ssDNA

$T_{(12\text{-}55)}$ 5′ $(T)_{12\text{-}55}$ 3′

dsDNA

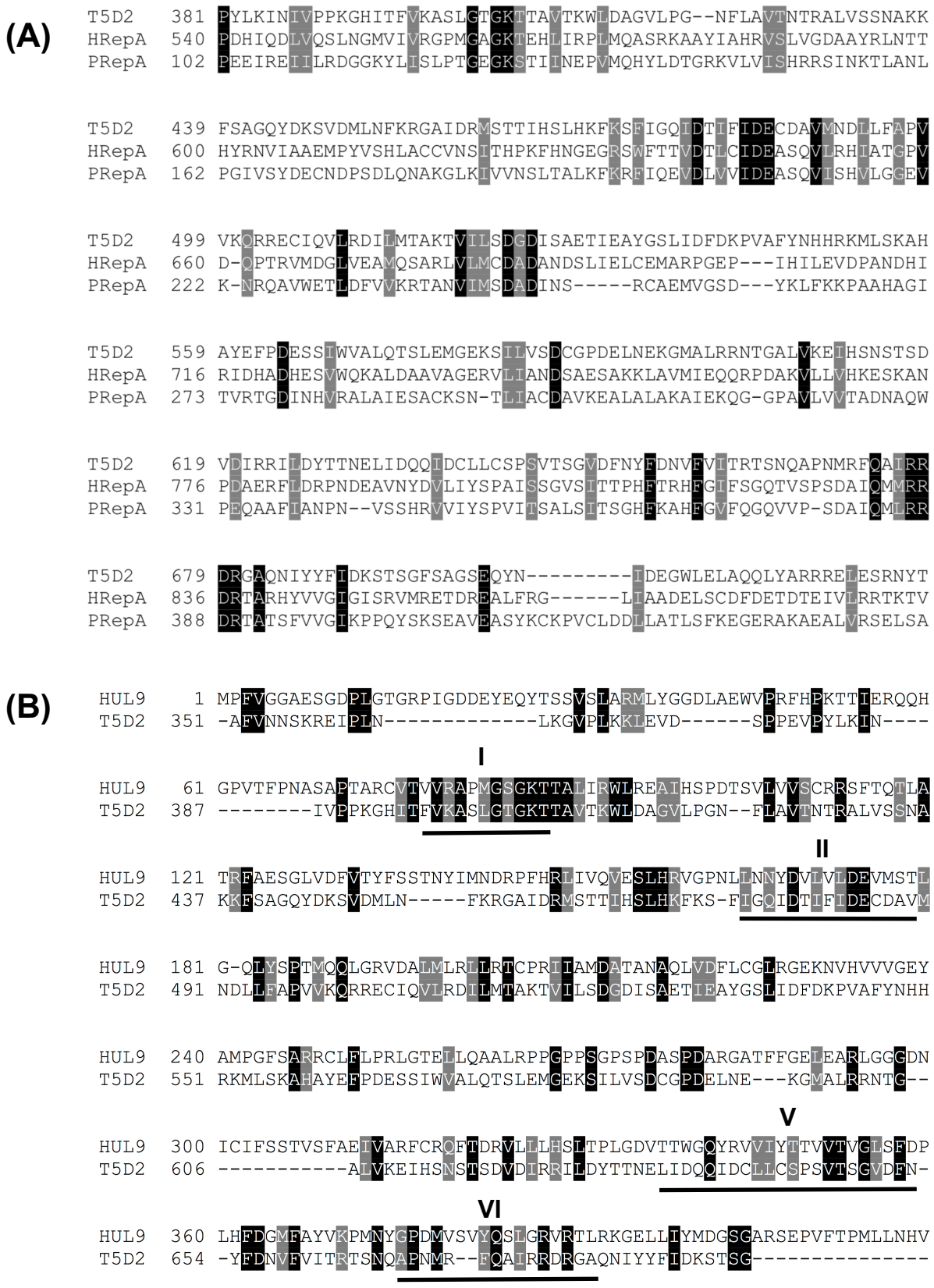
ds20
5′ CGAATTCGAGCTCGGTACCC 3′
3′ GCTTAAGCTCGAGCCATGGG 5′

ds40
5′ TTCGAAACGTGCGCGCCCAT CGAATTCGAGCTCGGTACCC 3′
3′ AAGCTTTGCACGCGCGGGTA GCTTAAGCTCGAGCCATGGG 5′

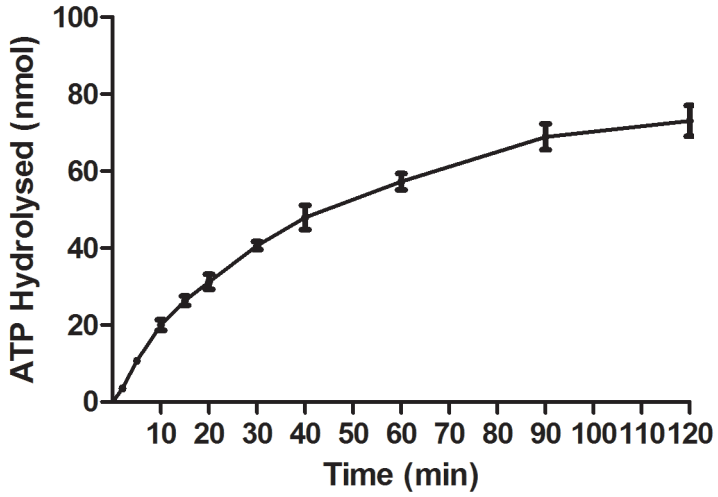
5'$T_{(5\text{-}55)}$-ds20
5′ $(T)_{5\text{-}55}$CGAATTCGAGCTCGGTACCC 3′
3′ GCTTAAGCTCGAGCCATGGG 5′

ds20-3'$T_{(5\text{-}55)}$
5′ GGGTACCGAGCTCGAATTCG $(T)_{5\text{-}55}$ 3′
3′ CCCATGGCTCGAGCTTAAGC 5′

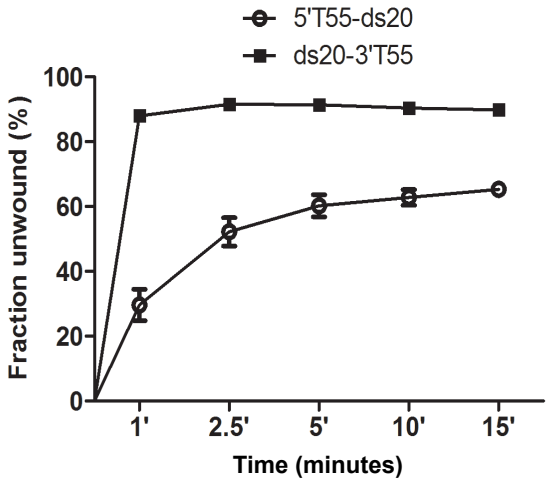
Supplementary Figure 1. Oligonucleotide substrates used in this study.



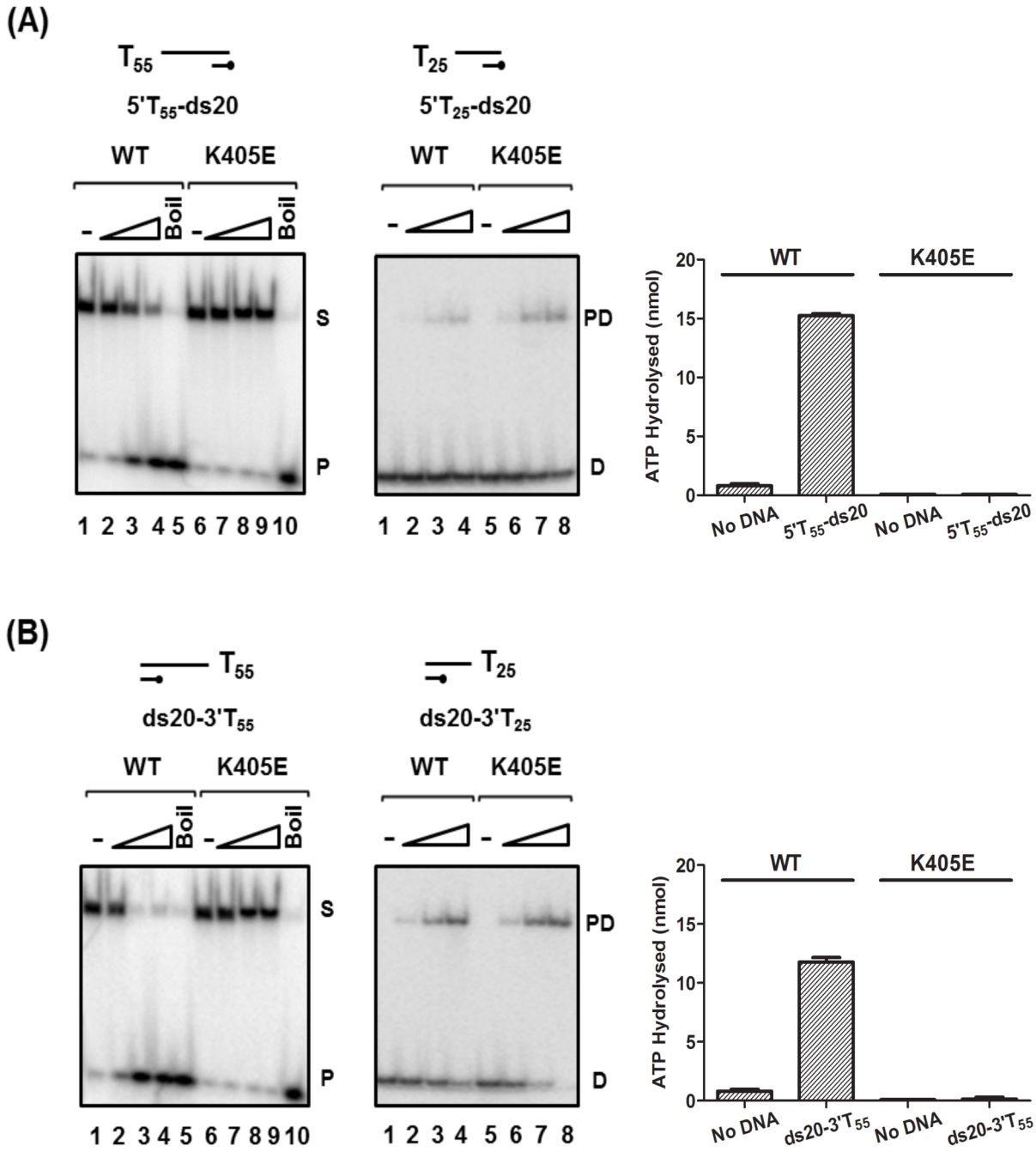
Supplementary Figure 2. Alignments of D2 protein with homologues. Alignment of the D2 protein (AAU05245) with RepA-proteins from *Halomonas* phage (YP_001686772) phiHAP-1 and *Pseudomona putida* (ADR59589) (**A**). Panel **B** shows the pairwise similarity between the T5 D2 protein and the herpesvirus UL9 protein (HUL9, AER38017). The helicase motifs I, II,V and VI are underlined. In both panels, identical residues are shown by black shading while similar residues are shown in gray.



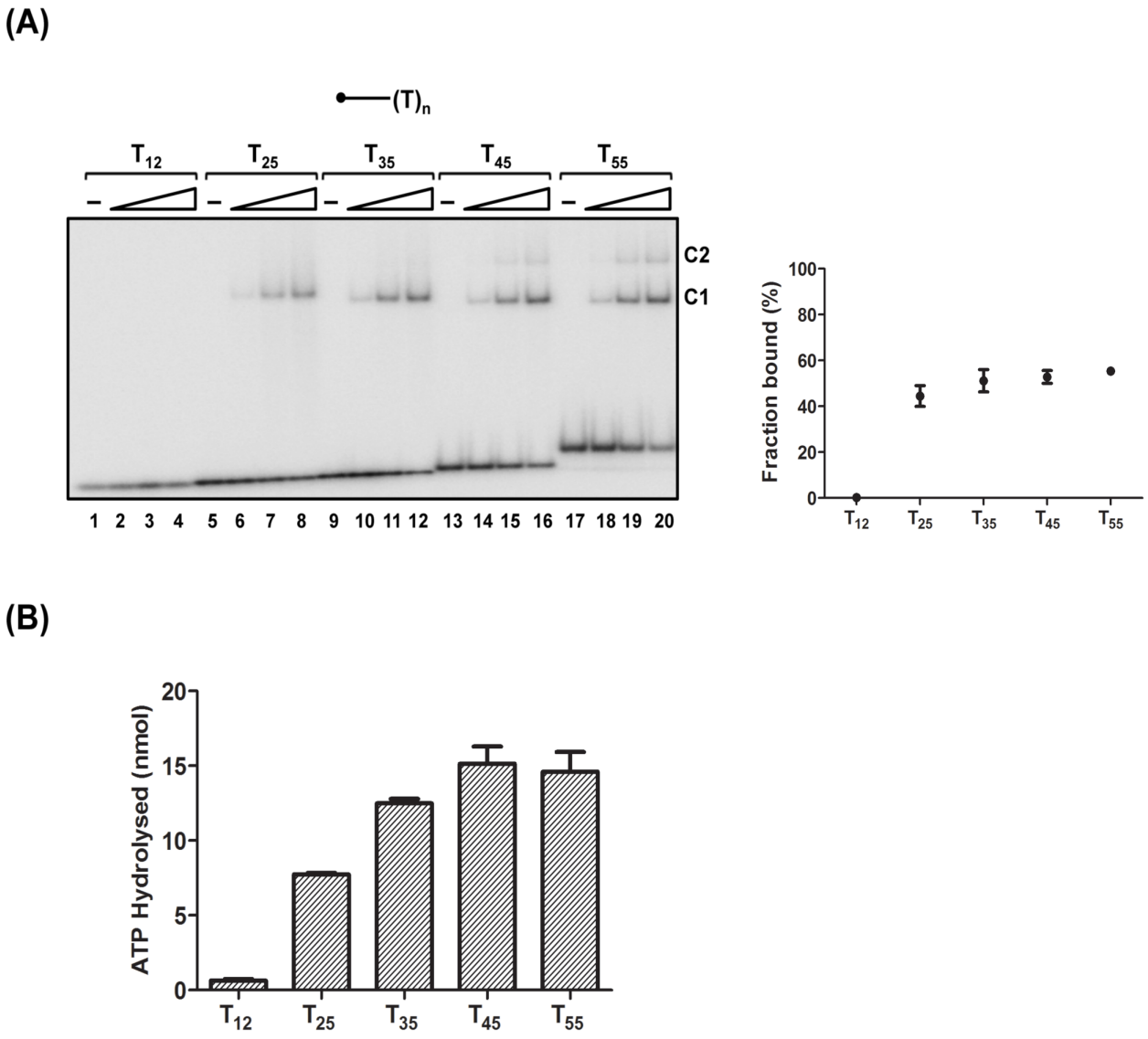
Supplementary Figure 3. Time course of ATP hydrolysis. Reactions (25 nM D2, 25 nM ds20-3′T_{55}, 100 mM NaCl) were assembled and processed as described in Materials and Methods. At the indicated times ATP hydrolysed was calculated from the release of free ^{32}P-phosphate.



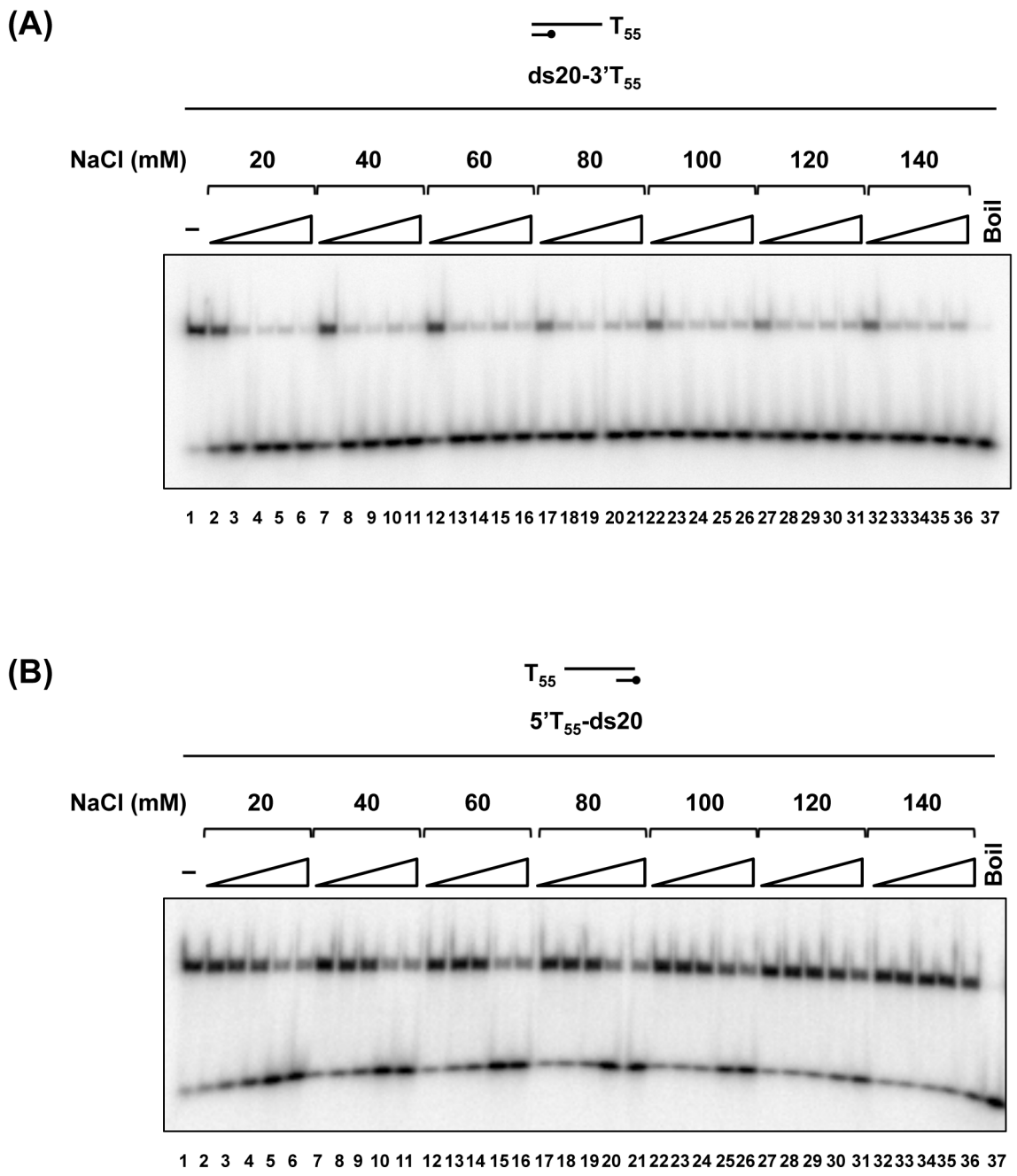
Supplementary Figure 4. Time course of 3′–5′ and 5′–3′ unwinding. Reactions were assembled under standard conditions (0.1 nM substrate, 10 nM D2) and the accumulation of product was determined as a function of time. Within 1 minute, near maximum extents (~90%) of ds20-3′T_{55} unwinding were achieved. For substrate 5′T_{55}-ds20 the extent of unwinding achieved at 15 minutes was lower (~65%), and this extent was approached after greater than 5 minutes' incubation (n = 3, mean and standard deviation shown).



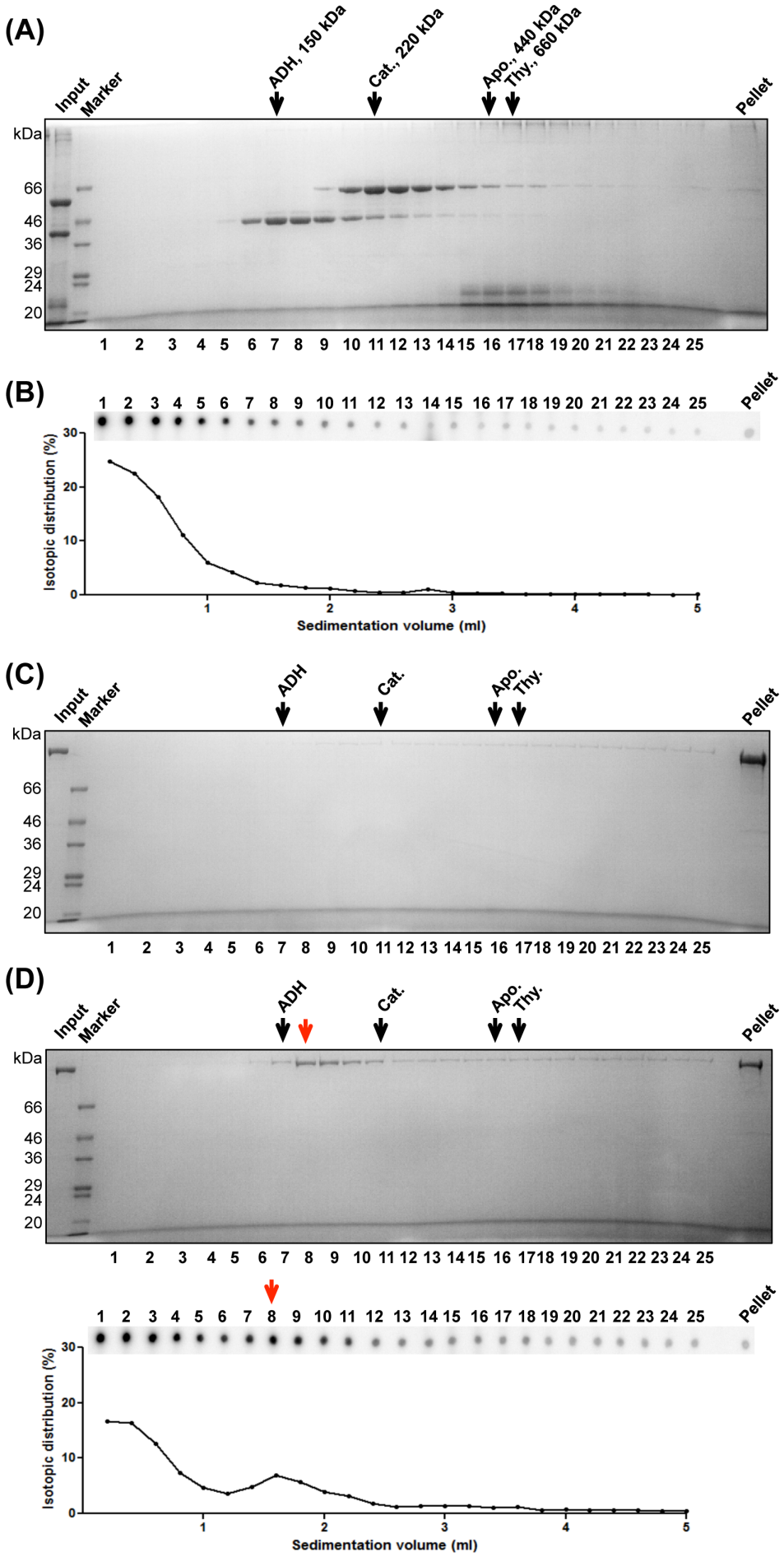
Supplementary Figure 5. Helicase, DNA-binding and ATP*ase* activities of D2 WT and K405E on partial duplex DNA, 20 bp with a 5′ T_{55} overhang. (**A**) or 3′ dT overhang (**B**). Helicase assays (0.1 nM 5′T_{55}-ds20 or ds20-3′T_{55}; 20 mM NaCl). Lanes 1 and 6, no protein control (-); lanes 5 and 10, heat-denatured substrate control (Boil); lanes 2-4, D2 WT (0.1, 1, 10 nM); lanes 7-9, D2 K405E (0.1, 1, 10 nM); S, native substrate; P, ssDNA product as indicated by boiling the substrate. Gel-shift assays (0.05 nM 5′T_{25}-ds20 or ds20-3′T_{25}), Lanes 1 and 5, no protein control (-); lanes 2-4, D2 WT (0.01, 0.05 and 0.1 nM); lanes 6-8, D2 K405E (0.01, 0.05 and 0.1 nM); PD, protein-DNA complex; D, free DNA probe. ATP*ase* activities of D2 WT and K405E (25 nM) were determined in the absence or presence of the DNA substrates (25 nM). In comparison with D2 WT, the mutant (K405E) retained DNA-binding activity, but totally lost helicase and ATP*ase* activities (n=3, mean and SD).

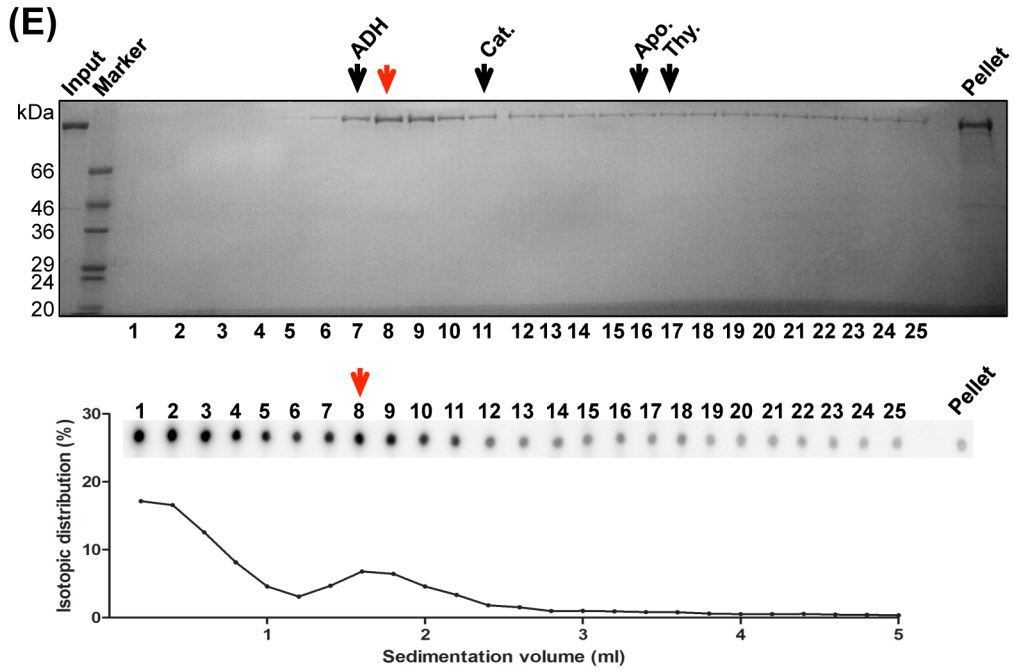


Supplementary Figure 6. D2 binding to ssDNA. (**A**) The oligo-dT substrates of increasing length (from 12 to 55 bases, 0.05 nM) were radiolabelled at their 5′ ends. Reactions (0.01, 0.05, 0.1 nM D2) were incubated at 22°C for 15 min and assessed by gel-shift assay in the absence of ATP/Mg^{2+}. Lanes 1, 5, 9, 13 and 17, no protein control (-); lanes 2-4, lanes 6-8, lanes 10-12, lanes 14-16 and lanes 18-20, D2 protein titrations (0.01, 0.05, 0.1 nM). Significant binding was observed with probes of 25 bases or longer (lanes 5-20) and two protein-DNA complexes (C1 and C2) formed on probes of 35 bases or longer. The graph to the right shows that the binding affinity of D2 for ssDNA increases when the length of the substrates is extended from 12 to 55 bases (data for 0.1 nM D2, n=3, mean and SD). **(B)** Stimulation of D2 ATP*ase* activity by oligo-dT substrates of increasing length. D2 (25 nM) was incubated with T_{12}, T_{25}, T_{35}, T_{45} and T_{55} (25 nM) at 37°C for 15 min and the activity correlated with the length of the substrates (n=3), reflecting the results of the DNA binding assays in (A). For the data n=3, mean and SD.



Supplementary Figure 7. Effect of NaCl concentration on the bipolar DNA unwinding activity of D2. The unwinding of 5´T_{55}-ds20 (**A**) and ds20-3´T_{55} (**B**) by D2 at various [NaCl] concentrations (20-140 mM). Lane 1, no protein control (-); lane 37, heat-denatured substrate control (Boil); lanes 2-6, lanes 7-11, lanes 12-16, lanes 17-21, lanes 22-26, lanes 27-31 and lanes 32-36, D2 protein titrations (0.1, 0.5, 1, 10, 100 nM) at the indicated [NaCl].


(A)
Input
Marker
ADH, 150 kDa
Cat., 220 kDa
Apo., 440 kDa
Thy., 660 kDa
Pellet
kDa
66
46
36
29
24
20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
(B)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Pellet
Isotopic distribution (%)
30
20
10
0
1
2
3
4
5
Sedimentation volume (ml)
(C)
Input
Marker
ADH
Cat.
Apo.
Thy.
Pellet
kDa
66
46
36
29
24
20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
(D)
Input
Marker
ADH
Cat.
Apo.
Thy.
Pellet
kDa
66
46
36
29
24
20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Pellet
Isotopic distribution (%)
30
20
10
0
1
2
3
4
5
Sedimentation volume (ml)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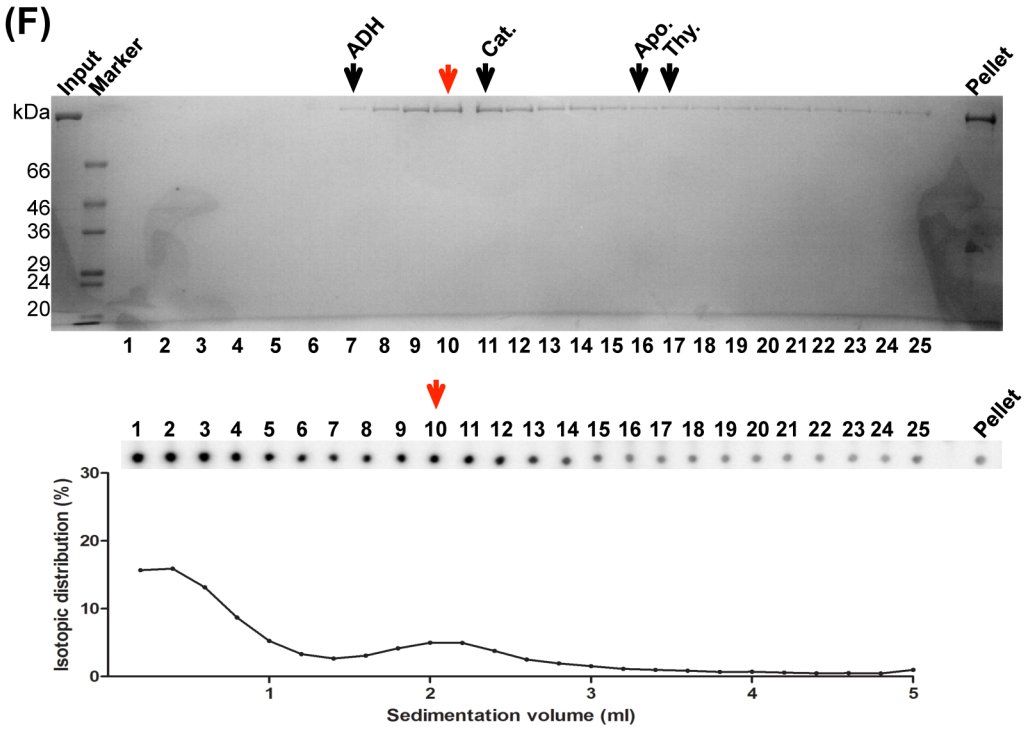
Pellet
Isotopic distribution (%)
Sedimentation volume (ml)


(F)
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20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Pellet
Isotopic distribution (%)
Sedimentation volume (ml)

Supplementary Figure 8. Glycerol gradient sedimentation of D2. 20%-40% glycerol gradients (20 mM HEPES-NaOH pH 7.5, 100 mM NaCl, 0.1% v/v NP40 alternative, 2 mM DTT, 1 mM EDTA) were spun at 237,000 x g at 4°C using a Beckman SW55 Ti rotor and harvested top to bottom (0.2 ml fractions), according to the general guidelines of Li and Desiderio (65). The pellet fraction was solubilised in SDS-PAGE loading buffer (0.2 ml). Fractions were analysed on 12% SDS-PAGE gels, stained with coomassie blue. Where indicated, the buffer contained 3 mM Mg^{2+} and 1 mM ADP or ATP. Samples of D2 (100 µl, 0.5 mg/ml) were pre-incubated with or without ^{32}P-end labelled T_{25} oligonucleotide (1:1 protein:DNA) at 22°C for 30 minutes in the same buffer except for the addition of 10% glycerol and 5 mM ADP/Mg^{2+} or 5 mM ATP/Mg^{2+} as indicated. The markers used were Alcohol dehydrogenase (ADH, 150 kDa), Catalase (Cat., 220 kDa), Apoferritin (Apo., 440 kDa) and Thyroglobulin (Thy., 660 kDa) and they were always run in parallel with the D2 analysis. (**A**) Sedimentation profile of the markers. Peak sedimentation volumes are: ADH, 1.4 ml; Cat., 2.2 ml; Apo., 3.2 ml and Thy., 3.4 ml. Fraction numbers are indicated, as in subsequent panels. Identical profiles were obtained when each marker was analysed in isolation, with or without co-factors. "Input" is a sample of the protein mixture before sedimentation. "Marker", standard SDS-PAGE markers). (**B**) Sedimentation of the T_{25} oligonucelotide. Samples of the gradient were spotted on DE81 paper and exposed to a phosphorimaging plate to determine the isotopic distribution in the gradient. (**C**) Sedimentation of D2. The majority of D2 pelleted but a small percentage of the protein was distributed in all gradient fractions after the ADH sedimentation peak. This showcases the difficulties encountered in trying to obtain molecular weight measurements for D2 and complexes. Using an analytical gel filtration column (Superdex S200 HR) we were unable to obtain flow of D2 through the column under any conditions, precluding an accurate mass determination by the Siegel-Monty calculation. However, upon addition of T_{25} ssDNA, the properties of D2 changed. (**D**) With T_{25} ssDNA, a significant fraction of D2 migrated between the ADH (150 kDa, 1.4 ml sedimentation volume) and catalase (220 kDa, 2.2 ml sedimentation volume) markers with a peak sedimentation of 1.6 ml, indicated with the red arrow as in subsequent figures below. D2 (MBP-D2) has a calculated monomeric molecular weight of 150 kDa. T_{25} ssDNA co-sedimented with the peak fraction of D2, indicating stable binding to monomeric D2 (graphed data below the analysis of protein distribution by SDS-PAGE). No T_{25} ssDNA was observed in the pellet above the levels seen for T_{25} analysed in the absence of protein, (B). Increasing the ratio of DNA to protein did not alter the distribution pattern of D2 (not shown). (**E**) Identical results were observed in the presence of ADP/Mg^{2+} (SDS-PAGE gel above graph of T_{25} distribution). (**F**) In the presence of ATP/Mg^{2+} we observed a broader D2 peak distribution in the gradient between the ADH and catalase sedimentation peaks and a shift in the peak sedimentation volume to 2 ml, less than the 2.2 ml observed for catalase (220 kDa). This suggests that ATP is unlikely to induce stable oligomersation of D2 in the presence of ATP/Mg^{2+} (SDS-PAGE gel above graph of T_{25} distribution).

Supplementary Table

Sample	Purity (%)	Specific activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)
D2 wild-type		
S	~20%	1.6±0.2
M	~96%	10.4±0.3
H	~96%	11.9±0.2
Q	~99%	13.1±1.2
D2 K405E		
S	~11%	0.8±0.1
M	~86%	0.2±0.07
H	~93%	0.2±0.1
Q	~98%	0.1±0.03
His-MBP		
His-MBP tag	~99%	0.1±0.1

Supplementary Table. A summary of the purification of T5 D2 WT and K405E from cell lysate of induced *E. coli* BL21(DE3). S, supernatant of cleared lysate; M, eluate from amylose resin; H, eluate from HisTrap™ column and Q, anion exchange chromatography. The purity of D2 protein (1 μg sample) was estimated by densitometry of the SDS-PAGE gel in Figure 2 using GelQuant.NET software provided by biochemlabsolutions.com. ATP*ase* activity was determined by the charcoal binding method (see materials and methods) and the specific activity expressed in $\mu mol \cdot min^{-1} \cdot mg^{-1}$ ATP hydrolysed.