

# $\gamma$ -Hemolysin Nanopore is Sensitive to Guanine-to-Inosine Substitutions in Double-Stranded DNA at the Single-Molecule Level

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## 1. Experimental methods

**DNA Preparation and Purification.** DNA was synthesized from commercially available phosphoramidites (Glen Research, Sterling, VA) by the DNA/peptide core facility at the University of Utah. After synthesis, each DNA sample was purified using an anion-exchange HPLC column with a linear gradient of B from 1% to 100% over 30 min (A = 10% CH<sub>3</sub>CN/90% ddH<sub>2</sub>O, B = 20 mM NaPi, 1 M NaCl, pH 7, in 10% CH<sub>3</sub>CN/90% ddH<sub>2</sub>O, flow rate = 3 mL/min) while monitoring the UV absorbance at 260 nm. The DNA sample was dialyzed in ddH<sub>2</sub>O for 36 h at 4 °C to remove the purification salts, and then dried by lyophilization. The concentrations of the resuspended DNA solutions in ddH<sub>2</sub>O were obtained by measuring the absorbance at 260 nm and using the primary sequence to estimate the extinction coefficient.<sup>1</sup>

**Oligomerization of  $\gamma$ -HL octamer.** Octamers were formed at room temperature according to the procedure reported by Bayley and co-workers for  $\alpha$ -HL oligomerization.<sup>2</sup> The wild-type  $\gamma$ -HL monomers (HlgB and HlgC) were purchased from IBT Bioservices (Gaithersburg, MD). A solution of 100 mM deoxycholate (DOC) was treated with the  $\gamma$ -HL monomer (HlgB/HlgC/DOC mass ratio 1:1:20), with stirring, in a stepwise fashion, to a final concentration of 6.25 mM DOC and 3.5  $\mu$ M  $\gamma$ -HL monomers, in 100 mM Tris-HCl buffer (pH 8.3). After a two-hour incubation at the room temperature, the octamers were purified by removing the monomers via a 100 kDa cut-off centrifugal filter (Amicon Ultra 100 K device). The preformed channels were stored in solution at -80 °C.

**Reagents and Chemicals for Nanopore Measurement.** 1,2-diphytanoyl-*sn*-glycero-3-phospho-choline (DPhPC) powder (Avanti Polar Lipids, AL) was dissolved in decane at 10 mg/mL, and used to form the lipid bilayer on the orifices (700 - 900 nm in radius) of the glass nanopore membranes (GNMs). The conical shaped GNM was fabricated using a previously reported bench-top method,<sup>3</sup> and pretreated with a 2% solution of (3-cyanopropyl)dimethylchlorosilane in acetonitrile (v/v) to produce a modified hydrophobic glass surface to allow for formation of a lipid bilayer across the orifice.<sup>3</sup> Unless otherwise specified, the ion

channel recordings were performed using 3 M KCl solution buffered by 20 mM HOAc/KOAc at pH 5.0 at  $20.0 \pm 0.5$  °C.

**Current–Time Recordings.** Current–time ( $I-t$ ) traces were recorded using a custom-built high-impedance and low-noise system (Electronic BioSciences Inc., San Diego, CA). The  $I-t$  traces for open channel optimization experiments and single-stranded DNA (ssDNA) translocation events were sampled at 500 kHz and filtered using a 4-pole Bessel low-pass filter at 100 kHz. For the double-stranded DNA (dsDNA) detection experiments, the current was sampled at 50 kHz and filtered at 10 kHz. A potential was applied across the bilayer using two Ag/AgCl electrodes placed inside and outside the capillary. A gas-tight syringe is used to apply a pressure of 40 to 80 mmHg inside of the GNM capillary to facilitate protein channel insertion.<sup>3</sup> The pre-oligomerized  $\gamma$ -HL protein was carefully added into the chamber outside of the GNM; insertion of  $\gamma$ -HL protein decreased the conductance to  $\sim 6.3$  nS ( $-120$  mV) in 3 M KCl. The ssDNA or dsDNA sample was added into the  $\sim 350$   $\mu$ L reservoir, resulting in  $I-t$  signatures corresponding to DNA translocation or capture in the vestibule.

**Reversal Potentials Measurements.** The ion selectivity was calculated from the measured reversal potentials ( $V_r$ ) using the Goldman-Hodgkin-Katz (GHK) equation. Each experiment was performed using asymmetric KCl solutions (3 M KCl (*cis*) and 1 M KCl (*trans*), with 20 mM buffer salts (PB or KOAc)). Single-channel current-voltage ( $I-V$ ) relationships of  $\gamma$ -HL nanopore were recorded to determine  $V_r$  value at zero current. In addition, the potential offset ( $\sim -27.5$  mV) between two Ag/AgCl electrodes was measured by a voltmeter with asymmetric KCl solution across the GNMs (700 - 900 nm in radius), prior to the addition of lipid layer and protein to the *cis* chamber. The reported values of  $V_r$  in Figure 2 and Section S2 were corrected by the measured potential offsets at each pH.

**Data Analysis.** The electrical current amplitude and noise were recorded and analyzed as described in the Results and Discussion section in the main text. QuB software (version 2.0.0.33) was used to extract open channel current and event blockade current levels. The  $I-t$  traces were post filtered and plotted using either

QuB (version 2.0.0.33) or Origin Pro (version 9.1). Population density plots were generated using data analysis programs provided by Electronic Biosciences Inc., San Diego, CA. Current, translocation duration histograms, current-voltage plots, and voltage-dependent translocation durations were plotted using Origin Pro (version 9.1).

## 2. Sequences of oligomers studied

Table S1. Sequences of DNA and RNA strands used in this study

Molecule Name	Sequence
<b>dA<sub>60</sub>-Btn</b>	5'- AAA-3' biotin
<b>ssDNA</b>	5'- AAA A-3'
<b>9bp dsDNA</b>	5'-(T) <sub>24</sub> TGG AGC TGG TGG CGT AG-3' 3'- CAC CGC ATC-5'
<b>17bp dsDNA</b>	5'-(T) <sub>24</sub> TGG AGC TGG TGG CGT AG-3' 3'-ACC TCG ACC ACC GCA TC-5'
<b>30bp dsDNA G:C</b>	5'-(A) <sub>30</sub> AGT TGC CAC CTA ATG CGT CGT CGG TCT ATC-3' 3'-TCA ACG GTG GAT TAC GCA GCA GCC AGA TAG-5'
<b>30 bp dsDNA I:C</b>	5'-(A) <sub>30</sub> AGT TGC CAC CTA ATG CGT CGT CGG TCT ATC-3' 3'-TCA ACI ITI IAT TAC ICA ICA ICC AIA TAI -5'
<b>60bp dsDNA</b>	5'-(A) <sub>30</sub> AGT TGC CAC CTA ATG CGT CGT CGG TCT ATC AAA AAG CCT ACA CAG AAA AAT CAG TTG TCG-3' 3'- TCA ACG GTG GAT TAC GCA GCA GCC AGA TAG TTT TTC GGA TGT GTC TTT TTA GTC AAC AGC-5'

### 3. Calculation of ion-selectivity of the $\gamma$ -HL nanopore

Ion selectivity of the  $\gamma$ -HL was measured at different pH in asymmetrical KCl solutions (3 M KCl (*cis*) and 1 M KCl (*trans*)). The *trans* side was held at ground potential. The reversal potential ( $V_r$ ), also known as the Nernst potential, corresponds to the applied potential at zero current. Experiments were carried out at 20.0 °C. The ion selectivity was calculated from  $V_r$  using the modified Goldman-Hodgkin-Katz (GHK) equation:<sup>4</sup>

$$V_r = \frac{RT}{F} \ln \left( \frac{\sum_i^N P_{M_i^+} [M_i^+]_{trans} + \sum_j^N P_{A_j^-} [A_j^-]_{cis}}{\sum_i^N P_{M_i^+} [M_i^+]_{cis} + \sum_j^N P_{A_j^-} [A_j^-]_{trans}} \right) \quad (1)$$

where  $V_r$  is the applied potential on the *cis* side,  $R$  is the universal gas constant,  $T = 293$  K,  $F$  is Faraday's constant,  $P_{ion}$  is the permeability of ion, and  $[ion]$  is the ion concentration on the *trans* or *cis* side of the protein channel. At pH 8.0,  $V_r$  is measured to be  $\sim -13.9$  mV, corrected by the measured potential offset ( $-27.5$  mV), and we obtained

$$-13.9 \text{ mV} = \frac{RT}{F} \ln \left( \frac{1P_{K^+} + 3P_{Cl^-}}{3P_{K^+} + 1P_{Cl^-}} \right) \quad (2)$$

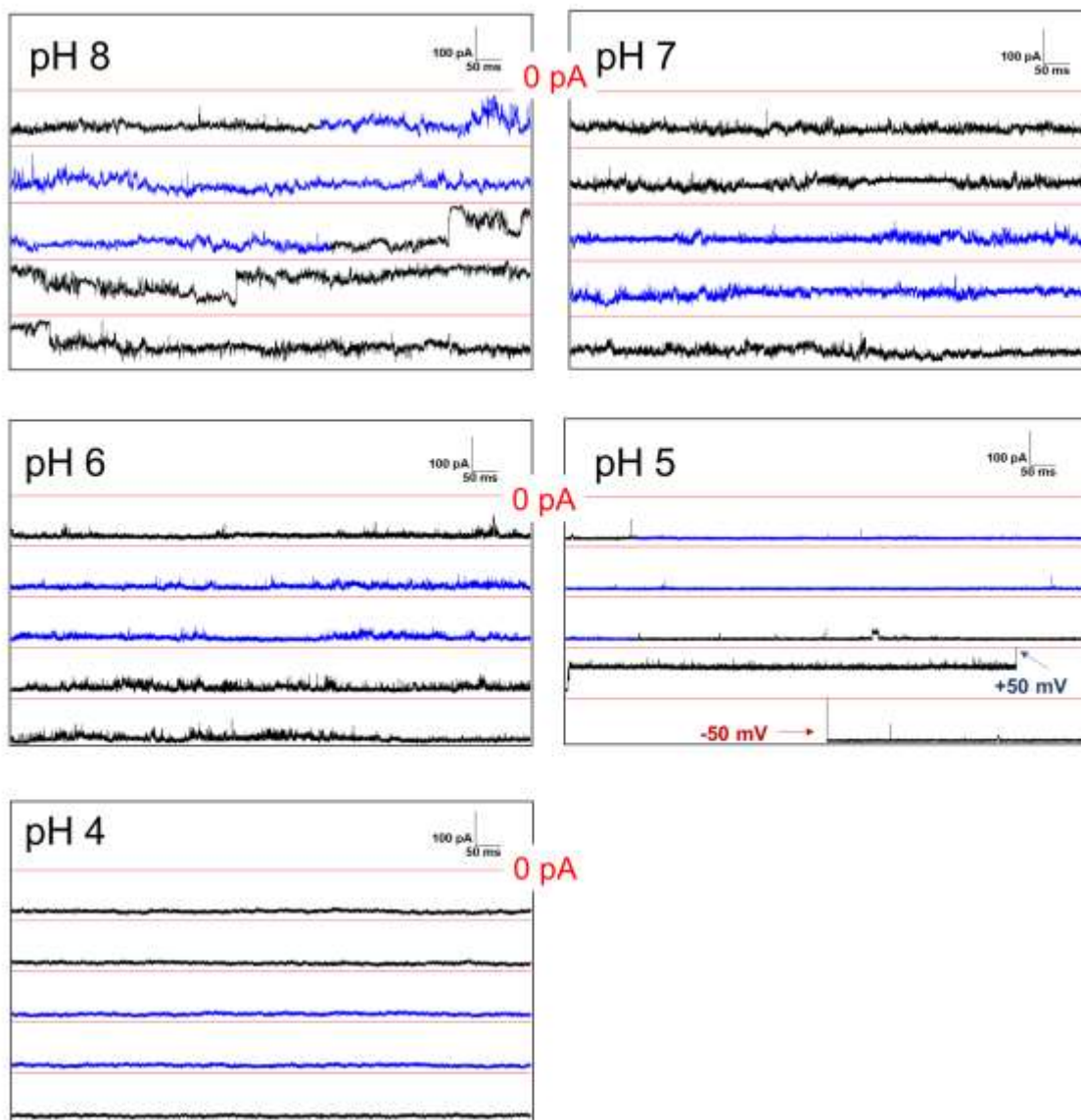
corresponding to an ion selectivity ( $P_{K^+}/P_{Cl^-}$ ) equal to 3.4.

Table S2. Effect of pH on the ion-selectivity of  $\gamma$ -HL protein channel.

<b>pH</b>	<b><math>V_r</math> (mV)</b>	<b><math>P_{K^+}/P_{Cl^-}</math></b>
4	0.74	0.94
5	-0.77	1.1
6	-1.35	1.1
7	-3.3	1.3
8	-13.9	3.4

#### 4. Current fluctuation and voltage-induced gating behavior of the $\gamma$ -HL ion channel

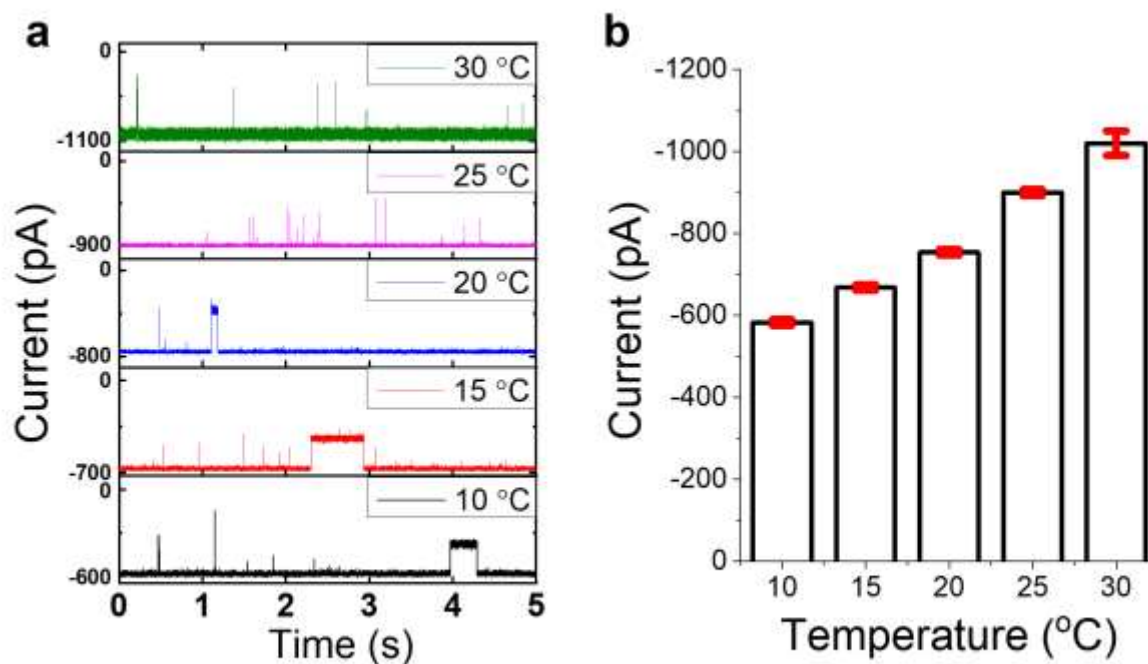
Presented as an accompaniment to Figure 2 in the main text.



**Figure S1.** Representative single-channel  $I$ - $t$  traces measured at  $-50$  mV from pH 8.0 to 4.0 in 1 M KCl, 20 mM HOAc/KOAc or PB, at  $20.0$  °C.  $I$ - $t$  traces were sampled at 50 kHz and filtered at 10-kHz using a low-pass Bessel filter. The portions highlighted in blue as shown in Figure 2c in the main text.



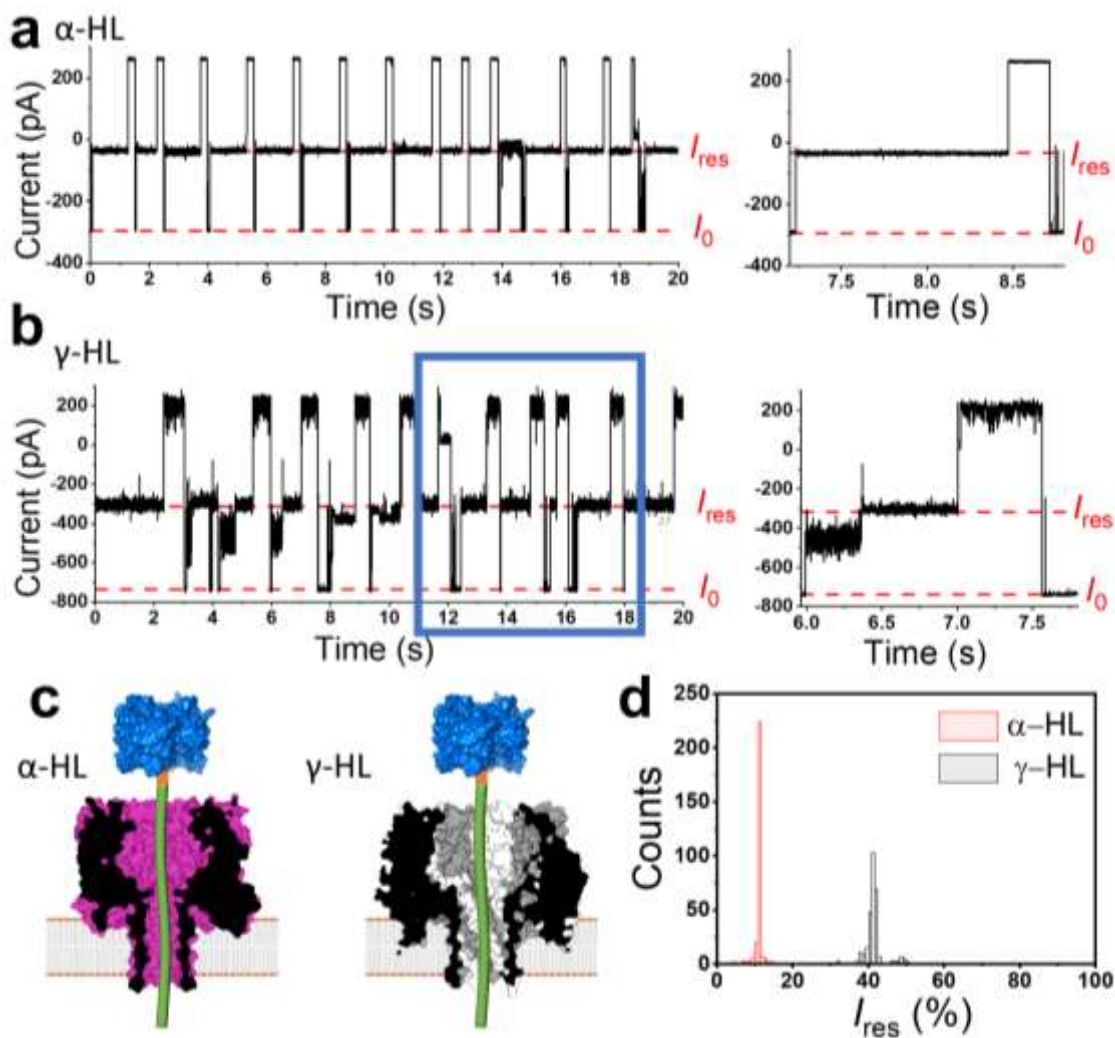
5. Effect of temperature on the current fluctuation and gating frequency.



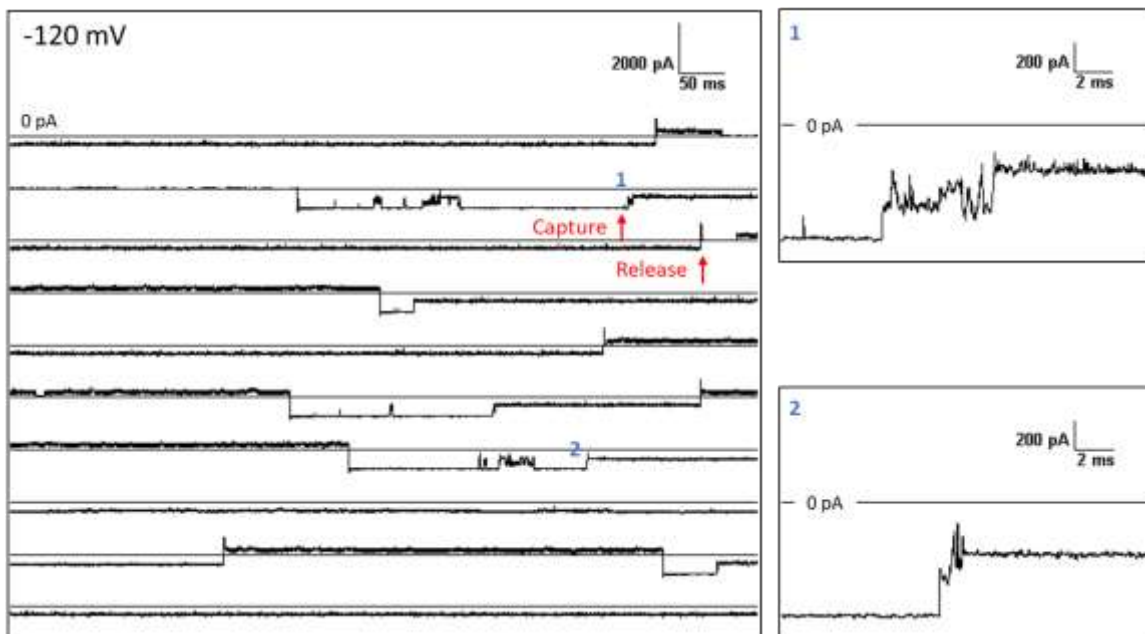
**Figure S2.** Effect of temperature on open-channel current. (a) Typical single-channel  $I$ - $t$  traces showing the open channel current for  $\gamma$ -HL as a function of temperature (10, 15, 20, 25, and 30 °C). (b) Average open-channel current obtained from the  $I$ - $t$  traces in (a) and the error bars represent the standard deviations of the recorded open-channel currents at five temperatures. Experiments were performed in 3 M KCl, 20 mM HOAc/KOAc, pH 5.0, at -120 mV. Note the current scale of each trace is different.  $I$ - $t$  traces were sampled at 50 kHz and filtered at 10 kHz using a low-pass Bessel filter.

## 6. $I$ - $t$ traces of immobilized ssDNA using $\gamma$ -HL protein nanopores

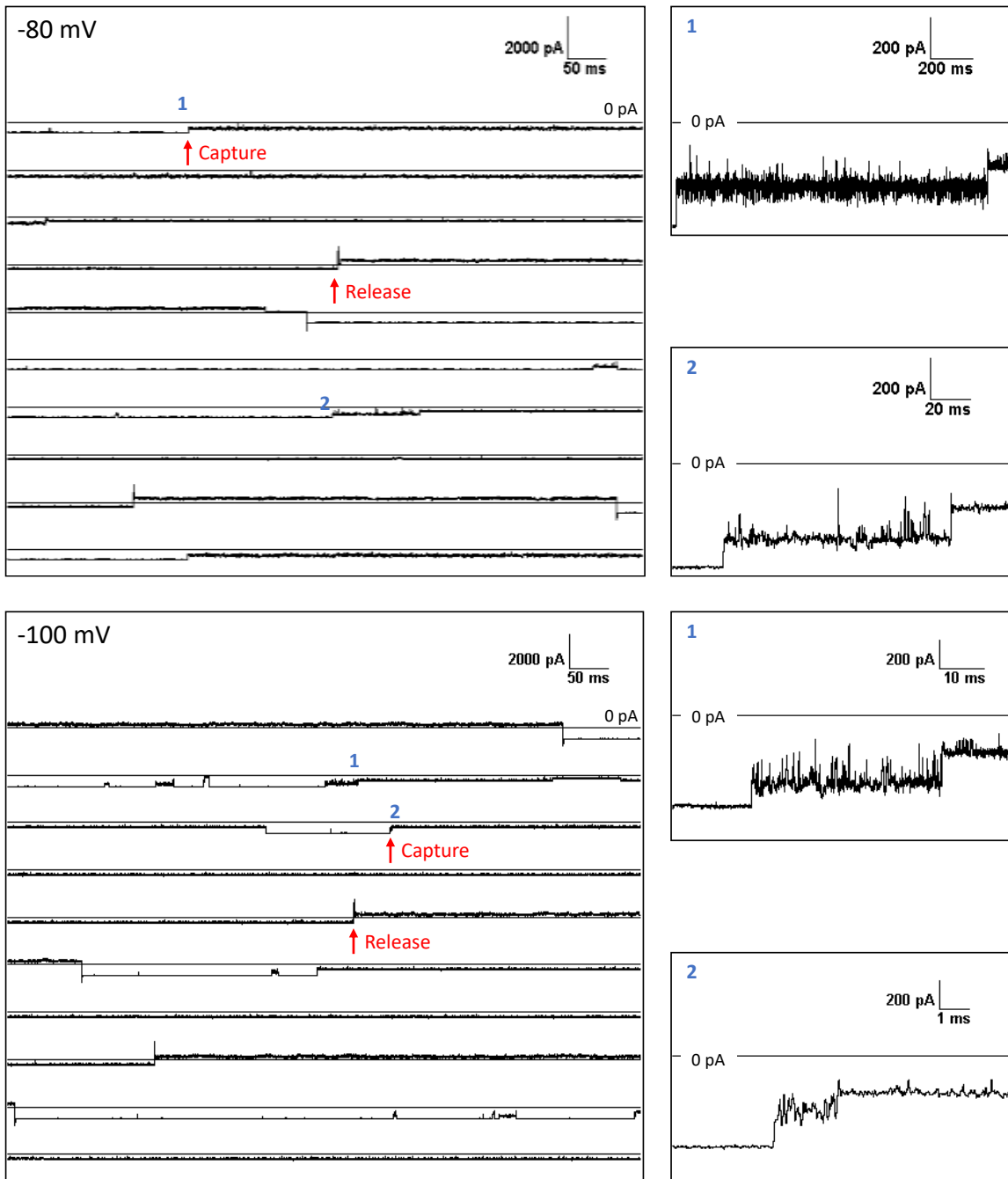
Presented as an accompaniment to Figure 4 in the main text.



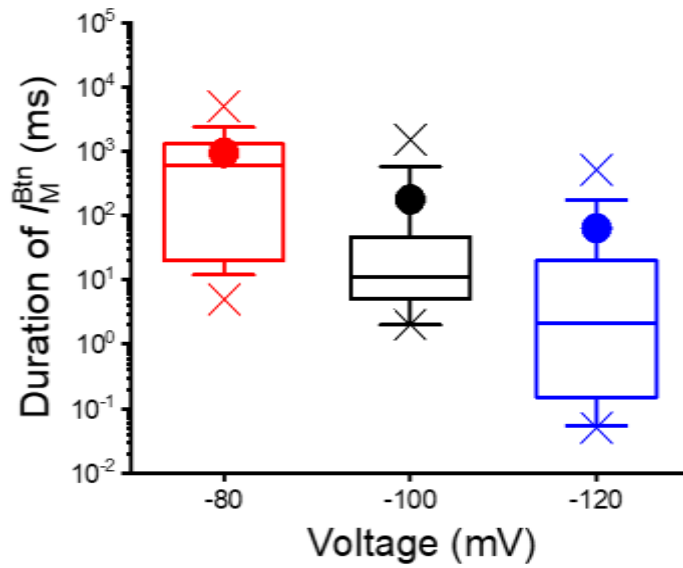
**Figure S3.** (a-b) Left: sample  $I$ - $t$  traces for immobilized  $A_{60}$ -Btn-Strep molecules in  $\alpha$ - and  $\gamma$ -HL protein nanopores. Right: expanded views of one capture/release  $I$ - $t$  trace to show the  $A_{60}$ -Btn-Strep molecule consistently yielded a single level of blockade current in  $\alpha$ -HL, but produced two levels of blockade current in  $\gamma$ -HL. The portion in the blue box in (b) is expanded and shown in Figure S4. (c) Scheme of the immobilized  $A_{60}$ -Btn-Strep molecules in the protein nanopores. (d) Residual current histograms of  $A_{60}$ -Btn-Strep in  $\alpha$ - (N = 288) and  $\gamma$ -HL (N = 294). Experiments were performed in 3 M KCl, 20 mM HOAc/KOAc, pH 5.0, at -120 mV. The  $I$ - $t$  traces were post-filtered at 1 kHz for presentation.



**Figure S4.** An expanded view of representative *I-t* trace outlined in the blue box in Figure S3 for immobilized A<sub>60</sub>-Btn-Strep molecules in  $\gamma$ -HL. The mid-level blockade current in the *I-t* trace shows that the ssDNA is captured in the vestibule and then attempts to thread into the  $\beta$ -barrel. Experiments were performed in 3 M KCl, 20 mM HOAc/KOAc, pH 5.0, at -120 mV. The *I-t* traces were post-filtered at 1 kHz for presentation.



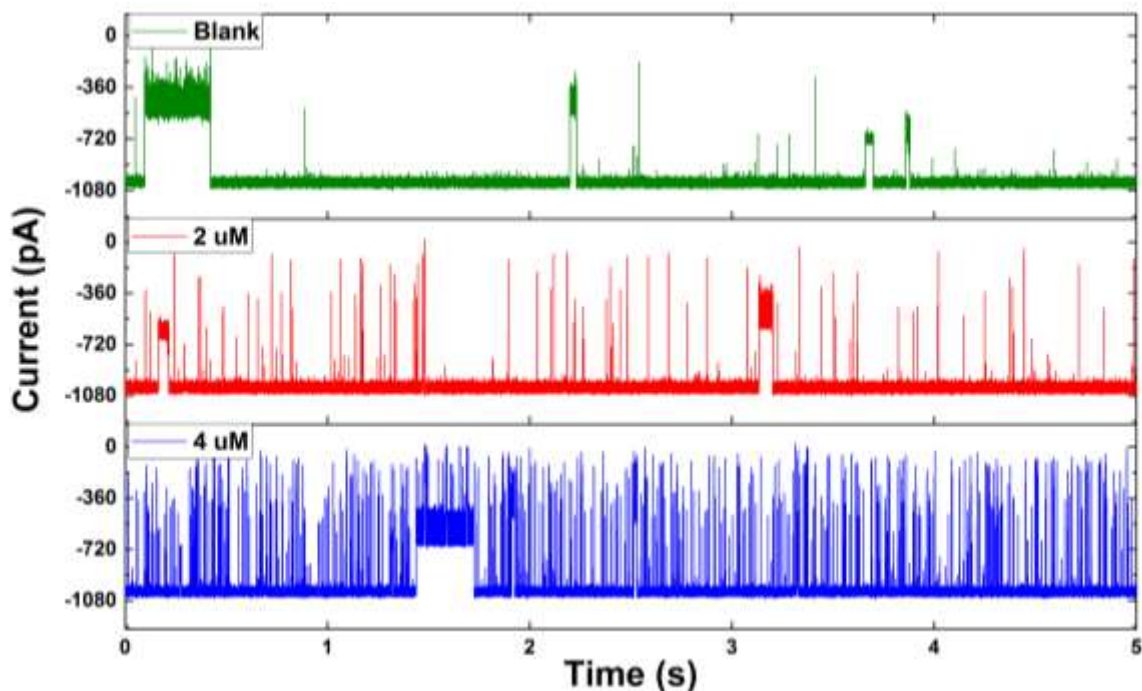
**Figure S5.** *I-t* traces for the capture/release of tethered ssDNA over an 8 s period using voltage toggling between  $\pm 80$  mV or  $\pm 100$  mV. The solution contained 3 M KCl and 20 mM HOAc/KOAc (pH 5.0). Expanded *I-t* traces showing the mid-level blockade current as the ssDNA is captured in the vestibule of the protein channel. The *I-t* traces were post-filtered at 1 kHz for presentation.



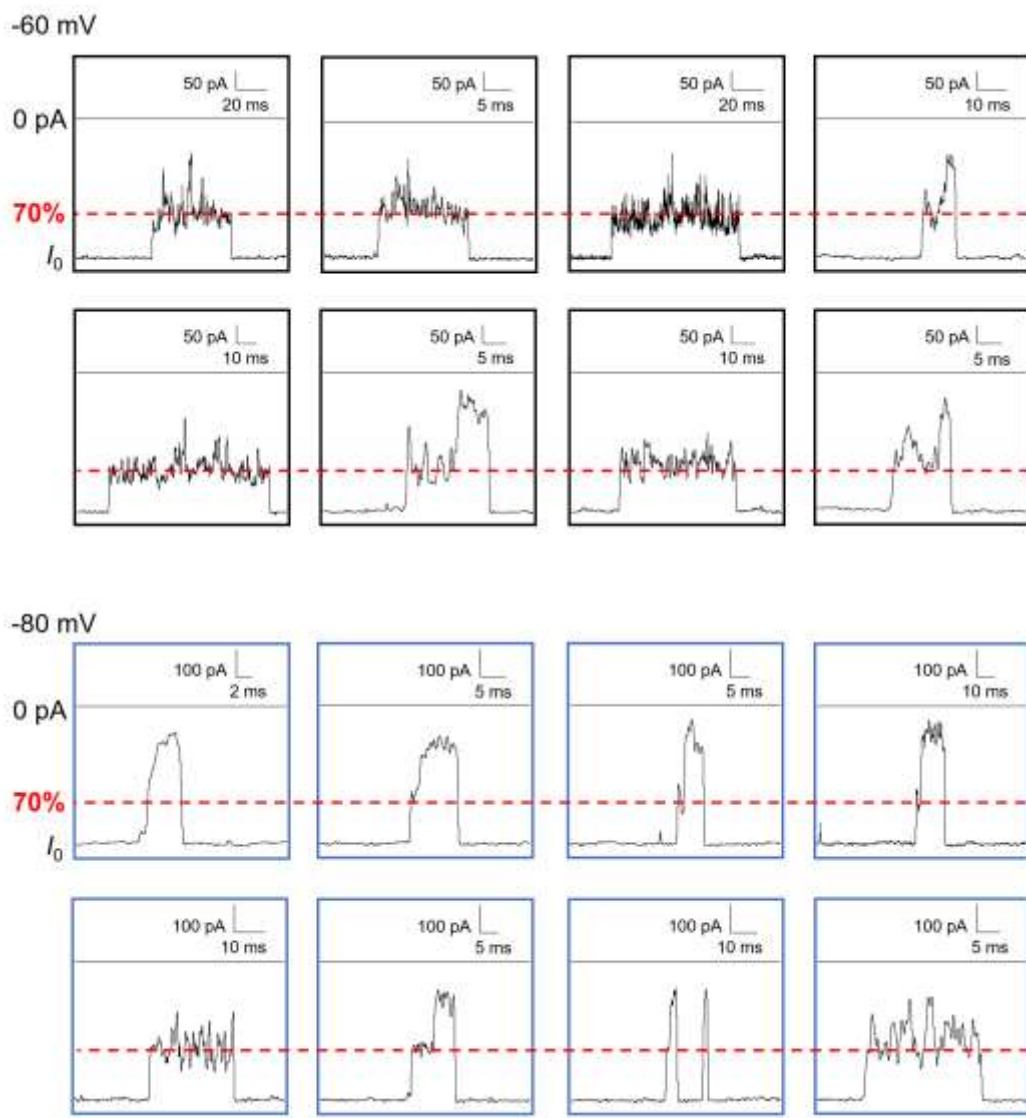
**Figure S6.** Box chart showing that durations of mid-level blockade  $I_M^{Btn}$  at three voltages (N = 20 for each). The Xs are the minima and mixima values; the height of the box displays 25-75% of all data falling within the box; the vertical lines outside the box display 10-90% of all data; the solid circles are the mean values.

## 7. Translocation of ssDNA using the $\gamma$ -HL protein channel

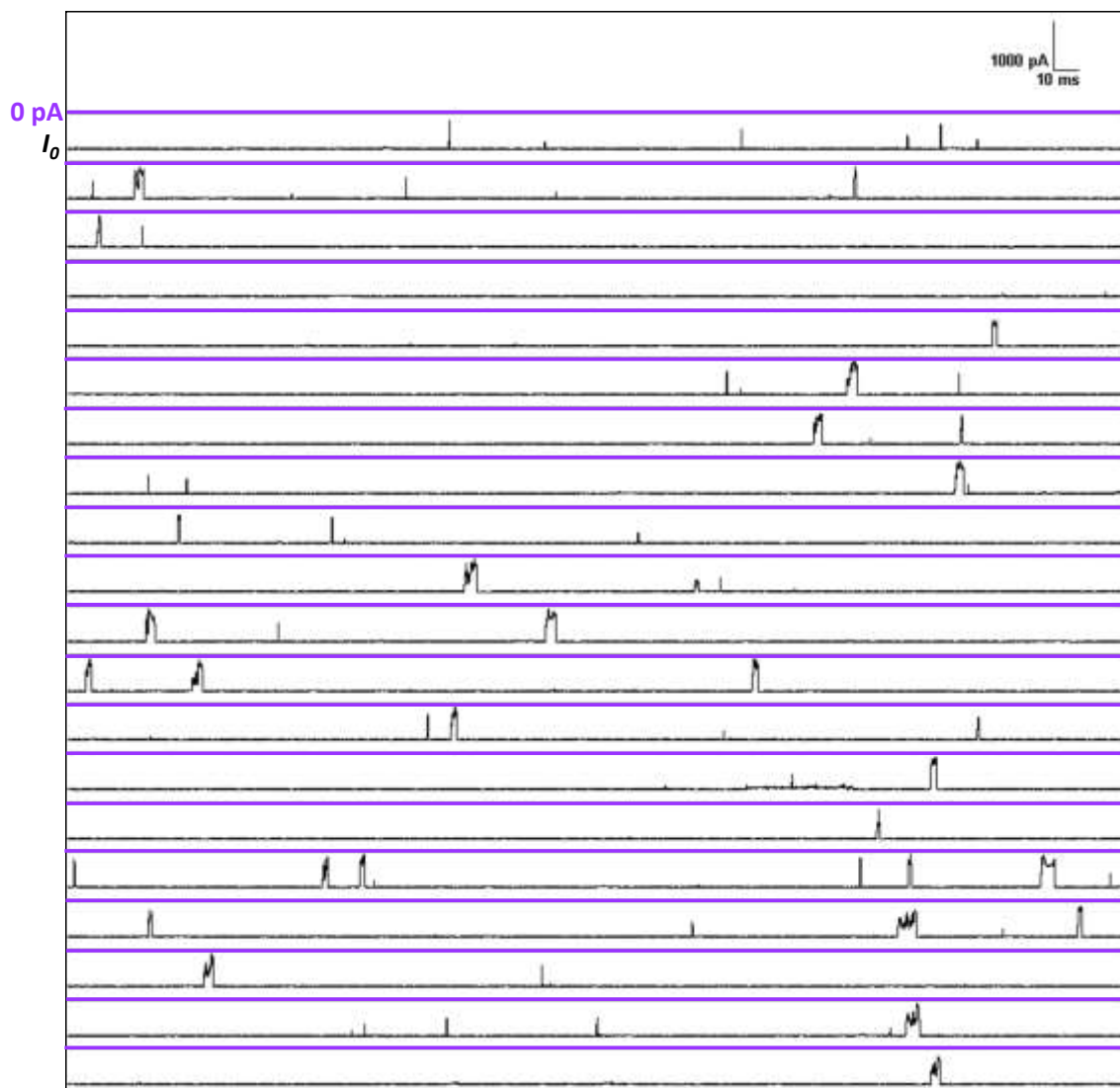
Presented as an accompaniment to Figure 5 in the main text.



**Figure S7.** Sample *I-t* traces of poly(dA)<sub>100</sub> translocation at different concentrations (0, 2 and 4  $\mu$ M). The green *I-t* trace presents a typical single-channel current recording of  $\gamma$ -HL protein channel at -140 mV. The channel switches between open and various closed states. The frequency of the gating events is reduced at lower applied potentials. Recordings were carried out in 3 M KCl, 20 mM HOAc/KOAc, pH 5.0, 20 °C, at -140 mV. Data were recorded using a 100 kHz Bessel filter at a 500 kHz sampling rate.

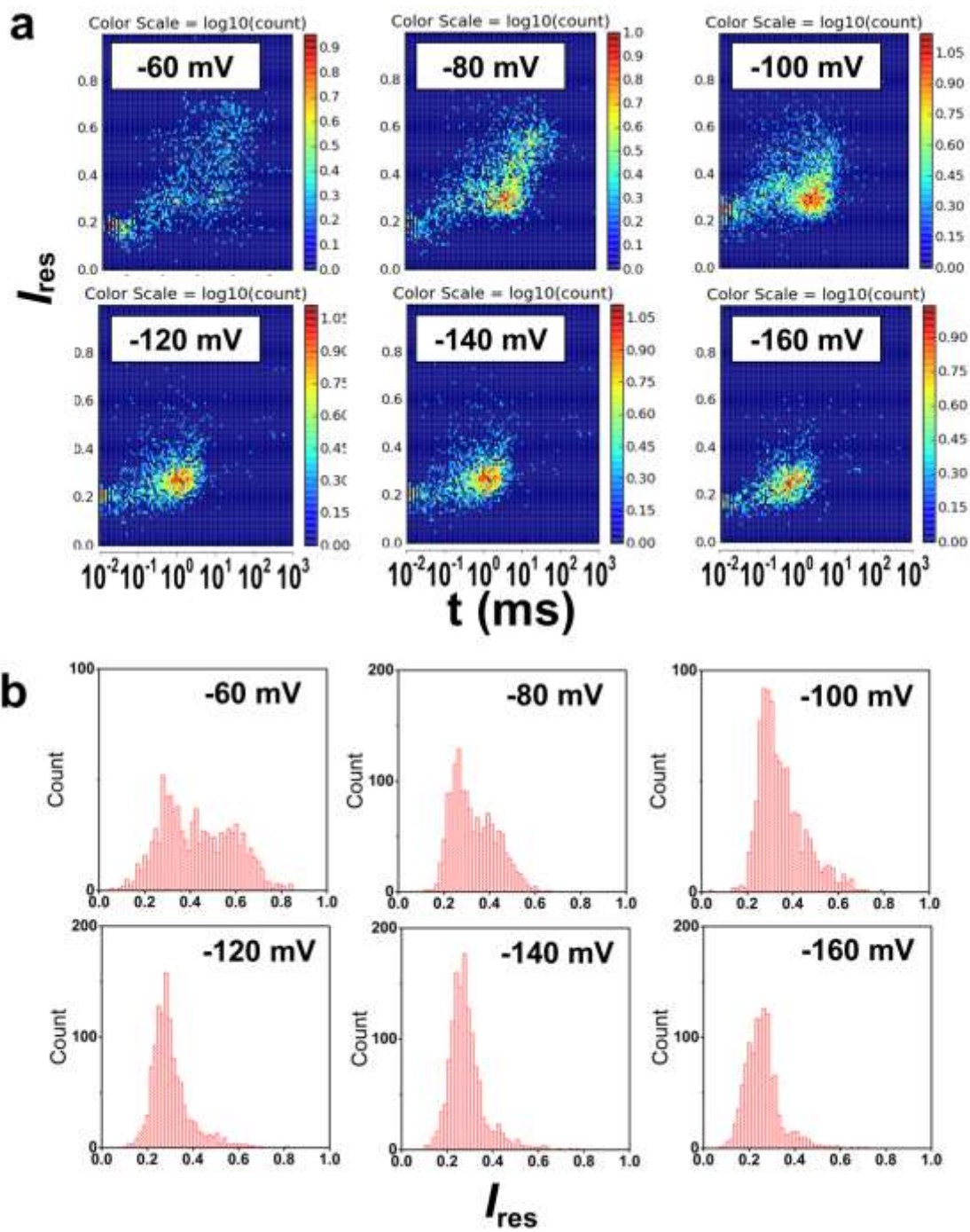


**Figure S8.** Sample  $I$ - $t$  traces of poly(dA)<sub>100</sub> in  $\gamma$ -HL at -60 and -80 mV. Recordings were carried out in 3 M KCl, 20 mM HOAc/KOAc, pH 5.0, at 20 °C. Data were recorded using a 100-kHz Bessel filter and post-filtered to 1 kHz. The mid-level blockade of ssDNA events shows consistent blockade current and upwards spikes of tethered-ssDNA poly (dA)<sub>60</sub> captured by the  $\gamma$ -HL (Figure S4-5), resulting from the ssDNA occupying the vestibule but not threading into the  $\beta$ -barrel yet. The deep-blockade current, which terminates the event, is corresponding to the poly(dA)<sub>100</sub> strand passing through the protein channel.



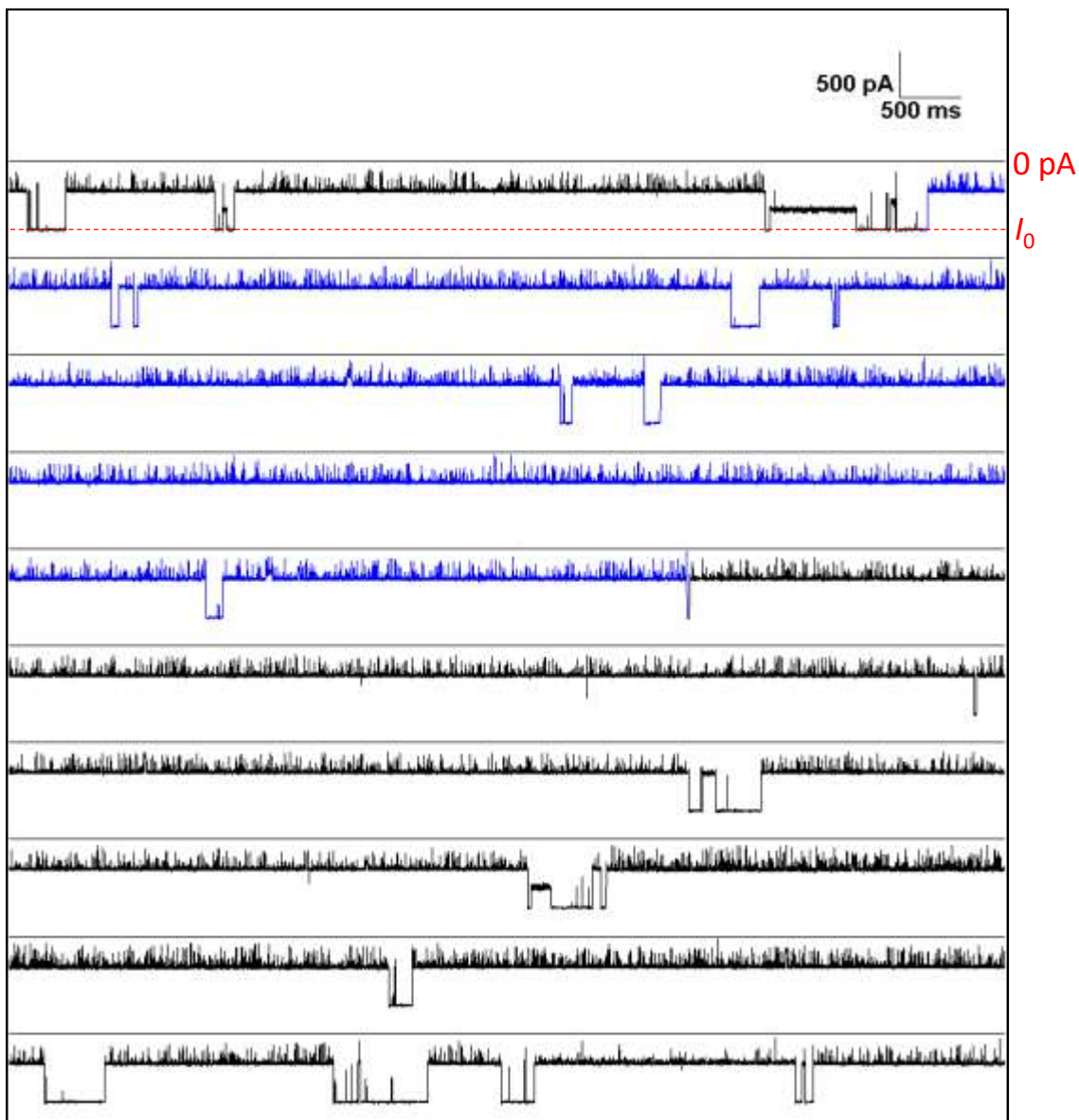
**Figure S9.** An expanded view of the  $I$ - $t$  trace shown in Figure 5 in the main text. Recordings were carried out in 3 M KCl, 20 mM HOAc/KOAc, pH 5.0 at 20 °C at an applied voltage -120 mV. The  $I$ - $t$  traces were post-filtered at 5 kHz for presentation.



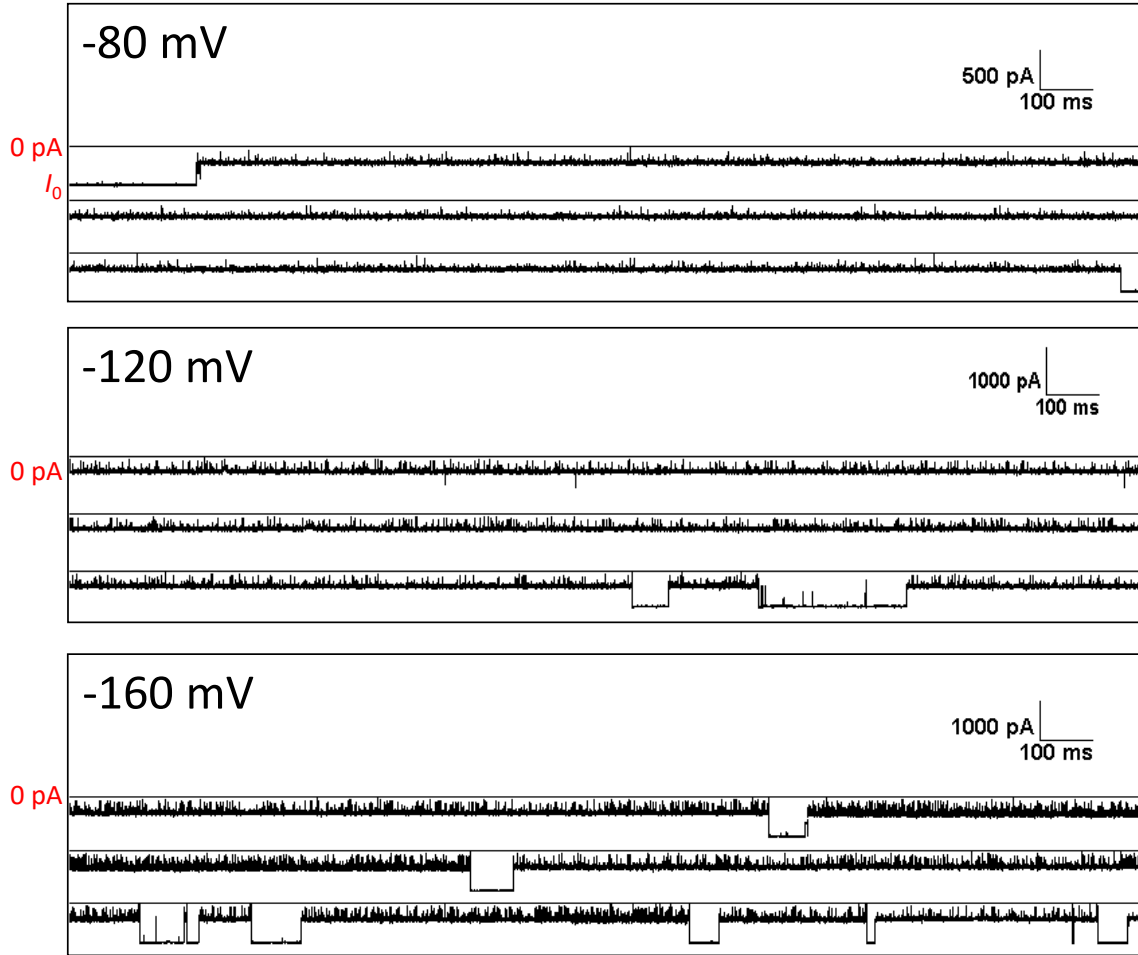


**Figure S10.** Effect of voltage on the ssDNA translocation rate. (a) Population densities of the current blockade as a function of duration time. (b) Histograms of current blockade of ssDNA events, including both complete translocation and protein-DNA collision/incomplete translocation events.

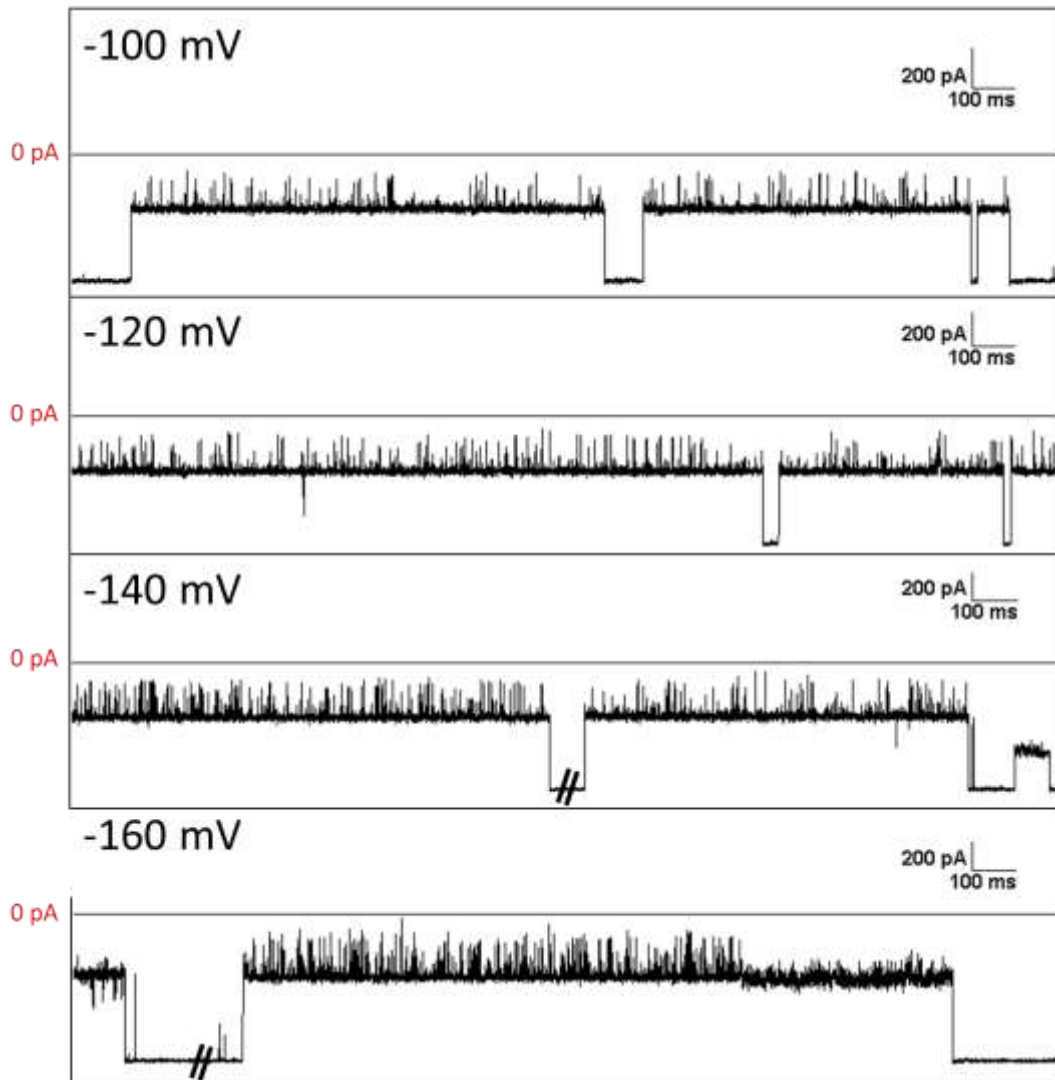
8. *I-t* trace of 30-bp G:C-containing dsDNA captured by the  $\gamma$ -HL nanopore



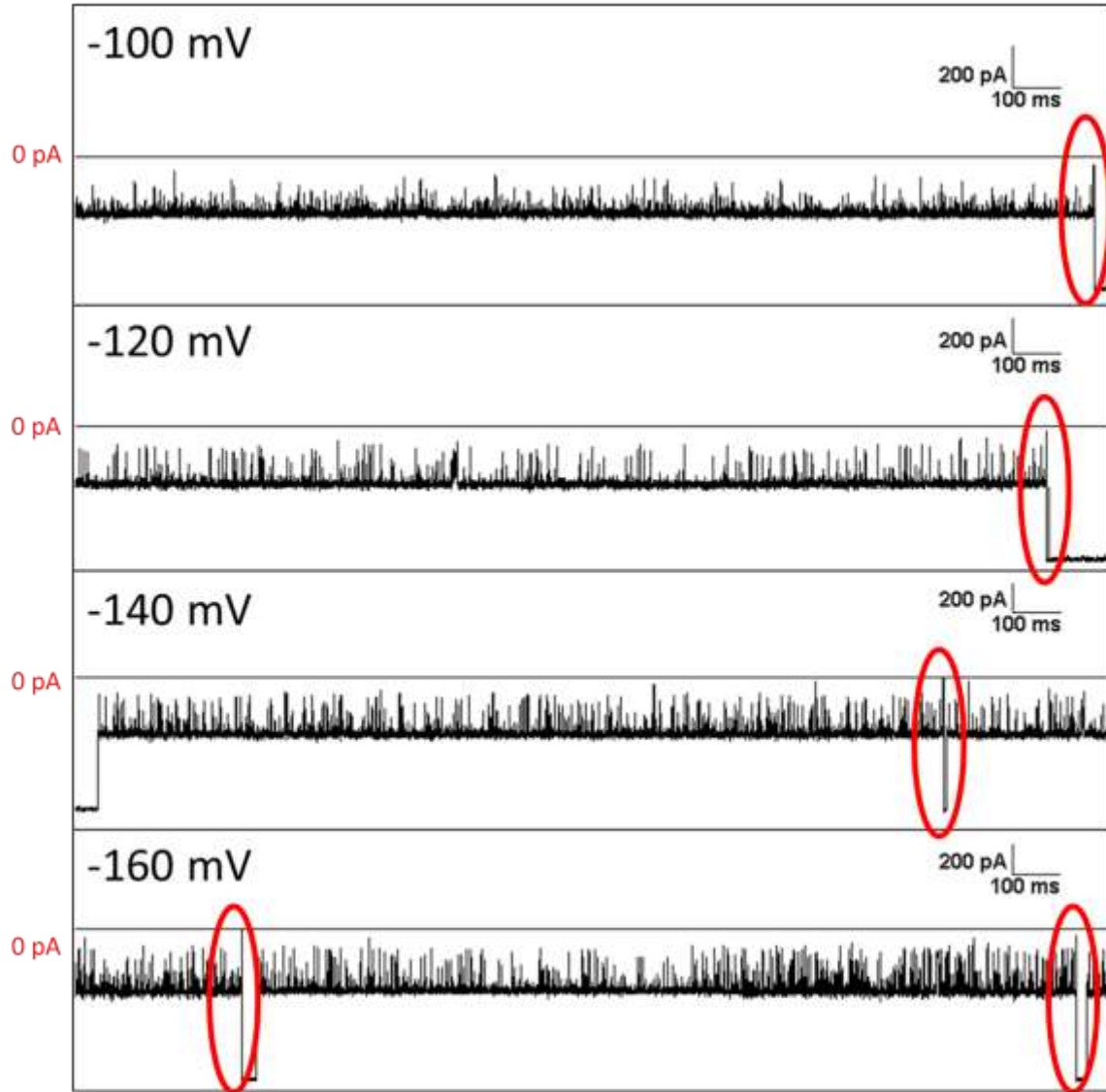
**Figure S11.** An 80 s-long *I-t* trace demonstrating capture and then release/or translocation of 30-bp dsDNA through the  $\gamma$ -HL channel. The portion highlighted in blue is shown in Figure 6b in the main text. The data were collected in 3 M KCl, 20 mM HOAc/KOAc, pH 5.0, at -120 mV, and filtered using a 10 kHz Bessel filter. The *I-t* traces shown here were post-filtered at 1 kHz for presentation.



**Figure S12.** Voltage dependence of event duration and mid-level current fluctuation. Six-second  $I-t$  traces demonstrating capture and release/or translocation of 30-bp dsDNA through the  $\gamma$ -HL channel at  $-80$ ,  $-120$ , and  $-160\text{ mV}$ . As the applied potential increases, the mid-level current fluctuation frequency of both Type 1 (capture and release, see Figure S13) and Type 2 (capture and translocation, see Figure S14) events increases. The  $I-t$  traces were post-filtered at  $3\text{ kHz}$  for presentation.

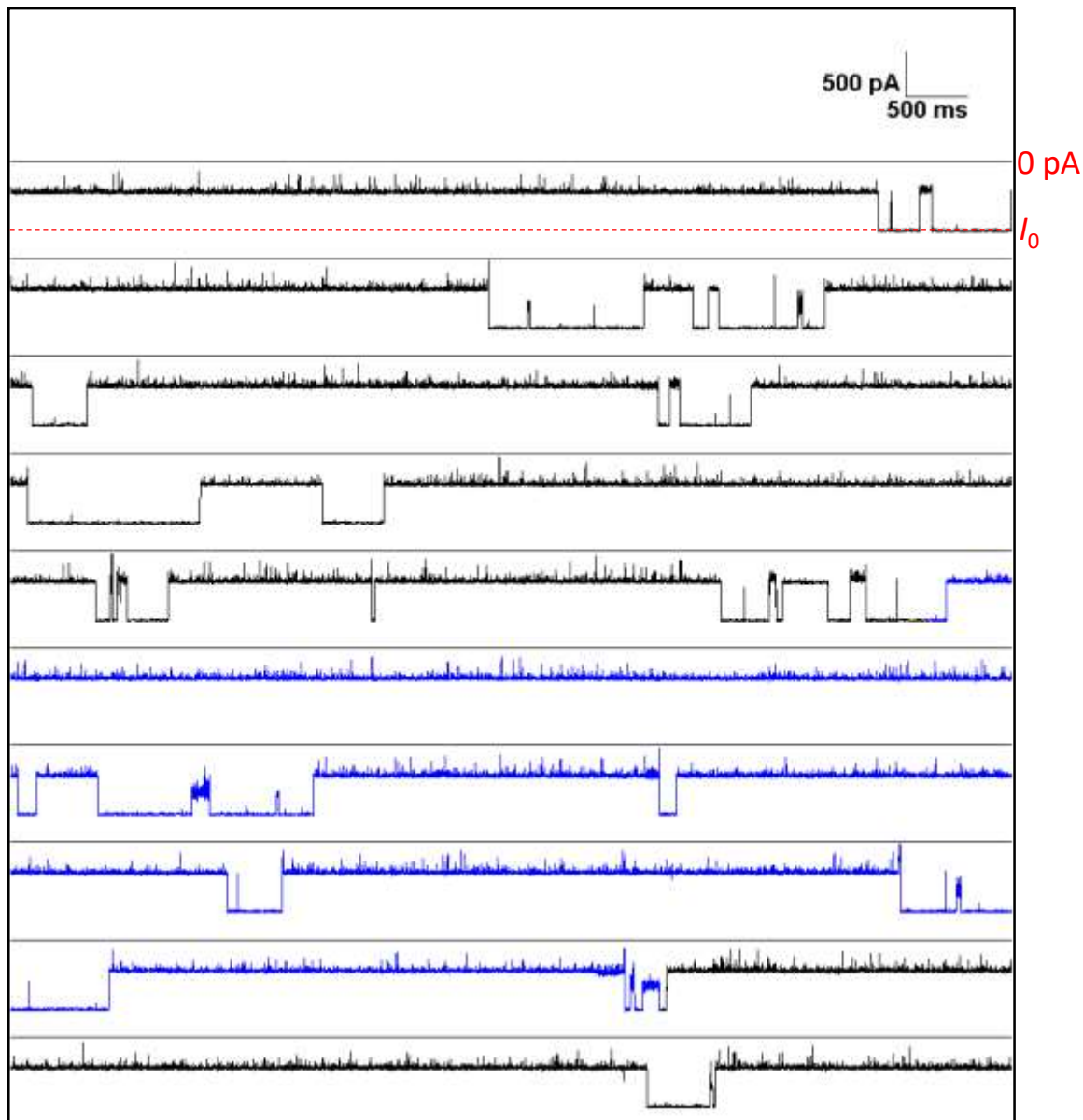


**Figure S13.** Mid-level current fluctuation frequency of Type 1 events increases as a function of applied voltage. Two-second long sample  $I$ - $t$  traces showing that the 30-bp dsDNA molecules are captured and then released from the  $\gamma$ -HL channel without unzipping at -80, -120, -140 and -160 mV. For clarity, Type 2 events data have been removed, as denoted by the breaks (//) in the  $I$ - $t$  traces recorded at -140 and -160 mV. The  $I$ - $t$  traces were post-filtered at 3 kHz for presentation.



**Figure S14.** Mid-level current fluctuation frequency of 30-bp dsDNA translocation events (Type 2) increases as a function of applied voltage. The 2-s *I-t* trace shows that the 30-bp dsDNA molecules are captured and then translocate through the  $\gamma$ -HL channel, resulting in a deep-blockade current at the end of each event (red circles). The *I-t* traces were post-filtered at 3 kHz for presentation.

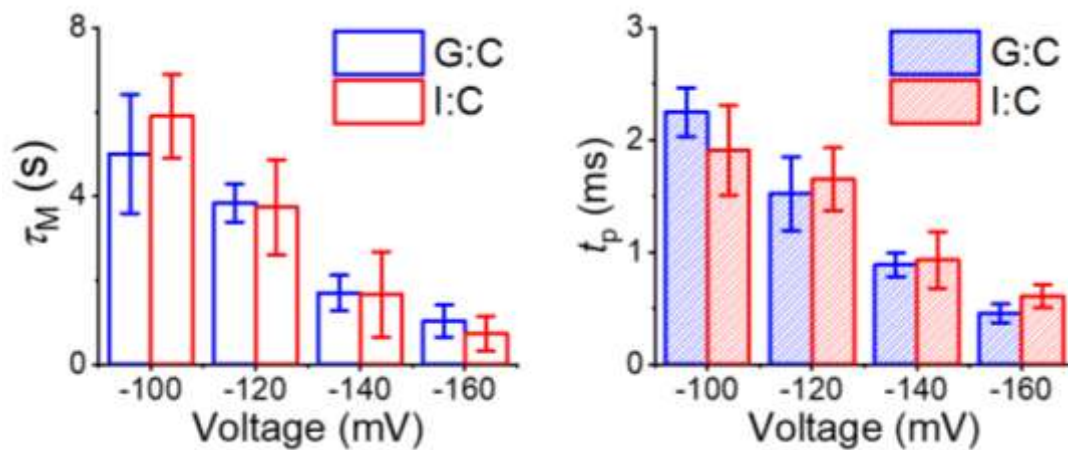
9. *I-t* trace corresponding to 30-bp inosine-substituted dsDNA capture and release/translocation using the  $\gamma$ -HL nanopore



**Figure S15.** An 80 s-long *I-t* trace of I:C-containing duplex. The portion highlighted in blue is shown in Figure 8b in the main text. Recordings were performed in 3 M KCl, 20 mM HOAc/KOAc, pH 5.0, at -120 mV. The *I-t* traces were post-filtered at 1 kHz for presentation.

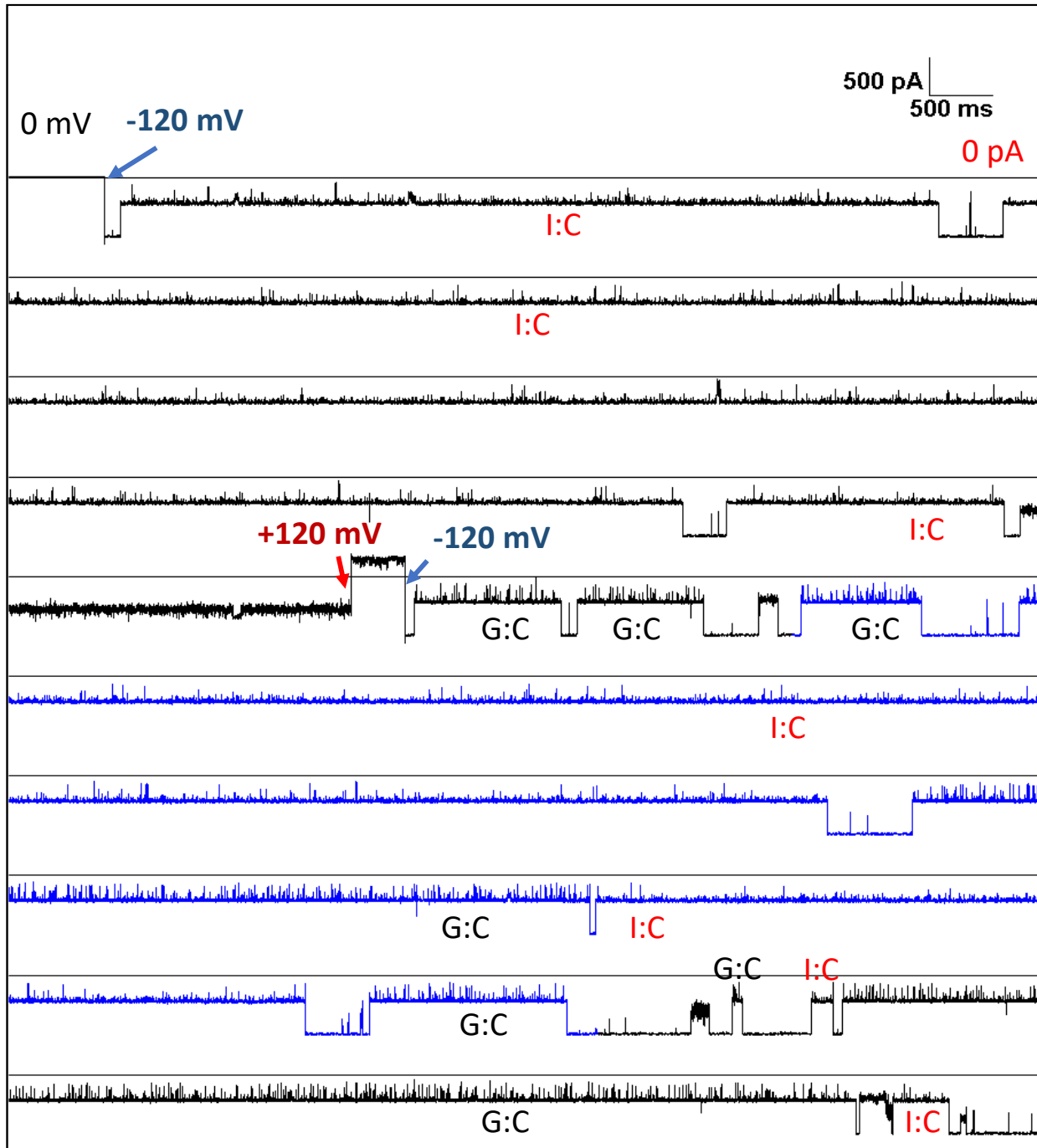


10. Dependence of translocation time on voltage for G:C- and I:C- containing duplexes.

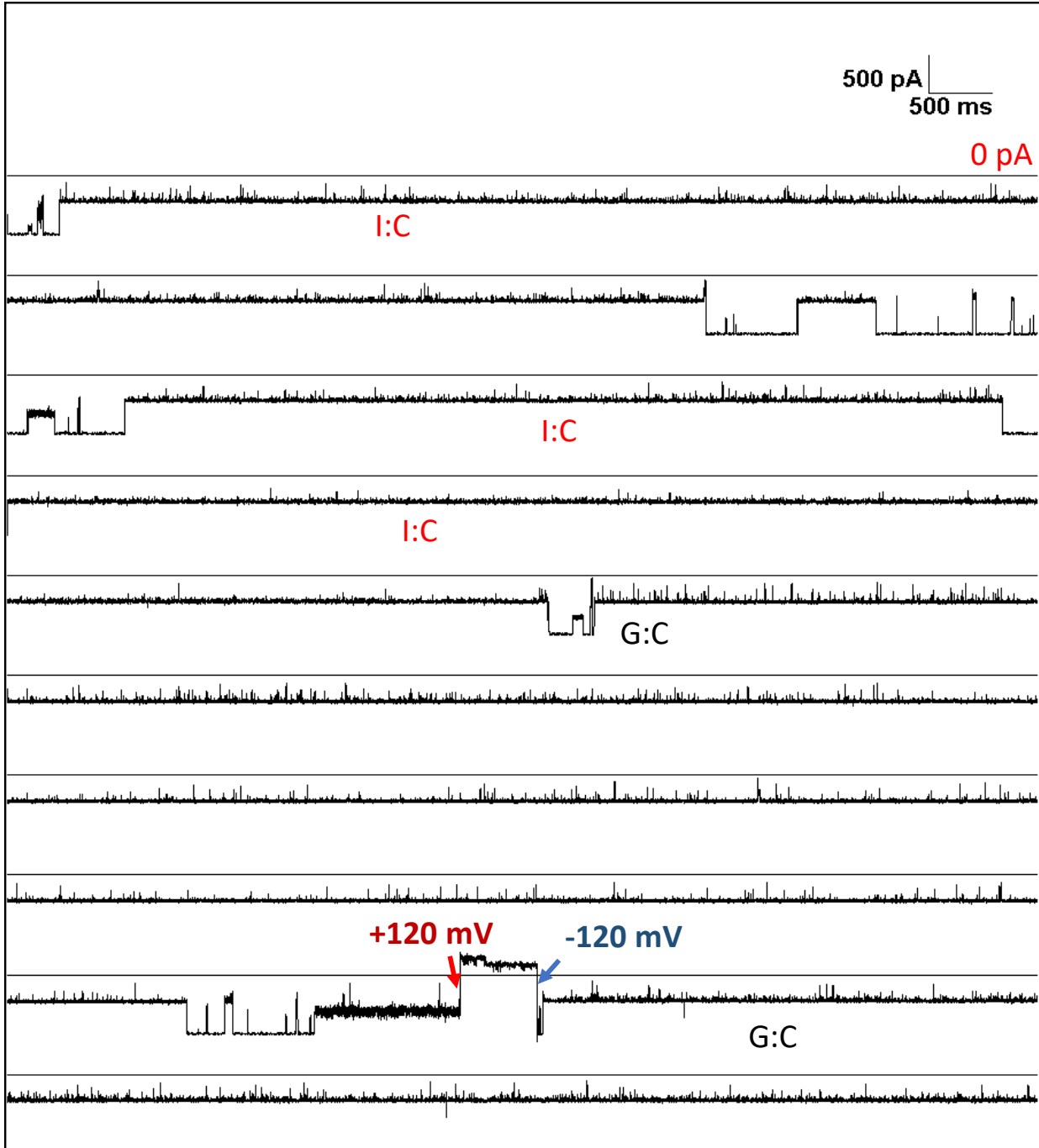


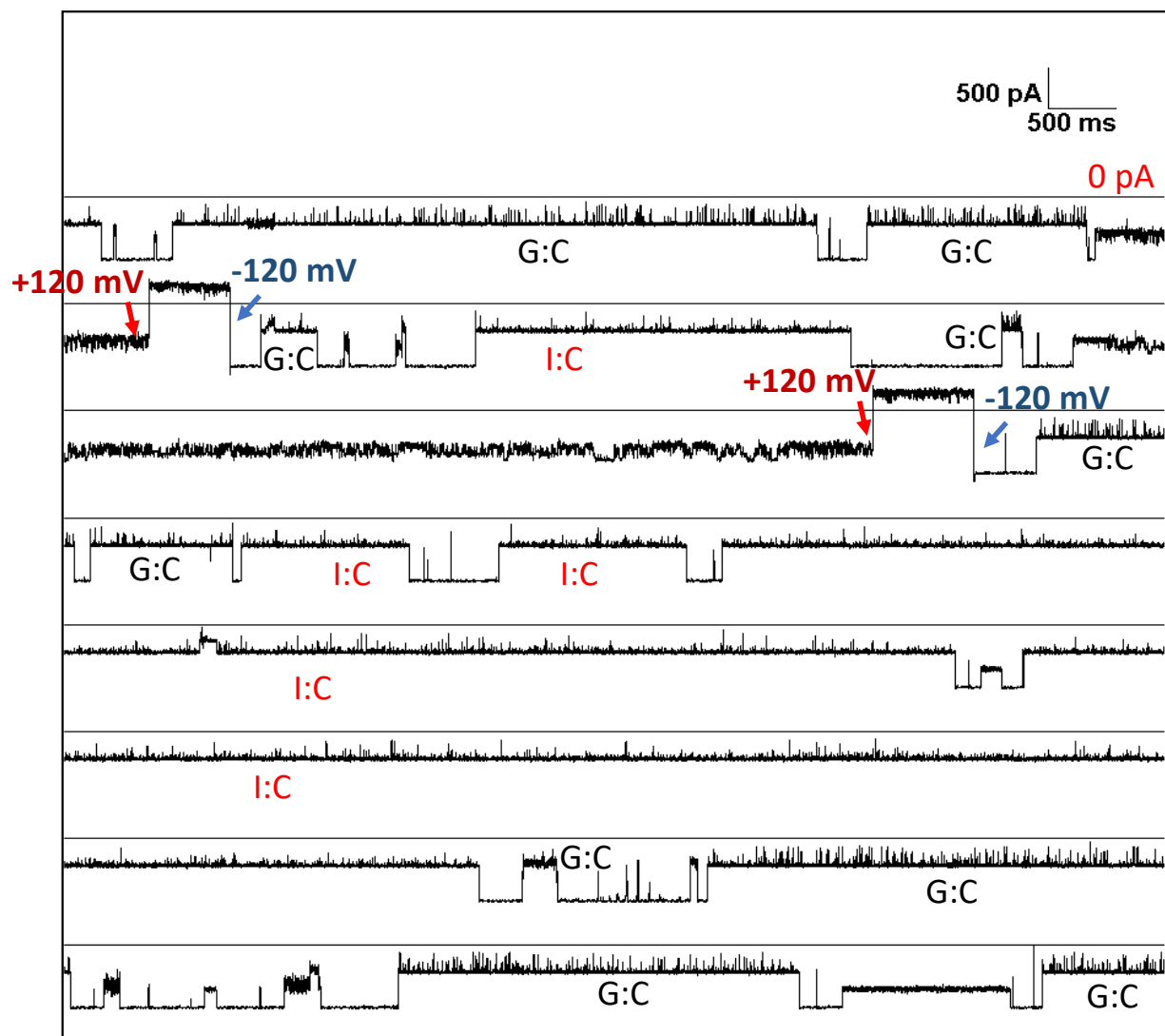
**Figure S16.** Effect of voltage on mid-blockade duration (a) and deep-blockade translocation time (b) of G:C- and I:C-containing duplexes. Error bars represent standard deviation from 3 or 4 repeated measurements (N = 30-500 in each experiment). Experiments were carried out in 3 M KCl, 20 mM HOAc/KOAc, pH 5.0, at 20.0 °C.

11. *I-t* trace demonstrating capture and release/translocation of G:C- and I:C-containing dsDNA







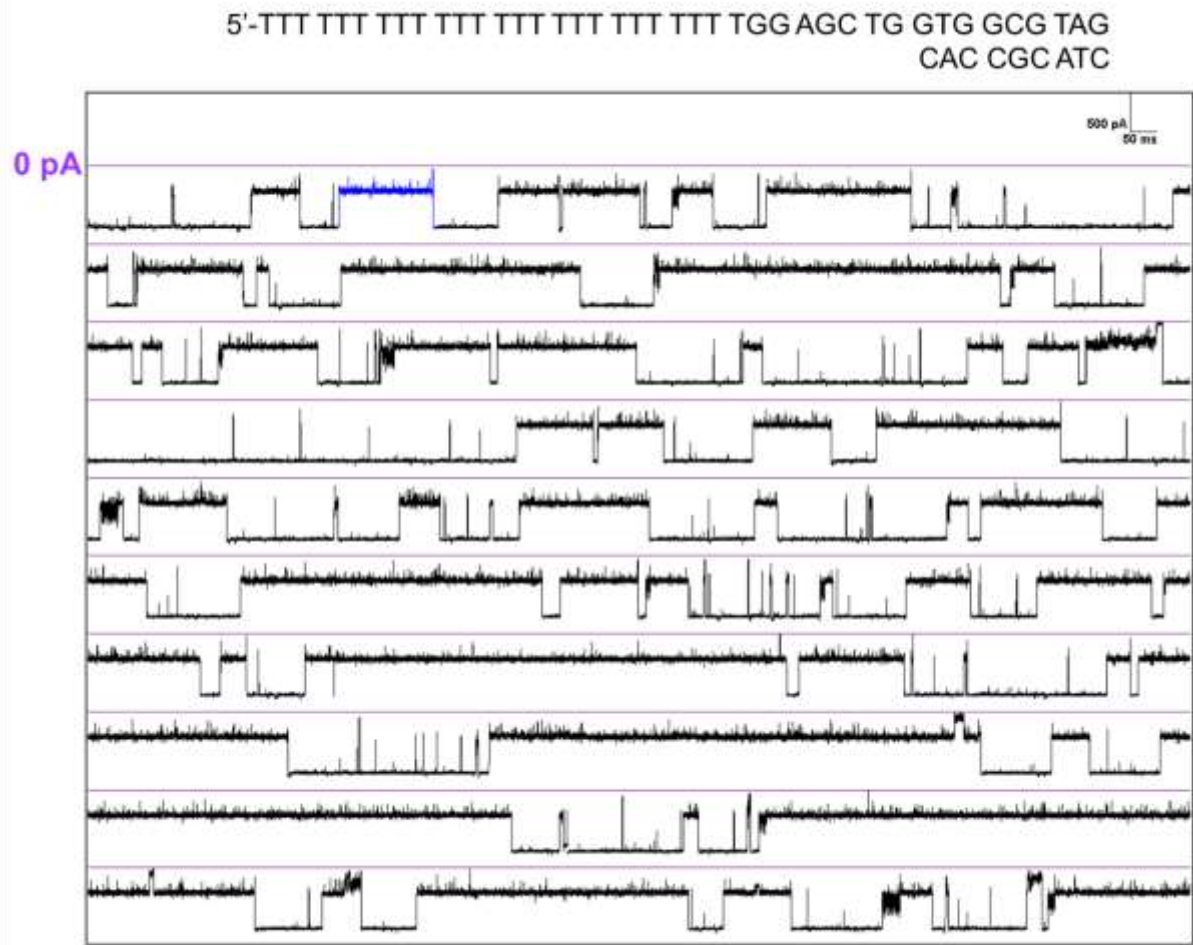


**Figure S17.** A 230 s of *I-t* trace demonstrating the capture and release/translocation of individual G:C- and I:C- containing duplexes in the same experiment. The portion highlighted in blue is shown in Figure 11c in the main text. Recordings were performed in 3 M KCl, 20 mM HOAc/KOAc, pH 5.0, 20.0 °C, at -120 mV. The *I-t* traces were post-filtered at 1 kHz for presentation.

## **12. Dependence of the translocation time on dsDNA length.**

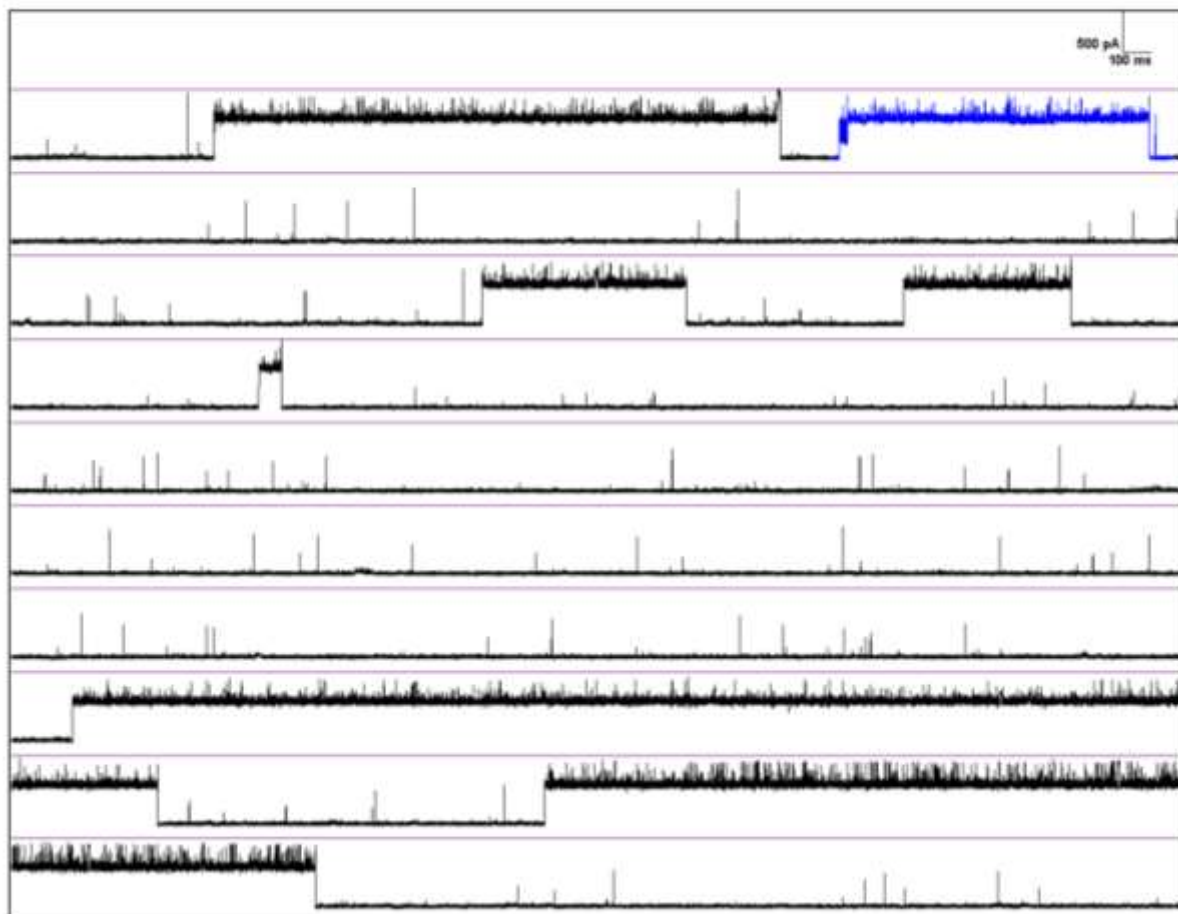
Presented as an accompaniment to Figure 11 in the main text.

This section contains uninterrupted  $I-t$  traces corresponding to capture and release/translocation of dsDNA.



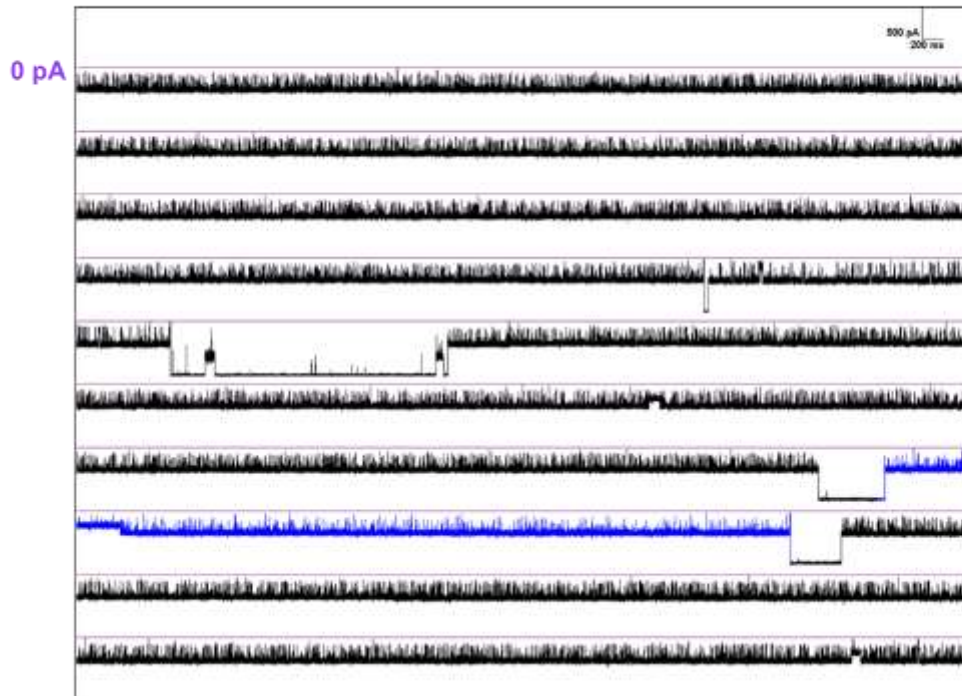
**Figure S18.** A 40 s *I-t* trace demonstrating capture and release/translocation events of a 9-bp dsDNA (~2  $\mu\text{M}$ ) using the  $\gamma$ -HL channel. The portion highlighted in blue is shown in Figure 11 in the main text. The data were collected in 3 M KCl, 20 mM HOAc/KOAc, pH 5.0, at -120 mV, and filtered using a 100-kHz Bessel filter. The *I-t* trace shown here was post-filtered at 3 kHz for presentation.

5'-TTT TTT TTT TTT TTT TTT TTT TTT TGG AGC TGG TGG CGT AG  
ACC TCG ACC ACC GCA TC



**Figure S19.** An 80 s *I-t* trace demonstrating capture and release/translocation events of a 17-bp dsDNA ( $\sim 2 \mu\text{M}$ ) using the  $\gamma$ -HL channel. The portion highlighted in blue is shown in Figure 11 in the main text. The data were collected in 3 M KCl, 20 mM HOAc/KOAc, pH 5.0, at -120 mV, and filtered using a 10-kHz Bessel filter. The *I-t* trace shown here was post-filtered at 3 kHz for presentation.

5'-A<sub>30</sub> AGT TGC CAC CTA ATG CGT CGT CGG TCT ATC AAA AAG CCT ACA CAG AAA AAT CAG TTG TCG  
TCA ACG GTG GAT TAC GCA GCA GCC AGA TAG TTT TTC GGA TGT GTC TTT TTA GTC AAC AGC



**Figure S20.** An 80 s *I-t* trace demonstrating capture and release/translocation events of a 60-bp dsDNA ( $\sim 2$   $\mu\text{M}$ ) using the  $\gamma$ -HL channel. The portion highlighted in blue is shown in Figure 11 in the main text. The data were collected in 3 M KCl, 20 mM HOAc/KOAc, pH 5.0, at -120 mV, and filtered using a 10-kHz Bessel filter. The *I-t* trace shown here was post-filtered at 3 kHz for presentation.

### 13. References

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