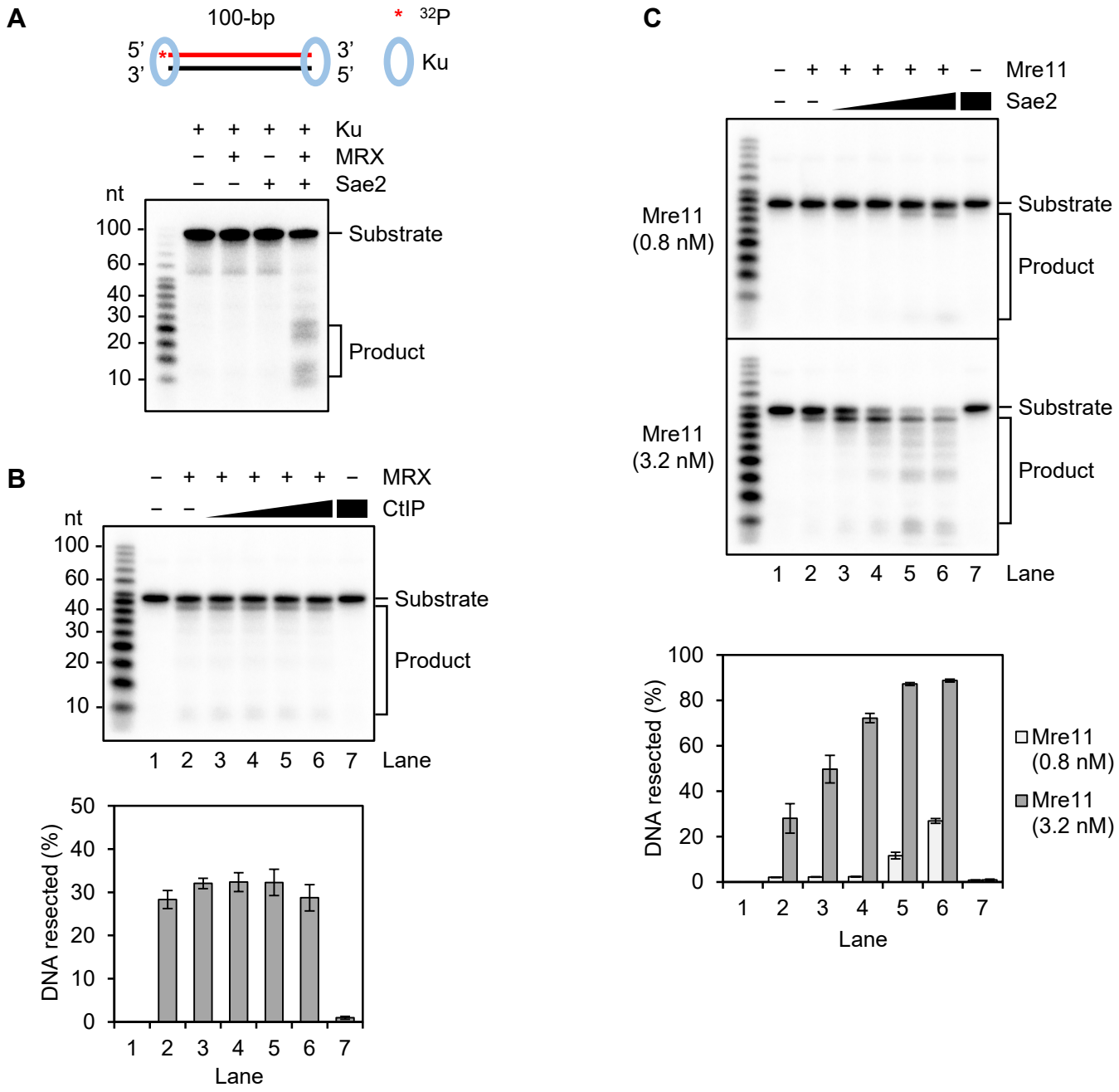
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Substrate
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(0.8 nM)
Substrate
Product
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(3.2 nM)
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