# SUPPORTING INFORMATION

## Individual RNA base recognition in immobilized oligonucleotides using a protein nanopore

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**αHL Protein Preparation.** Heptameric αHL WT was produced as described[1] by E. Mikhailova in our laboratory. Aliquots of the purified protein were collected and stored at -80 °C. The mutant αHL genes were prepared from αHL NN (E111N/K147N) and NNY (E111N/K147N/M113Y) by using a kit for site-directed mutagenesis (QuikChange II XL, Catalog no. 200522-5, Stratagene), and their DNA sequences were verified as before[1].

**Electrical Recordings.** Lipid bilayers were formed from 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC, Avanti Polar Lipids) across a 40-μm aperture in a PTFE film (20-μm thickness, Goodfellows, UK) that separated two compartments of a recording chamber[2]. Both compartments contained 500 μL of 1 M KCl, 25 mM Tris-HCl, pH 7.5, with 100 μM EDTA. Current recordings were performed by using a patch clamp amplifier (Axopatch 200B, Axon Instruments, CA) with the *cis* compartment connected to ground. The recording chamber was enclosed in a grounded aluminium Faraday cage. Under the buffer conditions used, the αHL pores remained open, without gating during measurements.

The αHL pores and the oligonucleotides (Table S1) were added to the *cis* compartment. ssDNA/ssRNA molecules, with a biotinyl group covalently attached to the 3' end through a linker, were obtained from Dharmacon (Thermo Fisher, UK) (Figure S1). Solutions of the biotinylated oligonucleotides, at 100 μM in 10 mM Tris-HCl, pH 7.5, 100 μM EDTA, were mixed with equal volumes of 25 μM streptavidin (SA) (New England Biolabs, Catalog No. N7021S) in the same buffer. Each oligonucleotide (pre-incubated with SA for at least five minutes) was added to the *cis* compartment to a final concentration of 200 nM. With the episodic stimulation mode in the pClamp software (version 10.1, Molecular Devices), the Digidata 1440A digitizer (Molecular Devices) was used to apply repeated voltage steps to drive single oligo-btn•streptavidin complexes into the pore (the electrical protocol used for these measurements is illustrated in Figure S2a).

Briefly, +200 mV was applied to the *trans* side for 900 ms to drive the negatively charged, biotinylated oligonucleotides into the pore. The capture of a strand by an αHL pore was observed as a stepwise decrease in the open pore current level ($I_O$) to a lower, but stable, current level ($I_{RES}$). A voltage of -140 mV was then applied for 50 ms to eject the immobilized strand from the pore. Subsequently, the applied potential was stepped to 0 mV for 50 ms. The one second sequence was repeated for at least 400 cycles for each ssDNA/ssRNA species added, with >90% of cycles giving current blockades. In general, when comparing several oligonucleotide species, one of the set was first added to the *cis* chamber and the currents were recorded. Subsequently, a second, third, fourth and if required, a fifth species was added and additional currents were recorded. For example, the data in Figures 2, 3, 4 and 5 come from >4 oligonucleotide species, with sequences that differ by a single nucleotide. When such experiments were repeated, the oligonucleotides were added to the chamber in a different order. The amplified signal (arising from the ionic current passing through the pore) was low-pass filtered at 5 kHz and sampled at 25 kHz.

**Data Analysis.** Data were analyzed and prepared for presentation with pClamp software (version 10.2, Molecular Devices). Single channel searches were performed to obtain the average current level for each ssDNA/ssRNA blockade ($I_{RES}$). The mean $I_{RES}$ value for each oligonucleotide was determined by performing a Gaussian fit to a histogram of the $I_{RES}$ values. The current blockade for each oligonucleotide was also described by a residual current ($I_{RES\%}$), in which the average remaining current during a blockade ($I_{RES}$) is expressed as a percentage of the open pore current ($I_O$): $I_{RES\%} = (I_{RES}/I_O) \times 100$. To determine how well a particular pore can discriminate between single nucleobase changes, two additional criteria were used (Figure S2b-d): 1. The overall dispersion was measured (the difference between the most widely separated residual current levels in the histogram: $\Delta I_{RES\%}^{OVERALL}$); 2. The product of the sequential differences between each of the residual current levels (one level for each oligonucleotide) was calculated (δ). An αHL pore that is unable to discriminate between all nucleobases in an oligonucleotide set has δ = 0 (i.e. the current levels of two or more oligonucleotides overlap)[3].

**Force encountered by the immobilized oligonucleotide.** When the RNA•streptavidin complex is immobilized inside the αHL pore, the ssRNA is elongated compared with its conformation in solution, because of the force arising from the applied potential. The experimentally determined effective charge on each base is ~0.1e[4-6]. The ~15 nt present in the barrel[7] have an overall charge of ~2.4 x $10^{-19}$ C. By assuming that the applied potential of +200 mV drops over the 5-nm length of the barrel, the electric field is estimated to be 4.0 x $10^7$ V $m^{-1}$. Therefore, the force ($F = QE$) on the RNA is estimated to be ~10 pN.

## REFERENCES


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**Table S1.** Sequences of the oligonucleotides used in this paper. Btn represents the 3' Biotin-TEG tag and linker (Figure S1), [d] represents a DNA background and [r] represents an RNA background. The substituted ribobases at position 9 are highlighted in red.

| Oligonucleotide | Sequence 5'→3' |
|---|---|
| oligo(rC)$_{30}$ | 5'-[r]CCCCCCCCCCCCCCCCCCCCCCCCCCCCCC[Btn]-3' |
| oligo(rA)$_{30}$ | 5'-[r]AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA[Btn]-3' |
| oligo(rU)$_{30}$ | 5'-[r]UUUUUUUUUUUUUUUUUUUUUUUUUUUUUU[Btn]-3' |
| | |
| oligo(dC)$_{30}$ | 5'-[d]CCCCCCCCCCCCCCCCCCCCCCCCCCCCCC[Btn]-3' |
| rG-oligo(dC)$_{30}$ | 5'-[d]CCCCCCCCCCCCCCCCCCCCCrGCCCCCCCC[Btn]-3' |
| rA-oligo(dC)$_{30}$ | 5'-[d]CCCCCCCCCCCCCCCCCCCCCrACCCCCCCC[Btn]-3' |
| rC-oligo(dC)$_{30}$ | 5'-[d]CCCCCCCCCCCCCCCCCCCCCrCCCCCCCCC[Btn]-3' |
| rU-oligo(dC)$_{30}$ | 5'-[d]CCCCCCCCCCCCCCCCCCCCCrUCCCCCCCC[Btn]-3' |
| rI-oligo(dC)$_{30}$ | 5'-[d]CCCCCCCCCCCCCCCCCCCCCrICCCCCCCC[Btn]-3' |
| m$^6$A-oligo(dC)$_{30}$ | 5'-[d]CCCCCCCCCCCCCCCCCCCCCm$^6$ACCCCCCCC[Btn]-3' |
| m$^5$C-oligo(dC)$_{30}$ | 5'-[d]CCCCCCCCCCCCCCCCCCCCCm$^5$CCCCCCCCC[Btn]-3' |
| | |
| oligo(rA)$_{30}$ | 5'-[r]AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA[Btn]-3' |
| rG-oligo(rA)$_{30}$ | 5'-[r]AAAAAAAAAAAAAAAAAAAAArGAAAAAAAA[Btn]-3' |
| rA-oligo(rA)$_{30}$ | 5'-[r]AAAAAAAAAAAAAAAAAAAAArAAAAAAAAA[Btn]-3' |
| rC-oligo(rA)$_{30}$ | 5'-[r]AAAAAAAAAAAAAAAAAAAAArCAAAAAAAA[Btn]-3' |
| rU-oligo(rA)$_{30}$ | 5'-[r]AAAAAAAAAAAAAAAAAAAAArUAAAAAAAA[Btn]-3' |
| rI-oligo(rA)$_{30}$ | 5'-[r]AAAAAAAAAAAAAAAAAAAAArIAAAAAAAA[Btn]-3' |
| m$^6$A-oligo(rA)$_{30}$ | 5'-[r]AAAAAAAAAAAAAAAAAAAAAm$^6$AAAAAAAAA[Btn]-3' |
| m$^5$C-oligo(rA)$_{30}$ | 5'-[r]AAAAAAAAAAAAAAAAAAAAAm$^5$CAAAAAAAA[Btn]-3' |
| | |
| rC-het$_{30}$ | 5'-[r]UAGCUAAACCGAUAGCUUCAGrCCAUGUAAC[Btn]-3' |
| rG-het$_{30}$ | 5'-[r]UAGCUAAACCGAUAGCUUCAGrGCAUGUAAC[Btn]-3' |
| rA-het$_{30}$ | 5'-[r]UAGCUAAACCGAUAGCUUCAGrACAUGUAAC[Btn]-3' |
| rU-het$_{30}$ | 5'-[r]UAGCUAAACCGAUAGCUUCAGrUCAUGUAAC[Btn]-3' |

**Table S2.** Voltage dependencies of the residual currents ($I_{RES\%}$) for the WT, NN and NNY αHL pores threaded with oligo(rA)$_{30}$, oligo(rC)$_{30}$ or oligo(rU)$_{30}$. The $I_O$ and $I_{RES\%}$ values are mean values (± S.D.) taken from Gaussian fits to event histograms at various applied potentials (n ≥ 3 experiments). $I_{RES\%} = (I_{RES}/I_O) \times 100$. $\Delta I_{RES\%}^{OVERALL}$ is the difference in residual current between the two most widely separated current peaks. The δ value is the product of the successive differences between the peaks. If any two peaks overlap, then the δ value is zero. The $I_{RES\%}$ values versus the applied potential are plotted in Figure S4.

| WT | | | | | | |
|---|---|---|---|---|---|---|
| mV | $I_O$ (pA) | Oligos | | | $\Delta I_{RES}^{OVERALL}$ (%) | δ (%) |
| | | Oligo(rA)$_{30}$ | Oligo(rC)$_{30}$ | Oligo(rU)$_{30}$ | | |
| | | $I_{RES}$ (%) | $I_{RES}$ (%) | $I_{RES}$ (%) | | |
| +100 | 98 ± 6.0 | 16.3 ± 0.4 | 13.1 ± 0.6 | 18.1 ± 0.4 | 4.9 ± 0.4 | 5.5 ± 0.2 |
| +120 | 117 ± 4.0 | 18.5 ± 0.5 | 15.8 ± 0.3 | 19.3 ± 0.3 | 3.5 ± 0.4 | 2.2 ± 0.1 |
| +140 | 139 ± 6.0 | 19.9 ± 1.0 | 17.6 ± 1.2 | 20.7 ± 1.0 | 3.1 ± 0.4 | 1.8 ± 0.2 |
| +160 | 159 ± 7.0 | 20.8 ± 0.2 | 18.9 ± 0.4 | 22.0 ± 0.3 | 3.1 ± 0.4 | 2.2 ± 0.4 |
| +180 | 181 ± 4.0 | 22.4 ± 0.7 | 21.3 ± 0.6 | 23.0 ± 0.7 | 1.7 ± 0.6 | 0.7 ± 0.2 |
| +200 | 199 ± 6.0 | 22.8 ± 0.4 | 21.8 ± 0.2 | 23.7 ± 0.4 | 1.5 ± 0.4 | 0.6 ± 0.2 |

| NN | | | | | | |
|---|---|---|---|---|---|---|
| mV | $I_O$ (pA) | Oligos | | | $\Delta I_{RES}^{OVERALL}$ (%) | δ (%) |
| | | Oligo(rA)$_{30}$ | Oligo(rC)$_{30}$ | Oligo(rU)$_{30}$ | | |
| | | $I_{RES}$ (%) | $I_{RES}$ (%) | $I_{RES}$ (%) | | |
| +100 | 113 ± 7.0 | 13.4 ± 0.3 | 15.5 ± 0.6 | 16.9 ± 0.2 | 3.6 ± 0.4 | 3.1 ± 0.1 |
| +120 | 129 ± 4.0 | 15.8 ± 0.5 | 17.0 ± 0.8 | 19.2 ± 0.5 | 3.4 ± 0.6 | 2.7 ± 0.2 |
| +140 | 149 ± 8.0 | 18.1 ± 0.2 | 19.7 ± 0.3 | 21.9 ± 0.3 | 3.8 ± 0.4 | 3.5 ± 0.1 |
| +160 | 171 ± 6.0 | 21.0 ± 0.6 | 23.1 ± 0.6 | 25.3 ± 0.8 | 4.3 ± 0.2 | 4.6 ± 0.2 |
| +180 | 192 ± 9.0 | 24.5 ± 0.4 | 26.1 ± 0.4 | 27.5 ± 0.4 | 3.0 ± 0.4 | 2.2 ± 0.2 |
| +200 | 214 ± 7.0 | 27.9 ± 0.3 | 28.5 ± 0.3 | 30.4 ± 0.2 | 2.4 ± 0.2 | 1.1 ± 0.1 |

| NNY | | | | | | |
|---|---|---|---|---|---|---|
| mV | $I_O$ (pA) | Oligos | | | $\Delta I_{RES}^{OVERALL}$ (%) | δ (%) |
| | | Oligo(rA)$_{30}$ | Oligo(rC)$_{30}$ | Oligo(rU)$_{30}$ | | |
| | | $I_{RES}$ (%) | $I_{RES}$ (%) | $I_{RES}$ (%) | | |
| +100 | 108 ± 4.0 | 17.3 ± 0.4 | 19.6 ± 0.4 | 21.7 ± 0.4 | 4.4 ± 0.4 | 4.8 ± 0.1 |
| +120 | 127 ± 6.0 | 21.5 ± 0.4 | 22.8 ± 0.4 | 24.9 ± 0.3 | 3.4 ± 0.3 | 2.7 ± 0.2 |
| +140 | 151 ± 5.0 | 25.8 ± 0.5 | 27.7 ± 0.6 | 29.3 ± 0.5 | 3.5 ± 0.2 | 3.0 ± 0.2 |
| +160 | 167 ± 4.0 | 28.4 ± 0.3 | 29.9 ± 0.3 | 32.4 ± 0.2 | 4.0 ± 0.3 | 3.9 ± 0.1 |
| +180 | 188 ± 6.0 | 31.3 ± 0.4 | 33.0 ± 0.2 | 34.7 ± 0.4 | 3.4 ± 0.3 | 2.8 ± 0.1 |
| +200 | 210 ± 8.0 | 32.8 ± 0.4 | 33.7 ± 0.3 | 34.1 ± 0.3 | 2.8 ± 0.4 | 1.8 ± 0.2 |

**Table S3.** Voltage dependencies of residual currents, $I_{RES\%}$ and $\Delta I_{RES\%}$ for the WT, NN and NNY αHL pores threaded with oligo(dC)$_{30}$ with a single substitution (rG, rA rC or rU) at position 9. The $I_O$ and $I_{RES\%}$ values are mean values (± S.D.) taken from Gaussian fits to event histograms at various applied potentials (n = 3 experiments). $I_{RES\%} = (I_{RES}/I_O) \times 100$. $\Delta I_{RES\%}^{OVERALL}$ is the difference in residual current between the two most widely separated current peaks. The δ value is the product of the successive differences between the peaks. If any two peaks overlap, then the δ value is zero. The values from these data are plotted in Figure S5a and S6. $\Delta I_{RES\%}$ values were determined for individual experiments and the mean value is used at each potential. $\Delta I_{RES}^{rX\text{-}oligo(dC)} = I_{RES\%}$ for the rX oligonucleotide - $I_{RES\%}$ of oligo(dC)$_{30}$. X = rG, rA, rC or rU.

| WT | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| mV | $I_O$ (pA) | oligo(dC)$_{30}$ | rA | | rC | | rG | | rU | | $\Delta I_{RES}^{OVERALL}$ (%) | δ (%) |
| | | $I_{RES}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(dC)}$ (%) | | |
| +100 | 100 ± 2.0 | 13.9 ± 0.4 | 14.5 ± 1.0 | 0.6 ± 0.2 | 12.9 ±0.4 | -1.0 ± 0.2 | 15.9 ± 0.6 | 2.0 ± 0.1 | 15.1 ± 0.6 | 1.2 ± 0.2 | 3.0 ± 0.4 | 0.8 ± 0.02 |
| +120 | 123 ± 4.0 | 14.5 ± 0.6 | 16.1 ± 0.3 | 1.6 ± 0.2 | 14.8 ± 0.6 | 0.3 ± 0.1 | 17.8 ± 0.4 | 3.3 ± 0.1 | 17.2 ± 0.3 | 2.7 ± 0.2 | 3.0 ± 0.3 | 0.9 ± 0.03 |
| +140 | 145 ± 6.0 | 17.1 ± 0.4 | 17.8 ± 0.6 | 0.7 ± 0.3 | 16.9 ± 0.6 | -0.2 ± 0.1 | 19.2 ± 0.4 | 2.1 ± 0.2 | 18.7 ± 0.4 | 1.6 ± 0.1 | 2.3 ± 0.3 | 0.4 ± 0.02 |
| +160 | 168 ± 4.0 | 18.6 ± 0.4 | 19.8 ± 0.4 | 1.2 ± 0.1 | 18.4 ± 0.4 | -0.2 ± 0.1 | 20.9 ± 0.4 | 2.3 ± 0.2 | 20.5 ± 0.4 | 1.9 ± 0.1 | 2.5 ± 0.3 | 0.4 ± 0.02 |
| +180 | 191 ± 3.0 | 20.5 ± 0.2 | 21.5 ± 0.4 | 1.0 ± 0.1 | 20.7 ± 0.4 | 0.2 ± 0.1 | 23.3 ± 0.4 | 2.8 ± 0.2 | 22.3 ± 0.3 | 1.8 ± 0.1 | 2.6 ± 0.3 | 0.6 ± 0.02 |
| +200 | 202 ± 6.0 | 23.1 ± 0.3 | 23.4 ± 0.6 | 0.3 ± 0.2 | 23.0 ± 0.3 | -0.1 ± 0.1 | 24.3 ± 0.4 | 1.2 ± 0.1 | 24.0 ± 0.3 | 0.9 ± 0.2 | 1.3 ± 0.4 | 0.1 ± 0.01 |

| NN | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| mV | $I_O$ (pA) | oligo(dC)$_{30}$ | rA | | rC | | rG | | rU | | $\Delta I_{RES}^{OVERALL}$ (%) | δ (%) |
| | | $I_{RES}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(dC)}$ (%) | | |
| +100 | 108 ± 6.0 | 20.5 ± 0.3 | 21.6 ± 0.3 | 1.1 ± 0.2 | 21.0 ± 0.4 | 0.5 ± 0.2 | 22.3 ± 0.3 | 1.8 ± 0.2 | 23.0 ± 0.6 | 2.5 ± 0.2 | 2.0 ± 0.4 | 0.3 ± 0.03 |
| +120 | 129 ± 8.0 | 22.5 ± 0.6 | 23.6 ± 0.3 | 1.1 ± 0.3 | 22.7 ± 0.4 | 0.2 ± 0.1 | 24.1 ± 0.4 | 1.6 ± 0.1 | 24.6 ± 0.4 | 2.1 ± 0.2 | 1.9 ± 0.3 | 0.2 ± 0.02 |
| +140 | 151 ± 6.0 | 23.9 ± 0.4 | 24.8 ± 0.6 | 0.9 ± 0.3 | 24.4 ± 0.6 | 0.5 ± 0.1 | 25.1 ± 0.4 | 1.2 ± 0.1 | 25.4 ± 0.4 | 1.5 ± 0.1 | 1.0 ± 0.3 | 0.0 ± 0.00 |
| +160 | 173 ± 5.0 | 24.9 ± 0.3 | 25.5 ± 0.2 | 0.6 ± 0.2 | 25.1 ± 0.6 | 0.2 ± 0.1 | 25.9 ± 0.6 | 1 ± 0.2 | 26.4 ± 0.6 | 1.5 ± 0.1 | 1.3 ± 0.3 | 0.1 ± 0.02 |
| +180 | 195 ± 6.0 | 25.5 ± 0.3 | 26.4 ± 0.6 | 0.9 ± 0.4 | 25.8 ± 0.3 | 0.3 ± 0.2 | 26.8 ± 0.3 | 1.3 ± 0.3 | 27.2 ± 0.4 | 1.7 ± 0.2 | 1.4 ± 0.3 | 0.1 ± 0.02 |
| +200 | 214 ± 6.0 | 26.7 ± 0.3 | 27.9 ± 0.6 | 1.2 ± 0.4 | 26.8 ± 0.4 | 0.1 ± 0.1 | 28.6 ± 0.6 | 1.9 ± 0.2 | 28.9 ± 0.4 | 2.2 ± 0.3 | 2.1 ± 0.4 | 0.2 ± 0.02 |

| NNY | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| mV | $I_O$ (pA) | oligo(dC)$_{30}$ | rA | | rC | | rG | | rU | | $\Delta I_{RES}^{OVERALL}$ (%) | δ (%) |
| | | $I_{RES}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX-oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX-oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX-oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX-oligo(dC)}$ (%) | | |
| +100 | 108 ± 4.0 | 33.7 ± 0.3 | 33.4 ± 0.3 | -0.3 ± 0.1 | 34.2 ± 0.6 | 0.5 ± 0.1 | 32.2 ± 0.6 | -1.5 ± 0.2 | 35.3 ± 0.3 | 1.6 ± 0.2 | 3.1 ± 0.4 | 1.1 ± 0.02 |
| +120 | 126 ± 9.0 | 34.6 ± 0.3 | 34.5 ± 0.3 | -0.1 ± 0.1 | 35.8 ± 0.4 | 1.2 ± 0.2 | 33.8 ± 0.4 | -0.8 ± 0.1 | 36.6 ± 0.4 | 2.0 ± 0.1 | 2.8 ± 0.3 | 0.7 ± 0.02 |
| +140 | 143 ± 6.0 | 38.3 ± 0.6 | 36.8 ± 0.4 | -1.5 ± 0.4 | 38.5 ± 0.6 | 0.2 ± 0.1 | 35.8 ± 0.3 | -2.5 ± 0.2 | 39.3 ± 0.4 | 1.0 ± 0.1 | 3.5 ± 0.3 | 1.4 ± 0.02 |
| +160 | 168 ± 4.0 | 38.4 ± 0.3 | 39.1 ± 0.4 | 0.7 ± 0.2 | 40.1 ± 0.6 | 1.7 ± 0.2 | 37.9 ± 0.3 | -0.5 ± 0.2 | 41.0 ± 0.3 | 2.6 ± 0.2 | 3.1 ± 0.3 | 1.1 ± 0.02 |
| +180 | 177 ± 8.0 | 40.5 ± 0.4 | 40.6 ± 0.5 | 0.1 ± 0.1 | 41.3 ± 0.6 | 0.8 ± 0.2 | 39.9 ± 0.4 | -0.6 ± 0.1 | 42.7 ± 0.6 | 2.2 ± 0.2 | 2.8 ± 0.3 | 0.7 ± 0.01 |
| +200 | 210 ± 7.0 | 42.6 ± 0.4 | 41.9 ± 0.7 | -0.7 ± 0.1 | 42.6 ± 0.8 | 0.0 ± 0.0 | 41.2 ± 0.7 | -1.4 ± 0.2 | 44.0 ± 0.6 | 1.4 ± 0.2 | 2.8 ± 0.4 | 0.7 ± 0.01 |

**Table S4.** Voltage dependencies of residual currents, $I_{RES\%}$ and $\Delta I_{RES\%}$ for the WT, NN and NNY αHL pores threaded with oligo(rA)$_{30}$ with a single substitution (rG, rA rC or rU) at position 9. The $I_O$ and $I_{RES\%}$ values are mean values (± S.D.) taken from Gaussian fits to event histograms at various applied potentials (n = 3 experiments). $I_{RES\%} = (I_{RES}/I_O) \times 100$. $\Delta I_{RES\%}^{OVERALL}$ is the difference in residual current between the two most widely separated current peaks. The δ value is the product of the successive differences between the peaks. If any two peaks overlap, then the δ value is zero. If any two peaks overlap, then the δ value is zero. The values from these data are plotted in Figure S5b and S7. $\Delta I_{RES\%}$ values were determined for individual experiments and the mean value is used at each potential. $\Delta I_{RES}^{rX\text{-}oligo(rA)} = I_{RES\%}$ for the rX oligonucleotide - $I_{RES\%}$ of oligo(rA)$_{30}$. X = rG, rA, rC or rU.

WT

| mV | $I_O$ (pA) | oligo(rA)$_{30}$ | rA | | rC | | rG | | rU | | $\Delta I_{RES}^{OVERALL}$ (%) | δ (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $I_{RES}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | | |
| +100 | 97 ± 4.0 | 11.2 ± 0.3 | 11.2 ± 0.3 | 0.0 ± 0.0 | 10.4 ± 0.2 | -0.8 ± 0.2 | 13.6 ± 0.4 | 2.4 ± 0.2 | 12.0 ± 0.4 | 0.8 ± 0.1 | 3.2 ± 0.3 | 1.0 ± 0.02 |
| +120 | 123 ± 8.0 | 13.0 ± 0.4 | 13.0 ± 0.4 | 0.0 ± 0.0 | 12.3 ± 0.4 | -0.7 ± 0.2 | 15.2 ± 0.4 | 2.2 ± 0.2 | 13.8 ± 0.4 | 0.8 ± 0.1 | 2.9 ± 0.4 | 0.8 ± 0.03 |
| +140 | 142 ± 7.0 | 15.3 ± 0.4 | 15.3 ± 0.4 | 0.0 ± 0.0 | 14.6 ± 0.4 | -0.7 ± 0.1 | 17.5 ± 0.3 | 2.2 ± 0.2 | 16.0 ± 0.5 | 0.7 ± 0.1 | 2.9 ± 0.4 | 0.7 ± 0.03 |
| +160 | 166 ± 9.0 | 17.0 ± 0.4 | 17.0 ± 0.4 | 0.0 ± 0.0 | 16.3 ± 0.3 | -0.7 ± 0.1 | 19.5 ± 0.3 | 2.5 ± 0.1 | 18.0 ± 0.4 | 1.0 ± 0.3 | 3.2 ± 0.3 | 1.1 ± 0.02 |
| +180 | 185 ± 6.0 | 20.1 ± 0.3 | 20.1 ± 0.3 | 0.0 ± 0.0 | 19.7 ± 0.3 | -0.4 ± 0.2 | 21.0 ± 0.4 | 0.9 ± 0.1 | 20.6 ± 0.4 | 0.5 ± 0.1 | 1.3 ± 0.4 | 0.1 ± 0.02 |
| +200 | 205 ± 7.0 | 22.9 ± 0.4 | 22.9 ± 0.4 | 0.0 ± 0.0 | 22.2 ± 0.4 | -0.7 ± 0.1 | 23.9 ± 0.3 | 1.0 ± 0.2 | 23.2 ± 0.4 | 0.3 ± 0.1 | 1.7 ± 0.4 | 0.1 ± 0.01 |

NN

| mV | $I_O$ (pA) | oligo(rA)$_{30}$ | rA | | rC | | rG | | rU | | $\Delta I_{RES}^{OVERALL}$ (%) | δ (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $I_{RES}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | | |
| +100 | 112 ± 8.0 | 19.1 ± 0.2 | 19.1 ± 0.2 | 0.0 ± 0.0 | 21.6 ± 0.4 | 2.5 ± 0.1 | 14.1 ± 0.2 | -5.0 ± 0.4 | 16.1 ± 0.4 | -3.0 ± 0.2 | 7.5 ± 0.3 | 15.0 ± 0.3 |
| +120 | 130 ± 7.0 | 21.2 ± 0.3 | 21.2 ± 0.3 | 0.0 ± 0.0 | 24.0 ± 0.2 | 2.8 ± 0.2 | 17.2 ± 0.2 | -4.0 ± 0.2 | 19.0 ± 0.3 | -2.2 ± 0.2 | 6.8 ± 0.2 | 11.1 ± 0.2 |
| +140 | 145 ± 4.0 | 24.0 ± 0.3 | 24.0 ± 0.3 | 0.0 ± 0.0 | 25.9 ± 0.2 | 1.9 ± 0.1 | 21.0 ± 0.4 | -3.0 ± 0.2 | 22.7 ± 0.3 | -1.3 ± 0.1 | 4.9 ± 0.3 | 4.2 ± 0.2 |
| +160 | 171 ± 6.0 | 26.9 ± 0.4 | 26.9 ± 0.4 | 0.0 ± 0.0 | 27.9 ± 0.4 | 1.0 ± 0.1 | 23.5 ± 0.5 | -3.4 ± 0.2 | 25.0 ± 0.2 | -1.9 ± 0.1 | 4.4 ± 0.5 | 2.9 ± 0.02 |
| +180 | 189 ± 7.0 | 29.9 ± 0.3 | 29.9 ± 0.3 | 0.0 ± 0.0 | 31.1 ± 0.2 | 1.2 ± 0.1 | 27.5 ± 0.4 | -2.4 ± 0.2 | 29.1 ± 0.4 | -0.8 ± 0.2 | 3.6 ± 0.3 | 1.5 ± 0.02 |
| +200 | 209 ± 8.0 | 33.8 ± 0.3 | 33.8 ± 0.3 | 0.0 ± 0.0 | 34.6 ± 0.6 | 0.8 ± 0.1 | 31.7 ± 0.4 | -2.1 ± 0.2 | 32.2 ± 0.4 | -1.6 ± 0.1 | 2.9 ± 0.5 | 0.6 ± 0.02 |

| NNY | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| mV | $I_O$ (pA) | oligo(rA)$_{30}$ | rA | | rC | | rG | | rU | | $\Delta I_{RES}^{OVERALL}$ (%) | δ (%) |
| | | $I_{RES}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX-oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX-oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX-oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX-oligo(rA)}$ (%) | | |
| +100 | 99 ± 7.0 | 25.6 ± 0.4 | 25.6 ± 0.4 | 0.0 ± 0.0 | 27.4 ± 0.3 | 1.8 ± 0.1 | 24.3 ± 0.3 | -1.3 ± 0.1 | 29.4 ± 0.3 | 3.8 ± 0.2 | 5.1 ± 0.3 | 4.7 ± 0.2 |
| +120 | 114 ± 4.0 | 27.1 ± 0.4 | 27.1 ± 0.4 | 0.0 ± 0.0 | 28.9 ± 0.3 | 1.8 ± 0.1 | 25.8 ± 0.3 | -1.3 ± 0.2 | 30.7 ± 0.2 | 3.6 ± 0.1 | 4.9 ± 0.3 | 4.2 ± 0.2 |
| +140 | 139 ± 3.0 | 29.1 ± 0.3 | 29.1 ± 0.3 | 0.0 ± 0.0 | 30.7 ± 0.3 | 1.6 ± 0.2 | 27.4 ± 0.2 | -1.7 ± 0.2 | 31.6 ± 0.4 | 2.5 ± 0.1 | 4.2 ± 0.3 | 2.4 ± 0.2 |
| +160 | 157 ± 6.0 | 30.4 ± 0.4 | 30.4 ± 0.4 | 0.0 ± 0.0 | 31.8 ± 0.4 | 1.4 ± 0.2 | 29.3 ± 0.4 | -1.1 ± 0.2 | 33.0 ± 0.6 | 2.6 ± 0.1 | 3.7 ± 0.5 | 1.8 ± 0.02 |
| +180 | 176 ± 7.0 | 32.3 ± 0.3 | 32.3 ± 0.3 | 0.0 ± 0.0 | 33.4 ± 0.3 | 1.1 ± 0.1 | 31.2 ± 0.3 | -1.1 ± 0.1 | 34.5 ± 0.4 | 2.2 ± 0.1 | 3.3 ± 0.4 | 1.3 ± 0.02 |
| +200 | 206 ± 8.0 | 34.8 ± 0.3 | 34.8 ± 0.3 | 0.0 ± 0.0 | 35.6 ± 0.4 | 0.8 ± 0.2 | 34.2 ± 0.2 | -0.6 ± 0.1 | 36.3 ± 0.3 | 1.5 ± 0.2 | 2.1 ± 0.3 | 0.3 ± 0.02 |

**Table S5.** Reproducibility experiments at +200 mV for the WT, NN and NNY αHL pores threaded with oligo(dC)$_{30}$ with a single substitution at position 9 (rG, rA rC, rU, inosine (rI), N$^6$-methyladenine (m$^6$A) or 5-methylcytosine (m$^5$C)). The $\Delta I_{RES\%}$ values are plotted in Figure 4a. Standard deviations are given for three experiments (n = 3). $\Delta I_{RES}{}^{rX\text{-}oligo(dC)}$ = $I_{RES\%}$ for the rX oligonucleotide - $I_{RES\%}$ of oligo(dC)$_{30}$. X = rG, rA, rC, rU, rI, m$^6$A or m$^5$C.

| WT | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Exp | $I_O$ (pA) | oligo(dC)$_{30}$ | rA | | rC | | rG | | rU | | rI | | m$^6$A | | m$^5$C | |
| | | $I_{RES}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}{}^{rX\text{-}oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}{}^{rX\text{-}oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}{}^{rX\text{-}oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}{}^{rX\text{-}oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}{}^{rX\text{-}oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}{}^{rX\text{-}oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}{}^{rX\text{-}oligo(dC)}$ (%) |
| 1 | 214.0 | 23.3 | 23.6 | 0.3 | 23.3 | 0.0 | 25.1 | 1.8 | 24.1 | 0.8 | 23.8 | 0.5 | 23.0 | -0.3 | 21.7 | -1.6 |
| 2 | 205.0 | 22.7 | 23.1 | 0.4 | 22.8 | 0.1 | 24.6 | 1.9 | 23.9 | 1.2 | 23.4 | 0.7 | 22.4 | -0.3 | 21.6 | -1.1 |
| 3 | 198.0 | 22.1 | 22.4 | 0.3 | 22.0 | -0.1 | 24.1 | 2.0 | 23.8 | 1.7 | 22.7 | 0.6 | 22.0 | -0.1 | 21.0 | -1.1 |
| Mean | 206.0 | 22.7 | 23.0 | 0.3 | 22.7 | 0.0 | 24.6 | 1.9 | 23.9 | 1.2 | 23.3 | 0.6 | 22.5 | -0.2 | 21.7 | -1.3 |
| SD | 8.0 | 0.6 | 0.6 | 0.1 | 0.7 | 0.1 | 0.5 | 0.1 | 0.2 | 0.5 | 0.6 | 0.1 | 0.5 | 0.1 | 0.2 | 0.3 |

| NN | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Exp | $I_O$ (pA) | oligo(dC)$_{30}$ | rA | | rC | | rG | | rU | | rI | | m$^6$A | | m$^5$C | |
| | | $I_{RES}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}{}^{rX\text{-}oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}{}^{rX\text{-}oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}{}^{rX\text{-}oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}{}^{rX\text{-}oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}{}^{rX\text{-}oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}{}^{rX\text{-}oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}{}^{rX\text{-}oligo(dC)}$ (%) |
| 1 | 200.0 | 27.4 | 27.6 | 0.2 | 27.3 | -0.1 | 27.9 | 0.5 | 28.2 | 0.8 | 27.7 | 0.3 | 27.3 | -0.1 | 27 | -0.4 |
| 2 | 211.0 | 26.8 | 27.1 | 0.3 | 26.8 | 0.0 | 27.6 | 0.8 | 27.7 | 0.9 | 27.3 | 0.5 | 26.9 | 0.1 | 26.4 | -0.4 |
| 3 | 201.0 | 25.3 | 25.8 | 0.5 | 25.2 | -0.1 | 26.1 | 0.8 | 26.2 | 0.9 | 25.9 | 0.6 | 25.6 | 0.3 | 24.8 | -0.5 |
| Mean | 204.0 | 26.5 | 26.8 | 0.3 | 26.4 | -0.1 | 27.2 | 0.7 | 27.4 | 0.9 | 27.0 | 0.5 | 26.6 | 0.1 | 26.1 | -0.4 |
| SD | 6.0 | 1.1 | 0.9 | 0.2 | 1.1 | 0.1 | 1.0 | 0.2 | 1.0 | 0.1 | 0.9 | 0.2 | 0.9 | 0.2 | 1.1 | 0.1 |

| NNY | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Exp | $I_O$ (pA) | oligo(dC)$_{30}$ | rA | | rC | | rG | | rU | | rI | | m$^6$A | | m$^5$C | |
| | | $I_{RES}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}{}^{rX\text{-}oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}{}^{rX\text{-}oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}{}^{rX\text{-}oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}{}^{rX\text{-}oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}{}^{rX\text{-}oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}{}^{rX\text{-}oligo(dC)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}{}^{rX\text{-}oligo(dC)}$ (%) |
| 1 | 210.0 | 43.1 | 41 | -2.1 | 43.1 | 0 | 39.6 | -3.5 | 45.4 | 2.3 | 42.7 | -0.4 | 41.4 | -1.7 | 43.6 | 0.5 |
| 2 | 204.0 | 42.6 | 40.8 | -1.8 | 42.5 | -0.1 | 39.5 | -3.1 | 45.1 | 2.5 | 42.6 | 0 | 41.2 | -1.4 | 43 | 0.4 |
| 3 | 209.0 | 42.4 | 40.3 | -2.1 | 42.4 | 0 | 38.8 | -3.6 | 44.4 | 2 | 42.1 | -0.3 | 40.9 | -1.5 | 42.9 | 0.5 |
| Mean | 207.0 | 42.7 | 40.7 | -2.0 | 42.7 | 0.0 | 39.3 | -3.4 | 45.0 | 2.3 | 42.5 | -0.2 | 41.2 | -1.5 | 43.2 | 0.5 |
| SD | 3.0 | 0.4 | 0.4 | 0.2 | 0.4 | 0.1 | 0.4 | 0.3 | 0.5 | 0.3 | 0.3 | 0.2 | 0.3 | 0.2 | 0.4 | 0.1 |

**Table S6.** Reproducibility experiments at +200 mV for the WT, NN and NNY αHL pores threaded with oligo(rA)$_{30}$ with a single substitution at position 9 (rG, rA rC, rU, inosine (rI), N$^6$-methyladenine (m$^6$A) or 5-methylcytosine (m$^5$C)). The $\Delta I_{RES\%}$ values are plotted in Figure 4b. Standard deviations are given for three experiments (n = 3). $\Delta I_{RES}$ $^{rX\text{-}oligo(rA)}$ = $I_{RES\%}$ for the rX oligonucleotide - $I_{RES\%}$ of oligo(rA)$_{30}$. X = rG, rA, rC, rU, rI, m$^6$A or m$^5$C.

| WT | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | oligo(rA)$_{30}$ | rA | | rC | | rG | | rU | | rI | | m$^6$A | | m$^5$C | |
| Exp | $I_O$ (pA) | $I_{RES}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) |
| 1 | 203.0 | 22.7 | 22.7 | 0.0 | 21.9 | -0.8 | 23.9 | 1.2 | 23.6 | -0.9 | 22.5 | -0.2 | 22.2 | -0.5 | 21.7 | -1.0 |
| 2 | 196.0 | 22.1 | 22.1 | 0.0 | 21.2 | -0.9 | 23.4 | 1.3 | 23.1 | -1.0 | 21.9 | -0.2 | 21.6 | -0.5 | 21.0 | -1.1 |
| 3 | 194.0 | 21.8 | 21.8 | 0.0 | 21.0 | -0.8 | 23.0 | 1.2 | 22.9 | -1.1 | 21.7 | -0.1 | 21.4 | -0.4 | 20.8 | -1.0 |
| Mean | 198.0 | 22.2 | 22.2 | 0.0 | 21.4 | -0.8 | 23.4 | 1.2 | 23.2 | -1.0 | 22.0 | -0.2 | 21.7 | -0.5 | 21.2 | -1.0 |
| SD | 5.0 | 0.5 | 0.5 | 0.0 | 0.5 | 0.1 | 0.5 | 0.1 | 0.4 | 0.1 | 0.4 | 0.1 | 0.4 | 0.1 | 0.5 | 0.1 |

| NN | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | oligo(rA)$_{30}$ | rA | | rC | | rG | | rU | | rI | | m$^6$A | | m$^5$C | |
| Exp | $I_O$ (pA) | $I_{RES}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) |
| 1 | 209.0 | 33.8 | 33.8 | 0.0 | 34.6 | 0.8 | 31.7 | -2.1 | 32.2 | -1.6 | 34.1 | 0.3 | 33.6 | -0.2 | 34.3 | 0.5 |
| 2 | 206.0 | 34.2 | 34.2 | 0.0 | 35.2 | 1.0 | 32.0 | -2.2 | 33.0 | -1.2 | 34.6 | 0.4 | 34.0 | -0.2 | 35.0 | 0.8 |
| 3 | 212.0 | 34.8 | 34.8 | 0.0 | 35.7 | 0.9 | 32.6 | -2.2 | 33.6 | -1.2 | 34.9 | 0.1 | 34.4 | -0.4 | 35.3 | 0.5 |
| Mean | 209.0 | 34.3 | 34.3 | 0.0 | 35.2 | 0.9 | 32.1 | -2.2 | 32.9 | -1.3 | 34.5 | 0.3 | 34.0 | -0.3 | 34.9 | 0.6 |
| SD | 3.0 | 0.5 | 0.5 | 0.0 | 0.6 | 0.1 | 0.5 | 0.1 | 0.7 | 0.2 | 0.4 | 0.2 | 0.4 | 0.1 | 0.5 | 0.2 |

| NNY | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | oligo(rA)$_{30}$ | rA | | rC | | rG | | rU | | rI | | m$^6$A | | m$^5$C | |
| Exp | $I_O$ (pA) | $I_{RES}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}oligo(rA)}$ (%) |
| 1 | 208.0 | 34.4 | 34.4 | 0.0 | 35.2 | 0.8 | 33.3 | -1.1 | 35.8 | 1.4 | 35.3 | 0.9 | 33.9 | -0.5 | 36.0 | 1.6 |
| 2 | 201.0 | 34.9 | 34.9 | 0.0 | 35.7 | 0.8 | 33.6 | -1.3 | 36.8 | 1.9 | 35.8 | 0.9 | 34.2 | -0.7 | 36.3 | 1.4 |
| 3 | 206.0 | 34.8 | 34.8 | 0.0 | 36.2 | 1.4 | 34.4 | -0.4 | 37.0 | 2.2 | 35.6 | 0.8 | 34.2 | -0.6 | 36.2 | 1.4 |
| Mean | 205.0 | 34.7 | 34.7 | 0.0 | 35.7 | 1.0 | 33.8 | -0.9 | 36.5 | 1.8 | 35.6 | 0.9 | 34.1 | -0.6 | 36.2 | 1.5 |
| SD | 4.0 | 0.3 | 0.3 | 0.0 | 0.5 | 0.3 | 0.6 | 0.5 | 0.6 | 0.4 | 0.3 | 0.1 | 0.2 | 0.1 | 0.2 | 0.1 |

**Table S7.** Voltage dependencies of residual currents, $I_{RES\%}$, for NNY αHL pores threaded with a heteropolymeric strand substituted at position 9 with rA, rC, rG and rU. The $I_O$ and $I_{RES\%}$ values are mean values (± S.D.) taken from Gaussian fits to event histograms at various applied potentials (n = 3 experiments). $I_{RES\%} = (I_{RES}/I_O) \times 100$. $\Delta I_{RES\%}^{OVERALL}$ is the difference in residual current between the two most widely separated current peaks. The δ value is the product of the successive differences between the peaks. If any two peaks overlap, then the δ value is zero. If any two peaks overlap, then the δ value is zero. $\Delta I_{RES\%}$ values were determined for individual experiments and the mean value is used at each potential. $\Delta I_{RES}{}^{rX\text{-}rA}$ = $I_{RES\%}$ for the rX oligonucleotide - $I_{RES\%}$ for rA-het$_{30}$ oligonucleotide. X = rG, rA, rC or rU. The $I_{RES\%}$ and $\Delta I_{RES\%}$ data from the table are plotted in Figure 5b and c.

| NNY | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| mV | $I_O$ (pA) | rA | | rC | | rG | | rU | | $I_{RES}^{OVERALL}$ (%) | δ (%) |
| | | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}rA}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}rA}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}rA}$ (%) | $I_{RES}$ (%) | $\Delta I_{RES}^{rX\text{-}rA}$ (%) | | |
| +100 | 99.0 ± 4.0 | 26.4 ± 0.3 | 0.0 ± 0.0 | 27.4 ± 0.3 | 1.0 ± 0.3 | 25.9 ± 0.3 | -0.5 ± 0.3 | 28.1 ± 0.3 | 1.7 ± 0.3 | 2.2 ± 0.3 | 0.4 ± 0.02 |
| +120 | 114.0 ± 6.0 | 28.3 ± 0.3 | 0.0 ± 0.0 | 29.0 ± 0.3 | 0.7 ± 0.3 | 27.6 ± 0.3 | -0.7 ± 0.3 | 29.7 ± 0.4 | 1.4 ± 0.3 | 2.1 ± 0.3 | 0.3 ± 0.01 |
| +140 | 132.0 ± 8.0 | 29.3 ± 0.4 | 0.0 ± 0.0 | 30.0 ± 0.3 | 0.7 ± 0.3 | 28.6 ± 0.4 | -0.7 ± 0.2 | 30.5 ± 0.3 | 1.2 ± 0.3 | 1.9 ± 0.4 | 0.2 ± 0.01 |
| +160 | 158.0 ± 6.0 | 30.2 ± 0.4 | 0.0 ± 0.0 | 30.9 ± 0.5 | 0.7 ± 0.3 | 29.4 ± 0.5 | -0.8 ± 0.2 | 31.4 ± 0.3 | 1.2 ± 0.3 | 2.0 ± 0.4 | 0.3 ± 0.02 |
| +180 | 179.0 ± 9.0 | 30.5 ± 0.2 | 0.0 ± 0.0 | 31.0 ± 0.3 | 0.5 ± 0.1 | 30.0 ± 0.2 | -0.5 ± 0.2 | 31.8 ± 0.2 | 1.3 ± 0.2 | 1.8 ± 0.2 | 0.2 ± 0.01 |
| +200 | 204.0 ± 7.0 | 31.0 ± 0.2 | 0.0 ± 0.0 | 31.7 ± 0.2 | 0.7 ± 0.1 | 30.6 ± 0.2 | -0.4 ± 0.2 | 32.4 ± 0.3 | 1.4 ± 0.3 | 1.8 ± 0.3 | 0.2 ± 0.01 |

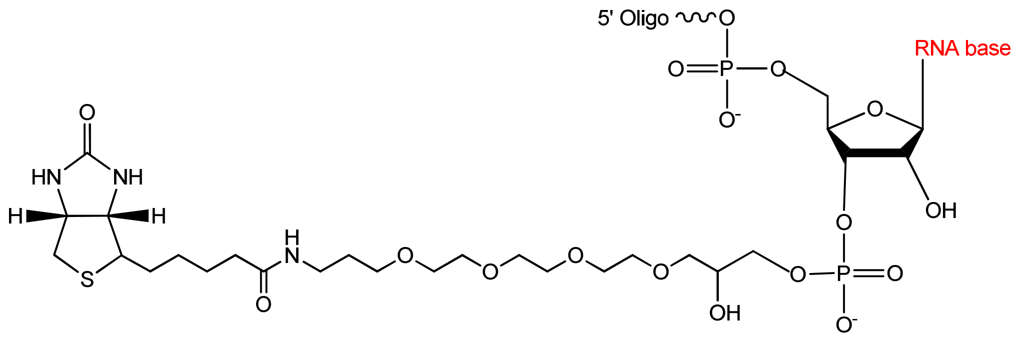


**Figure S1.** Chemical structure of the Biotin-TEG linker used to biotinylate the 3' terminus of the oligonucleotides (Table S1). Structure produced with ChemBioDraw Ultra software (version 12.02). TEG: triethylene glycol.

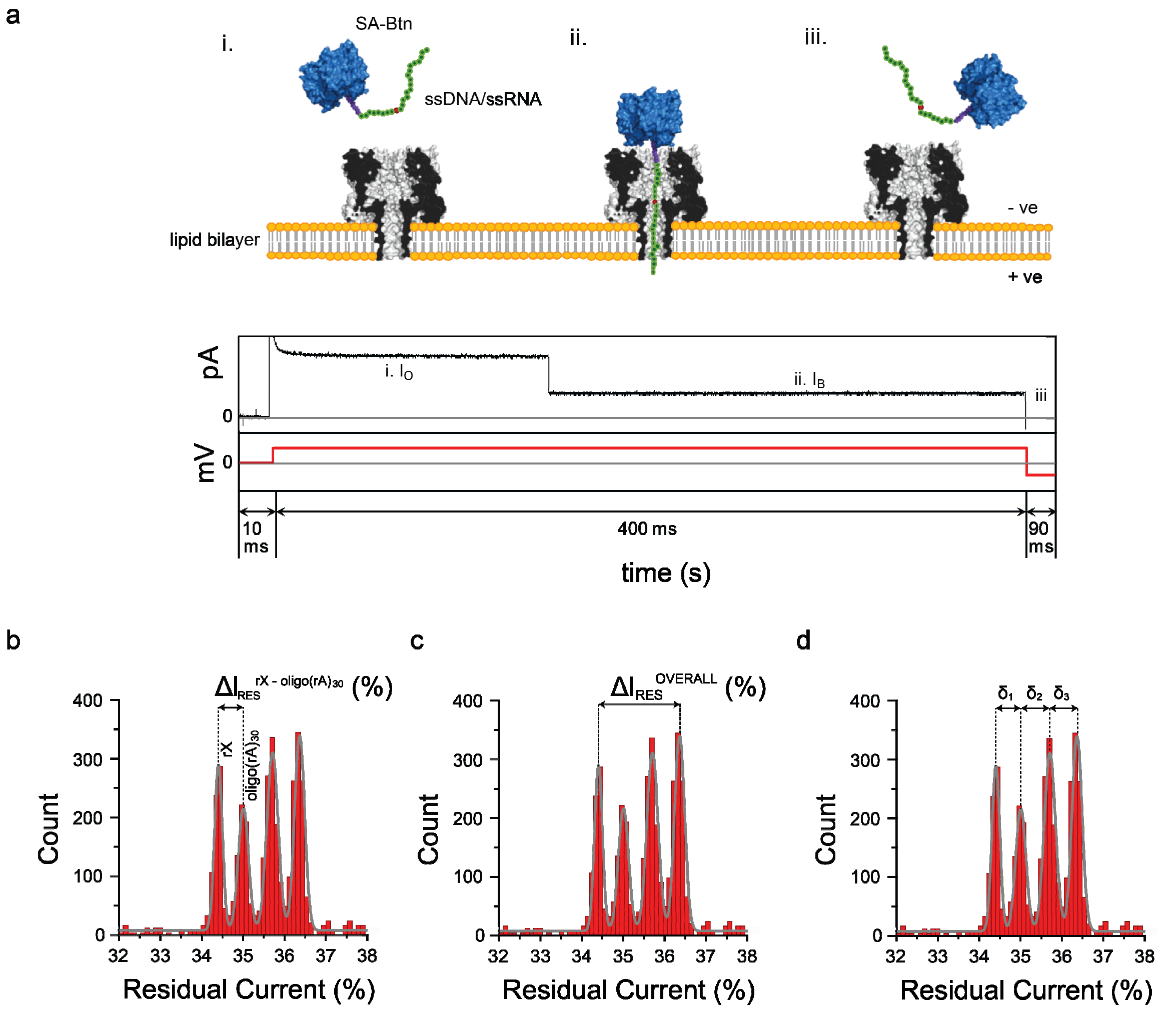


**Figure S2.** (a) The voltage protocol used to immobilize oligonucleotides inside the αHL pore and the corresponding current trace. $I_O$ is the open pore current; $I_{RES}$ is the remaining current when the oligonucleotide is immobilized inside the pore; SA-Btn is the streptavidin-biotin complex. (b-d) Histogram showing the $I_{RES\%}$ levels observed when an NNY pore is probed with four oligo(rA)$_{30}$ oligonucleotides that contain a single substituted nucleotide at position 9 (relative to the 3' biotin-tag). The parameters used to determine the discrimination of single nucleobase substitutions are shown. The $I_{RES\%}$ value of each oligonucleotide sequence, in this case, rX-oligo(rA)$_{30}$ and oligo(rA)$_{30}$ were taken from Gaussian fits to the blockade events, and the $\Delta I_{RES\%}$ value is calculated as the difference between them: $\Delta I_{RES\%}^{rX\text{-}oligo(rA)} = I_{RES\%}$ for the rX oligonucleotide - $I_{RES\%}$ for oligo(rA)$_{30}$. X = rG, rA, rC or rU[3]. $\Delta I_{RES\%}^{OVERALL}$ is the difference in residual current between the two most widely separated current peaks. The δ value is the product of the successive differences between the peaks, $\delta = \delta_1 \times \delta_2 \times \delta_3$. If any two peaks overlap, then the δ value is equal to zero[3, 7].

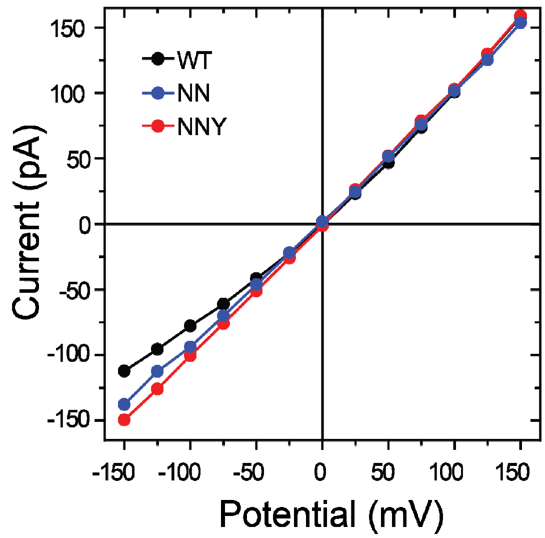


**Figure S3.** Typical current-voltage (I-V) traces for WT (*black*), E111N/K147N (NN, *blue*) and E111N/K147N/M113Y (NNY, *red*) αHL pores, in 1 M KCl, 25 mM Tris-HCl, pH 7.5, containing 100 µM EDTA.

**Figure S4.** Plot of $I_{RES\%}$ versus the applied potential (+100 to +200 mV) for the homopolymers (oligo(rA)$_{30}$, oligo(rC)$_{30}$ and oligo(rU)$_{30}$) immobilized in WT, NN and NNY αHL pores. Lines are for guidance only. Data are compiled in Table S2.

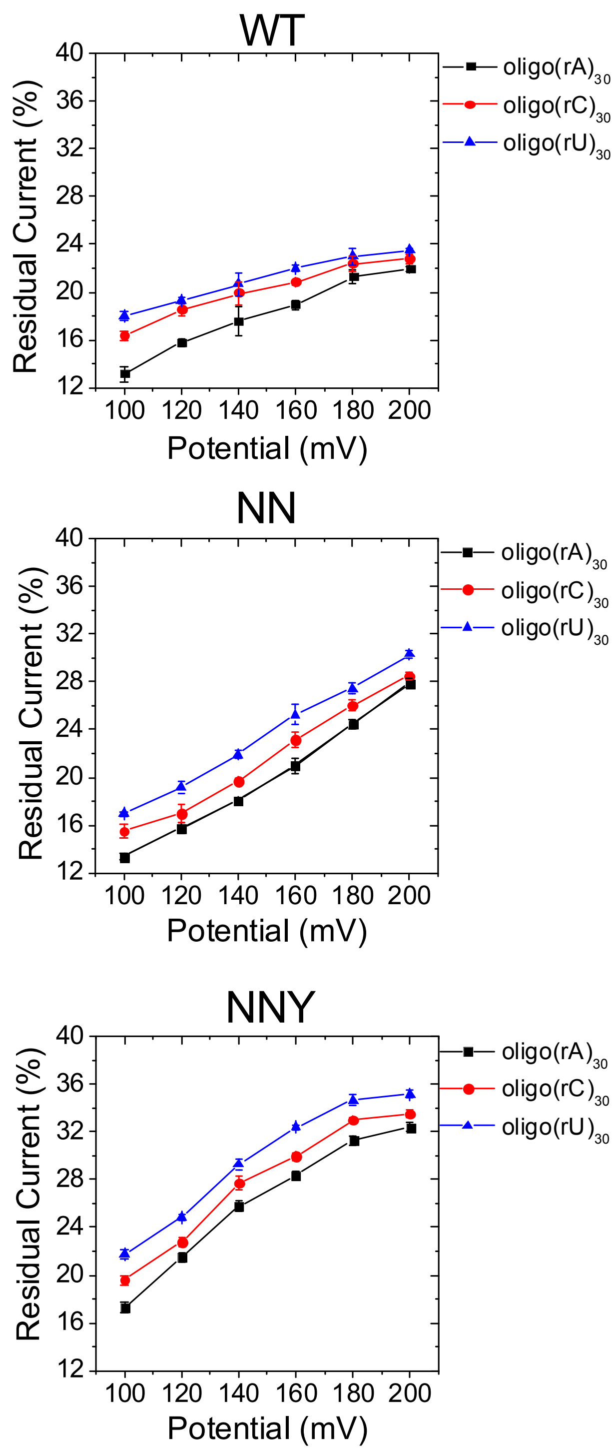

**Figure S5.** Plots of $I_{RES\%}$ versus the applied potential (+100 to +200 mV) for WT, NN and NNY αHL pores in the presence of (a) ssDNA or (b) ssRNA strands with a single base substitution at position 9 (X= rG, rA, rC or rU). The data for the graphs were obtained by taking mean values from Gaussian fits to peaks of histograms of residual current levels ($I_{RES}$) for multiple blockades by each oligonucleotide, at various applied potentials (Tables S3 and S4). $I_{RES\%}$ is calculated by using the mean value from at least three experiments. $I_{RES\%} = (I_{RES}/I_O) \times 100$.

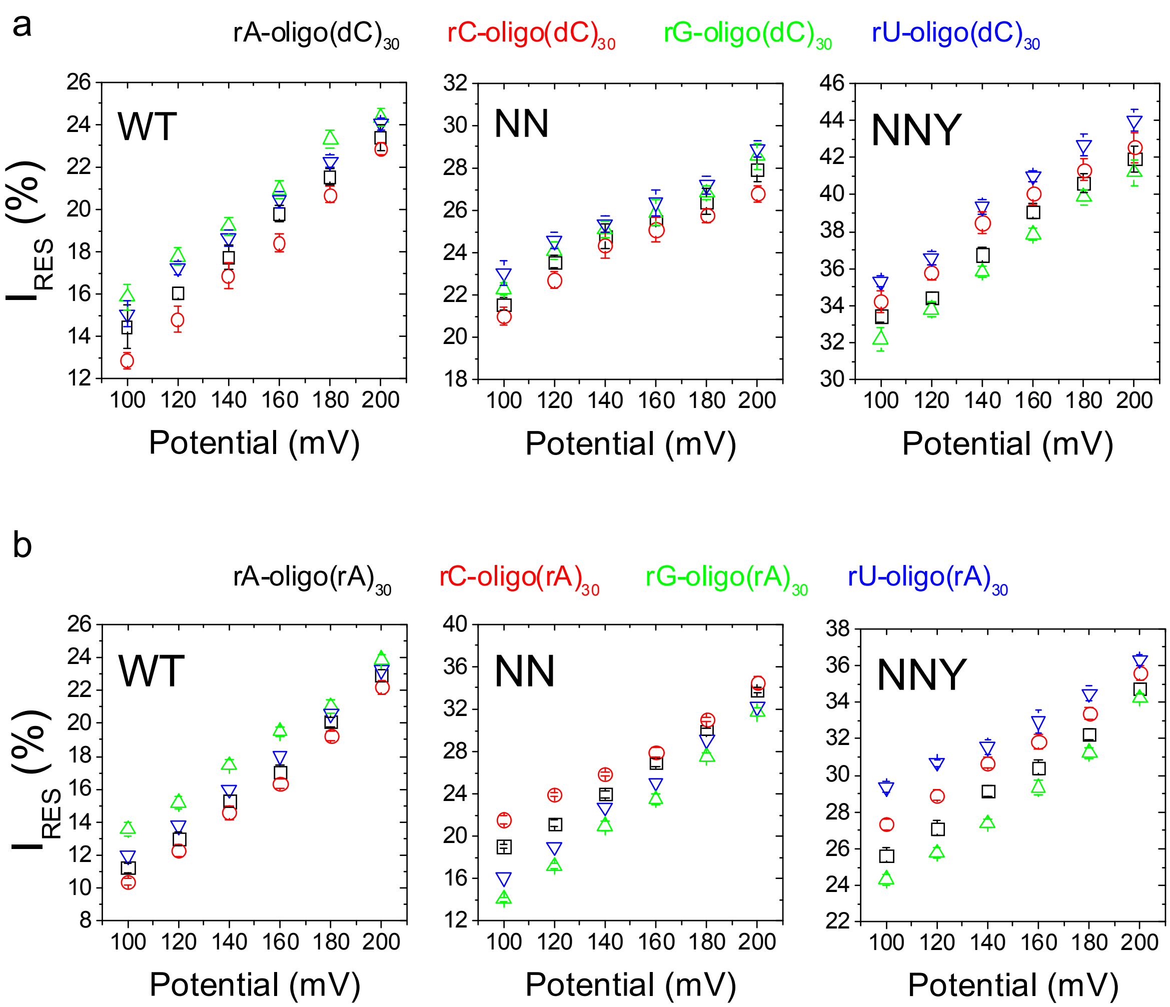

**Figure S6.** Plots of residual current differences, $\Delta I_{RES\%}$, between blockades by substituted oligonucleotides and oligo(dC)$_{30}$ for (a) WT (b) NN and (c) NNY αHL pores. The data were obtained by taking mean values from Gaussian fits to histograms of residual current levels ($I_{RES}$) for multiple blockades by each oligonucleotide, at various applied potentials, Table S3. $I_{RES}$ is calculated by using the mean value from at least three experiments. $I_{RES\%} = (I_{RES}/I_O) \times 100$. $\Delta I_{RES}{}^{rX\text{-}oligo(dC)} = I_{RES\%}$ for the rX oligonucleotide - $I_{RES\%}$ for oligo(dC)$_{30}$. X = rG, rA, rC or rU.

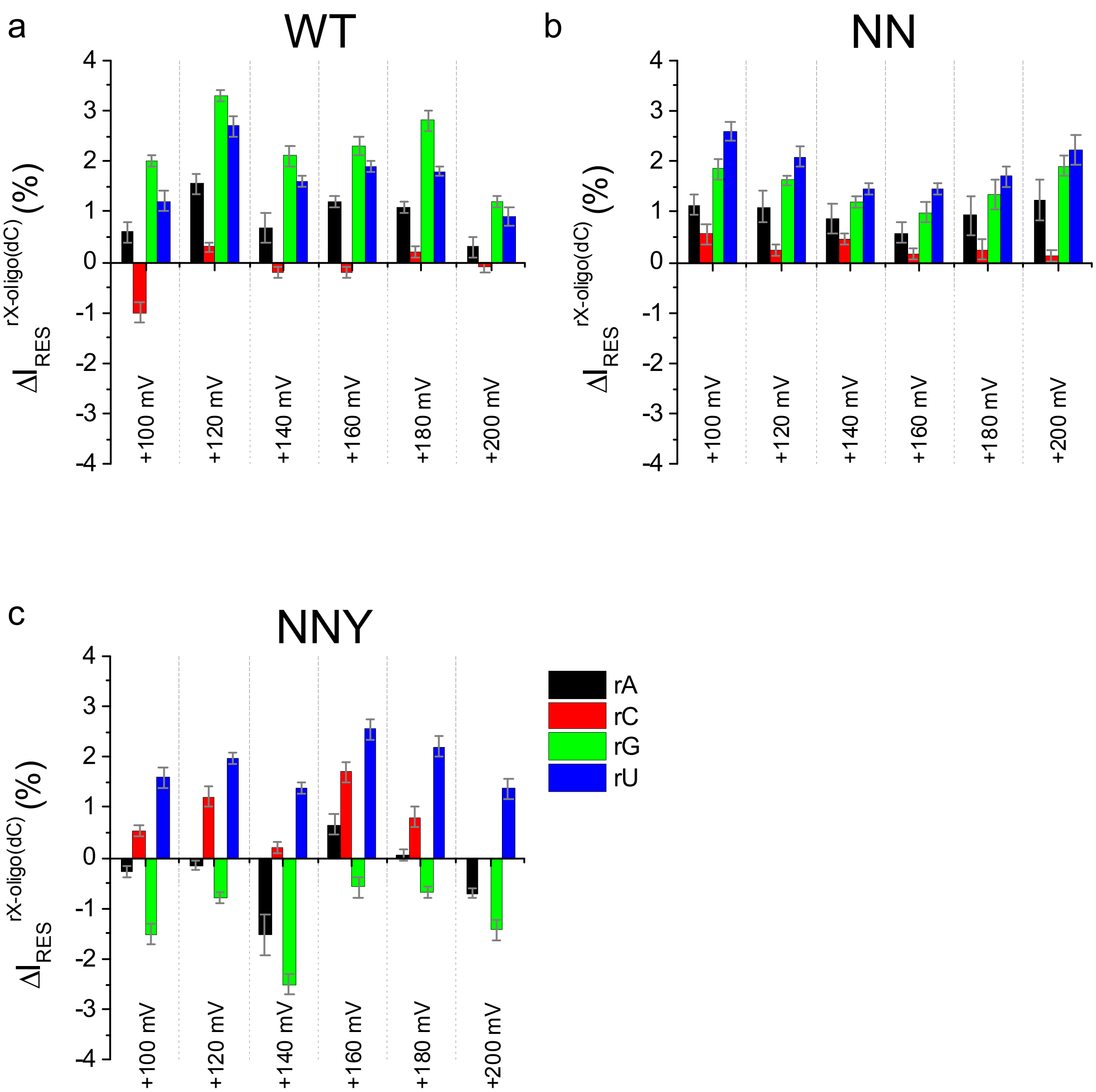

**Figure S7.** Plots of residual current differences, $\Delta I_{RES\%}$, between blockades by substituted oligonucleotides and oligo(rA)$_{30}$ for (a) WT (b) NN and (c) NNY αHL pores. The data were obtained by taking mean values from Gaussian fits to histograms of residual current levels ($I_{RES}$) for multiple blockades by each oligonucleotide, at various applied potentials, Table S4. $I_{RES}$ is calculated by using the mean value from at least three experiments. $I_{RES\%} = (I_{RES}/I_O$ x 100. $\Delta I_{RES}{}^{rX\text{-}oligo(rA)} = I_{RES\%}$ for the rX oligonucleotide - $I_{RES\%}$ for oligo(rA)$_{30}$. X = rG, rA, rC or rU.