Unfolding kinetics of the human telomere i-motif under a 10 pN force imposed by the alpha- hemolysin nanopore identify transient folded state lifetimes at physiological pH	
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## SI1

## **METHODS**

**Ion channel recording:** A custom-built, high-impedance, low-noise amplifier and data acquisition system, designed and constructed by Electronic Biosciences (EBS), San Diego, CA, was used for the current–time (*i*-*t*) recordings. For all studies, the i-motif DNA (2 nmol, 10  $\mu$ M) was added, and > 200 events were collected for each voltage with a 100 kHz low pass filter and a 500 kHz data acquisition rate. The composition of the buffered electrolyte solution was 1.00 M KCl, 10 mM KP<sub>i</sub>, 5 mM citrate, and 1 mM EDTA. Data were collected at -100 mV, -120 mV, -140 mV, -160 mV, and -180 mV (*cis* vs. *trans*).

**DNA Preparation and Purification Procedures:** The oligodeoxynucleotides were synthesized from commercially available phosphoramidites (Glen Research, Sterling, VA) by the DNA-Peptide Core Facility at the University of Utah, followed by purification using a semi-preparation ion-exchange HPLC column with a linear gradient of 5% to 100% B over 30 min while monitoring absorbance at 260 nm (B = 20 mM Tris, 1 M NaCl, pH 8 in 10% CH<sub>3</sub>CN/90% ddH<sub>2</sub>O; A = 10% CH<sub>3</sub>CN/90% ddH<sub>2</sub>O; flow rate = 3 mL/min). The purities of the oligodeoxynucleotides were determined by reinjecting the purified samples on an analytical ion-exchange HPLC running the previously mentioned buffers and method with the exception that the flow rate was 1 mL/min. After purification, the DNA was dissolved in the nanopore buffer and annealed at 90 °C for 5 min followed by cooling down overnight in a water bath.

**Ion channel measurements:** The glass nanopore membrane (GNM; r ~ 800 nm) was fabricated as previously reported.<sup>1</sup> 1,2-Diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) bilayers spanning across the orifice of the GNM were prepared as previously described.<sup>2</sup> The protein  $\alpha$ -HL was diluted to a 0.2 mg/mL solution in ultra-pure water (18 M $\Omega$ \*cm) and the DPhPC was dissolved in decane to a concentration of 10 mg/mL, both of which were stored at -80 °C. A pipette holder with a pressure gauge and a 10-mL gas-tight syringe were used to attach the GNM to the DC system. Two Ag/AgCl electrodes were positioned inside and outside of the GNM to apply a voltage. A plastic pipette tip was used to paint the DPhPC solution (1  $\mu$ L, 10 mg/mL) on the GNM surface. After addition of monomer  $\alpha$ -HL (0.5  $\mu$ L, 0.2 mg/mL), a pressure was applied to form a suspended bilayer, followed by reconstitution of a single  $\alpha$ -HL nanopore in the bilayer. Populations of >2000 deep blockage events were collected for most of experiments and >200 deep blockage events were collected for those i-motifs that last longer than 2 s. The voltages were generally -120 and -160 mV (*cis* vs. *trans*).

The pH-dependent CD studies: The CD spectra were recorded on a 10  $\mu$ M solution of the i-motif sequences in a Britton-Robinson buffer (25 mM each of sodium acetate, sodium phosphate, and sodium borate) with 1.0 M KCl at 20 °C. The pH values were adjusted from 5.0 to 7.5 in 0.25 pH unit increments. The measured spectra were normalized by the i-motif concentration to arrive at molar ellipticity values vs. wavelength that were plotted for each measurement. The transition pH (pH<sup>T</sup>) was determined by plotting and fitting the molar ellipticity at 286 nm vs. pH.

**Data analysis**: Density plots were analyzed with software donated by EBS. Events were extracted using QUB 1.5.0.31 and fitted using Origin 9.1. Individual translocation *i-t* traces were refiltered to 2 kHz or 10 kHz for presentation depending on the duration of single events. Due to the fact that different hairpins may have very different unzipping times and distributions, different numbers of bins (30 - 100) were used to fit the current or time histograms.

Figure S1. The long blocking event for the tailless i-motif at -120 mV and pH 5.0. The events lasted longer than 2 min.

100 pA 500 ms

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100 pA 500 ms

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Figure S2. Sample i-t traces and Type 1 events for the tailless i-motif. A: Sample *i-t* traces of the tailless i-motif at -120 mV at pH 5.0.



SI6

B: Sample of Type 1 events. In each event, the polarity was reversed to eject the i-motif from the nanopore.



C: Details of Type 1 events in the first 20 ms of the event. In each event, the open channel current is at the bottom and the 0 pA current is at the top (thin black line).

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Figure S3. Type 2 event for the tailless i-motif. A: In each event, the polarity was reversed to eject the i-motif from the nanopore.



B: Blow up of *i*-*t* traces for Type 2 events in first 20 ms of each event. In each event, the open channel current is at the bottom and the 0 pA current is at the top.



Figure S4. pH-Dependent CD spectra for the i-motif with 2 nt tails.



Figure S5. Type 1 events for the i-motif with 2 nt tails at -120 mV. After 2s, the polarity was reversed to eject the i-motif from the nanopore.



B: Blow up of Type 1 events in the first 20 ms of each event. In each event, the open channel current is at the bottom and the 0 pA current is at the top.

Figure S6. Type 1 events for i-motif with 2 nt tail that last longer than 2 min.

500 ms

50 pA





Figure S8. pH-Dependent CD study for the long-tail i-motif.



SI16

Figure S9. Deep current blockage event for the long-tail i-motif at -120 mV (longer than 3 min) and pH 5.0. Data were refiltered at 2 kHz for the presentation.

100 pA

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100 pA 500 ms

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Figure S10. Type 1' and 2' events for the long-tail i-motif at -160 mV. The events with less noise were the Type 1' that have a median lifetime of 21 s at pH 5.0. The events that were noisier were the Type 2' that cannot translocate and were ejected from the channel by reversal of the bias.







Figure S11. Type 1' and 2' events for the long-tail i-motif at -180 mV and pH 5.0. The events with less noise are Type 1'. The events that are noisier are the Type 2' that cannot translocate and are followed by reversal of the bias to eject the i-motif out of the vestibule. A: *i-t* traces for the long-tail i-motif at -180 mV and pH 5.0.



B: Voltage-dependent study for the long tail i-motif at pH 5.0.

Voltage (mV)	120	160	180
Median unzipping	>180	21.6	7.4
time (s)			

100 pA 500 ms h ٦ T Т Π Π  $\|$ Γ

Figure S12. *i-t* traces for the long-tail i-motif at -120 mV and pH 5.0. The polarity was reversed at 2 s after capture of the i-motif to eject the i-motif from the channel and collect another one for study.











Figure S13. *i-t* traces for Type 1' and Type 2' events for the long-tail i-motif at -120 mV and pH 5.7. The polarity was reversed every 2 s to eject the i-motif from the nanopore and capture another one.

Figure S14. Details of Type 2' events at -120 mV and pH 5.0. Only the first 20 ms of each event are shown because they are > 2 min long.



Figure S15. Voltage-dependent study of the long-tail i-motif at pH 6.8. Top panel is -100 mV, middle panel is -120 mV, and the bottom panel is -160 mV (*cis* vs. *trans*).







Figure S17. Samples of *i*-*t* traces for the long-tail i-motif at pH 6.0 and -120 mV.

	200 pA 200 ms

Figure S18. Samples of *i-t* traces for the long-tail i-motif at pH 6.3 and -120 mV.





Figure S19. Samples of *i-t* traces for the long-tail i-motif at pH 6.8 and -160 mV.





**Figure S21**. Comparison of folded lifetimes for long-tail i-motif at 150 mM and 1.0 M salt. Both experiments were conducted at pH 6.8 at 160 mV but in different ionic strengths (1.00 M KCl and 150 mM KCl) buffered with potassium phosphate and citric acid.



## References

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