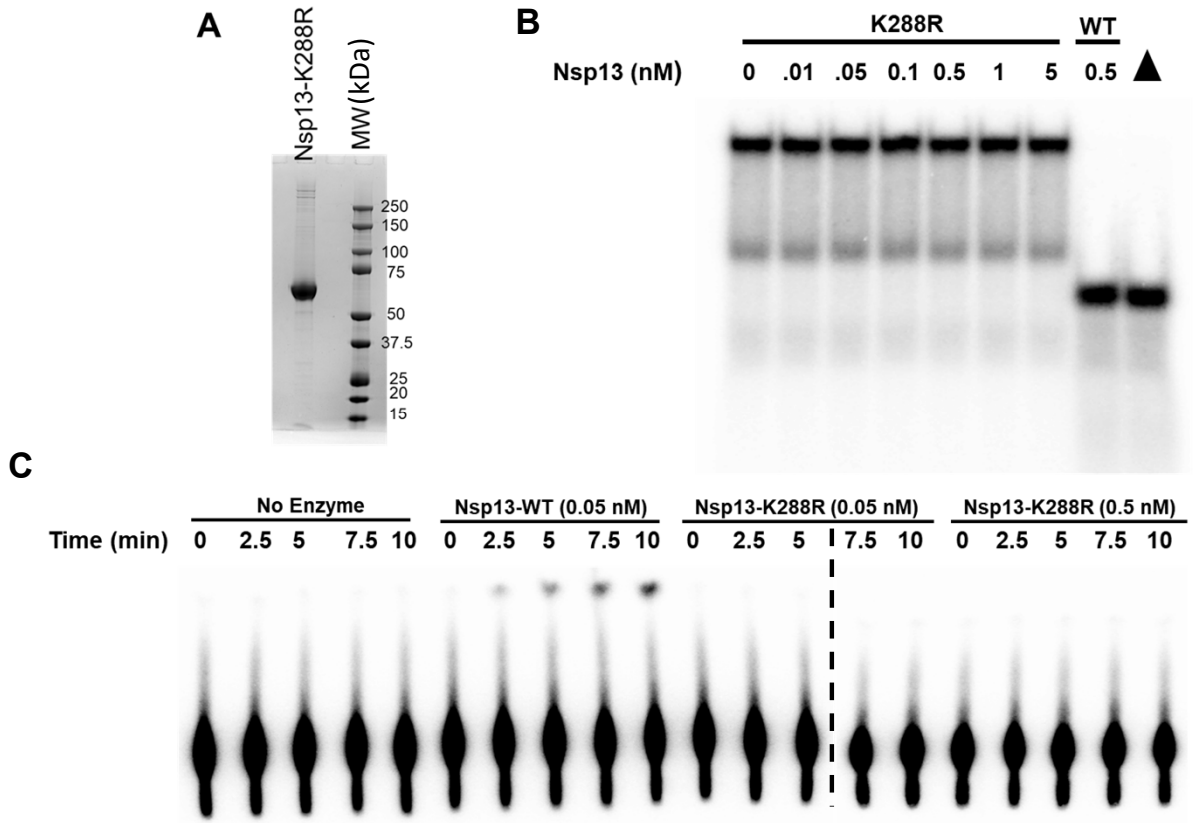

Fig. S1. Nsp13-K288R mutant protein has no detectable ATPase or helicase activity. (A) Purified recombinant Nsp13-K288R protein (5,000 ng) after final dialysis (see *Experimental Procedures*), resolved by SDS-PAGE and detected by Coomassie-staining. (B) Representative native polyacrylamide gel analysis of products from Nsp13-K288R reaction mixtures with a 19-bp RNA partial substrate conducted in the presence of 2 mM ATP and 1 mM Mg^{2+} for 15 min at 37 ℃. Filled triangle represents heat-denatured RNA substrate control. Products from reaction mixture containing Nsp13-WT is shown. (C) Representative thin layer chromatography of a kinetic analysis of Nsp13-K288R (0.05 and 0.5 nM) ATPase activity in the presence of a 60 nt ssRNA effector. The two chromatography sheets containing reactions from one experiment were spliced together and no lanes were omitted.

Fig. S1

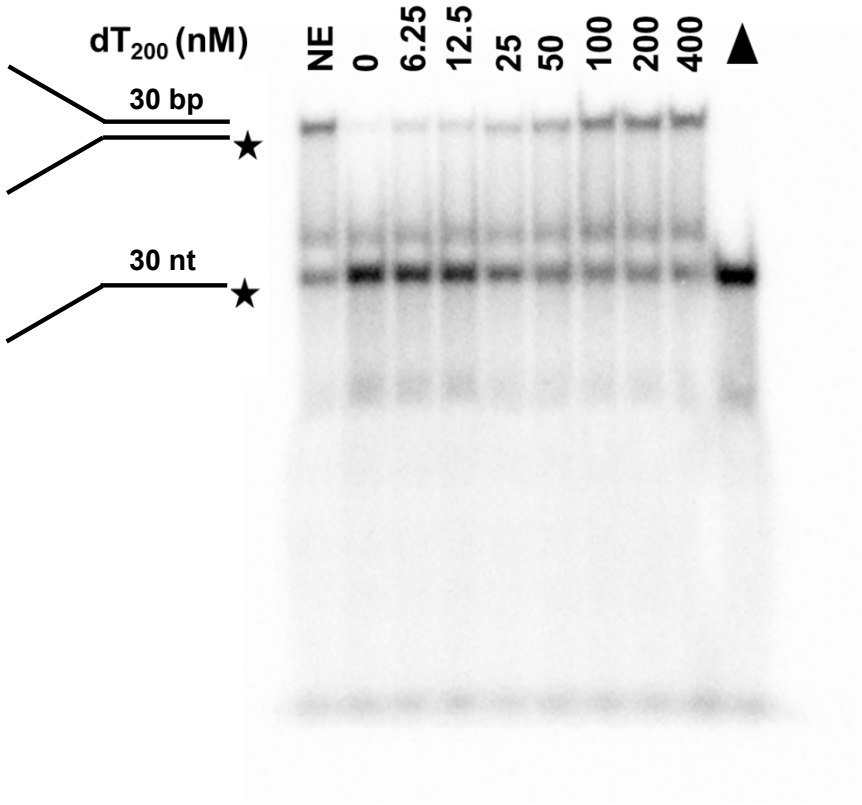

Fig. S2. dT_{200} titration to determine polynucleotide concentration suitable for helicase trap in single-turnover kinetic experiments. The indicated concentrations of dT_{200} were pre-incubated with Nsp13 (0.1625 nM) for 5 min at RT. Subsequently, ATP and radiolabeled 30-bp RNA partial duplex substrate (0.25 nM) was added to the reaction mixture and allowed to incubate at 37 ℃ for 60 min. Reactions were quenched and analyzed by native polyacrylamide gel electrophoresis and ImageQuant as described under *Experimental Procedures*.

Fig. S2

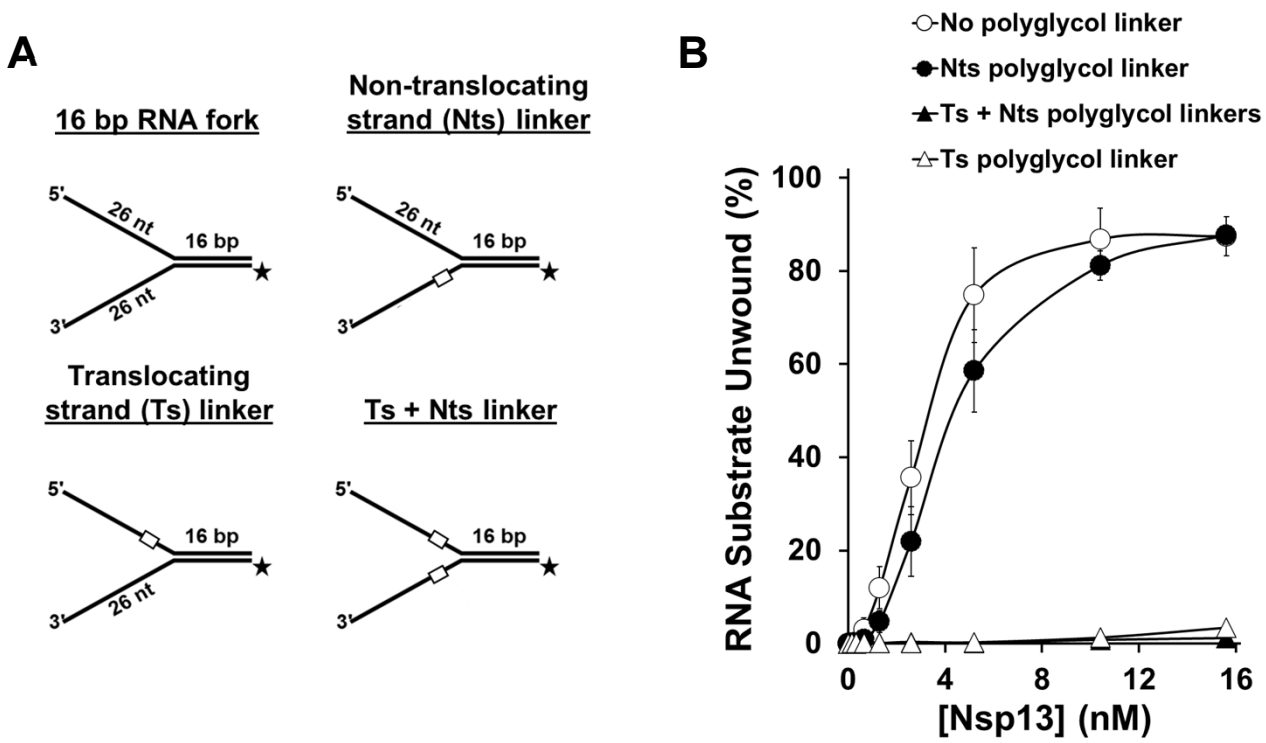

Fig. S3. Strand-specific inhibition of Nsp13 RNA helicase activity by sugar-phosphate backbone discontinuity. (A) Depiction of the partial duplex RNA substrates with a polyglycol linker (PGL) in the top or bottom strand. (B) Quantitative assessment of Nsp13 helicase activity from a 15-min incubation at 37 ℃ as a function of protein concentration in the presence of 2 mM ATP and 5 mM Mg^{2+} on the PGL backbone-modified substrates (0.25 nM) shown in (A). Data represent the average of at least three independent experiments with SD indicated by error bars.

Fig. S3

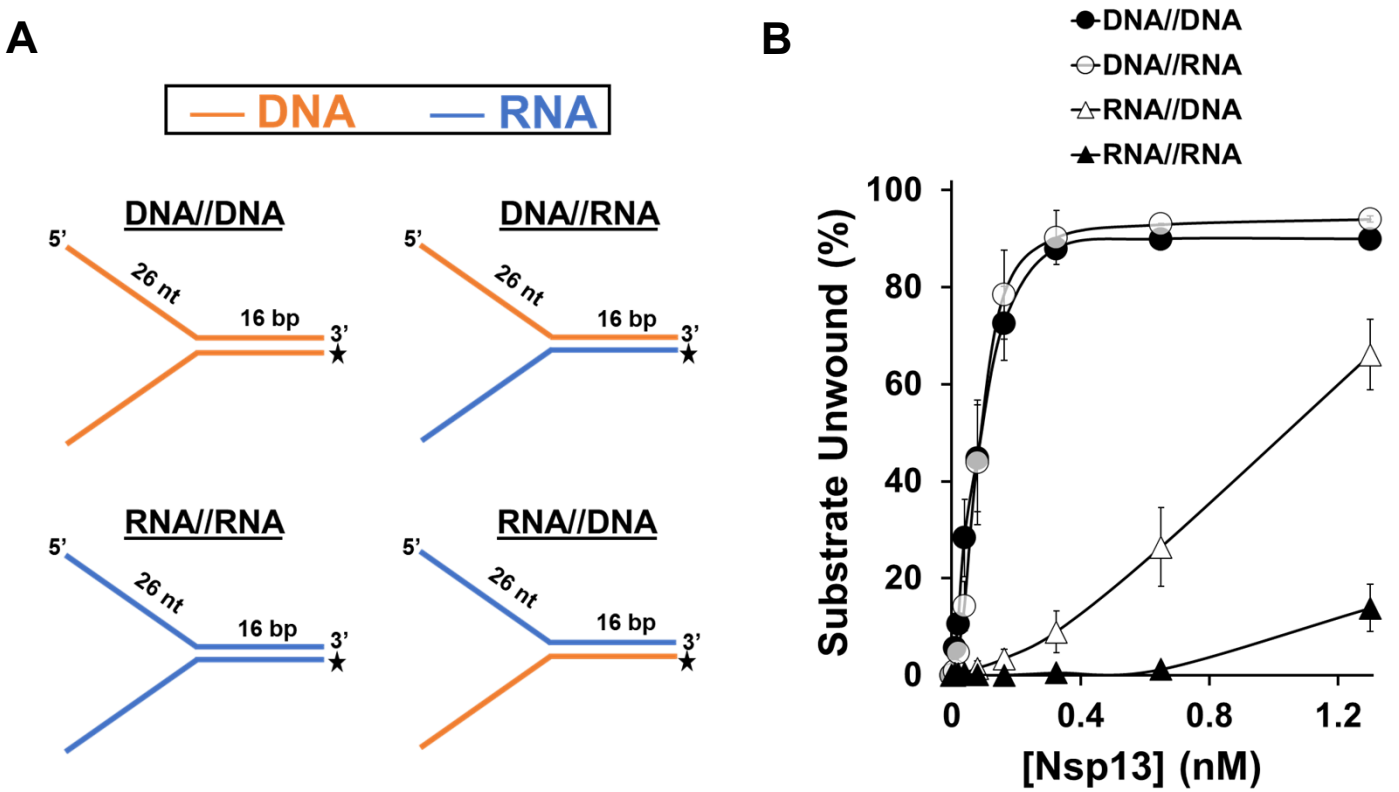

Fig. S4. Chemical identity of nucleic acid strands dictates Nsp13 helicase unwinding efficiency. (A) Depiction of the partial duplex substrates with DNA or RNA in the top or bottom strand. (B) Quantitative assessment of Nsp13 helicase activity from a 15-min incubation at 37 ℃ as a function of protein concentration in the presence of 2 mM ATP and 5 mM Mg^{2+} on the nucleic acid substrates (0.25 nM) shown in (A). Data represent the average of at least three independent experiments with SD indicated by error bars.

Fig. S4

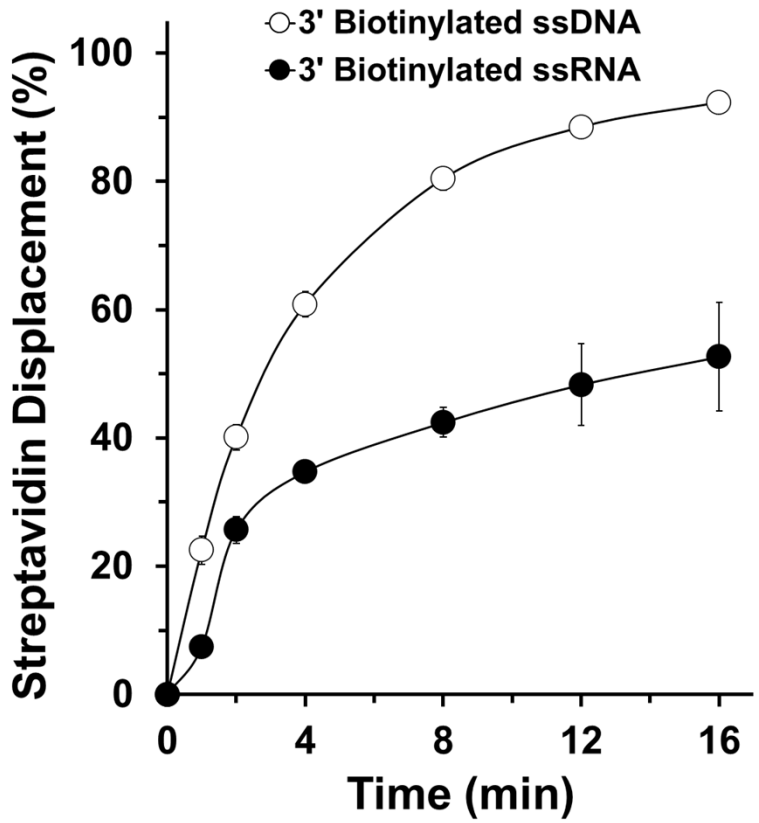

Fig. S5. Nsp13 disrupts high affinity interaction of streptavidin bound to 3' biotinylated ssDNA or ssRNA. Quantitative assessment of Nsp13-catalyzed streptavidin displacement kinetic reactions incubated at various time points (1 - 16 min) at 37 °C in the presence of 1 mM $MgCl_2$ and 2 mM ATP using 39-mer single-stranded RNA and DNA substrates (0.25 nM) with a biotin moiety at the 3' end. Biotinylated oligonucleotide was preincubated with 0.75 nM streptavidin monomer for 10 min and reactions were initiated by the addition of 1 μM biotin immediately followed by Nsp13 (1.6 nM). Data represent the average of at least three independent experiments with SD indicated by error bars.

Fig. S5

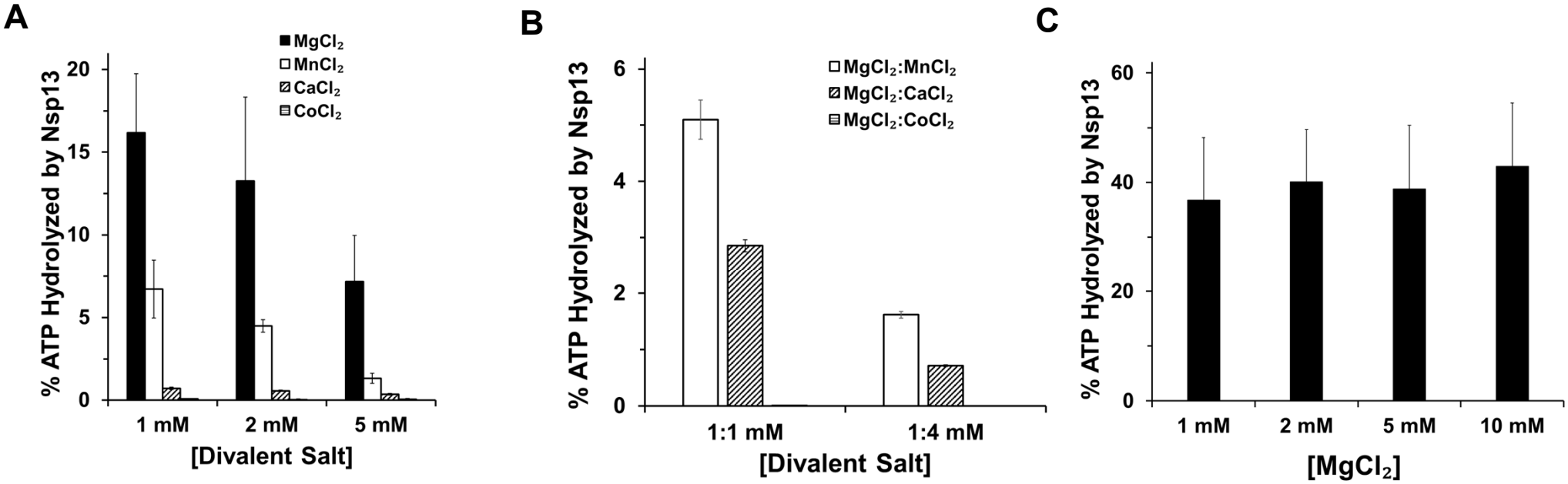

Fig S6. Effect of Divalent Salts on Nsp13 ATPase Activity. (A) 0.4 nM Nsp13 was incubated with 1 mM ATP in Nsp13 reaction salts with either $MgCl_2$, $MnCl_2$, $CaCl_2$ or $CoCl_2$ and 1.6 μM 60 nt RNA effector. Reactions were quenched at 0 and 10 minutes. (B) 0.4 nM Nsp13 was incubated with 1 mM ATP in Nsp13 reaction salts with 1.6 μM 60 nt RNA effector and mixtures of divalent salts either in a 1:1 or a 1:4 ratio resulting in a final concentration of either 2 or 5 mM divalent salt in each reaction. (C) 4 nM Nsp13 was incubated with 1 mM ATP in Nsp13 reaction salts with increasing amounts of $MgCl_2$ in the absence of effector RNA. Reactions from these ATPase assays were quenched at 0 and either 10 (A,B) or 30 (C) minutes and run on PEI-cellulose TLC sheets for 20 minutes in 1M Formic Acid and 0.8 M LiCl. % ATP hydrolyzed was calculated by dividing the released phosphate by the total of ATP and phosphate in each lane. Background from the t=0 was subtracted out from each measurement. Data represent the average of at least three independent experiments with SD indicated by error bars.

Fig. S6

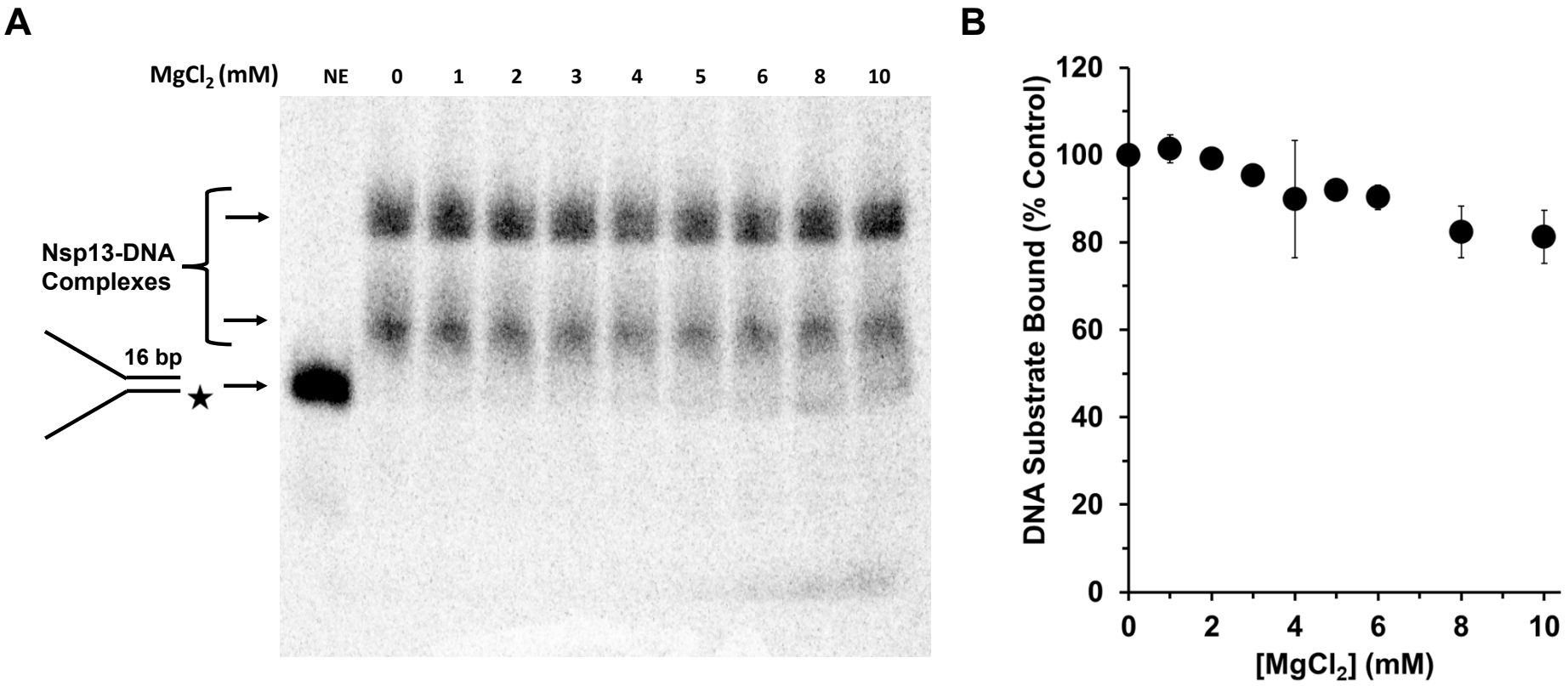

Fig S7. Effect of $MgCl_2$ on Nsp13's binding to the DNA substrate. Nsp13 (2.5 nM) was incubated with the 16-bp DNA forked duplex substrate (0.25 nM) for 15 min at RT. (A) Binding mixtures were assessed by EMSA, as described in *Experimental Procedures*. (B) Quantitative analysis is shown. Data represent average of at least three independent experiments with SD indicated by error bars.

Fig. S7

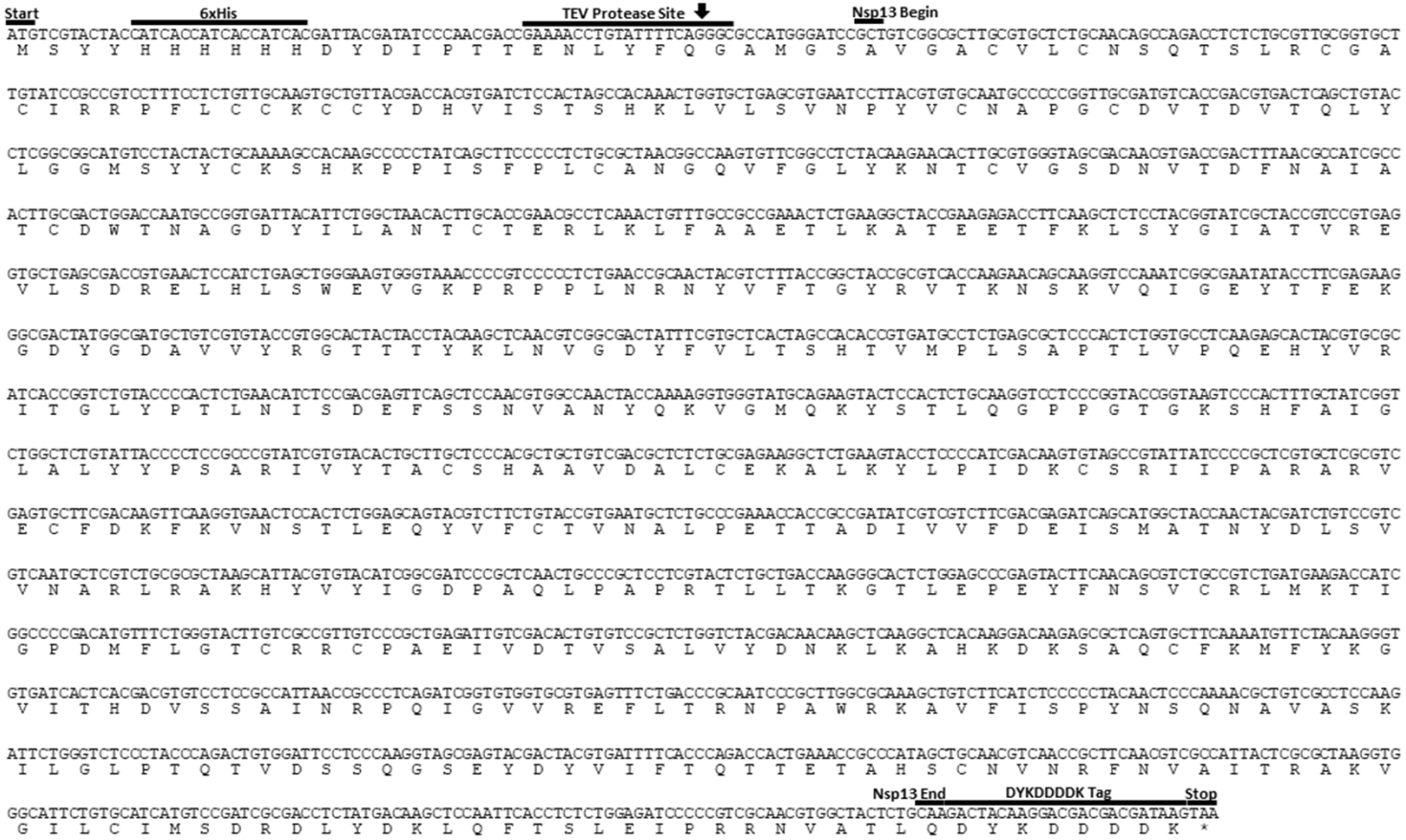

Fig. S8. Nsp13 coding sequence optimized for insect cell expression. Sequence of the insert coding for Nsp13 which was codon-optimized for insect cell expression. The insert was inserted into the pFastBac HT B vector using BamHI and XhoI restriction sites as noted in *Methods*. Lines above the DNA sequence indicate regions of interest and amino acids are below the sequence.

Fig. S8

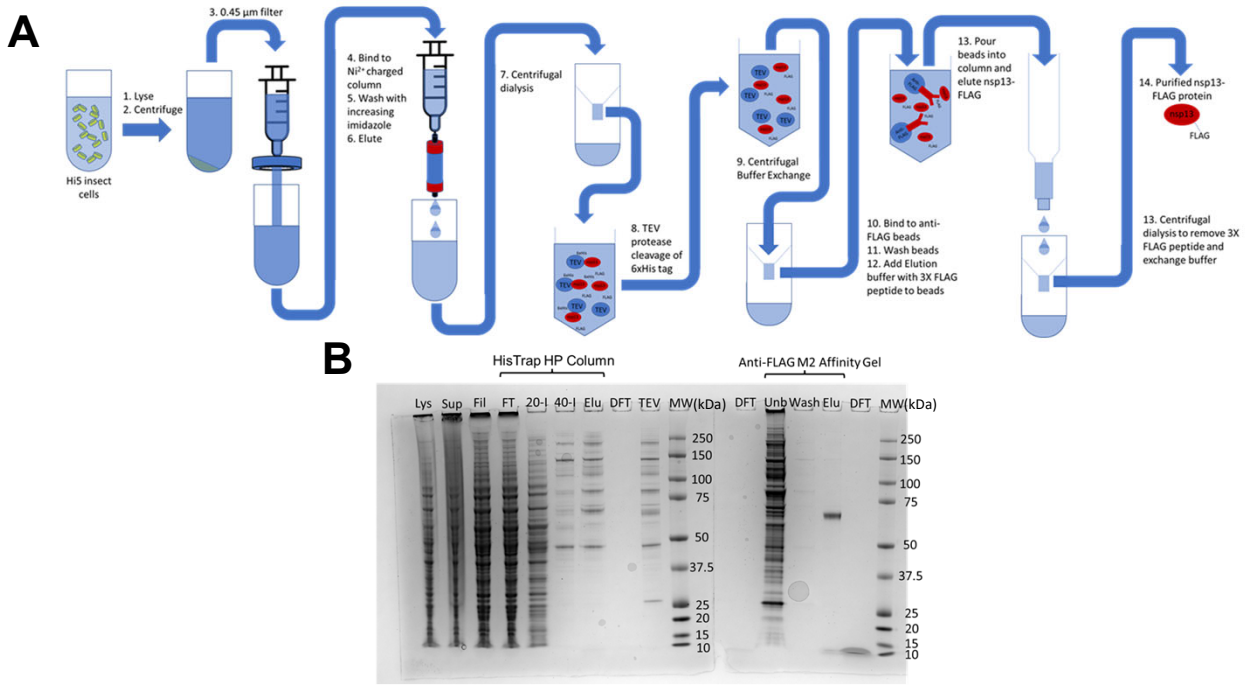

Fig. S9. Recombinant SARS-CoV-2 Nsp13 purification. (A) Scheme for purification of recombinant SARS-CoV-2 Nsp13 protein. ① Hi5 insect cells previously infected with baculovirus containing recombinant Nsp13 were lysed and cell debris pelleted after a centrifugation step. ② The supernatant was filtered through a 0.45 micron PVDF filter. ③ The filtered lysate was injected into a 1 ml HisTrap HP column followed by imidazole washes and elution with a high concentration of imidazole. ④ The eluted protein was dialyzed to remove imidazole and high salt using a centrifugal MWCO filter device. ⑤ The 6xHis tag was cleaved off of the Nsp13 using TEV protease overnight at 4°C. ⑥ The cleaved protein was dialyzed to remove β-mercaptoethanol. ⑦ Nsp13 was bound to anti-FLAG beads followed by washes and elution with excess 3XFLAG peptide. ⑧ The elution was poured into a column and eluted Nsp13 was collected. ⑨ The eluted Nsp13 was dialyzed to exchange into the storage buffer and remove any 3XFLAG peptide. (B) Coomassie-stained SDS polyacrylamide gel showing Nsp13 fraction from affinity purification. Lysate (Lys), Supernatant (Sup), Filtered (Fil), 20 mM Imidazole wash (20-I), 40 mM imidazole wash (40-I), Elution (Elu), Dialysis flow-through (DFT), TEV protease cleaved protein (TEV), molecular weight markers (MW), Unbound (UB), washes (Wash). The final dialyzed recombinant Nsp13 protein is shown in **Fig. 1**.

Fig. S9